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CONTENTS

FARNESOID X RECEPTOR IN HUMAN MALIGNANCIES: AN OVERVIEW

C. GIORDANO[1], I. BARONE[2], D. BONOFIGLIO[2], S. CATALANO[2] and S. ANDÒ[2]

[1]Centro Sanitario, University of Calabria, Rende (CS), Italy; [2]Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende (CS), Italy

Cancer represents a leading cause of death in developed and developing countries, accounting for 8.8 million deaths in 2015. These numbers are expected to continue rising with 13.1 million deaths estimated in 2030. Although the earlier diagnosis and increasing treatment options have improved the survival rates for the majority of cancers, many tumors exhibit therapeutic resistance and severe adverse effects remain major therapeutic hurdles. Thus, the main challenge of the cancer research area is the definition of additional treatment options tailored on the distinct tumor characteristics and the expression of appropriate targets. Nuclear receptors, controlling many biological features of cancers as proliferation, survival, apoptosis, and differentiation, possess a significant clinical relevance in disease management, representing a "druggable" class of molecules useful for potential therapeutic intervention. In the last years, among the nuclear receptors, the metabolic sensor Farnesoid X Receptor was recognized to play a role in carcinogenesis, acting either as an oncogene or as a tumor suppressor gene. Here, we will summarize the current knowledge on the FXR role in human cancers, highlighting its ligands as new potential anticancer tools.

Nuclear receptors (NRs) are a superfamily of ligand-activated transcription factors that regulate essential physiological functions such as differentiation, development, reproduction and metabolism. NRs can be classified in hormonal receptors (estrogen-ER, progesterone-PR, androgen-AR, glucocorticoid-GR, mineralcorticoid receptor-MR), metabolic sensors/receptors (Peroxisome Proliferator-Activated Receptors-PPARs, Liver X Receptor-LXR, Retinoid X Receptor-RXR, Farnesoid X Receptor-FXR) and orphan nuclear receptors for which endogenous ligands have not yet been identified.

Beside their role in physiological conditions, a large amount of literature have highlighted the importance of NR activities in several pathological conditions including malignant diseases (1). In this scenario, great attention has been given in the last decades to the role of the metabolic sensor FXR in several cancer types. Initially identified as mammalian orphan receptor activated by high levels of farnesol and its metabolites able to form heterodimeric complex with RXR (2), FXR is now well recognized as a bile acid (BA) receptor (3). Indeed, FXR is highly expressed in tissues involved in the enterohepatic circulation of BAs such as the liver and the gastrointestinal tract, where it modulates expression of proteins controlling BA metabolism and transport (4). Moreover, FXR is engaged in enteroprotection, by mediating antibacterial activity of BAs in intestine (5), in liver regeneration and protection against hepatocarcinogenesis (6), and it appears to act as an oncosuppressor in several malignancy (7-12). Indeed, several *in vitro* and

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Mailing address:
Dr Stefania Catalano,
Full Professor Clinical Pathology,
Department of Pharmacy, Health and Nutritional Sciences,
University of Calabria,
Arcavacata di Rende (CS) 87036, Italy
Tel.: +39 0984 496207
Fax: +39 0984 496203.
e-mail: stefania.catalano@unical.it

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in vivo studies demonstrate the role of FXR as a cell protector, strongly supporting the idea that therapeutic modulation of FXR activity could be attractive as new pharmacological intervention in different malignant diseases.

Farnesoid X Receptor: gene, protein, mechanisms of action and ligands

Farnesoid X Receptor is encoded by two different genes FXRα (*NR1H4*) and FXRβ (*NR1H5*). In humans, the FXRα gene, mapped in the chromosome 12q23.1, encodes four isoforms (FXRα1, FXRα2, FXRα3, and FXRα4) due to the use of different promoters, and to the alternative RNA splicing in exon 5 (13). FXRα1 and FXRα2 have a shorter N terminus compared to the FXRα3 and FXRα4 isoforms. In addition, FXRα1 and FXRα3 contain, in the hinge region closely adjacent to the DNA binding domain (DBD), a four amino acid motif (MYTG) lacking in both FXRα2 and FXRα4 isoforms (13, 14).

FXRβ gene encode for a functional receptor in mice, rats, rabbits, and dogs, while it encodes a nonfunctional protein in humans and primates (15). FXR is prevalently expressed in the gastrointestinal tract, liver, kidney and adrenals, highlighting its biological relevance in metabolic homeostasis regulation (2, 14), but it has been also detected in a variety of nonenterohepatic compartments including heart, vasculature, ovary, testes, breast, thymus, eye, spleen, and adipose tissue (9, 11, 13, 15-17). As other members of NR's family, FXR contains a NH_2-terminal region that holds a ligand-independent activation function (AF-1), a highly conserved DNA binding domain (DBD), a hinge region responsible for receptor dimerization, a COOH-terminal region containing the ligand binding domain (LBD) and a ligand-dependent activation function (AF-2) (Fig. 1A).

FXR works as a transcription factor that in the absence of ligands binds, either as a monomer or as a heterodimer with the obligated partner RXRα, to a specific DNA consensus sequence (FXREs) consisting of two inverted repeat of six nucleotide AGGTCA separated by one nucleotide (IR-1). Additional FXREs exist, such as IR-0, IR-8, everted repeats (ER-8) or directed repeats (DR-1), but FXR binds these alternative FXREs with lower affinity compared to IR-1 sequence (18). In the

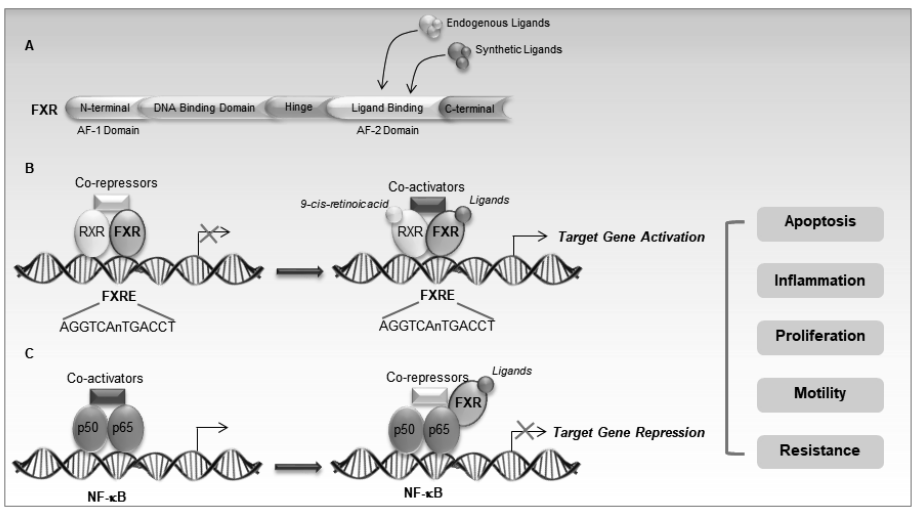

Fig. 1. *FXR mechanism of action in cancer.* ***A)****. Structural organization of Farnesoid X Receptor.* ***B)****. In the absence of ligand, FXR forms an obligate heterodimer with retinoid X receptor (RXR) that binds to specific FXR responsive elements (FXREs) in the promoter region of target genes. Upon ligand binding, FXR undergoes conformational changes, which results in dissociation of corepressor and recruitment of coactivators, thus inducing the activation of the transcriptional machinery.* ***C)****. Activated FXR might exert trans-repression by inhibiting the activity of different transcription factors (e.g. NF-kB, AP-1) on target gene expression. These events result in the modulation of the expression of many target genes involved in the control of apoptosis, inflammation, proliferation, motility and resistance on several cancers.*

inactive state, FXR interacts with corepressors (e.g. NCoR and SMRT) to the specific DNA sequences preventing the activation of the transcriptional machinery, while ligand-activated FXR undergo a conformational change that determines the dissociation of corepressors and the recruitment of several coactivators inducing the transcriptional activation of target genes.

Activated FXR is also able to trans-repress the expression of genes such as IL-1b, endothelin-1 and HER2 by several mechanisms including the direct interaction with NF-kB and AP-1 complexes, thus inhibiting their transcriptional activity (11, 19, 20) (Fig. 1B, C). Natural endogenous FXR ligands are BAs, classified in primary BAs, chenodeoxycholic acid (CDCA) and cholic acid (CA), end product of liver cholesterol catabolism, and secondary BAs deoxycholic acid (CDA) and lithocholic acid (LCA), products of the gut bacterial transformation of primary BAs (3, 21).

Based on the knowledge of the structure-activity relationship between BAs and FXR, either chemical manipulation of the BA structures or *de novo* development of synthetic FXR ligands were assessed to generate molecules with higher affinity, selectivity, and an augmented bioavailability. The obethicholic acid (6-ECDCA or INT-747), a 6α-ethyl derivative of CDCA, showed a greatest FXR agonistic effect compared to CDCA (22). Among the synthetic FXR modulators, the non-steroidal GW4064 was shown as the most selective and potent ligand (23), and the compound FXR-450 (WAY-362450 or XL335) displayed high efficacy, good oral bioavailability and prolonged half-life (24). Additional FXR agonists are the 2.2-dimethylbenzopyran scaffold derivatives (25), among which the most potent fexaramine has been demonstrated to induce a diverse subset of target gene expression compared to CDCA and GW4064, supporting the idea that different FXR ligands can selectively activate a specific gene signature (26).

FXR in cancer

Cancer occurrence is continuously growing worldwide (27) because of population aging, environmental exposures and lifestyle factors, thus representing a major global public health concern. The majority of cancers can be treated, but clinical improvements are often associated to severe adverse effects on normal cells and tissues and many tumors might exhibit *de novo* or acquired therapeutic resistance. Since the biology of cancer has become progressively deciphered on a molecular level, therapeutic research has focused on newer target-based agents able to inhibit specifically tumor outgrowth and progression. In the last decades, accumulating evidences indicate that activated FXR might function as a cell protector in target tissues, and could exert anticancer activities in many tumors.

Liver cancer

Several *in vivo* evidences highlight a tumor suppressor role for FXR in hepatocellular carcinoma (HCC), representing the prevalent form of primary liver cancer. FXR-null mice, with whole body FXR deficiency, exhibiting elevated hepatic BAs, sustained chronic inflammation as well as hepatic fibrosis, spontaneously developed hepatocellular carcinoma and the rare hepatocholangio cellular carcinoma at 12 months of age with a high incidence of 38% (28, 29). Otherwise, $FXR^{hep-/-}$ mice, with hepatocyte-specific FXR deficiency, did not show spontaneous liver carcinogenesis with aging, while developed tumors when treated with cholic acid (30). *In vitro* activated-FXR protects liver HepG2 cells from apoptosis induced by serum deprivation, and *in vivo* FXR-null mice displayed an impaired liver apoptosis (31), thus FXR showed a role in controlling liver homeostasis. In addition, FXR is able to control liver inflammation by inhibiting the activity of NF-kB and STAT3 inflammatory signaling.

Wang et al. found that activation of FXR antagonized NF-kB–induced expression of inflammatory mediators *in vitro* in hepatic cancer cells, whereas *in vivo* in $FXR^{-/-}$ livers they showed an increased sensitivity to LPS-induced inflammation compared to the WT mice (32). In HepG2 and Huh7 HCC cells treatment with GW4064 inhibited cancer cell proliferation and induced cell cycle arrest by increasing the expression of a negative regulator of STAT3 signaling, the suppressor of cytokine signaling 3 (SOCS3) (33).

In line with these evidences, Attia et al. reported

that INT-747 inhibited HCC cell proliferation, migration and invasion through an up-regulation of SOCS3 expression and a significant inhibition of IL6/STAT3 signaling (34). Activated FXR also controlled liver cancer cell proliferation by inhibiting the mTOR/S6K pathway (35). Clinical observations further support the protective role of FXR in liver carcinogenesis. Indeed, FXR expression was down-regulated at both mRNA and protein levels in human hepatocellular carcinoma specimens, and this down-regulation was positively correlated with multiple malignant clinicopathological characteristics (e.g. larger tumor size, poor differentiation, absence of encapsulation) (36).

Colorectal cancer

FXR deficiency in APC^{min} mice, a model of genetically induced intestinal carcinogenesis, resulted in an enhanced colon cell proliferation along with an increased expression of β-catenin, c-Myc, Cyclin D1 and IL-6 (37). Furthermore, APC^{min} female mice without FXR displayed higher adenocarcinoma multiplicity and larger size compared to mice expressing functional FXR (37).

Analysis of FXR expression in surgical specimens of human colon cancer revealed a reduction of FXR mRNA levels compared to normal colorectal mucosa (38). FXR protein and mRNA expression were found to be down-regulated in malignant compared to normal intestinal tissues of mice and human samples (7). Moreover, a direct correlation between FXR levels and degree of differentiation in colon cancer cells was detected (38).

Modica et al. demonstrated that FXR deficiency resulted in early mortality and tumor progression in different mouse models of intestinal tumorigenesis, suggesting that sustained Wnt signaling, increased TNFα production and infiltration of activated neutrophils and macrophages might be responsible for the increased tumor susceptibility to FXR loss. These authors also demonstrated that reintroduction of FXR in colon cancer xenograft mouse models resulted in a decreased tumor proliferation and induction of apoptosis (7).

FXR down-regulation during the development of colon cancer has been attributed to DNA methylation events and increased KRAS signaling (39). *In vitro* and *in vivo* studies using human colon cancer cell lines proposed a model in which FXR, inhibiting EGFR phosphorylation at Tyr845 and ERK1/2 activation, was able to control intestinal tumor cell proliferation by a Src-dependent cross-talk with EGFR (40).

Breast cancer

FXR expression was found in both normal and malignant breast tissues, with elevated levels in epithelial cells lining the ducts of normal breast and in infiltrating ductal carcinoma cells (8). Analysis of 65 breast cancer samples revealed that FXR levels were moderately higher in the ER-positive compared to the ER-negative cancers, with a significant correlation between FXR levels and ER, Ki-67 and topoisomerase-II alpha expression (41).

More recently, FXR was identified as a strong and independent prognostic marker of favorable overall and disease-free survival in invasive breast carcinoma (42). Up to now, only a study reported that farnesol-activated FXR stimulates ER-positive MCF-7 cell proliferation in steroid-free medium, while did not affect ER-negative MDA-MB-231 cells (41). Otherwise, Swales et al. demonstrated that activated-FXR induced apoptosis, in both ER-positive and triple negative breast cancer cells, and negatively regulated aromatase activity reducing local estrogen production (8). In addition, the authors showed the ability of activated FXR to modulate the expression of several P-glycoproteins (MDR3, MRP1, and SLC7A5), known as multidrug resistance proteins, suggesting a role for this receptor in drug-resistant state of breast cancer (8).

Indeed, we demonstrated that FXR ligands inhibited Tamoxifen resistant breast cancer cell growth, negatively modulating the expression of the transmembrane tyrosine kinase HER2 receptor, a signaling amplifier of the HER family network involved in the occurrence of endocrine resistance. Regulation of HER2 expression occurs at transcriptional level by an FXR-mediated inhibition of the NF-kB binding on the human HER2 promoter (11). We have also demonstrated that FXR counteracts tumor-promoting activities of

Cancer-Associated Fibroblasts in the complex breast microenvironment (12, 43). Moreover, it has been reported that FXR induced cell death by apoptosis in different breast cancer cells by activating the intrinsic apoptotic pathway (44).

Genitourinary Cancers

Ligand-activated FXR also exerted anti-proliferative effects on testicular tumors, the most frequent solid malignant tumor diagnosed in young men. We demonstrated in rat Leydig tumor R2C cells that activated-FXR, by competing with SF-1 binding to aromatase promoter, inhibited the expression of aromatase enzyme (9), responsible of increased local estrogen production able to induces R2C cell proliferation (45). In addition, in a xenograft mouse model of Leydig tumor, treatment with GW4064 reduced tumor volume and decreased expression of aromatase (10). In prostate cancer the role of FXR appears conflicting. Indeed, it has been proposed that FXR might have a procarcinogenic activity reducing androgen glucuronidation and thus sustaining the accumulation of androgens, the main regulators of prostate cancer cell growth (46). On the other hand, it has been demonstrated that FXR inhibited cell proliferation in human prostate adenocarcinoma LNCaP cells, up-regulating the expression of the tumor suppressor gene PTEN (47). In line with these evidences, *in vitro* studies demonstrated that activated FXR inhibited lipid metabolism via sterol response element binding protein 1 (SREBP1) pathway and suppressed growth in LNCaP cells (48).

CONCLUSIONS

Increased knowledge of cancer biology is essential for the definition of predictive biomarkers and specific molecular targets to choose an appropriate tailored therapy. Although the role of FXR in the regulation of many biological features of cancers such as apoptosis, proliferation, motility, and resistance needs to be further explored, the revised literature has shown its ability to work as a cell protector in many target tissues and highlighted the anticancer activity of FXR ligands in different human malignancies.

The advances in the understanding the functions of FXR in cancer, strongly supported by the use of specific transgenic mouse models, led us to conclude that activated FXR may negatively impact initiation and progression of the most common male and female types of tumors. Moreover, the possibility to use selective FXR agonists able to induce specific gene signature is an attractive feature to be exploited in order to obtain clinically useful compounds with reduced undesirable side effects.

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THE SIGNATURE OF LONGEVITY IN SICILY

G. ACCARDI [1], L. SCOLA [1], A. AIELLO[1], S. APRILE[1], M. BULATI[1], G. CANDORE[1], C. CARUSO[1], L. CRISTALDI[1,2], G. DURO[2], C.M. GAMBINO[1], M.E. LIGOTTI[1] and S. VASTO[2,3]

[1]Department of Pathobiology and Medical Biotechnologies (DIBIMED), University of Palermo, Palermo, Italy; [2]Institute of Biomedicine and Molecular Immunology "Alberto Monroy", National Research Council, Palermo, Italy; [3]Department of Biological Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy

The first two authors contributed equally to this work.

Ageing is a natural and physiological condition that is the result of compromised stress response, homeostatic imbalance and increased risk of developing diseases. However, if aging with good health and functions (successful ageing) and aging with disease and disability (unsuccessful ageing) depends on a combination of "positive features", including genetic, epigenetic and phenotypic characteristics in combination with favourable environment, economic status and social involvement. In our study, we summarize some key points for the identification of a longevity signature, with a particular focus on long-living Sicilian individuals and centenarians. Analysing three different Sicilian cohorts (young, people with no centenarian parents and long-living individuals (LLI) aged >90) we found APOE ε3\ε3 in our LLIs and no presence of ε4. Regarding FOXO rs2802292 G-allele (G>T) we did not observe an association with longevity, probably because of the small sample of centenarians studied. Regarding haematological and anthropometric results, it is still difficult to point specific longevity features and so far, we cannot specify a single one. On the other hand, we believe that the synergy among genetics and environment might create successful interaction to achieve and obtain effective longevity.

Background

Ageing results in compromised stress response, homeostatic/homeodynamic imbalance, and elevated risk of disease, making organism less, and eventually, non-resilient. All these elements lead people to reach different ages in different conditions (with and without success), and with different life-spans.

Centenarian people represent the best model to study successful ageing and longevity. Demographic selection has permitted to identify centenarians as healthy survivors, offering a "natural" selected population in which studying the effect of specific polymorphisms and genetic loci associated or not with longevity. Undoubtedly, they are able to respond well to the stressors and to repair damages thanks to a combination of "positive features", including genetic, epigenetic and phenotypic characteristics with favourable environment, economic status and social involvement (1). Although many studies exist on centenarians, it has not yet been possible to identify the longevity signature. However, a favourable

Key words: bioimpedance, body composition, centenarians, APOE, FOXO3A, longevity, successful ageing

Mailing address:
Prof. Calogero Caruso,
Immunosenescence Unit,
Department of Pathobiology and Medical Biotechnologies,
University of Palermo, Corso Tukory 211,
90134 Palermo, Italy
Tel.: +390916555911
e-mail: calogero.caruso@unipa.it

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genetic background is essential to live longer. Indeed, siblings and offspring of centenarian (CO), but not their spouses, show an increased odd ratio between 4- and 17-fold for longevity compared with appropriate controls thus they have a good chance to live approximately 100 years or over compared with the average population. Moreover, CO show reduction or delay in cardio-vascular diseases (CVDs) and all-cause morbidity and mortality (2-5).

The aim of this review is to summarize some important key points for the identification of a longevity signature, with a particular focus on Sicilian centenarians.

Genetics of longevity: APOE and FOXO3A

There is growing evidence that genetic component of longevity becomes more influent in the attainment of 100+ years old beyond the age of 90. Accordingly, the heritability of living to at least 100 has been estimated at 0.33 in women and 0.48 in men (6).

There are many possible candidate genes for human longevity but up to now, only two have shown positive results in different studies and populations, including a recent meta-analysis: the APOE and the FOXO3A genes (7-9).

The APOE ε4 allele has been linked to elevated cholesterol, CVDs, age-related cognitive decline, and dementia. It is strongly associated with Alzheimer disease and to a less extent, with CVDs. On the contrary, APOE ε2 allele, encoding a protein with less affinity to cholesterol, has been found more frequently in centenarians, also in Italy, although this datum was not replicated in all studies, as demonstrated by a recent meta-analysis (10, 11).

Our unpublished preliminary results in Sicilian population, including centenarians and their offspring and age-matched people without centenarian parents, showed a frequency of APOE ε3 ranging from 0.81 and 0.90, with a prevalence of ε3/ε3 genotype. This could be lead to hypothesize that in our population there is no specific association of APOE ε2 allele with longevity. But, looking at ε4, the detrimental allele, we found no presence in our long-living individuals (LLIs; ≥90 years old), so confirming the data existing on centenarians about the low frequency of this allele in extremely longevous European people rather than the presence of other specific pro-longevity allele (9,11).

The other replicable association is with FOXO3A rs2802292 G-allele (G>T). Dietary intervention and genetic alterations in gene encoding proteins that take part in metabolic nutrient-sensing pathways can modulate lifespan, as reviewed by Aiello et al. (12). It might depend on the hyper- o hypo-activation of those signalling pathways due to genetic mutations that under or overexpress regulative molecules leading to different expression of homeostatic genes. FOXO3A probably is the gene that more influences longevity in different ethnic populations. FOXO3A might act as a transcription factor on multiple homeostatic genes in response to decreased insulin/insulin growth factor-1 signalling, hence increasing life-span. A meta-analysis of over 7900 cases and 9500 controls confirms the association of the G allele of the single-nucleotide polymorphism (SNP) rs2802292 with exceptional longevity, especially in male (9).

However, in our analysis of this SNP in three different Sicilian cohorts (young, people with no centenarian parents and LLIs) we did not observe association with longevity and we measured G-allele frequencies in all the three cohorts lower that the expected from the data registered in dbSNP (NCBI) from a Tuscany population (expected G frequency 0.5093, measured G frequency: 0.40). In LLIs the frequency of T was double than G (0.66 VS 0.33). This result might be linked to the small sample of centenarians studied.

Longevity in Sicily: overview

Looking at the Italian ratio of centenarians per inhabitants, in some areas of Sicily, in particular in the countryside, there is more than a four-fold increase. In particular, the area of the Sicani Mountain was extensively studied in its dietary habits leading to the conclusion that this high rate of centenarians is strictly related to the adherence to the Mediterranean diet (MD) (13).

New unpublished data obtained from another mountainous population of Sicily (Madonie area), confirmed the results obtained in Sicani Mountain. In this area the probability to reach 90 is 4.6% and

the one to reach 100 is 0.22%. Our preliminary analysis of the nutritional habits confirmed the possible association of longevity phenotype with a Mediterranean lifestyle but not during ageing or extreme longevity, rather during young age. In fact, our LLIs followed MD during life but not during ageing, suggesting an interesting and effective role of epigenetics in the attainment of longevity.

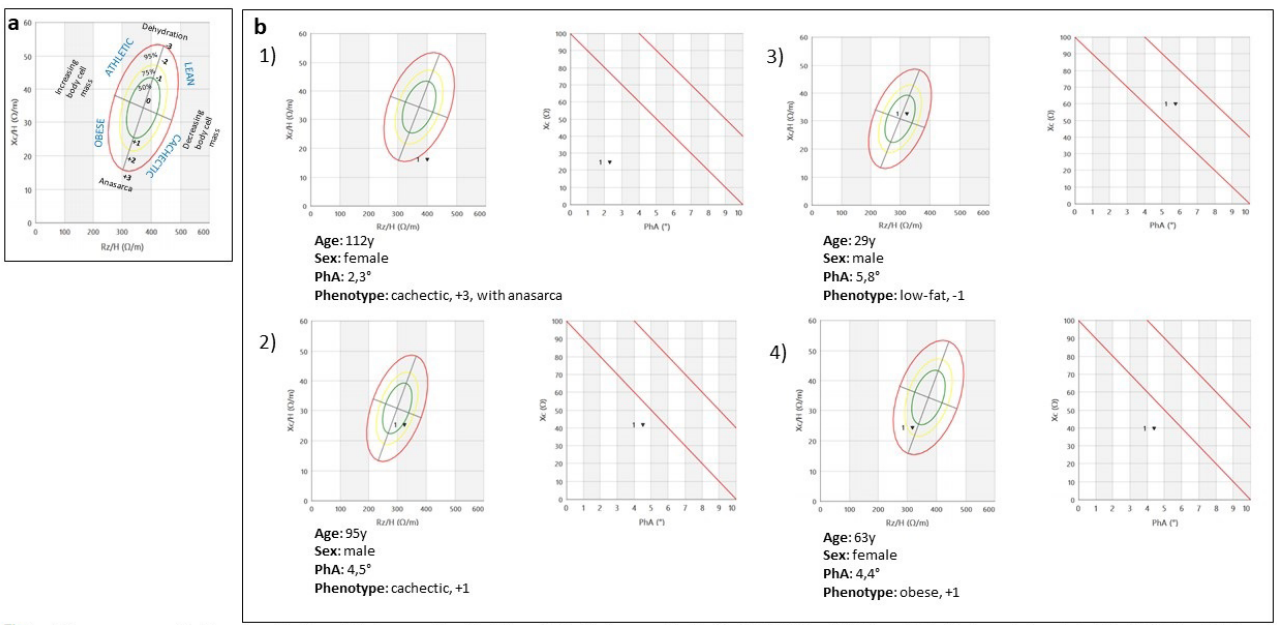
Overall, these data suggest that longevity concerns subjects living in small towns, without pollution, with different working conditions and lifestyles compared to the big towns, and close adherence to MD. Accordingly, these municipalities share low mortality from cancer and CVDs. In addition, old individuals have greater access to social support and family network; hence, they have better health and lower levels of mortality, particularly when living at home with adult daughters.

Phenotypic signature and hematochemical values

Scientific reports show that some values change during ageing, whereas there are some other parameters, which seems do not change significantly with age (14). A study published in 2008, about Italian population, reported laboratory parameters obtained from 120 healthy centenarians and 381 younger subjects (between 65 and 85 years old), to evaluate the possible influence of longevity on these values. Although the majority of values were similar, some differences were identified between the two groups. In particular, these were in urea nitrogen (increased), platelets count (lower in centenarians compared to elderly), ALT, total cholesterol and glycaemia (significantly reduced) (15). These data seem to be confirmed by recent survey on Sicilian centenarians, taking into account the differences with people aged 65+ (Ciaccio and Caruso, unpublished observations).

Body composition

Our study in Sicilian centenarians, with a special focus on anthropometric measurement, are trying to understand the differences in body composition between young-adult and LLIs, to propose reference values of anthropometric measurements and to identify a Longevity Anthropometric Phenotype,



including body mass index (BMI), body composition, body fat distribution, resting metabolic rate and waist-hip ratio. Contemporary, we developed a specific questionnaire to collect anamnestic, lifestyle and nutritional information to try to address the longevity phenotype through a characteristic footprint.

Our preliminary results (unpublished) confirmed the adherence to MD in LLIs, especially during young rather that in old age. Mean BMI was 24.35 Kg/m^2 so not associated with mortality risk. The analysis of body composition demonstrated hyperhydration, especially in extra-cellular compartment and low phase angle (Pa; mean: 3.2°). Mean value of Pa in our LLIs (mean age 101.5) classify them as cachectic (Pa 3.5° or lower). There is a significant difference in Pa between healthy condition and disease. The higher the Pa value, the better is healthy condition (17-19). Pa depends on cell membrane integrity and on body cell mass. Lower Pa appears to be consistent with either cell death or a breakdown in the selective permeability of the cell membrane, in accordance with hyper extracellular hydration (oedema). Although its biological meaning is still not clear, Pa appears to have an important prognostic role for disease, especially for sarcopenia and cachexia (16). One of the problems of its interpretation and use is linked to reference values. In fact, probably it is not possible to use the normal range of values (mean 6.5°) for the oldest old that have their "normal" body composition totally different from young and adults.

Immunophenotype

In aged people, several changes of both innate and acquired immunity have been described and viewed as deleterious, hence the term immunosenescence. On the other hand, centenarian immune system shares characteristics both of young and elderly people. In particular, number and function of natural killer cells are well conserved and comparable to those observed in young, whereas T and B cell number and function are similar to those observed in elderly (20).

We have performed several studies in Sicilian CO with the aim to track their immune signatures to test the hypothesis that these individuals might have immunological advantage, which may explain their longevity. The major finding of our studies is that CO have intermediate features between younger and elderly control groups. In fact, data show that CO retain more youthful immunological parameters and that the exhaustion of the immune system is less evident than in elderly without centenarian parents (references in 21).

CONCLUSION

Human population is very heterogeneous because of the different genetic background and different environmental stimuli, so it has not been yet possible to identify a clear signature of longevity. Longevity is a complex trait influenced by genetic and epigenetic component and other determinants as the environmental conditions during the prenatal and early postnatal period as well as life circumstances at adult and old age. In addition, chance also plays a role (22). Unfortunately, the intrinsic complexity and the heterogeneity among people make studies on ageing and longevity difficult to standardized, also because it is not easy to collect an adequate population in terms of number and information related to previous event happened in life.

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CLINICAL APPLICATIONS OF MICROENVIRONMENT-CONTROLLED IMMUNOSUPPRESSIVE PROPERTIES OF MESENCHYMAL STEM CELLS-DERIVED EXOSOMES: A REVIEW

R. DOMENIS[1], A. CIFÙ[1], M. FABRIS[2] and F. CURCIO[1,2]

[1]Dipartimento di Area Medica, Università degli Studi di Udine, Udine, Italy; [2]Istituto di Patologia Clinica, Azienda Sanitaria Universitaria Integrata di Udine, Udine, Italy

The discovery that mesenchymal stem cells (MSCs) modulate inflammation and promote tissue regeneration has revolutionized stem cells-based therapy. However, some observations have raised questions about the limitations of their clinical use. On the other hand, recent findings have demonstrated that exosomes released by MSCs contribute significantly to their therapeutic effect and may be used as an alternative MSCs-based therapy in regenerative medicine. In this review, we summarize the current knowledge about the immunosuppressive capacity of MSCs-derived exosomes and we discuss the critical role of the surrounding microenvironment in the modulation of their regenerative and therapeutic potential.

Mesenchymal stem cells secretome

Mesenchymal stem cells (MSCs) are described as multipotent non-hematopoietic adult stem cells, which express specific surface markers such as CD44, CD73, CD90, CD105, but not others such as CD45, CD34, and CD14. They are capable of self-renewal, colony forming and differentiation into mesodermal lineage such as osteocytes, adipocytes and chondrocytes as well ectodermal (neurocytes) and endodermal lineages (hepatocytes) (1). MSCs have been isolated from a wide range of sources, including bone marrow (BM), umbilical cord (UC), adipose tissue, liver, multiple dental tissues, and induced pluripotent stem cells (iPSC) (2).

For a long time the therapeutic effect of MSCs has been attributed to their capacity for local engrafting and differentiation into multiple tissue, but the lack of correlation between functional improvement and cell engraftment or differentiation at the site of injury, has led to the proposal that MSCs exert their effects through a different mechanism. Recent studies have revealed that MSCs release a massive array of bioactive factors, collectively defined as secretome, that play an important role in the regulation of key biological processes. MSCs secretome is composed by many cytokines, chemokines, growth factors and micro-RNAs, together with extracellular vesicles (EVs). MSCs secretome promotes a regenerative microenvironment through the inhibition of cell apoptosis, stimulation of angiogenesis and modulation of the immune response3, having direct influence in being able to suppress the classical pro-inflammatory markers and shift the immune system toward an anti-inflammatory phenotype.

Specifically, MSCs secreted factors are known to have a role in regulating the immune response by 1): suppressing T-cell proliferation, cytokines secretion and cytotoxicity; 2): regulating the balance of Th1/Th2; 3): modulating the function of regulatory T cells (Tregs); 4): inhibiting maturation, activation and antigen-presenting capacity of dendritic cells; 5): affecting the secretion of antibodies and production

Key words: mesenchymal stem cells, exosomes, immunosuppression, inflammatory microenvironment, cell-free regenerative medicine

Mailing address:
Dr Francesco Curcio,
Dipartimento di Area Medica,
Università degli Studi di Udine,
Udine, 33100, ITALY
Tel.: +39 0432 559201
e-mail: francesco.curcio@uniud.it

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of co-stimulatory molecules by B cells; 6): reducing interleukin-2 (IL-2)-induced natural killer cell activation; 7): affecting macrophage migration and polarization (4, 5).

MSCs-derived exosomes as new tool for regenerative medicine

Thanks to their multi-lineage potential, immunomodulation and secretion of anti-inflammatory molecules, MSCs are considered a promising tool for regenerative medicine, exhibiting great potential in most preclinical and clinical studies (6). However, the full range of MSCs potential remains incompletely understood and many questions are unanswered. Indeed, the clinical outcome of stem cell transplantation remains poor and the use of MSCs has some drawbacks, such as the need of a consistent supply of cells with stable phenotype, high costs and time delays for the generation and handling of these cells (7, 8). Moreover, the large variability in cell quality derived from different donors and tissues, inconsistent protocols, varying dosages and differing transfusion patterns can limit their therapeutic benefit (9).

Recent research has focus on exosomes secreted by MSCs as a possible non–cellular therapy (3, 10). Increasing evidences indicate that MSCs, in addition to soluble factors, produce massive amount of extracellular vesicles (EVs), which take part in tissue regeneration thought transferring information to damaged cells or tissues and exert biological activities similar to the originating cells (11). Specifically, exosomes are a subgroup of extracellular vesicles (EVs) with a diameter of 30-150 nm, derived from endosomes and released into the extracellular milieu upon the fusion of multivesicular bodies with the plasma membrane. MSCs-derived exosomes transport a complex cargo of bioactive molecules that includes nucleic acids, proteins and lipids (12) and are central mediators of intercellular communications. Indeed, exosomes are involved in many different biochemical and cellular processes, such as bioenergetics, immune regulation, tissue repair and regeneration (13).

The use of MSCs-derived exosomes as therapeutic tool may have significant advantages3. Replacing the administration of live cells with their secreted exosomes mitigates many of the safety concerns and limitations associated with the transplantation of viable replicating cells. Moreover, compared to living cells, exosomes are more stable, can be stored for some time and by virtue of their molecular characteristics are able to protect their cargo from *in vivo* degradation. Finally, in a manner similar to biopharmacological conventional products, exosomal therapeutic dose can be standardized and tested in terms of biological activity.

In light of the high therapeutic potential, the regenerative capacity of MSCs-derived exosomes are a subject of ongoing investigation. MSCs-derived exosomes are thought to have similar functions compared to MSCs, but little is known about the immunomodulatory effect of these vesicles and results reported in literature are sometimes discordant. It has been demonstrated that MSCs-derived exosomes are able to induce anti-inflammatory signals and attenuate pro-inflammatory molecules in monocytic cell line (14) and have an inhibitory effect towards the differentiation and activation of T cells (15). Moreover, MSCs-derived exosomes derived from healthy donors' bone marrow are able to suppress PBMCs' secretion of pro-inflammatory factors, such as TNF-α and IL-1β, and increase the release of the anti-inflammatory cytokine TGF-β. In addition, these vesicles may induce switching of T helper type 1 (Th1) into T helper type 2 (Th2) cells and reduce the capacity of T cells to differentiate into IL-17-producing effector T cells (Th17) (16). On the other hands, it has been also reported that exosomes secreted by MSCs fail to suppress lymphocyte proliferation (17), suggesting that cell-cell contact plays an important role in their immunosuppressive potential.

In order to identify the molecular components of exosomes that drove the observed immunomodulatory effects, MSCs-derived exosomes proteomic profiling has been analyzed. Data have revealed >200 proteins with immunomodulatory functions (18) and published studies have implicated miRNAs as one of the main functional effectors (19). MiR-21, miR-146a and miR-181 have been found having the most significant and highest expression in MSCs-derived

exosomes and could modulate the expression of pro-inflammatory genes, thus reducing or delaying inflammation and enhance wound healing (20).

Does the microenvironment influence the therapeutic potential of MSCs-derived exosomes?

MSCs seem to exert variable immunomodulatory effects on the same types of immune cells depending on the local microenvironment or disease status. Interestingly, recent data strongly support the concept that the immunoregulatory potential of MSCs are highly plastic and are critically influenced by an inflammatory microenvironment. Indeed, it has been proposed that the immunosuppressive potential of MSCs is not innate, but is conditioned by microenvironmental factors (21, 22). Hypoxic preconditioning, addition of an inflammatory stimuli, and growing cells in spheres or tri-dimensional scaffolds (23) have all been shown to modulate the production and excretion of different potentially therapeutic factors (24, 25).

Recent studies have demonstrated that culture under hypoxia has beneficial effects on MSCs since they maintain stem-like features (26), increase the production of growth factors (27-29) and increase their immunomodulatory capacity to stimulate the proliferation of regulatory T cells (30). It has been proposed that MSCs require the "licensing" by proinflammatory cytokines, such as IFNγ, TNFα, IL-α and IL-1β, to acquire a immunosuppressive phenotype (31-33). For example, the exposure of MSCs to IFNγ and TNFα stimulates the release of the enzyme IDO (34) and inhibitor Factor H (35), and endowed the cells with superior angiogenic activity (36).

Pre-treatment with IL-1β enhanced the efficacy of MSC transplantation in DSS-induced colitis, modulating the balance of immune cells through elevating COX-2 and IL-8 expression (37). Likewise, the regenerative activity of MSCs can be stimulated by toll-like receptors agonists through the release of soluble factors, which promote angiogenesis, by increasing proliferation, migration and tube formation of endothelial cells (38).

Therefore, it is possible to hypothesize that also the regenerative potential of MSCs-derived exosomes may be modulated by exposure of the producing cells to microenvironmental stimuli. To date, there is still very little knowledge about the influence of microenvironment on MSCs-derived exosomes composition and functionality. It has been shown that treating MSCs with lipopolysaccharide (LPS) modulates the abundance of miR-let-7b in exosomes, which in turn enhances wound healing and decreases inflammation via signaling to macrophages (39). Ischemic preconditioning was shown to potentiate the protective effect of exosomes released by MSCs in mouse acute lung injury (40) and myocardial infarction models (41). Moreover, the exposure of MSCs to ischemic tissue-simulated microenvironment increases expression of exosomes containing a robust angiogenic signaling

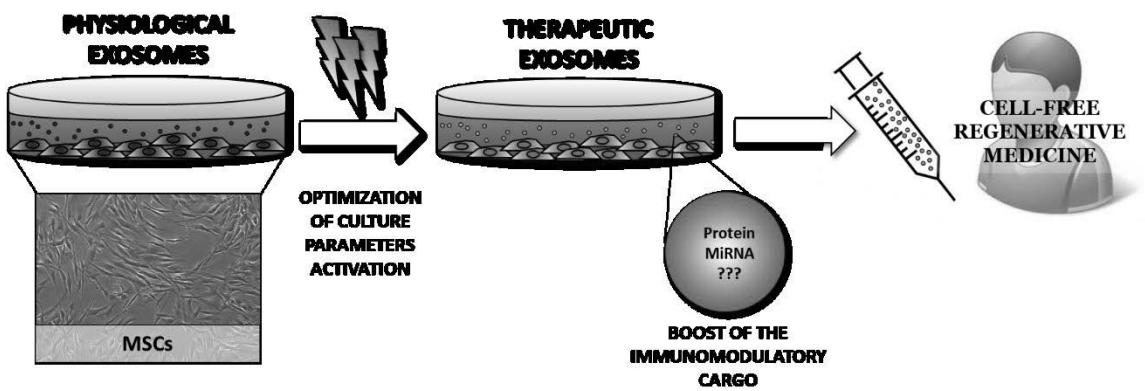

Fig. 1. *The activation of MSCs as a strategy to boost up the therapeutic efficacy of MSC-derived exosomes.*

profile, which are capable of inducing angiogenesis in vitro via NFkB pathway (42).

Recently, we demonstrated that treatment with inflammatory cytokines increases the immunosuppressive and anti-inflammatory potential of MSCs-derived exosomes, which acquire the ability to shift macrophages from M1 to M2 phenotype by shuttling miRNA regulating macrophages polarization. Specifically, exosomes produced by pre-activated AMSCs shuttled miR-146, which promote M2 macrophage polarization by targeting expression of IRAK1 (43). In accordance with our data, it has been reported that miR-146a was strongly upregulated in exosomes released by MSCs stimulated with IL-1β, which in turn contributes to mitigate inflammation and increase survival in septic mice (44). It has also been reported that MSC exosomes alone failed to polarize T cells to Tregs or Th17 cells 14, but became capable of such effect when cells were pre-primed with TGF-β and IFN-γ. This effect may be related to important changes of the exosomal cargo, characterized by increases cytokines and IDO expression (45).

CONCLUSIONS

The regenerative potential of MSCs-derived exosomes is a subject of intensive investigation by the scientific community. There are remarkable future opportunities to successfully use MSC-derived exosomes as therapeutic tool. However, to be able to really use MSC-derived exosomes in cell-free regenerative medicine, we urgently need to better define the mechanisms by which microenvironment modulates MSCs secretome, to finally standardize the optimal culture conditions to obtain vesicles with precise immune-regulatory functions, to develop safe and robust production processes and to define the regulatory framework.

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DYSFUNCTIONAL EPICARDIAL ADIPOSE TISSUE (EAT) AND MALADAPTIVE HEART REMODELING IN PATIENTS WITH INCREASED VISCERAL ADIPOSITY: THE ST2/IL-33 CARDIO-FAT SIGNALING

E. VIANELLO[1], E. DOZIO[1,2], L. TACCHINI[1], J. LAMONT[3], F. BANDERA[1,4] and M.M. CORSI ROMANELLI[1,5]

[1]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy; [2]Laboratory of Molecular Pathology, IRCCS Policlinico San Donato, San Donato Milanese, Italy; [3]Randox Laboratories LTD, Research and Development, Crumlin-Antrim, Belfast,Northen Ireland, UK; [4]Cardiology University Department, Heart Failure Unit, IRCCS Policlinico San Donato, Milan, Italy; [5]U.O.C. SMEL-1 of Clinical Pathology, IRCCS Policlinico San Donato, San Donato Milanese, Italy

Recent clinical evidences suggested that the expansion of epicardial adipose tissue (EAT) and its transformation into a pro-inflammatory organ in visceral obesity are related to maladaptive heart remodeling and heart failure (HF). However, the sustained molecular mechanisms are poorly understood. The ST2/IL33 system has recently obtained great attention in the field of cardiovascular diseases due to its cardio-protective role in different stress conditions that can promote cardiomyocyte hyperthrophy and deposition of extracellular proteins (fibrosis). Any imbalance in ST2/IL33 signaling may thus lead toward HF. Discussing how dysfunctional EAT may interfere with the protective role of ST2/IL33 pathway and its association with heart remodeling in visceral obesity are the aims of the present review.

The epicardial adipose tissue (EAT) is a visceral fat depot surrounding myocardium, with a well-established role in cardiovascular disease (CVD) pathogenesis through the release of active adipokines and inflammatory mediators. In this regard, EAT secretome has been widely investigated and currently associated to changes in left ventricular (LV) morphology and hypertrophy (1). Results from imaging studies reported that EAT amount is positively correlated with LV mass and end-diastolic measurements in heart failure (HF) patients and significantly associated to arrhythmias, atrial fibrillation and the severity of global cardiac fibrosis (2). Although clinical evidences identified EAT as an important risk factor for HF, pathological mechanisms linking EAT expansion and heart remodeling are really poorly understood.

Increased triglyceride storage is the basic mechanism of EAT cell enlargement and EAT expansion. In this condition, EAT become predominantly composed by dysfunctional adipocytes and, together with infiltrated immune cells, release pro-inflammatory mediators, vasoconstrictors and pro-fibrotic modulators (3). As a matter of the fact, secretory products of EAT may affect cardiomyocyte functions both through passive diffusion into interstitial spaces and directly secretion into the shared coronary circulation (4-6).

In physiological conditions, EAT adipokines exert important cardio-protective effects. Among different mediators, orosomucoid mediator inhibits cardiomyocyte apotosis and adiponectin exerts important anti-oxidant and anti-inflammatory effects by reducing O_2^- production and improving nitric

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Mailing address:
Elena Vianello, Ph.D.,
Department of Biomedical Sciences for Health,
Università degli Studi di Milano,
Via Luigi Mangiagalli 31, 20133 Milan, Italy
Tel.: +39 02 50315342 - Fax: +39 02 50315338
e-mail: elena.vianello@unimi.it

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oxide bioavailability (2, 7). At increasing EAT, a shift from anti- toward pro-inflammatory mediator release can be observed. In particular, EAT cells produce and release activin A, a member of the TGFβ superfamily, one of the main mediators of cardiac collagen deposition and promoters of tissue fibrosis. Activin A interferes with anti-fibrotic signals in cardiomyocytes and promotes extracellular matrix deposition (8, 9). Furthermore, by activating NF-kB, activin A exerts important pro-inflammatory effects: it increases the production of different cytokines, like IL-6 and TNF-α, induces M2 to M1 macrophage polarization and promotes monocyte activation and infiltration with a further amplification of the pro-fibrotic signaling (9).

Cardiac dysfunction and arrhythmias are the main cardiac consequences associated to EAT-released adipo-fibrokines (8-10). Different studies in human and in animal model confirmed that EAT expansion is directly associated with the production of other pro-fibrotic mediators, including thrombospondin 2, vascular endothelial growth factor and different metalloproteinase isoforms, which induce heart and vessels remodeling (2, 11).

In the light of this existing relationship between increased fibro-adipokine release by dysfunctional EAT and heart remodeling, in the next sections we will discuss in detail the role of EAT amount in affecting the ST2/IL33 system, a newer discovered fat pathway associate to maladaptive remodeling.

Role of ST2/IL-33 signaling in the heart and in adipose tissue

ST2/IL-33 signaling has been implicated in a wide range of fibrotic diseases, with beneficial effects in some and detrimental consequences in others. Regarding cardiac fibrosis, ST2/IL-33 signaling is the main cardiac pathway expressed in response to cardiac pressure overload (12, 13). ST2 is a member of the IL-1 receptor family. It is constitutively expressed by cardiomyocytes and exists in two main isoforms, the transmembrane ST2L receptor and the soluble sST2 receptor, obtained through alternative splicing.

Both ST2 isoforms are biomechanically induced in cardiomyocytes in response to hydrodynamic load but they mediate opposite functions. Under physiological conditions, cardiomyocyte stretching is supervised by the binding between ST2L and its natural ligand IL-33, the 11th member of IL-1 cytokine superfamily. IL-33 is principally expressed by myofibroblats and it is classified as an alarmin-like molecule since it is released into extracellular spaces during cell damage or tissue injury and acts as an endogenous warning signal to alert adjacent cells and tissues. In the heart, IL-33 promotes cardiomyocyte survival by sequestering NF-kB to dampen pro-inflammatory and pro-fibrotic signaling (14).

After physiological stretch, ST2L/IL-33 signaling is silenced by sST2 that, working as a decoy receptor, sequesters extracellular IL-33. Under pathological conditions characterized by altered hydrodynamic flows, the irregular ST2 gene activation up-regulates sST2 transcription and promotes cardiac hypertrophy and fibrosis (15). For these reasons, sST2 is currently used by physicians as a biomarker of cardiomyocyte stress and fibrosis and it is also used as a prognostic factor for HF([13]).
Due to the emblematic role of ST2/IL-33 signaling in cardiac fibrosis and the link between visceral adiposity and CVD, it was assumed that sST2 may be produced also from other tissues, like the adipose tissue. In fact, in obese individuals, plasma levels of sST2 are increased, whereas no alterations has been reported in IL-33 levels (16). However, the role of adipose tissue both as a source and a target of IL-33 is strongly debated.

In this regard, it has been reported that leptin, whose levels are increased in obesity, induces adipose tissue macrophages to produce the alarmin IL-33 which try to reduce the pro-inflammatory fat environment by promoting M1 to M2 macrophages polarization (16, 17).

Recent studies on leptin-deficient ob/ob mice as well as on adipocyte cell cultures suggested that exogenous administration of IL-33 reduces adipose tissue weight, total fat mass and adipocyte size in mice (18) and adipocyte differentiation and lipid storage *in vitro* (19, 20). These data seem to confirm the anti-obesity role of IL-33.
Notably, the activation of ST2L has been reported as

the main molecular mechanism activated by IL-33 in adipose tissue (16). As a result, IL-33 up-regulates the expression of both ST2 isoforms via alternative splicing thus increasing the local expression of ST2L and sST2 release into systemic circulation where it can reduce the availability and thus the protective role of IL-33.

Role of ST2/Il-33 signaling in maladaptive heart remodeling

sST2 is now considered the most promising marker of early myocardial remodeling and several studies reported that sST2 may be produce by adipose tissue competent cells. Recently, Gruzdeva et al. demonstrated that EAT thickness is associated with cardiac fibrosis development 1-year post-myocardial infarction in patients with visceral adiposity (21).

The novelty of this study deals with the observation, for the first time, that ST2/IL-33 signaling is also related to EAT thickness. The authors found a positive correlation between ST2/IL-33 expression, EAT thickness measured both on left and right ventricle, and the degree of myocardial fibrosis in obese post-myocardial infarction patients.

No molecular mechanisms underlying this association have been explored. However, considering that endothelial cells are the main source of ST2, the authors hypothesized that the increase in ST2 levels is mainly due to endothelial activation induced by adipocyte-derived leptin. Of

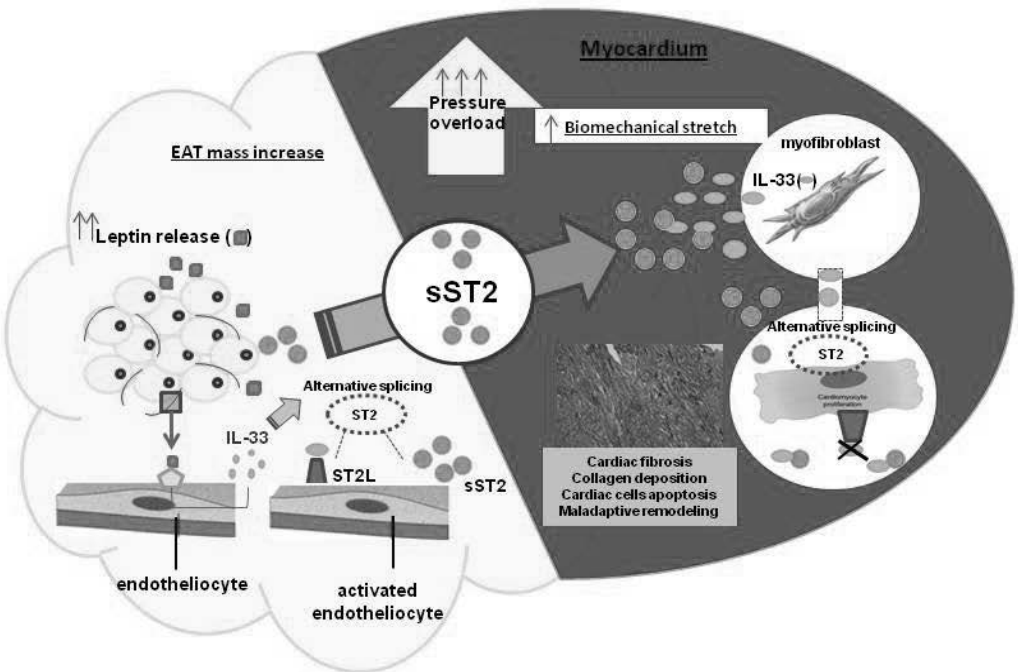

Fig. 1. *The increase of visceral adiposity promotes an unbalnce of ST2 proteins production by both dysfucntional EAT cells and cardiomyocytes resulting maladaptive heart remodeling. In visceral obesity, cardiac stretching induced by hydrodinamic pressure overload, occuring in case of CVD and HF, promotes cardic fibroblast activation in myofibroblasts that release alarmin IL-33, aimed to reduce biomechanical stretch damage. Cardiac IL-33 induces ST2 gene expression and through alternative splicing both ST2L and sST2 are expressed by cardiomyocytes. An unbalance of cardiac ST2L and sST2 by cardic cell promotes maladaptive cardiac remodeling. In dysfunctional EAT cells, leptin adipocytes release induces endothelial cells to produce IL-33 that directly activates ST2 expression and production of ST2 isoforms. It suggested that sST2 from EAT cell through the common microcirculation can reach the heart and can contribute to the unbalanced concentration of cardiac sST2. Fat/cardiac sST2 scavenger of alarmin IL-33 block ST2L/IL-33 signaling in both EAT and heart tissue promoting fat inflammation and maladaptive cardiac remodeling.*

course future molecular studies on EAT will help to better clarify these interesting findings.

CONCLUSIONS

Dysfunctional visceral fat depots, including EAT, are sources of adipo-fibrokines which are involved in maladaptive heart remodeling. In this field, the ST2/IL-33 system is getting much attention due its role in inflammation, fibrosis and HF. The adipose tissue has been suggested both a source and the target of this pathway.

The ST2/IL-33 system seems quite complex due to the existence of different ST2 isoforms with different roles, protective for ST2L and detrimental for sST2. Notably, in the clinical setting, sST2 is considered a promising prognostic marker for HF. Dysfunctional EAT may promote heart remodeling and HF by directly secreting into the coronary circulation sST2 which, binding to IL-33, abrogates its protective effect (figure 1). The mechanisms which drive sST2 production in EAT are poorly understood but may deal with the increased production of adipokines, like leptin, and other pro-inflammatory mediators by dysfunctional EAT.

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GAMMA-GLUTAMYLTRANSFERASE (GGT) IN TUMOR PROGRESSION, DRUG RESISTANCE AND TARGETED THERAPIES

A. POMPELLA and A. CORTI

Dipartimento di Ricerca Traslazionale NTMC, Scuola Medica dell'Università di Pisa

The biological role of a protein seldom coincides with the function first described, i.e. the easiest to observe (the two aspects usually coexist). First observations usually settle down and address subsequent research during years to follow. However, it often happens, that from an out of frame data, indications later emerge that something else (possibly more interesting) may hide beneath the surface. An analysis free from preconceptions may therefore allow other aspects such as unexpected processes and novel functions of the protein under investigation, to emerge. This is well illustrated in the case of gamma-glutamyltransferase (GGT), whose pathophysiological significance we could specify and redefine in experimental studies carried out in our laboratories, first in Siena and then in Pisa.

MATERIALS AND METHODS

GGT activity participates in the conservative metabolism of glutathione, and was therefore regarded as central for the maintenance of the antioxidant defences of the cell. At variance, we highlighted however, some important prooxidant effects capable of modulating selected intracellular signal transduction pathways (1). Over the years, we have documented the occurrence of such redox reactions in a whole series of pathophysiological processes. For instance, the prooxidant action of GGT actually imposes a continuous oxidative damage on DNA, which can be somehow considered as constitutive in GGT-expressing cells, and as such can obviously contribute to tumor progression of initiated cells (2).

The expression of GGT is often increased both in solid tumors and haematological malignancies, and this can contribute to drug resistance first of all through an increase of intracellular levels of glutathione. However, it should be taken into account that GGT is active at tle plasma membrane level, where it degrades glutathione present in the extracellular milieu. We have shown that the metabolites thus originated are able to form adducts with cisplatin and prevent its entry into the cell, what actually constitutes a yet unsuspected mechanism of drug resistance – sort of an extracellular detoxication (3). Cisplatin should therefore be avoided in the case of GGT-expressing tumors, in which the selective GGT-mediated activation can be obtained instead of suitable pro-drugs bearing gamma-glutamyl residues (the specific substrate of the enzyme). We have in fact shown that a glutathione-based compound, 4-(N-(S-Glutathionylacetyl) amino) phenylarsonous acid (GSAO), is by far more effective than cisplatin in preventing the growth of GGT-expressing pancreatic carcinoma cell lines (4).

RESULTS

Soluble GGT activity is better known, especially in the clinical setting, and is currently interpreted as the result of passive shedding in blood following cell injury at the hepato-biliary level. We have

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however documented that GGT is actively released from several other cell types. Tumor cell lines can do it, notably those expressing high levels of the enzyme, and GGT is released in association with exosomes. Equally interesting is the observation that GGT is secreted by phagocytic cells (neutrophils, macrophages) upon their activation. It is difficult to precise the exact significance of such exocytic GGT, especially in an inflammatory context, also due e.g. to the participation of the enzyme in the metabolism of leukotrienes. We observed anyway that in inflammatory exudates – where GGT levels are significantly increased – the action of the enzyme can produce a decrease in the levels of glutathione and antioxidant defenses. Clinical implications of such phenomenon were the subject of a recent study we have conducted on patients with cystic fibrosis, in whom the inhalatory administration of glutathione for therapeutic purposes appeared instead capable of igniting undesired prooxidant effects (5).

CONCLUSION

A perspective of sure interest would be thus represented by the development of competitive, non-absorbable GGT inhibitors, suitable for the treatment of such conditions deriving from an extracellular pathogenesis.

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INVITED SPEAKER

CHEMOTHERAPY RESISTANCE-ASSOCIATED EPITHELIAL TO ENDOTHELIAL TRANSITION IN GASTRIC CANCER

S. PERI[1], A. BIAGIONI[2], F. CIANCHI[3], I. SKALAMERA[3], F. STADERINI[3], N. SCHIAVONE[2], L. PAPUCCI[2] and L. MAGNELLI[2]

[1]Department Of Biotechnology, Chemistry And Pharmacy, University of Siena - Department of Experimental and Clinical Biomedical Sciences "Mario Serio", university of Florence; [2]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence; [3]Department of Surgery and Translational Medicine (DCMT), University of Florence

Gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of cancer-related deaths. To date, gastrectomy and chemotherapy are the only therapeutic options, but drug resistance is the main cause for treatment failure. Vasculogenic mimicry (VM) is a new model of neovascularization in aggressive tumors and has been correlated with poor prognosis in GC patients. Our group has developed chemotherapy-resistant GC cells using the Caucasian adenocarcinoma cell line AGS and three drugs among the most used in clinic (5-fluorouracil, cisplatin and paclitaxel) henceforward denominated 5FUr, CISr, TAXr. Our study has highlighted phenotypical differences among chemo-sensitive and chemo-resistant cell lines such as acquisition of stem-like phenotype and increased capacity to form vessels.

MATERIALS AND METHODS

Establishment of AGS resistant cell lines exposing cells to increasing dilution of drugs for over 9 months up to dilutions higher than IC50 values, initially verified on AGS cells through MTT analysis; Quantitative RT-PCR, flow cytometry and western blot analysis for stemness and VM markers; Vasculogenic mimicry assay.

RESULTS

AGS cells acquired chemoresistance as indicated by the increase of IC50 values in drug-treated cells with respect to AGS. Furthermore, MTT assay highlighted that there is not cross-resistance among 5FUr, CISr and TAXr. Supportive data is that cells are MDR1 negative.

Resistant cells showed an upregulation of Yamanaka factors either in qPCR and flow cytometer analysis, and particularly interesting is ALDH overexpression in 5FUr.

Twist upregulation suggested the investigation of VM, which resulted particularly enhanced in 5FUr cells that demonstrated their ability to form and sustain vessels up to 96 hrs in the tube formation assay. Laminin 2 and Ephrin A2 showed an increase in resistant cells and especially in 5FUr.

CONCLUSION

One of the most interesting result is that 5FUr cells acquire stemness properties and are positive to the tube formation assay suggesting that VM might be one mechanisms adopted by cells to avoid drugs exposure.

These findings suggest that acquisition of chemoresistance could cause a relapse of disease in which tumor cells take advantage of their capability to perform VM in order to self-sustain their growth and that may be cause of poor outcomes.

SLC7A7/Y+LAT1, MUTATED IN LYSINURIC PROTEIN INTOLERANCE, HAS A SIGNIFICANT ROLE IN REGULATING THE INFLAMMATORY STATUS OF HUMAN MACROPHAGES

F. INGOGLIA, A. BARILLI, B.M. ROTOLI, R. VISIGALLI, F. FERRARI and V. DALL'ASTA

Unit of General Pathology, Dept. of Medicine and Surgery, University of Parma, Italy

Lysinuric protein intolerance (LPI) is a recessively inherited aminoaciduria caused by mutations of SLC7A7, the gene that encodes y+LAT1 light chain of system y+L for cationic amino acids (CAA; namely arginine, lysine, and ornithine) transport. Clinical signs of LPI are highly heterogeneous and only poorly understood: while hyperammonemia and protein intolerance can be explained by urea cycle slowdown due to the impairment of CAA absorption/reabsorption in intestinal and renal epithelial cells, little is known about the pathogenesis of the often fatal complications affecting lungs and immune system. Here, we explore the possibility that y+LAT1 protein directly exerts immunomodulatory functions and that LPI defects, besides affecting CAA transport, also activate inflammatory mononuclear phagocytes, ultimately leading to immunological complications.

MATERIALS AND METHODS

Human monocytes-derived macrophages (MDM) were obtained from blood circulating monocytes isolated from seven healthy subjects; the LPI defect of SLC7A7/y+LAT1 was reproduced *in vitro* by means of short interference RNA (siRNA). The inflammatory phenotype of silenced cells was evaluated by monitoring IL1ß cytokine production and release with RT-qPCR and ELISA, respectively, while the activation of inflammasome was assessed by mean of Western Blot analysis and caspase-1 activity assay.

RESULTS

Gene silencing with specific siRNA effectively lowered the expression of SLC7A7/y+LAT1 in human MDM (>70%). Under this condition, an induction of mRNA expression for the inflammatory mediator IL1ß was observed, along with a significant increase of cytokine release in the extracellular medium. This effect was mainly regulated through the activation of NFkB signalling pathway, while the activity of caspase-1 was comparable in scrambled and SLC7A7-silenced cells.

CONCLUSION

Our results point to a thus far unknown role for SLC7A7/y+LAT1 that, besides transporting arginine, is supposed to act as a brake to restrain the inflammatory status of immune cells through the regulation of NF□B signalling pathway. Then, SLC7A7 defects induce an inflammatory status in macrophages. LPI could, hence, be regarded as an autoinflammatory disease, and cytokine antagonists as promising therapies for the disease.

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UPREGULATION OF CIRCULATING LEVELS OF RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (SRAGE) IN OBESE RATS MAY PROTECT AGAINST ECTOPIC FAT ACCUMULATION IN THE HEART

E. DOZIO[1], F. BANDERA[2], E. VIANELLO[3], S. BRIZZOLA[4], L. TACCHINI[3] and M.M. CORSI ROMANELLI[5]

[1]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; Laboratory of Molecular Pathology, IRCCS Policlinico San Donato, San Donato Milanese, Milan, Italy; [2]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; Department of Cardiology, IRCCS Policlinico San Donato, San Donato Milanese, Milan, Italy; [3]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; [4]Department of Veterinary Medicine, Università degli Studi di Milano, Milan, Italy; [5]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; Service of Laboratory Medicine1-Clinical Pathology, I.R.C.C.S. Policlinico San Donato, San Donato Milanese, Milan, Italy;

Receptor for advanced glycation end-products (receptor for AGEs, RAGE) is a cell-surface protein identified as a receptor for AGEs. RAGE activation mainly induces oxidative stress and inflammation. Indeed, in different organs, such as the adipose tissue and the liver, RAGE engagement has also been shown to promote lipid accumulation. Besides the cell-membrane form, RAGE exists as a soluble circulating molecule (sRAGE), a decoy receptor able to prevent the detrimental effects of AGEs at cellular level. As most of the obesity-related complications are due to the ectopic fat accumulation and no data are available about RAGE, sRAGE and heart steatosis, our aim was to explore any potential association of these molecules with heart steatosis in obesity.

MATERIALS AND METHODS

Lean (L) (n=10) and obese non-diabetic (OB) (n=9) Zucker rats (age: 20 weeks) were used as a model of obesity (investigations have been conducted in conformance with the Principles for the use of Animals in Research and Education, Protocol Number 325/2015PR - 2015/04/05, Italian Ministry). Serum sRAGE levels were quantified by ELISA assay. Heart tissues were studied in terms of lipid accumulation, RAGE expression, activation/modification of specific genes (n=84) involved in lipid metabolism (RT2 Profiler PCR Array, PARN-007Z, QIAGEN). The following methods were used: red oil staining, real time RT-PCR and Western blot.

RESULTS

sRAGE levels were higher in OB than L rats (1869.00±890.80 pg/mL *vs* 1115.00±500.80 pg/mL; $p < 0.05$). Heart fat content did not differ between L and O rats as well as RAGE expression. Among the 84 genes evaluated, 6 genes were significantly up-regulated: 4 promote fatty acid metabolism (Acaa2, Acadl, Acadm, Acot9), 1 fatty acid transport (Crat) and 1 tryacilglycerol metabolism (Gpd1).

CONCLUSION

Uncomplicated obesity in 20-week old rats is not associated with fat accumulation in the heart. The increased serum levels of sRAGE, the lack of heart RAGE hyper-expression and the increased fatty acid metabolism seem to be protective mechanisms against fat-induced heart damage.

Whether fat accumulation may be promoted by aging and the onset of obesity-related complication (i.e. diabetes) needs to be explored.

SRAGE: A PROGNOSTIC FACTOR FOR MORTALITY IN END-STAGE RENAL DISEASE PATIENTS ON DIALYSIS

E. DOZIO[2], E. VIANELLO[1], F. AMBROGI[4], M. DE CAL[5], C. RONCO[5], L. TACCHINI[1] and M.M. CORSI ROMANELLI[3]

[1]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; [2]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; Laboratory of Molecular Pathology, IRCCS Policlinico San Donato, San Donato Milanese, Milan, Italy; [3]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; Service of Laboratory Medicine1-Clinical Pathology, IRCCS. Policlinico San Donato, San Donato Milanese, Milan, Italy; [4]Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy; [5]Department of Nephrology, Dialysis & Transplantation, San Bortolo Hospital, Vicenza, Italy; International Renal Research Institute Vicenza (IRRIV), San Bortolo Hospital, Vicenza, Italy

End stage renal disease patients on dialysis (CKD-G5D) have a high mortality rate mainly due to cardiovascular diseases (CVD). In addition to traditional CVD risk factors, which are common in these patients, excessive oxidative stress, uremia and chronic inflammation may further increase the production of advanced glycation end-products (AGEs) which in turn promote CVD and CVD-related morbidity and mortality. Much attention has been paid recently to the soluble receptor for AGEs (sRAGE) as a marker of inflammation, oxidative stress, atherosclerosis and heart failure (HF) in CKD-G5D. However, its relationship with patient outcomes is still debated.

MATERIALS AND METHODS

We evaluated in 56 hemodialysis (HD) and 67 peritoneal dialysis (PD) patients the potential role of sRAGE as a predictor of mortality at two-year follow-up. Biochemical and clinical data were recorded. sRAGE was quantified by ELISA assay.

RESULTS

Each increase of 100 pg/mL in sRAGE was associated with an increase of about 7% in mortality risk. sRAGE positively correlated with brain natriuretic peptide (BNP) both in the whole group ($r=0.353$, $p< 0.001$) and in HD ($r=0.281$, $p<0.05$) and PD ($r=0.334$, $p<0.01$) subgroups. The mortality rates (17.86% *vs* 19.40%) and sRAGE levels (4597.38±930.18 *vs* 3480.86±1538.62 pg/mL; $p=0.07$) in patients who died did not differ between the HD and PD groups.

CONCLUSION

Our results revealed for the first time that in patients with active cardiac remodeling, elevations in sRAGE levels may have a prognostic significance. Its quantifications seems a useful supportive tool that could help to identify high-risk patients.

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UPAR FUNCTION IN MELANOMA DERIVED-EXOSOMES: PRO-ANGIOGENIC EFFECTS ON ECFCS

F. MARGHERI, B. MENICACCI, A. LAURENZANA, A. BIAGIONI, A. CHILLÀ, A. MOCALI, G. FIBBI and M. DEL ROSSO

Department of Experimental and Clinical Biomedical Sciences, University of Florence

Tumor-derived exosomes are emerging mediators of tumorigenesis. Exosomes are small membrane vesicles (40-150nm) derived from the luminal membranes of multivesicular bodies, and are released via fusion with the cell membrane. Exosomes mediate local and systemic cell communication through the horizontal transfer of information (mRNAs, microRNAs and proteins). It is well recognized that uPAR, is one of the main systems involved in tumor invasion and metastasis. Several malignant tumors show a positive correlation between uPAR levels and a more aggressive phenotype together with a poor prognosis. We have showed that uPAR is strongly upregulated in A375 and in metastasis-prone A375M6 melanoma cells with respect to normal melanocytes. Here we explored the uPAR function in melanoma-derived exosomes and their pro-angiogenic capacity in ECFCs (Endothelial Colony Forming Cells).

MATERIALS AND METHODS

Exosomes were isolated by ultracentrifugation combined with filtration from media of A375 and A375 M6. The quality of exosomes was confirmed by dynamic light scattering and the expression of typical exosomal markers (CD63, CD9, CD81 and ALIX) and uPAR was quantified by Western Blotting. The morphology of exosomes was observed under TEM. The angiogenic properties of ECFCs were assessed by Matrigel invasion and capillary morphogenesis assays.

RESULTS

uPAR was found expressed in A375 and A375M6 derived exosomes and in particular the receptor is present only in these vesicles but not in ectosomes. The uPAR+/A375M6 derived-exosomes treatment of ECFCs enhance invasion and capillary morphogenesis through ERK 1/2 signaling activation. Downregulation of uPAR by siRNA or CRISPR-Cas9 uPAR gene editing results in a reduction of exosomes-dependent pro-angiogenic effects.

CONCLUSION

These results suggest that uPAR+/A375M6 exosomes plays an important role in ECFC mediated angiogenesis.

ST2 FIBRO-CITOKINE AND IL-33 ALARMIN PROTEIN ARE EXPRESSED IN OBESE FA/FA- ZUCKER RAT MODEL AND CORRELATED WITH PRO-FIBROTIC GENE PATHWAYS.

E. VIANELLO[1], E. DOZIO[2], S. BRIZZOLA[5], F. ACOCELLA[4], L. TACCHINI[1] and M.M. CORSI ROMANELLI[3]

[1]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy; [2]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy and Laboratory of Molecular Pathology, IRCCS Policlinico San Donato, San Donato Milanese, Italy; [3]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy and UOC SMEL-1 of Clinical Pathology, IRCCS Policlinico San Donato, San Donato Milanese, Italy; [4]Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy; [5]Department of Veterinary Medicine, University of Milan, Milan, Italy

The function of ST2 fibro-mediator and IL-33 alarmin protein has been mainly investigated on immunological aspects, but recent data suggest that this pathway plays also an important role in cardiovascular system and adipose tissue inflammation. Whereas IL-33 has been demonstrated to exert anti-inflammatory and protective effects, circulating ST2 (sST2) has emerged as a prognostic biomarker in patients with myocardial infarction and heart failure. Furthermore, the IL-33/ST2 expression system is increased in severe obesity, although its role in the pathogenesis of detrimental cardiac remodeling associated with obesity is still not well defined. The aim of this study is to investigate the molecular pattern of IL-33/ST2 system and pro-fibrotic genes activation in experimental model of obese fa/fa- Zucker rat model.

MATERIALS AND METHODS

By using lean fa/fa+ (n=5) and obese fa/fa- (n=5) Zucker rats (age:20 weeks) (protocol number 325/2015PR - 2015/04/05, Italian Ministry), we tested the hypothesis which in case of incremental visceral adiposity, cardiac hypertrophy may be mediated by the deregulation of ST2/IL-33 signaling, causing an up-regulation of genes involved in collagen synthesis and deposition associated to cardiac fibrosis. Heart tissues were studied in terms of collagen deposition, ST2 and IL-33 tissue expression and activation/modification of specific genes (n=84) involved in collagen synthesis and deposition (RT Profiler PCR Array, PARN-120Z, QIAGEN). The following methods were used: histological staining, real time RT-PCR and Western blot.

RESULTS

Masson's trichrome staining shows the presence of collagen deposition only in obese fa/fa- Zucker rats and both ST2 and IL-33 gene expression present a trend to increase although not significant than age matched lean fa/fa+ Zucker rats. Almost all 84 pro-fibrotic genes result up-regulated in obese fa/fa- Zucker rats than control lean rats and we found direct and indirect correlations between IL-33/ST2 mRNA and pro-fibrotic genes expression.

In our study, the effects of obesity on the pro-fibrotic IL-33/ST2 system is investigated in cardiac tissue from Zucker rats. Our results suggested an involvement for IL-33/ST2 system in molecular mechanism of obesity associated to cardiac remodeling. These data suggested that understanding IL-33/ST2 system in a more detailed manner could allow gaining insights on new molecular pathways associated with obesity, in order to perhaps identify new and more specific therapeutic strategies to prevent cardiovascular outcomes associate to obesity.

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TARGETING CHRONIC MYELOID LEUKEMIA STEM CELLS WITH ERK5 PATHWAY INHIBITORS

I. TUSA[2], G. CHELONI[3], M. POTETI[4], A. GOZZINI[6], X. DENG[1], N. S GRAY[1], S. LI[5], P. DELLO SBARBA[2] and E. ROVIDA[2]

[1]Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; [2]Department of Experimental and Clinical Biomedical Sciences, University of Florence and Istituto Toscano Tumori, Florence, Italy; [3]Department of Experimental and Clinical Biomedical Sciences, University of Florence and Istituto Toscano Tumori, Florence, Italy; [4]Department of Experimental and Clinical Biomedical Sciences, University of Florence, Italy and Department of Medical Biotechnologies, University of Siena, Italy; [5]Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA; [6]Hematology Unit, AOU Careggi, Florence, Italy

Tyrosine kinase inhibitors (TKi) targeting BCR/ABL are very effective for the treatment of Chronic Myeloid Leukemia (CML). However, discontinuation and/or inefficacy on CML leukemia stem cells (LSC) may lead to relapse. To identify new druggable targets alternative to BCR/ABL, we investigated the role of the Extracellular signal-Regulated Kinase 5 (ERK5) pathway in CML LSC maintenance.

MATERIALS AND METHODS

KCL22 and K562 CML cell lines, patient-derived CML cells or CD34+ peripheral blood cells from healthy donors were incubated in normoxic or hypoxic (0.1% O2) primary cultures (LC1) in the presence or the absence of drugs. At the end of incubation (day 7), cells were analyzed on a flow cytometer to determine the expression of stem cell markers or transferred to drug-free normoxic secondary cultures (LC2) to measure LC2 repopulation as a read-out of progenitor/stem cell potential (CRA assay). In the serial Colony Formation Ability (CFA) assay colonies were scored on day 7 of each passage (III passages). In the Long-Term Culture-Initiating Cells (LTC-IC) assay the number of colonies was scored after 14 days. Compounds: XMD8-92 (ERK5 inhibitor) and BIX02189 (MEK5 inhibitor); imatinib and dasatinib (BCR/ABL inhibitors).

RESULTS

In CML patient-derived cells and cell lines, we found that the MEK5/ERK5 pathway is necessary for the optimal proliferation in low oxygen, a condition typical of normal hematopoietic and leukemic stem cell niches. Treatment of primary CML cells with XMD8-92 or BIX02189, but not with TKi, strikingly reduced Culture Repopulation Ability (CRA), serial CFA and LTC-IC. Importantly, inhibition of MEK5/ERK5 was effective on CML cells regardless of the presence or absence of imatinib (IM), and did not reduce CRA or LTC-IC of normal CD34+ cells. Interestingly, in hypoxia, combined treatment XMD8-92/IM decreased the expression of genes relevant for stem cell maintenance such as p21, p27, c-MYC, SOX2 and NANOG and the expression of CD26, a CML LSC marker. Moreover, inhibition of MEK5/ERK5 reduced the percentage of CD34+/CD26+ cells on primary CML cells.

CONCLUSION

We propose ERK5 pathway inhibitors as a novel therapeutic approach to prevent CML relapse and, in combination with TKi, enhance induction of remission.

CIRCULATING BIOMARKERS IN PROSTETIC JOINT INFECTION

E.R. GALLIERA

Department of Biomedical Sciences for Health, University of Milan and IRCCS Galeazzi Orthopedic Institute, Milan, Italy

Post-operative prosthetic joint infection (PJI) is the most common cause of failure of total joint arthroplasty, but a gold standard for PIJ diagnosis is still lacking. Among the scenario of infections diagnosis, an emerging molecule is Presepsin, the soluble fraction of CD14, recently described as a powerful diagnostic tool, to detect sepsis and to discriminate of sepsis severity. Among the scenario of infections diagnosis, an emerging molecule is Presepsin, the soluble fraction of CD14, recently described as a powerful diagnostic tool, able not only to detect sepsis but also to discriminate different grade of sepsis severit. Presepsin is a fraction of the soluble form of CD14, which is shedded from monocytes surface during inflammatory response and then released into blood. Therefore, Presepsin can be used a circulation marker of infection, but so far little is known about the mechanism of this sCD14 fraction shedding. A better understanding the mechanism of action of Presepsin in the inflammatory response and its correlation with other inflammatory mediators could improve the diagnostic potential and clinical application of Presepsin.

Recent unpublished results of our group showed a marked increase of Presepsin in Prosthetic Joint Infection patients compared to aseptic prosthesis loosening, indicating that this molecule can be a powerful marker of early PJI detection. In particular, in PJI patients we observed a correlation of Presepsin with the two main inflammatory markers, IL-6 and CRP, a monocyte –specific chemokine CCL2, suggesting a possible mechanism of action of Presepsin involving this chemokine and other related inflammatory mediators. Moreover, in a recently published study, we showed a specific involvement in PJI of the soluble form of the Toll like Receptor TLR2, acting in the recognition and response to Gram-positive bacteria. There is a strict interaction between the soluble form of CD14 and the receptor TLR2, suggesting a possible mechanism of action of Presepsin the response to Gram-positive bacteria infection.

A connection between Toll-like receptors, monocytes, inflammatory cytokines, such as IL-1 and IL-6 and the chemokine CCL2, in the mechanism of action of Presepsin could be represented by the new infection marker TREM-1. TREM-1 is related to different aspects of the inflammatory response: TREM-1 is involved in TLR signaling, acting as an "amplifier of inflammatory response", it is over-expressed on monocytes in parallel with CD14 in septic condition, then it is shedded from monocytes by the action of the metalloprotease MMP-9 and then it induces the inflammatory cytokines IL-1, IL-6, TNFa and CCL2. The overexpression of CD14 and its shedding a soluble form during inflammatory response is also correlated with another infection marker, SuPAR, the soluble urokinase plasminogen activating receptor. Our group recently described that suPAR, similarly to Presepsin, has a good diagnostic value in PJI detection and it correlates with the same inflammatory cytokines and cytokines of Presepsin, suggesting that this two molecules could act together in the inflammatory host response to infection.

Another emerging serum marker of infection is the

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hemoglobin (Hb) scavenger receptor, CD163. This is a macrophage-specific protein and the upregulated expression of this receptor is one of the major changes in the macrophage switch to alternative activated phenotypes in inflammation. Accordingly, a high CD163 expression in macrophages is a characteristic of tissues responding to inflammation. In addition to this biological role in inflammation, CD163 is a potential inflammation biomarker and a therapeutic target. The biomarker form of CD163 is the soluble plasma CD163 that arises from the increased shedding of CD163 mediated by the tumor necrosis factor-α (TNF-α) cleaving enzyme.

The aim of this project was to investigate of the mechanism of action of Presepsin in the inflammatory response, in particular investigating the inflammatory mediators involved such as the cytokines CCL2 and the inflammatory cytokines, IL-6. The diagnostic and prognostic value of Presepsin on PIJ was also investigated correlating Presepsin values with other emerging infection marker such as TREM-1, CD-163 and SuPAR, OPN, and MMP-9 involved in the progression of PJI.

MATERIALS AND METHODS

Study population

A population of 100 selected patients undergoing prosthesis revision was enrolled from IRCCS Istituto Ortopedico Galeazzi (Milan), IRCCS Istituto Ortopedico G Pini (Milan), and IRCCS Policilinco San Donato (Milan), and subdivided into two groups according to the cause of prosthesis implant failure: 48 patients having bacterial infection (confirmed by positive culture test, with isolation of the causal agent in the infectious focus) and 52 patient with no infection, undergoing aseptic loosening of the implant. Biomarkers evaluation: CRP was measured using immunoturbidimetry on an automated biochemical analyzer (Olympus CRP-Latex assay, Central Valley, PA, CA, USA).

IL-6, TREM-1, CCL2, MMP-9, OPN were measured using an ELISA sandwich Quantikine Assay, according to manufacturer protocol (R&D System, Minneapolis, MN, USA). SuPAR was measured by Suparnostic ELISA Assay, according to manufacturer protocol (Virogates, Denmark). Presepsin levels were measured by Presepsin/ PATHFAST Immunoassay (Mitsubishi Chemical).

RESULTS

Presepsin has a greater diagnostic value than CRP and IL-6 in the diagnosis of PJI, OPN, CCL2, TLR2 and SuPAR displayed good diagnostic values in PJI, while CD163, TREM-1 and MMP-9 displayed very low diagnostic potential.

CONCLUSION

Presepsin can be considered a useful tool for the diagnosis and clinical monitoring of PJI, supported by a panel of new inflammatory makers involved in monocyte/macrophage mediated inflammatory response such as OPN, CCL2 and SuPAR.

THE ROLE OF TUMOR-DERIVED EXOSOMES IN CANCER IMMUNE EVASION MEDIATED BY TOLL-LIKE RECEPTOR 4 ACTIVATION

R. DOMENIS[1], A. CIFÙ[1], D. MARINÒ[1], M. FABRIS[2], K.R. NIAZI[3], P. SOON-SHIONG[3] and F. CURCIO[1]

[1]Dipartimento di Area Medica (DAME), Università degli Studi di Udine, ITALY; [2]Istituto di Patologia Clinica, Azienda Sanitaria Universitaria Integrata di Udine, ITALY; [3]NantBioScience, Inc Culver City, CA, USA

Although, it is widely recognized that cancer cells actively utilize exosomes to promote tumor growth and metastasis, the biology of tumor-derived exosomes (TEX) is incompletely understood and much remains to be study in order to define the molecular and genetic mechanism by which exosomes operate in cancer. Increased expression and activity of toll-like receptor 4 (TLR4) in chronic infectious and inflammatory conditions is associated with cancer progression: its activation induces an inflammatory signaling that increases the tumorigenic potential of cancer cells promoting their immune evasion. The purpose of this study is to investigate the immunosuppressive properties of TEX released upon TLR4 activation.

MATERIALS AND METHODS

Cancer cell lines were treated with LPS (1μg/ml) for 24h and then cultured in exosome-free medium for 48h. TEX were isolated from cells supernatants by polymer precipitation method, quantified by nanoparticles tracking analysis and validated by exosome-specific tetraspanins expression by western blot and flow cytometry analysis. The expression of immunosuppressive factors on TEX surface was evaluated by flow cytometry. To investigate the ability of immune cells to internalize TEX, exosomes were stained with DiD dye and their uptake by T cells and monocytes was monitored by flow cytometry. TEX immuneregulatory properties were assessed by *in vitro* functional assays on immune cell subpopulations isolated from healthy donor's blood.

RESULTS

We demonstrated that the treatment with LPS enhances the immunosuppressive potential of TEX, although differences were observed depending on the origin of the tumor. The expression of TGFα was increased on surface of TEX released from LPS-stimulated cells. CD14+ monocytes quickly and efficiently internalized DiD-labelled exosomes, while CD3+ T cells slightly internalized TEX, even after 48h of co-incubation. TEX released upon LPS treatment became able to inhibit the proliferation of activated CD3+ cells in dose-dependent manner and promote the conversion of CD4+ into regulatory T cells.

CONCLUSION

Our data suggest that the activation of TLR4 contributes to tumor progression by promoting the release of more effective immunosuppressive TEX, which allow tumor cells to escape immune surveillance.

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THE KERATINOCYTE DIFFERENTIATION INDUCED BY FGFR2B REQUIRES SEQUENTIAL INVOLVEMENT OF PKCδ AND PKCα

R. DANILO, M. NANNI, B. ROSATO, M.R. TORRISI and F. BELLEUDI

Department of Clinical and Molecular Medicine, Sapienza University of Rome, Rome

The fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases expressed, by tissue-specific alternative splicing, in epithelial IIIb or mesenchymal IIIc isoforms and controlling key physiological processes, such as cell proliferation, differentiation, survival and migration. We have previously demonstrated that the tumor suppressor epithelial isoform of the FGFR2 (FGFR2b) induces early differentiation of human keratinocytes. Since protein kinases C (PKCs) are known to regulate the differentiation program in several cellular contexts, including keratinocytes, aim of our present study was to clarify if FGFR2b could play a role also in the late steps of keratinocyte differentiation and to assess if this receptor-induced process would involve PKC isoforms.

MATERIALS AND METHODS

Immunofluorescence, biochemical and molecular analysis, combined with the use of specific PKC isoform inhibitors and/or specific small interfering RNA approaches, were performed on 2D cultures or 3D organotypic rafts of human keratinocytes stably overexpressing FGFR2b.

RESULTS

We found that FGFR2b overexpression and its signaling induced both the precocious onset and an the accelerated progression of keratinocyte differentiation. In addition, we also demonstrated that PKCδ controls the early steps of FGFR2b-triggered differentiation, while PKCα plays a role restricted to the terminal stages of the process. Finally, on the light of our previous findings indicating that FGFR2b exerts its differentiative function via p63 repression (Purpura et al., Oncotarget 2013; Ranieri et al., Mol. Carcinog. 2018), here we showed that the two PKC isoforms sequentially act via the induction of KLF4 and DLX3, two transcription factors linked by negative loops to p63.

CONCLUSION

Overall, our results suggest that FGFR2b play a general role in the control of the entire program of keratinocyte differentiation and that the function of FGFR2b in the different steps of the process appear to be regulated by a signaling network sequentially involving PKCδ and PKCα isoforms and their respective downstream transcription factors.

A LARGE BIOMARKER INVESTIGATION IN YOUNG ASYMPTOMATIC PATIENTS WITH CEREBRAL SMALL VESSEL DISEASE IDENTIFIES ADMA AS A POSSIBLE KEY MOLECULE DRIVING ENDOTHELIAL DAMAGE

A. CIFÙ[1], M. FABRIS[1], F. JANES[2], M.E. PESSA[2], R. DOMENIS[1], A.C. NILO[2], R. GARBO[3], R. GIACOMELLO[1], G.L. GIGLI[2] and F. CURCIO[1]

[1]Dipartimento di Area Medica (DAME), Università degli studi di Udine, Udine, Italy; [2]Dipartimento di Neuroscienze, S. Maria della Misericordia, Azienda Sanitaria Integrata di Udine, Udine, Italy; [3]Dipartimento di Scienze Mediche Sperimentali e Cliniche, Università degli studi di Udine, Udine, Italy

Cerebral small vessel disease (CSVD), detected as white matter hyperintensities (WMH) on brain MRI, could be a distinct pathological entity within cerebrovascular diseases. The increasing use of MRI has lead to more incidental finding also in young people without classical vascular risk factor. The purpose of this work was to investigate different pathways implicated in endothelial dysfunction (inflammation, autoimmunity, coagulation, nitric oxide pathway) in a well selected, homogeneous population of patients with WMH, without significant vascular risk factors or neurological diseases.

MATERIALS AND METHODS

We recruited 35 patients with incidental finding of CSVD and 35 controls age-sex matched identified among people attending our Neurologic Clinic for a brain MRI which displayed a normal scanning, and other neurological, vascular and chronic inflammatory diseases were excluded. An extended screening for thrombophilia (anti-thrombin III, protein C, protein S, fibrinogen, plasminogen activator inhibitor –1, tissue plasminogen activator, von Willebrand factor, homocysteine, C-reactive protein) and autoimmunity (ANA, ENA, anti-phospholipids antibodies, lupus anticoagulant) was conducted using diagnostic assays.

We analysed several markers of inflammation and endothelial activation. Platelet Activating Factor-Acetyl Hydrolase and nitric oxide were measured with a colorimetric assay. Interleukin 6, interleukin 10, E-selectin, P-selectin and asymmetrical dimethylarginine (ADMA) were assayed by ELISA, while vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 were assayed by Luminex® assay. Statistical analyses were conducted with SPSS 20 (SPSS Inc., Chicago USA).

RESULTS

We did not find any significant difference between patients and controls, except for ADMA. Indeed, patients displayed significantly higher levels of ADMA (71.97±36.49 *vs* 44.46±29.94, p=0.002) than controls.

CONCLUSION

ADMA is an endogenous inhibitor of nitric oxide (NO) synthase: it reduces NO production and consequently could lead to endothelial dysfunction and cardiovascular disease. In this very large biomarker study, ADMA results the only endothelial marker significantly associated to WMH, supporting the hypothesis of an important role of endothelial dysfunction in CSVD.

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NUCLEO-CYTOPLASMIC SHUTTLING OF PROGESTERONE RECEPTOR AND CELL CYCLE PROGRESSION OF BREAST CANCER CELLS

G. GALASSO[1], E. DI ZAZZO[2], M. DI DONATO[1], P. GIOVANNELLI[1], B. PERILLO[1], A. MIGLIACCIO[1] and G. CASTORIA[1]

[1] Dipartimento di Medicina di Precisione, Università Degli Studi Della Campania "L. Vanvitelli"; [2]Dipartimento di Medicina e di Scienze della Salute "V. Tiberio", Università Degli Studi Del Molise

Progesterone receptors (PRs) belong to the steroid receptor superfamily and regulate development, growth and transformation of breast tissues. Different mechanisms, such as ligand binding, post-transcriptional modifications, interaction with signalling effectors or scaffold proteins control the steroid receptor subcellular localization, thereby influencing their activity. Derangement of steroid receptor import/export process often causes receptor delocalization and proliferative diseases of target tissues, such as breast and prostate. However, while the mechanism of steroid receptor nuclear import is well documented, the mechanism of their nuclear export is still unclear.

MATERIALS AND METHODS

By combining different approaches, such as immunofluorescence analysis and biochemical techniques, we have analyzed the mechanism of PR nuclear export in breast cancer-derived T47D cells untreated or treated with the synthetic progestin, R5020 in the presence or not of leptomycin B (LMB), a nuclear export inhibitor. To disclose the signal transduction pathway controlling PR shuttling, we have used dominant negative forms of various signaling effectors. BrdU incorporation and cell cycle analysis were performed to uncover the effects of R5020 treatment on cell cycle progression mediated by PR.

RESULTS

Findings obtained by our experiments indicate that the PR is exported from nuclei through a CRM1-dependent mechanism. Such a process is linked with cell cycle progression of progestin-treated cells. Moreover, PR nuclear export depends, at least in part, on MAPK activation induced by R5020 in T47D cells.

CONCLUSION

Our study provides a new link between signaling activation by MAPK, nuclear export of PR and cell cycle progression regulated by progestin in breast cancer cells.

FOXO3A RE-EXPRESSION OVERCOMES TAMOXIFEN RESISTANCE IN BREAST CANCER

C. MORELLI[1], M. FIORILLO[2], M. PELLEGRINO[1], P. RIZZA[1], A. NIGRO[1], E. RICCI[1], M. LANZINO[1] and D. SISCI[1]

[1]Department of Pharmacy and Health and Nutritional Sciences, University of Calabria, Rende, Cosenza, Italy; [2]Translational Medicine, School of Environment and Life Sciences, Biomedical Research Centre (BRC), University of Salford, Greater Manchester, United Kingdom

The poor outcome of patients resistant to endocrine treatments is a major clinical challenge in the management of estrogen receptor positive (ER+) breast cancers (BC) and demands additional studies. In this context, the role of FoxO3a transcription factor was investigated.

MATERIALS AND METHODS

FoxO3a involvement in the acquisition and reversion of tamoxifen resistance was assessed *in vitro* in three parental ER+ BC cells, MCF-7, T47D and ZR-75, in the deriving Tamoxifen resistant models (TamR) and in Tet-inducible TamR/FoxO3a stable clones, by proliferation, siRNA, RT-PCR, Western blot (WB), Immunofluorescence (IF), Transmission Electron Microscopy (TEM), TUNEL, cell cycle and proteomics analyses. FoxO3a clinical relevance in the response to endocrine therapy was validated in silico by Kaplan-Meier (K-M) survival curves. FoxO3a ability to restore the responsiveness to tamoxifen has also been confirmed in animal models.

RESULTS

FoxO3a expression is reduced in TamR, very likely for its increased phosphorylation and degradation by a hyperactive ERK1/2 pathway. FoxO3a silencing counteracts Tamoxifen induced growth inhibition in wtMCF-7, demonstrating that FoxO3a is a mediator of cell response to Tamoxifen. Re-expression of an active (nuclear) FoxO3a in TamR tetracycline inducible clones (TamR/Tet-OnAAA) re-established the sensitivity to the antiestrogen. Proteomics analysis unveiled novel mediators of the anti-proliferative activity of the transcription factor. High levels of FoxO3a transcripts correlate to a positive response to endocrine therapy in BC patients (K-M curves). FoxO3a induction by the anti-convulsant drug Lamotrigine (LTG) strongly reduced tumor mass in TamR-derived mouse xenografts.

CONCLUSION

FoxO3a is a good prognostic factor in ER+ BC, predicting a positive response to endocrine therapy, and a key target to be exploited in combination therapy. LTG seems a valid candidate to be used as an adjuvant to Tamoxifen therapy in patients at risk.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

ERK5 INHIBITION ELICITS CELLULAR SENESCENCE IN HUMAN MELANOMA CELLS

A. LAZZERETTI[1], I. TUSA[1], P. DELLO SBARBA[1], B. STECCA[2] and E. ROVIDA[1]

[1]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Università degli studi di Firenze, Florence, Italy; [2]Istituto per lo studio, la prevenzione e la rete oncologica (ISPRO), Florence, Italy

Extracellular signal-Regulated Kinase 5 (ERK5) is a member of the Mitogen Activated Protein Kinase (MAPK) family. ERK5 is activated by MEK5 in response to numerous stimuli and is involved in several cellular processes including the proliferation of both normal and neoplastic cells. We previously demonstrated that ERK5 is required for the proliferation of melanoma cells, a neoplasia that, especially in the advanced stages, has a very poor prognosis. Human melanomas are frequently characterized by the loss or the reduced expression of markers of cellular senescence that results in tumor growth and progression. We evaluated the effects of the inhibition of the MEK5-ERK5 pathway on cellular senescence in melanoma cells.

MATERIALS AND METHODS

We used two different melanoma cell lines: A375 cells, expressing the oncogenic form of BRAF (BRAFV600E), and SSM2C cells, which express wild-type BRAF. The inhibition of the MEK5-ERK5 pathway was achieved using either small-molecules inhibitors of MEK5 (BIX02189) or ERK5 (XMD89-2; JWG-045) and gene silencing, using lentivirus encoding for ERK5-specific short hairpin RNA (shRNA). Cellular senescence was evaluated by SA-Galactosidase assay. The expression of ERK5 and senescence signaling proteins was evaluated by western blotting.

RESULTS

We found that both gene silencing and pharmacological inhibition of ERK5, but not of MEK5, induced cellular senescence in A375 and SSM2C cells. These results were confirmed by the increase of the cell area, the arrest of the cell cycle in G0/G1 phase, the reduced-expression of the cyclins E and D likely due to the increase of p21, and the hypo-phosphorylation of RB. The same findings were obtained in NIH/3T3 cells.

CONCLUSION

We have demonstrated that ERK5 inhibition, but not MEK5 inhibition, induced cellular senescence in human melanoma cells. Future studies will be directed to deepen the knowledge of the role of ERK5 pathway in the cellular senescence process with the aim to identify new therapeutic targets for melanoma and other tumors.

GENOTYPIC ASPECTS OF LONGEVITY. DATA FROM DESIGN PROJECT

A. AIELLO[1], G. ACCARDI[1], C. GIUSEPPINA[1], C.M. GAMBINO[1], M.E. LIGOTTI[1], S. VASTO[2] and C. CARUSO[1]

[1]Department of Pathobiology and Medical Biotechnologies (DIBIMED) - University of Palermo, Palermo, Italy; [2]Department of Biological Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy

Ageing is defined as a physiological and ineluctable process, characterised by a gradual decrease of the ability to adapt to stress. On the other side, longevity is a complex phenotype influenced by environmental factors (for 75%), and genetics (for 25-30%). The centenarian people represent the best model to study successful ageing and longevity. They reach the age of 100 or more years without any age-related disease, in good physical and mental condition. About genetic aspects, only two genes have been shown positive results in different studies and populations: APOE and FOXO3A. In particular, APOE ε4 allele has been linked to elevated cholesterol level, cardio-vascular diseases, and age-related cognitive decline. On the contrary, APOE ε2 allele, encoding a protein with less affinity to cholesterol, has been found more frequently in centenarians. Moreover, FOXO3A represents the other gene that influences longevity in different ethnic populations.

MATERIALS AND METHODS

We have recruited 120 Sicilian subjects, with an average age between 30 and 102 years, and we classified them in 4 groups: young, non-centenarians offspring (nCO), centenarians offspring (CO), and long-lived individuals (LLI). We performed the genotyping of APOE genes profile (ε2, ε3, ε4) using an APOE genotyping kit, and FOXO3A G/T polymorphisms (rs2802292 variant), in a sub-group of the recruited people.

RESULTS

We observed that the frequency of ε3/ε3 genotypes and ε3 genes are similar to Italian mean in all analyzed groups (between 81% and 90%). It was very interesting to note that, in our centenarians, ε4 allele is missing, and that the levels of LDL cholesterol in CO are low. About FOXO3A genotyping, in our population, we found that the T polymorphism is more frequent than the Italian frequency, and that the dominant genotypes are GT and TT, in particular in CO and nCO. We found the same result in Y, in CO, and LLI, in which T polymorphism is present at 66%.

CONCLUSION

Our data are collected in a relatively small sample of subjects. Accordingly, our data needed to be confirmed in the other zones of Sicily. In addition, it will interesting to associate APOE genotypes with HDL and LDL cholesterol levels, and FOXO3A genotypes with telomeres length, in all recruited subjects.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

ANTHROPOMETRIC SIGNATURE OF LONGEVITY IN SICILY

G. ACCARDI[1], A. AIELLO[1], C. CARUSO[1], C.M. GAMBINO[1], M.E. LIGOTTI[1], S. VASTO[2] and G. CANDORE[1]

[1]University of Palermo, Department of Pathobiology and Medical Biotechnologies (DIBIMED) - University of Palermo, Palermo, Italy; [2]Department of Biological Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Italy

The dramatic extension of maximum lifespan in Europe (110+ years) need action plans for long-living individuals (LLIs, aged 90). Analysis of body composition (fat mass, fat free mass, and total body water (TBW) divided in intra and extra cellular) by bioimpedance (BIA) is a non-invasive technique to monitor health status. Its variation seems to be linked to oedema, response to specific drug, clinical treatment, onset of age-related diseases, sarcopenia. The limit of BIA is the absence of range of values for restricted population, as LLIs, with different body composition. Our aim is to identify a longevity anthropometric phenotype of LLIs and reference values for them.

MATERIALS AND METHODS

We are performing a population study in Sicily. In 16 people (mean age of 101.5) we performed anthropometric evaluation with BIA 101. Skin electrodes are placed on hand and foot of the same side of body, applying low-voltage and giving two-measures: resistance (Rz, indirectly correlated with amount of body fluids. The higher is Rz, the lower is TBW) and reactance (Xc, directly correlated with cell density in tissues) by human tissues, associated with body composition. A vector quantity, Pa, is obtained from Rz and Xc. It permits to evaluate the quality of cell, depending on membrane integrity and body cell mass. Lower Pa is associated with low Xc and cell death or breakdown in selective permeability of membrane (people with low Pa have higher Na+/K+ ratio).

RESULTS

Preliminary results demonstrated that our population have mean BMI of 24.35 Kg/h2, mean Pa of 3.2° (mean ref. val. 6.5°). Na+/K+ ratio of subjects was above 1. Interestingly, all people followed a strict Mediterranean diet during ageing but no at present.

CONCLUSION

In aged people, abdominal fat accumulation is often a marker of resilience, better functional reserve and lower subclinical diseases prevalence, characterizing the “healthy cohort” effect. Results found in studies with adults and younger elderly should create an over- or underestimation of risks. Understanding the changes in body composition and distribution with ageing and their health implications is important for nutritional support and pharmacologic treatment and for the development of appropriate health guidelines for elderly.

CHK1 INHIBITION RESTORES INOTUZUMAB OZOGAMICIN CITOTOXICITY IN CD22-POSITIVE CELLS EXPRESSING MUTANT P53

E. TIRRÒ[1], M. MASSIMINO[1], C. ROMANO[1], M.S. PENNISI[1], S. STELLA[1], S. VITALE[1], A. FIDILIO[2], L. MANZELLA[1], N. PARRINELLO[3], F. STAGNO[3], G. PALUMBO[4], A. ROMANO[3], F. DI RAIMONDO[5] and P. VIGNERI[1]

[1]Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy and Center of Experimental Oncology and Hematology, A.O.U. "Policlinico-Vittorio Emanuele", Catania, Italy; [2]Department of Biomedical and Biotechnological Sciences, Section of Pharmacology, University of Catania, Catania, Italy; [3]Division of Hematology, A.O.U. "Policlinico-Vittorio Emanuele", Catania, Italy; [4]Department of Medical, Surgical Sciences and Advanced Technologies "G.F. Ingrassia", University of Catania, Catania, Italy; [5]Division of Hematology, A.O.U. "Policlinico-Vittorio Emanuele", Catania, Italy and Department of Surgery and Medical Specialties, University of Catania, Catania, Italy

Inotuzumab ozogamicin (IO) is an anti-CD22 calicheamicin immunoconjugate that has been recently approved for the treatment of relapsed or refractory B-Acute Lymphoblastic Leukemia (r/r B-ALL). We employed both immortalized and primary cells derived from CD22-positive lymphoproliferative disorders to investigate the signaling pathways contributing to IO sensitivity or resistance.

MATERIALS AND METHODS

To calculate IC50 values, BL-2, Sup-B15, Namalwa and primary CD22-positive cells were exposed to logarithmic dilutions of IO. Multiple DNA-damage-induced proteins, cell cycle distribution and apoptosis were evaluated after exposure to IO, alone or in combination with the Chk1 inhibitor UCN-01. Immortalized cells bearing wild-type or mutant p53 were lentivirally transduced with mutant or wild-type p53, respectively.

RESULTS

We found that the drug reduced the proliferation rate of CD22-positive cell lines expressing wild-type p53, but was remarkably less effective on cells exhibiting mutant p53. In addition, CD22-positive cells surviving IO were mostly blocked in the G2/M phase of the cell cycle because of Chk1 activation that, in the presence of a wild-type p53 background, led to p21 induction. When we combined IO with the Chk1 inhibitor UCN-01, we successfully abrogated IO-induced G2/M arrest regardless of the underlying p53 status, indicating that the DNA damage response triggered by IO is also modulated by p53-independent mechanisms.

To establish a predictive value for p53 in determining IO responsiveness, we expressed mutant p53 in cell lines displaying the wild-type gene and observed an increase in IO IC50 values. Likewise, overexpression of an inducible wild-type p53 in cells natively presenting a mutant protein decreased their IC50 for IO. These results were also confirmed in primary CD22-positive cells derived from B-ALL patients at diagnosis and from patients with r/r B-ALL. Furthermore, co-treatment with IO and UCN-01 significantly increased cell death in primary cells expressing mutant p53.

CONCLUSION

p53 status may represent a biomarker predictive of IO efficacy in patients diagnosed with CD22-positive malignancies.

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VITAMIN D PREVENTS HIGH GLUCOSE-INDUCED ENDOTHELIAL PERMEABILITY

R. SCRIMIERI[1], L. LOCATELLI[1], V. ROMEO[1], E. GIANI[2], J. MAIER[1], G.V. ZUCCOTTI[2] and A. CAZZANIGA[1]

[1]Dipartimento di scienze biomediche e cliniche L. Sacco, Università degli Studi di Milano; [2]Dipartimento pediatrico, Università degli studi di Milano, Ospedale dei Bambini V. Buzzi, Milan, Italy

The endothelium is the gate-keeper of vascular health. Accordingly, endothelial dysfunction is a crucial event in macro-vascular and in small vessel diseases. Since the morbidity and mortality due to micro- and macro-vascular complications in patients with diabetes is very high, understanding the molecular bases of endothelial dysfunction in response to high glucose is a major issue.

MATERIALS AND METHODS

HUVEC are seeded in transwell, and permeability is evaluated using fluorescent albumin. Griess assay is performed to measure the release of nitric oxide. siRNA targeting inducible-NOS (iNOS) and N6-(1-Iminoethyl)-lysine, hydrochloride (L-NIL) are used to inhibit iNOS.

RESULTS

HUVEC cultured in high glucose release higher amounts of nitric oxide through the induction of iNOS. Indeed, both genetic and pharmacological inhibition of iNOS restore normal nitric oxide synthesis and permeability in HUVEC exposed to high glucose. Interestingly, we found that very low concentrations of vitamin D prevent the accumulation of nitric oxide and the increase of endothelial permeability.

CONCLUSION

Increased permeability is the first step in endothelial dysfunction in general and in diabetes in particular. We show that i) high glucose increases endothelial permeability through the induction of iNOS; and ii) vitamin D prevents high glucose detrimental effects in HUVEC.

EDF-1 CONTRIBUTES TO PPARγ TRANSCRIPTIONAL ACTIVATION IN ENDOTHELIAL CELLS TREATED WITH VEGF

A. CAZZANIGA, L. LOCATELLI, V. ROMEO, R. SCRIMIERI, S. CASTIGLIONI and J. MAIER

Dipartimento di scienze biomediche e cliniche L. Sacco, Università degli Studi di Milano

The endothelium is the gate-keeper of vascular health. Accordingly, endothelial dysfunction is a crucial event in macro-vascular and in small vessel diseases. Since the morbidity and mortality due to micro- and macro-vascular complications in patients with diabetes is very high, understanding the molecular bases of endothelial dysfunction in response to high glucose is a major issue.

MATERIALS AND METHODS

Human Umbilical Vein Endothelial cells (HUVEC) were treated for different times with VEGF. The sub-cellular localization of EDF1 was investigated by immunofluorescence and western blot performed on nuclear and cytosolic fraction. HUVEC were stably silenced for EDF1 (αs1) and compared to HUVEC transfected with a scrambled non silencing sequence as controls. The transcriptional activity of PPARγ was detected using vector expressing luciferase (pDR1-Luc). The expression of Fatty Acid-binding protein (FAB4), a PPARγ inducible gene, was evaluated by Real Time PCR.

RESULTS

We demonstrate that VEGF stimulates the nuclear translocation of EFD1, which induces the transcriptional activity of PPARγ. Accordingly, in αs1 cells VEGF does not stimulate the transcriptional activation of PPARγ and does not induce the expression of FAB4.

CONCLUSION

Our results substantiate that PPARγ activation has a role in maintaining the integrity of the vasculature and highlight EDF1 as a novel player in the complex regulation of PPARγ transcriptional activity in the endothelium. On these bases, we propose that EDF1 is an important contributor to maintain endothelial integrity, and this may be crucial in the prevention of cardiovascular diseases.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

MESOPOROUS SILICA NANOPARTICLES AS A VEHICLE FOR HIGHLY SELECTIVE DELIVERY OF BORTEZOMIB TO FOLATE RECEPTOR EXPRESSING CANCER CELLS

M. PELLEGRINO[1], A. NIGRO[1], E. RICCI[1], M. GRECO[1], A. COMANDÈ[1], I. PERROTTA[2], A. LEGGIO[1], L. PASQUA[3], D. SISCI[1] and C. MORELLI[1]

[1]Department of Pharmacy and Health and Nutritional Sciences, University of Calabria, Rende, Italy; [2]Department of Biology, Ecology and Earth Sciences and Centre for Microscopy and Microanalysis (CM2),Transmission Electron Microscopy Laboratory, University of Calabria, Rende, Italy; [3]Department of Chemical Engineering and Material, University of Calabria, Rende, Italy

The major limitation of traditional chemotherapeutic agents is their poor selectivity for cancer cells and their severe toxicity to normal cells. Therefore, localized drug delivery would, ideally, improve the therapeutic efficacy, minimizing side effects. The properties of mesoporous silica nanoparticles (MSNs) seem to cope with this aim. A MSN-based device, FOL-MSN-BTZ, bearing the antineoplastic drug bortezomib (BTZ), linked to MSNs by means of a pH-sensitive bond and the folic acid (FOL, a targeting function), was then developed and tested on folate receptor (FR+ cells) overexpressing cancer cells and on FR- normal cells, in order to investigate MSNs behaviouor.

MATERIALS AND METHODS

The cellular uptake of FOL-MSN-BTZ was assessed by Transmission Electron Microscopy (TEM) investigations and Immunogold labelling (IG), while efficacy studies were conducted in FR+ tumor cells using growth experiments, TEM, TUNEL assay and Western Blotting analyses.

RESULTS

FOL-MSN-BTZ was able to induce death exclusively in FR+ tumor cells, while free BTZ resulted toxic for all cell lines tested, including FR- normal cells. TEM observations evidenced that MSNs uptake occurs in FR+ RPMI-8226 (RPMI) multiple myeloma cells only and not in FR- BJhTERT normal cells, where they remain confined in the intercellular spaces. In addition, IG labeling on RPMI confirmed that the recognition of MSN-FOL depends on FR expression and occurs through FR-mediated endocytosis. Moreover, both FOL-MSN-BTZ and free BTZ lead to comparable apoptotic rates in RPMI cells, while negligible apoptosis was detected in FOL-MSN-BTZ treated BJhTERT cells. Finally, both FOL-MSN-BTZ and free BTZ trigger the same apoptotic pathways in RPMI cells, inducing PARP-1 and caspases cleavage, as well as Cyt c translocation from the mitochondria to the cytoplasm. Moreover, the cleavage of both caspase-8 and caspase-9 suggests the concomitant activation of both the extrinsic and the intrinsic apoptosis pathway in these cells.

CONCLUSION

These data show the outstanding specificity of FOL-MSN-BTZ toward FR+ tumor cells and the highly promising preliminary *in vivo* safety data (ongoing studies in mice models), pave the way for a future exploitation of our MSNs technology for drug targeting applications, particularly in cancer therapy.

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CXCL12α IS A KEY FACTOR IN THE REGENERATION OF THE DAMAGED NEUROMUSCULAR JUNCTION BY ACTING ON THE CXCR4 RECEPTOR

C. MONTECUCCO[1], S. NEGRO[1], F. LESSI[2], E. DUREGOTTI[1], P. ARETINI[2], A. MEGIGHIAN[1], M. PIRAZZINI[1], C. MAZZANTI[2], M. RIGONI[1]

[1]Department of Biomedical sciences, University of Padova; [2]Fondazione Pisana per la Scienza

The neuromuscular junction (NMJ) is one of the few human tissues capable of complete regeneration after major damages (1). We want to identify the cross-talk taking place among the different cell type of the NMJ: neuron, muscle, Schwann cells.

MATERIALS AND METHODS

We have set up a reliable model of acute degeneration of the motor axon terminals followed by complete recovery of function based on the use of the neurotoxins alfa-latrotoxin that induces a rapid, but reversible complete degeneration of the axon terminal. We have performed a time resolved trascriptomic analysis of the NMJ treated with this toxin from injection to complete recovery of NMJ function.

RESULTS

We have found that the chemokine CXCL12α and other factors, which are currently under analysis, play a major role in NMJ regeneration after complete degeneration. CXCL12α acts via the CXCR4 receptor that is localized at the tip of the regrowing motor axons (2).

CONCLUSION

We are currently testing the activity of CXCR4 agonists and have found a very active, nontoxic, compound that strongly promotes the recovery of function of the NMJ completely degenerated by the spider neurotoxin α-latrotoxin. Moreover, we are extending the analysis of the pharmacological properties of the CXCR4 agonists to the sciatic nerve crush.

HLA-B51 ALLELE CORRELATION WITH AUTOIMMUNE DISEASES OTHER THAN BEHÇET'S: A RETROSPECTIVE ANALYSIS

G. DI SPIGNA[1], B. COVELLI[1], M.R. SANSONE[2], M.P. OTTAVIANO[2], P. PALMIERI[1], E. DE MARINIS[1], A. SANGERMANO[2], G. BENINCASA[2] and L. POSTIGLIONE[1]

[1]Department of Translational Medical Sciences, University of Naples "Federico II", Naples, Italy; [2]Dia-Chem Srl Biomedical Technologies, Pineta Grande Group SPA, Naples, Italy

Behçet's Disease (BD) is a systemic vasculitis, prevalent in males, with a chronic and inflammatory course and with multi-organ involvement. Are affected young subjects (20-30 years). The etiology remains unknown but appears to be mediated by exogenous factors (bacterials or virals) that, through immunopathological mechanisms, start humoral or cellular responses. The main clinical manifestations are represented by: oral ulcers, genital ulcers, cutaneous, ocular, articular, vascular and neurological involvement. Frequent is the association (about 72%) with HLA-B51 histocompatibility antigen, which suggested that the disease is present, following external factors, in genetically predisposed patients. HLA (Human Leukocyte Antigen) is a highly polymorphic gene with different haplotypes that can agree the response of the human adaptive immune system or predispose individuals to a particular immune system disease. The diagnostic utility of HLA-B51, associated with Behçet's disease, has been clearly identified; however, its correlation with other autoimmune diseases has not yet been clarified. This study evaluate the presence of the HLA-B51 allele with other autoimmune diseases.

MATERIALS AND METHODS

80 plasma samples have been collected from patients with different autoimmune diseases (LES, Rheumatoid Arthritis, Polymyalgia Rheumatica, Sjogren's Syndrome, Scleroderma, Dermatomyositis); no Behçet's patients have been enrolled. The research of the HLA-B51 allele has been performed by Real Time PCR High-Resolution Melt (HRM) technique. The method is based on detecting small differences in PCR melting (dissociation) curves. Data are analyzed using software designed specifically for HRM analysis.

RESULTS

The results obtained are the following: n. 16 samples (20%) present the HLA-B51 allele, compared to the reference health population (0.5-1.5%).

CONCLUSION

Our study shows that 20% of the patients studied affected by other classical autoimmune diseases have the HLA-B51 allele, compared to 0.5-1.5% of the reference population. Therefore, the results of this study confirm that HLA-B51 is associated not only with Behçet's disease, but that HLA-B51 may also play an unknown role in inducing, modulating or upregulating other forms of autoimmune disease.

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UNRAVELING THE ROLE OF MAPK ERK5 IN HUMAN MELANOMA

S. GAGLIARDI[1], A. TUBITA[2], I. TUSA[2], S. PANDOLFI[1], X. DENG[3], N. GRAY[3], J. WANG[3], E. ROVIDA[2] and B. STECCA[1]

[2]Istituto Toscano Tumori, Core Research Laboratory, Florence, Italy; [3]University of Florence, Department of experimental and clinical biomedical sciences, Florence, Italy; [1]Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA, USA;

Melanoma is the deadliest skin cancer, with a very poor prognosis in advanced stages. Available treatments for melanoma, including immunotherapy or inhibitors for BRAF-MEK1/2, have greatly improved the survival for this disease although they are not applicable to all patients and/or their long-term benefits are still unsatisfactory. As a member of Mitogen-Activated Protein kinase family, ERK5 could play a relevant role in melanoma regulating cell functions critical for tumor development, such as proliferation, invasion and angiogenesis.

MATERIALS AND METHODS

Cell lines and patient-derived primary melanoma cells (wild type B-RAF: SSM2c and M26c; BRAFV600E: A375, SK-Mel-5, SK-Mel-28, M42, 501-Mel, expressing; NRASQ61R: SK-Mel-2; MeWo) have been used for *in vitro* and *in vivo* experiments. ERK5 inhibition was achieved using ERK5 and MEK5 inhibitors (XMD8-92 and BIX02189, respectively) or lentiviral vectors encoding shRNA specific for ERK5. Vemurafenib was used as a Braf inhibitor.

RESULTS

Initially, we found that ERK5 is consistently expressed and active in commercial and patients derived melanoma cell lines. On that basis, we investigated the role of ERK5 in melanoma cell growth. *In vitro*, pharmacological or genetic inhibition of ERK5 decreased the number of viable cells in several melanoma cell lines. Moreover, xenografts performed using LV-shERK5-transduced A375 or SSM2c cells showed a reduced tumor growth when compared to those transduced with control LV-shC. We also found that oncogenic BRAF positively regulates expression, phosphorylation and nuclear localization of exogenous and endogenous ERK5. Accordingly, combined pharmacological inhibition of BRAFV600E and MEK5 is required to decrease nuclear ERK5 that is critical for the regulation of cell proliferation. Furthermore, the combination of MEK5 or ERK5 inhibitors with vemurafenib is more effective than single treatments in reducing 2D colony formation and growth of BRAFV600E melanoma cells and xenografts.

CONCLUSION

Our results identify ERK5 as a critical regulator of melanoma growth *in vitro* and *in vivo*, and point toward the possibility of targeting ERK5, alone or in combination with BRAF-MEK1/2 inhibitors, for the treatment of melanoma.

A NEW THERAPEUTIC STRATEGY IN MULTIPLE MYELOMA BASED ON SMALL MOLECULES DIRECTED TO NOTCH PATHWAY

N. PLATONOVA[1], C. PARRAVICINI[2], L. PALAZZOLO[2], S. SAPORITI[2], M. COLOMBO[1], V. VALLELONGA[1], D. GIANNANDREA[1], D. GIANA[1], A. NERI[3], I. EBERINI[2] and R. CHIARAMONTE[1]

[1]Department of Health Sciences, University of Milano; [2]Department of Oncology and Hemato-oncology, University of Milano; [2]Department of Pharmacological and Biomolecular Sciences, University of Milano; [3]Unit of Hematology, Fondazione Cà Granda IRCCS Policlinico, Milano

Multiple myeloma (MM) is the second most common hematological malignancy. Although clinical advances, it is still incurable disease, sustained by a tight interaction of malignant plasma cells with the bone marrow (BM) microenvironment that promotes tumor growth, immunosuppression, drug resistance, neoangiogenesis and bone destruction. The oncogenic Notch signaling plays a crucial role in MM. In particular, aberrant Notch2 receptor activation and Jag1 and 2 ligands overexpression stimulate MM cells to establish pathological interactions with BM that trigger MM progression. Our previous data showed that these effects can be interfered by knocking down of Jag1 and 2 expression. Indirect approaches to inhibit Notch signaling are mainly based on inhibition of γ-Secretase that catalyzes Notch activation along with other several γ-Secretase substrates. Moreover, inhibition of all four Notch receptors is associated with a gut toxicity. This evidence prompted us to develop a therapeutic tool to selectively inhibit Notch2 signaling triggered by Jag1 and 2.

MATERIALS AND METHODS

We applied in silico protein-protein docking and virtual high-throughput screening (HTS) of a chemoteque to select druglike small molecules. The biological activity was validated by a Notch responsive reporter assay and co-culture assay that allow measuring Notch2 transcriptional activity induced by either Jag or Dll ligands.

RESULTS

Based on previous setup of integrated in silico pipeline, we applied a strategy to exclusively uncouple Notch2::Jag1/2, leaving unaltered the interaction with Dll. 100 top-scoring compounds directed exclusively to Notch2::Jag2 surface were selected by HTS of the chemoteque. 2 of 100 compounds were validated *in vitro* by the Notch responsive reporter assay on HEK293T cells and showed a significant reduce in Notch transcriptional activity. The co-culture assay of Hela cells overexpressing Notch2 with NIH3T3 overexpressing Jag1 or Dll4 ligands identified one promising compound that specifically inhibited Notch2::Jag1 and not Notch2::Dll4 interactions.

CONCLUSION

Overall, our results showed that the identified compounds could selectively antagonize Notch activation providing a rationale for an effective and safe anti-Notch therapy in MM and other Notch-dependent tumors.

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ATYPICAL BCR-ABL BREAKPOINTS DISPLAY SELECTIVE RESPONSIVENESS TO TYROSINE KINASE INHIBITORS

M. MASSIMINO[1], E. TIRRÒ[1], S. STELLA[1], C. ROMANO[1], S. VITALE[1], A. PUMA[1], L. MANZELLA[1], F. DI RAIMONDO[2], F. STAGNO[3] and P. VIGNERI [1]

[1]Department of Clinical and Experimental Medicine, University of Catania and Center of Experimental Oncology and Hematology, A.O.U. Policlinico Vittorio Emanuele, Catania; [2]Division of Hematology and Bone Marrow Transplant, University of Catania and Department of Surgery, Medical and Surgical Specialties, University of Catania; [3]Division of Hematology and Bone Marrow Transplant, University of Catania

The BCR-ABL oncoprotein is the culprit of CML as it transforms the hematopoietic stem cell by altering its survival and proliferation properties. The efficacy of Tyrosine Kinase Inhibitors (TKIs) of the canonical BCR-ABL variants e1a2 (p190), e13a2 or e14a2 (p210) has been well established. Alternative breakpoints involving different BCR and/or ABL exons have been previously described but yet to be characterized. We analyzed 50 CML patients, not presenting the canonical isoforms, finding three atypical BCR-ABL breakpoints in five subjects: one e12a2$^{ins/del}$, three 13a3 and one e14a3 transcripts. These atypical isoforms (with the addition of two further deletion mutants lacking the BCR DC2 domain or the ABL SH3 domain) were investigated for their catalytic activity, transforming potential and TKI responsiveness.

MATERIALS AND METHODS

Canonical and atypical BCR-ABL variants were subjected to catalytic efficiency assay or lentivirally expressed in Ba/F3 and Rat1^{Myc} cells to assess their transforming ability and TKIs responsiveness. *Ex vivo* experiments were also performed using CD34+ progenitors expressing these atypical BCR-ABL transcripts.

RESULTS

The e14a3 isoform showed catalytic efficiency comparable to conventional BCR-ABL and significantly superior to e12a2ins/del, e13a3, BCR-ABL DC2 and BCR-ABL SH3. However, in Ba/F3 cells we found that canonical BCR-ABL and e13a3 were most effective in IL3-independent growth, while the remaining variants were less proficient. No differences were observed in anchorage-independent growth assessed in Rat1^{Myc} cell line. Interestingly, evaluating the TKI sensitivity we found that, all atypical rearrangements, compared to canonical BCR-ABL, were significantly less responsive to Imatinib and Dasatinib but not to Nilotinib. Similar data were obtained in LTC-IC assays using CD34-positive cells isolated from patients expressing the e14a3 variant. Finally, patients diagnosed with the atypical variants failed Imatinib as first line treatment and are currently benefiting from Nilotinib therapy.

CONCLUSION

Our findings suggest that BCR and ABL regions flanking the BCR-ABL breakpoint play a critical role in leukemic transformation, selectively modulating TKI responsiveness representing meaningful clinical consequences for CML patients displaying these atypical transcripts.

A NEW DOWEL IN ENDOTHELIAL ADAPTATION TO MICROGRAVITY: IMPACT ON MITOCHONDRIA

L. LOCATELLI, A. CAZZANIGA, V. ROMEO and J. MAIER

University of Milan, Department of Biomedical and Clinical Sciences L. Sacco

Blood vessels are lined by cells (ECs) that are crucial to maintain vascular and tissue homeostasis. Endothelial function is shaped by mechanical forces, i.e. shear stress and gravity (1G). Since life evolved under the influence of the earth's gravitational pull, it is not surprising that microgravity activates adaptive responses that impact also on the endothelium. Several studies have shown that real and simulated microgravity affect ECs behaviour in different aspects but very little is known about the metabolic adaptation of these cells. In particular, we focused our attention on the mitochondria, rod-shaped organelles that convert oxygen and nutrients into adenosine triphosphate (ATP).

MATERIALS AND METHODS

To simulate microgravity we used a bioreactor developed by NASA, i.e. the Rotating Wall Vessel (RWV). Experiments were performed on Human Endothelial Cells from the Umbelical Vein (HUVEC), a model of macrovascular ECs. Mitochondrial content was assessed by Real Time PCR for mtDNA, while mitochondrial function was measured using the O2K oxygraph chambers.

RESULTS

We show a decrease of the number of mitochondria in face of an increased respiratory capacity in HUVEC grown for 4 or 10 days in simulated microgravity. We also show that the reduction of mitochondria is due to an increased autophagy, as demonstrated by western blot for specific markers of this process, p62 ans LC3-B II.

CONCLUSION

This is the first evidence of a metabolic adaptation to microgravity in human ECs exposed to simulated microgravity.

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THE METABOLIC MICROENVIRONMENT IMPOSED BY GLUTAMINE SYNTHETASE-NEGATIVE MULTIPLE MYELOMA CELLS SHAPES THE BONE MARROW NICHE

M. CHIU, D. TOSCANI, M. BOLZONI, G. TAURINO, F. ACCARDI, N. GIULIANI and O. BUSSOLATI

Department of Medicine and Surgery (DiMeC), University of Parma, Parma, Italy

Altered cancer metabolism satisfies high demand for nutrients but also has an impact on non-cancer cells of tumor microenvironment. In the bone marrow (BM) of multiple myeloma (MM) patients, glutamine (Gln) is lowered, while glutamate (Glu) and ammonium increase (Bolzoni, Chiu et al., Blood 2016; 128:667-679). Glutamine Synthetase (GS), which catalyzes Gln synthesis from Glu and ammonium, is lowered in most MM cells and down-regulated during osteoblastogenesis. Since bone lesions of MM patients are characterized by low osteoblast viability and function, we hypothesize that MM cells negatively affect osteoblastogenesis through the peculiar low-Gln, high-Glu bone marrow microenvironment.

MATERIALS AND METHODS

Human MM cell lines (HMCLs) and the immortalized osteoblastic cell lines HOBIT and HOB-01 were grown in Glu-free medium supplemented with 2 mM Gln and 10 % fetal bovine serum. Gene expression profiles were obtained with dataset of 323-sample plasma cell dyscrasias. Extracellular Glu and Gln were measured with a commercial kit and their uptake assessed as described [Bianchi et al. Neuroscience 2008; 151(4):1042-52].

RESULTS

Gene expression profiling data revealed that GS expression is downregulated during MM progression so that less than 20% MM patients had GS-positive CD138+ cells. Consistently, most HMCLs, but not MM1.S and U266, had a negligible expression of GS. Both low- and high-GS HMCLs exhibited fast initial uptake and high consumption of Gln. However, while low-GS HMCLs exploited roughly 50% of Gln to secrete Glu in the extracellular space, Glu secretion was considerably less in high-GS HMCLs. Consistently, low-GS MM cells exhibited higher expression and activity of the Glu exchanger x-CT, the expression of which, interestingly, increases during MM progression. Osteoblasts were sensitive to Gln depletion and their viability was reduced when co-cultured with low-GS, but not with high-GS HMCLs.

CONCLUSION

All the HMCLs tested require high amounts of Gln to support their proliferation, but only low-GS lines secreted sizable amounts of Glu in the extracellular medium and negatively affected osteoblast viability. These preliminary results are consistent with the hypothesis that low GS expression and Gln addiction of MM cells are functional to osteoblast impairment in the BM niche.

MEDITERRANEAN DIET AS PREVENTION STRATEGY OF AGE-RELATED DISEASE

L. CRISTALDI[1], S. VASTO[2], M. DI CARLO[3] and C. CARUSO[2]

[1]Department of Pathobiology and Medical Biotechnology (DI.BI.MED), University of Palermo, Italy; [2]Department of Biological Chemical and Pharmaceuticals Sciences and Technologies, University of Palermo, Italy; [3]Institute of Biomedicine and Molecular Immunology "Alberto Monroy" (IBIM), CNR - Palermo, Italy

Several evidence indicates a strong correlation between healthy lifestyle and age-related pathology, in particular neurodegenerative diseases, including Alzheimer's disease (AD). This pathology is characterized by two histo-pathological hallmarks, the amyloid plaques, formed by Aβ oligomers, and neurofibrillary tangles, due to hyperphosphorylation of tau protein. At molecular level, Aβ induces mitochondrial and endoplasmatic reticulum dysfunctions, oxidative stress and apoptosis. The brain is susceptible to oxidative stress and several prevention strategy based on use of antioxidant molecules have been proposed. Traditional Mediterranean Diet (MD) includes high consumption of vegetables, fruits and herbs, food rich in antioxidant molecules, such as polyphenols. Some studies indicate that MD influences longevity as confirmed by studies on centenarian subjects. *In vitro* studied have demonstrated that Caffeic Acid (CA), one of the main polyphenol present in several foods that make up the MD, has high anti-inflammatory and antioxidant properties.

MATERIALS AND METHODS

We have analysed the properties of CA both in differentiated LAN5 neuronal cells and peripheral blood mononuclear cells (PBMCs). The antioxidant properties of CA were demonstrated by treatment of the cells with Tert-butyl hydroperoxide (TBH) and with Aβ. We have used cell proliferation assay, cellular reactive oxygen species detection assay, and western blot assay to detect oxidative stress markers (phospho-ERK1/2, SOD2, phospho-FoxO3a).

RESULTS

The administration of CA in neuronal cells results in almost complete recovery of cell viability and morphology. The ROS generation induced by TBH, both in neuronal cells and PBMCs, was reduced, as well as the expression of several oxidative stress markers after CA administration.

CONCLUSION

The results indicate that CA could be a good biomolecule to be employed for reducing the risk of developing several oxidative stress damages. Future prospective will consider the analysis of antioxidant properties on PBMCs of successful and unsuccessful ageing individuals.

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CYTOPLASMIC CYCLIN D1 MEDIATES PROGESTERONE/PR-B INHIBITION OF HUMAN BREAST CANCER CELL INVASION

F.I. MONTALTO[1], C. CHIODO[1], D. NAIMO[1], M. SANTORO[2], L. MAURO[1], S. AQUILA[1], F. DE AMICIS[1], M. LANZINO[1] and S. ANDÒ[1]

[1]Dpt. of Pharmacy, Health and Nutritional Sciences University of Calabria, Italy; [2]Health Center University of Calabria, Italy

Progesterone Receptor (PR) positivity is associated with a good prognosis and better response to breast cancer treatment. Conversely, cyclin D1 (CD1) is retained a marker of poor outcome since it has been associated with breast cancer metastasis in clinical studies.

MATERIALS AND METHODS

17α-Hydroxyprogesterone (OHPg) was from Sigma-Aldrich. Antibodies and Protein A/GPLUS-Agarose were from Santa Cruz Biotechnology. T47-D, MCF-7 and MDA-MB-231 human breast cancer cells from the American Type Culture Collection; Total real-time RT-PCR assay; Western blotting and immunoprecipitation; Transfections and luciferase assays; Lipid-Mediated Transfection of siRNA Duplexes; Chromatin immunoprecipitation (ChIP) assays and realtime ChIP; Wound-healing assays; Transmigration assays; Cell invasion assay; Phalloidin staining

RESULTS

Herein we provide evidences that OHPg through PR-B isoform reduces motility and invasion of T47-D and MCF-7 breast cancer cells, by a double mechanism targeting CD1. Specifically, OHPg reduces CD1 expression through the occupancy of CD1 promoter at a canonical half progesterone responsive element by PR-B. This allows the recruitment of HDAC1, influencing a less permissive chromatin conformation for gene transcription and release of RNA Pol II. CD1 has an active role in the control of cell migration and metastasis through the interaction with key components of focal adhesion such as Paxillin (Pxn). In untreated T47-D and MCF-7 cells, a specific co-immunoprecipitation of endogenous cytoplasmic CD1 with Pxn was observed. Interestingly, OHPg exposure reduced the interaction between these proteins although total Pxn expression was substantially unaffected. Moreover a concomitant reduction of p-Pxn levels was observed and these effects were required for OHPg/PR-B dependent delay in cell invasion, as evidenced by assays carried out with the phoshomimetic mutants of Pxn.

CONCLUSION

Collectively these findings support the importance of PR-B expression in breast cancer cells behavior, suggesting potentiating of PR-B signaling as a prospective useful strategy to restrict breast tumour cells invasion and metastasis.

SIGNATURE OF LONGEVITY

G. ACCARDI[1], L. SCOLA[1], A. AIELLO[1], S. APRILE[1], M. BULATI[1], G. CANDORE[1], C. CARUSO[1], L. CRISTALDI[1], G. DURO[2], C. GAMBINO[1], M. LIGOTTI[1] and S. VASTO[1]

[1]University of Palermo; [2]CNR

Ageing is a natural and physiological condition that is the results of compromised stress response, homeostatic imbalance and increased risk of developing diseases. However, if we age having a good health and functioning (successful aging) or with disease and disability (unsuccessful aging), it depends on a combination of "positive features", including genetic, epigenetic and phenotypic characteristics in combination with favorable environment, economic status and social involvement. In our study, we summarize some key points for the identification of a longevity signature, with a particular focus on Sicilian long-lived individuals and centenarians.

MATERIALS AND METHODS

We have analyzed genetic biomarkers as Apo E ε2-3-4 and FOXO3A rs2802292 G-allele (G>T). Hematochemical biomarkers: Glycaemia, ALT, AST, total Cholesterol, HDL-CHO, LDL-CHO, urea Nitrogen. Antropometric biomarkers: Anthropometric Phenotype (LAP), BMI, body composition, body fat distribution, resting metabolic rate and waist-hip ratio.

RESULTS

We have analyzed in three different Sicilian cohorts (young, people with no centenarian parents and long-lived individuals aged >90) and found APOE ε3 with a frequency ranging from 0.81 and 0.90, with a prevalence of E3/E3 genotype, no presence in our long-living population of ε4.

Regarding FOXO rs2802292 G-allele (G>T) we did not observe an association with longevity but, we observed a higher frequency of the anti-inflammatory alleles of TLR4, CCR5, Cox2, 5-Lo and cytokine genes associated to longevity.

Regarding hematochemical results: In our three cohorts, urea nitrogen (increased), platelets count (lower in centenarians compared to elderly), ALT, total cholesterol and glycaemia (significantly reduced).

In our population, the mean BMI was 24.35 Kg/m2 so not associated to mortality risk. The analysis of body composition demonstrated hyperhydration (phase angle PA; mean: 3.2°).

CONCLUSION

In Sicily, we are studying longevity features in some mountainous area (Sicani and Madonie area). In the former, centenarians are present above the Italian statistical ratio (2.4/10.000) while in the latter we have a high density of long-living individuals. So far, it is difficult to specify common longevity features due to the intrinsic complexity and heterogeneity of the studied population.

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ADIPOCYTE-RELEASED FACTORS AFFECT VIABILITY, MIGRATION AND RESPONSE TO DOCETAXEL IN PROSTATE CANCER CELLS

A. LIOTTI[1], E. LA CIVITA[1], D. LIGUORO[1], L. DE STEFANO[2], A. PALMIERI[2], V. MIRONE[2], F. BEGUINOT[1], P. FORMISANO[1] and D. TERRACCIANO[1]

[1]Department of Translational Medical Sciences, Federico II University, Naples, Italy; [2]Department of Urology, Federico II University, Naples, Italy

The prevalence of obesity is increasing at an alarming rate in developed countries throughout the world. Epidemiologic studies indicate that obesity is important risk factors for diabetes, cardiovascular disease and cancer. In particular, obesity is associated with a greater increase of aggressive prostate cancer (PCa) with increased local dissemination. Prostatic gland is surrounded by periprostatic adipose tissue (PPAT). PPAT is an active endocrine organ able to secrete molecules known as adipokines. The high prevalence of both obesity and PCa highlights the importance of understanding the biological features of this relationship. Recent studies uncovered that the secretion of mature adipocytes can affect the early stage of PCa progression by promoting the spread of cancer cells outside the prostate gland. It is clear that tumor-surrounding adipocytes might affect cancer phenotype, but the molecular mechanisms involved in this cross-talk are still unknown.

MATERIALS AND METHODS

Adipose-derived mesenchymal stem cells (Ad-MSCs) were obtained from biopsy of PPAT from patient undergoing radical prostatectomy. Ad-MSCs were differentiated in mature adipocytes and then serum starved to obtain conditioned media (Ad-CM). Two prostate cancer cell line, PC3 and DU145, were used to assess cell viability, migration and drug response to docetaxel

RESULTS

The treatment for 48h with Ad-CM from PPAT induced 20% increase of cell viability of PC3 cells. In addition, adipocyte-released factors (ARFs) from PPAT induced migration of PC3, while no effects were observed in DU145. Next, we assessed the effect of Ad-CM on response to docetaxel, a chemotherapic agent used for the treatment of castrate-resistant prostate cancer. PC3 and DU145, pretreated with Ad-CM, showed a reduced response to docetaxel of about 40% and 20%, respectively. This effect is associated with a higher mRNA expression of genes related to drug resistance, such as ABCB1, TUBB2B and TUBB4A.

CONCLUSION

ARFs form PPAT affect malignant phenotype of prostatic cancer cells, suggesting that the study of cross-talk between adipocytes and prostate cancer cells promotes the identification of new prognostic biomarkers and pharmacological targets for the treatment of PCa.

ROLE OF ANDROGEN RECEPTOR IN BREAST CANCER-ASSOCIATED FIBROBLASTS: MAY ANDROGEN SHAPE BREAST TUMOR MICROENVIRONMENT?

C. CHIODO[1], C. MORELLI[1], F. DE AMICIS[1], C. GIORDANO[2], D. SISCI[1], S. ANDÒ[1] and M. LANZINO[1]

[1]Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende, Italy; [2]Health Center, University of Calabria, Rende, Italy

Interaction between breast tumor epithelial and stromal cells is central for tumor growth and progression. Indeed, while providing a scaffold for the breast, the stroma also regulates epithelial cell function through physical and hormonal paracrine exchanges, providing a favorable environment for proliferation and metastasis. There is extensive knowledge of androgen receptor (AR) signaling in breast epithelial cancer cells, but less regarding AR-mediated action in breast tumor stroma. In the present report, we provide evidence of AR expression in breast cancer-associated fibroblasts (CAFs) isolated from breast cancer patients. We also examined the effect of CAFs exposure to androgens on: 1) secretory phenotype and 2) the migratory behavior of the estrogen-responsive breast cancer MCF-7, T47D and ZR-75 cells.

MATERIALS AND METHODS

CAFs were from biopsies of primary breast tumors (n=3); qRT-PCR, IHC, IF and WB were used for evaluating AR expression; CAFs and MCF-7 or T47D or ZR-75 cells were employed for co-colture experiments; cytokine array was performed to analyze specific cytokines secreted by CAFs; wound healing and transmigration assays were used for evaluating cell migration.

RESULTS

CAFs do express the AR as demonstrated by RT-PCR and WB. IHC and IF studies further demonstrated that AR nuclear localization is more pronounced upon androgen treatment. Moreover, androgens administration affects CAFs secretory phenotype as demonstrated by cytokine array performed on CAFs conditioned medium (CM).

The relationship between AR expression in the tumor stroma and epithelial cancer cells behavior was explored by co-colture experiments using CAFs and MCF-7 or T47D or ZR-75 cells. CM from androgen-treated CAFs was less effective in stimulating breast cancer cells motility compared to CM from untreated ones, indicating that androgens, via AR, may influence CAFs secretion of paracrine soluble factors involved in tumor cell motility sustainment.

CONCLUSION

Androgens, via AR, may drive stromal paracrine effects on epithelial breast cancer cells. Disclosing the effectors of androgen response in fibroblast and their impact on tumor microenvironment may provide an alternative perspective on breast cancer progression and therapeutic requirements of patients.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

INHIBITING FOCAL ADHESION KINASE (FAK) REDUCES THE GROWTH OF UVEAL MELANOMA GNAQ/GNA11-MUTATED CELLS BY TARGETING THE PI3K/AKT/MTOR PATHWAY

D.C. RIGIRACCIOLO[1], X. FENG[2], R. LAPPANO[1], M. MAGGIOLINI[1] and J.S. GUTKIND[2]

[2]Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende, Italy; [1]Department of Pharmacology and Moores Cancer Center, University of California San Diego, La Jolla, California, United States of America

Uveal melanoma (UM) represents the most common intraocular malignancy in adults and approximately 50% of patients develops metastatic disease. The new genomic sequencing technologies on UM tumors allowed to identify mutations in the Gq alpha subunits GNAQ and GNA11. These mutations appear in a mutually exclusive manner and are consequently of prognostic importance (Van Raamsdonk et al., Nature 2009). FAK (focal adhesion kinase) is a cytoplasmatic tyrosine kinase localized at the sites of cell adhesion to the extracellular matrix. FAK is overexpressed in several tumors and mediates diverse signaling promoting cancer growth and metastasis. To date, it has been considered as a potential target for cancer therapeutics (Sulzmaier et al., Nat Rev Canc 2014).

MATERIALS AND METHODS

A computational framework carried out on Gaq-activated tumors and the analysis of whole-genome sequencing of UM samples (TCGA data) were used to identify novel vulnerabilities and gene targets in UM. Immunoblotting and Immunofluorescence assays were performed to dissect the mechanisms by which GNAQ leads to FAK activation. Knockdown experiments and small molecule FAK inhibitors were utilized to investigate the growth promoting pathways regulated by Gaq through FAK and how interfering with FAK signal transmission may provide new therapeutic options for UM. Finally, the potential clinical benefit of targeting FAK in UM *in vivo* was investigated using the CRISPR/Cas9 genome editing strategy.

RESULTS

We identified PTK2 (the gene encoding FAK) as the top predicted gene target in GNAQ-expressing tumors, and UM as the human cancer harboring the highest mRNA levels of FAK. Next, we found that Gaq promotes FAK activation involving the guanine nucleotide exchange factor (TRIO)/Rho-A signaling independently from the PKC pathway. We also assessed the involvement of FAK in the control of the PI3K/AKT/mTOR survival pathway, and in affecting transiently the MEK/ERK signaling resulting in UM cell growth inhibition. Lastly, we demonstrated that targeting FAK *in vivo* abolishes UM tumor formation

CONCLUSION

Our findings suggest that FAK may represent a novel therapeutic target for the treatment of UM and other human diseases resulting from mutually exclusive mutations in GNAQ and GNA11 oncogenes.

ESTROGENIC GPER SIGNALING PROMOTES FGF2/FGFR1 ACTIVATION BETWEEN BREAST CANCER-ASSOCIATED FIBROBLASTS (CAFS) AND TUMOR CELLS

M.F. SANTOLLA, A. VIVACQUA and M. MAGGIOLINI

Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende (CS), Italy

Many cancers acquire aberrant growth and invasion capacity through the dysregulation of fibroblast growth factors (FGFs)-mediated signaling (Carter EP, Trends Cell Biol. 2015). In particular, FGFs secreted by the tumor cells or stromal compartment activate the cognate receptors leading to cancer progression (Babina IS, Nat Rev Cancer. 2017). For instance, FGF2-FGFR1 autocrine and/or paracrine loop activation has been involved in the migration and invasion of cancer cells (Coleman SJ, EMBO Mol. Med. 2014). Remarkably, estrogens induced via the estrogen receptor (ER) the up-regulation and secretion of FGF2 in breast and lung cancer (Fillmore CM, PNAS. 2010, Siegfried JM, Oncotarget. 2017). Likewise, the G protein estrogen receptor (GPER) was also involved in the regulation of FGF2 by estrogens toward the activation of downstream signaling pathways in astroglial cells (Huang C, Neuroscience. 2016).

MATERIALS AND METHODS

By qPCR, western blot, immunofluorescence analysis, ELISA and ChIP assays, we ascertained the estrogen-mediated regulation of FGF2 expression in GPER-positive but ER-negative primary cancer-associated fibroblasts (CAFs) derived from mammary ductal carcinomas. Then, we investigated the genetic alteration of FGFRs family members in both METABRIC (n=2051) and TCGA (n=818) patients data sets and we generated FGFR1 knockout (KO) MDA-MB-231 cells using CRISPR/Cas9 genome editing strategy in order to assess the functional FGF2/FGFR1 interplay triggered by CAFs and breast cancer cells.

RESULTS

We first determined that estrogenic GPER signaling mediates the up-regulation and secretion of FGF2 in CAFs. Next, we ascertained that FGFR1 is the most amplified FGFRs in human breast tumors. Then, using conditioned medium from estrogen-stimulated CAFs we established that FGF2/FGFR1 paracrine activation increases CTGF and CYR61 levels through the ERK1/2 and AKT signaling cascades toward the migration and invasion of MDA-MB-231 cells.

CONCLUSION

These findings shed new light on the role elicited by estrogens through GPER in FGF2/FGFR1 paracrine signaling leading to breast tumor progression. Therefore, our data may provide further biological targets useful in innovative combination strategy halting breast cancer development.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

GLUTAMINE METABOLISM AND ITS INHIBITION IN THE TARGETING OF STEM CELLS OF CHRONIC MYELOID LEUKAEMIA REFRACTORY TO STANDARD THERAPY

M. POTETI[1], N. MAZURE[2], E. ROVIDA[1] and P. DELLO SBARBA[1]

[1]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Università di Firenze, Florence, Italy; [2]Institute for Research on Cancer and Ageing of Nice, Université de Nice-Sophia-Antipolis, Nice, France

We previously found that the adaptation of Chronic Myeloid Leukaemia (CML) cells to energy restrictions in paralleled by the suppression of BCR/Ablprotein, the oncogenic driver of CML, notably in a subset of leukaemia stem cells (LSC). These LSC, while remaining genetically leukaemic, are independent of BCR/Abl signaling for maintenance in tissues (within the "stem cell niches") and therefore refractory to tyrosine kinase inhibitors (TKi) used for CML therapy. We envisioned on these bases a "metabolic" stem cell niche model to explain the long-term persistence of LSC responsible for Minimal Residual Disease (MRD) of CML. In this model, glutamine plays an important role.

MATERIALS AND METHODS

K562 and KCL22 CML cell lines were incubated in primary cultures (LC1) in normoxia (21% O2) or low-oxygen (0.1% O2) where glutamine, and/or drugs (2-DG and Metformin) were administered or not at time zero. At the end of incubation, protein or mRNA were determine in cell lysates and pH and metabolite concentrations in culture medium. To estimate stem cell potential LC1, cells were transferred to secondary cultures (LC2) incubated in normoxia in drug-free standard medium, to measure LC2 repopulation.

RESULTS

In glutamine-containing cultures incubated in low oxygen, glucose was time-dependently consumed driving BCR/Ablprotein suppression, lactate was produced and extracellular pH decreased. In the absence of glutamine, BCR/Ablprotein and signaling were maintained, and lactate production and medium acidification were reduced. Furthermore, in the presence of glutamine, the expression of glucose transporters was reduced. Incubation with 2-DG prevented BCR/Ablprotein suppression, confirming that glucose metabolism drives this suppression (in the presence of glutamine). On the contrary, metformin didn't have any effect. When stem cell potential was evaluated, LC1 cells incubated in the presence of glutamine repopulated LC2 after a lag-phase, a pattern typical of BCR/Ablprotein-negative LSC refractory to TKi. On the contrary, cells from glutamine-free LC1 repopulated LC2 immediately, a kinetics typical of TKi-sensitive cells where BCR/Abl signaling is maintained.

CONCLUSION

Glutamine emerges as a crucial metabolite for the selection of CML cells refractory to TKi.

IDENTIFICATION OF NEW BIOMARKERS FOR CARDIAC DISORDERS EXPLOITING TISSUE-SPECIFIC METHYLATION PATTERN OF CIRCULATING FREE DNA: A PILOT STUDY

C. DI RESTA[1], M.S. SPANÒ[2], S. MERELLA[3], M. FERRARI[4] and S. BENEDETTI[3]

[1]Vita-Salute San Raffaele University, Genomic Unit for the Diagnosis of Human Disorders, Division of Genetics and Cell Biology, IRCCS San Raffaele Hospital, Milan Italy; [2]Vita-Salute San Raffaele University, Milan, Italy; [3]Laboratory of Clinical Molecular Biology and Cytogenetics, IRCCS San Raffaele Hospital, Milan, Italy; [4]Vita-Salute San Raffaele University, Genomic Unit for the Diagnosis of Human Disorders, Division of Genetics and Cell Biology, IRCCS San Raffaele Hospital, Milan, Italy

Cardiomyopathies are a group of diseases leading to adverse outcome. Due to clinical heterogeneity and phenotypic overlapping, the identification of specific non-invasive diagnostic and prognostic markers can be useful and remains a challenge. Our aim is to exploit cardiac cfDNA as new cardiac biomarker based on the following assumptions: i) methylation patterns are unique and conserved in a specific tissue, hence their analysis could be used to identify the tissue of origin of circulating cell free DNA (cfDNA); ii) cfDNA levels of cardiac origin may be higher in the presence of cardiac disorder.

MATERIALS AND METHODS

To identify cardiac-specific methylation regions, we analyzed data obtained by bisulphite sequencing approaches performed on 12 human tissues available in ENCODE database and compared the methylation status of cardiac tissues respect to others.

RESULTS

Overall, we have identified 12 cardiac-specific methylation regions with a significant value of Z-score and we selected 6 of them containing at least 2 CpGs with the highest/lowest Z-score values. Bioinformatic results were first validated by analyzing genomic DNA extracted from peripheral blood and treated with sodium bisulphite with PCR amplification and Sanger sequencing.

CONCLUSION

To further verify the sensitivity of the detection method and confirm the expected methylation state of these regions in cardiac and blood cells, we will analyze genomic DNA from cardiac tissue biopsies and cfDNA extracted from control plasma samples. We will subsequently quantify cardiac cfDNA in blood samples from both affected and healthy subjects using droplet digital PCR and next generation sequencing on Illumina MiSeq platform. The presence of cardiac-specific cfDNA will be correlated with different types and severity of cardiac disorders in order to identify new biomarkers for early diagnosis and disease progression.

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NOTCH 1 PATHWAY AND EPCS IN BICUSPID AORTIC VALVE: IS THEIR INTERPLAY INVOLVED IN THE ASCENDING AORTIC ANEURYSM ONSET?

C.R. BALISTRERI[1], F. CRAPANZANO[1], A. ALLEGRA[2], C. PISANO[3], G. RUVOLO[4], D. LIO[1], L. SCHIRONE[5],
E. CAVARRETTA[5], S. SCIARRETTA[6] and G. FRATI[6]

[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Palermo, Italy; [2]Department of Surgery and Oncology, University of Palermo, Italy; [3]Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, Rome, Italy; [4]Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, Rome, Italy; [5]Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy; [6]Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy, IRCCS Neuromed, Pozzilli, IS, Italy

Bicuspid aortic valve (BAV) is frequently associated with development of ascending aortic aneurysm, even if the underlying mechanisms remain to be clarified. Here, we investigated if a deregulation of Notch1 signaling pathway and endothelial progenitor cells (EPCs) number is associated with BAV disease and an early ascending aortic aneurysm (AAA) onset.

MATERIALS AND METHODS

To this aim, 70 subjects with BAV (M/F 50/20; mean age: 58.8±14.8 years) and 70 subjects with tricuspid aortic valve (TAV) (M/F 35/35; mean age: 69.1±12.8 years), AAA complicated or not, were enrolled. Multiparametric flow cytometry analyses, plasma amount assessments, gene expressions, and tissue protein semi-quantitative evaluations were also performed.

RESULTS

Surprisingly, patients with AAA showed a significant increase in circulating Notch1 levels and EPC number with respect to subjects without AAA. However, circulating Notch1 levels and EPC number were significantly lower in subjects with BAV than patients with TAVeither in the presence or absence of AAA. Finally, Notch pathway was activated in aortic aneurysmportions with respect to healthy aortic fragments in both BAV and TAV patients. However, the expression of these genes in aortic tissues was significantly lower in BAV subjects than TAV subjects, as well as the semi-quantitative levels of the related encoding proteins.

CONCLUSION

Our study demonstrates that subjects with BAV are characterized by a significant decrease in both tissue and circulating levels of Notch pathway, and in blood EPC number with respect toTAV patients, either in the presence or absence of AAA disease.

IGA NEPHROPATHY IN A 12-YEAR-OLD GIRL WITH PERSISTENT HAEMATURIA AND PROTEINURIA: A CASE REPORT

R. MILANIC, G. STEL, L. SIGALOTTI, A.D. SPATOLA and F. CURCIO

Institute of Clinical Pathology, ASUIUD, Udine, Italy

Immunoglobulin A nephropathy (IgAN) is an immune complex disease and a major cause of glomerulonephritis in pediatric patients. Clinical picture includes hematuria and proteinuria, and can progress into renal failure. Urinalysis, as well as renal biopsy, serve to confirm the diagnosis. We report a case of a 12-year-old girl with persistent haematuria and severe proteinuria, in whom subsequently an IgAN was diagnosed.

MATERIALS AND METHODS

Our hospital laboratory was asked to perform a standard set of blood tests and urinalysis, following an admission of a 12-year-old girl with the symptoms of fever and haematuria. Urinalysis was performed by automated urine analyser (IriCELL, Beckman Coulter, USA). Renal tissue specimen obtained by biopsy was examined by light microscopy. Direct immunofluorescence was used to detect renal mesangial deposits of IgA, C3, Kappa and Lambda chains of Ig.

RESULTS

A 12-year-old girl was admitted to our hospital in May 2017, presenting with fever, vomiting and macroscopic haematuria. Upper respiratory tract infection was preceding the symptoms. Inital blood tests revealed leukocytosis (22.3 x10^3/μL) and high CRP (203 mg/L). On urinalysis haematuria (+++, erythrocytes: 3186/μL), proteinuria (7557 mg/L) and leukocyturia (370/μL) were detected. 24h-urine protein level was within nephrotic range (5382 mg/24h). Dysmorphic erythrocytes (27%) with acanthocytes (15%) indicated glomerular origin of haematuria. Subsequently, she developed a transient renal failure with oliguria (Creatinine: 1.61 mg/dl) and was treated with diuretics. After a second episode of macroscopic haematuria, a renal biopsy exam was performed. It showed mesangial deposits of IgA (+++), C3 (+++), Kappa and Lambda chains of Ig (++) and confirmed the diagnosis of IgAN. In one year of follow-up clinical improvement was assessed. Values of proteinuria and haematuria decreased markedly, while creatinine level was within reference range.

CONCLUSION

Recent studies have shown that end-stage renal disease can develop frequently in children with IgAN. Therefore, suspecting and diagnosing IgAN early in children who present with persistent haematuria and proteinuria in concomitance with mucosal infection is important, since regular monitoring and initiation of treatment may prevent progression of the disease.

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INVOLVEMENT OF ANNEXIN A3 ISOFORMS IN MODULATION OF LIPID STORAGE INTO CLEAR CELL RENAL CELL CARCINOMA (CCRCC) CELLS

C. BIANCHI[1], S. BOMBELLI[1], S. DE MARCO[1], B. TORSELLO[1], N. ZUCCHINI[2], P. VIGANÒ[3] and R. PEREGO[1]

[1]School of Medicine and Surgery, Milano-Bicocca University, Monza; [2]Pathology Unit, ASST Monza, San Gerardo Hospital, Monza; [3]Urology Unit, ASST North Milan, Bassini Hospital, Cinisello Balsamo

ccRCC cells show an adipocyte-like morphology due to neutral lipid accumulation. The molecular mechanism responsible for this phenotype has yet to be clarify. Notably, ccRCC cells show a gene expression signature consistent with adipogenesis and can undergo adipogenic transdifferentiation. The Ca^{2+}-dependent phospholipid-binding praotein Annexin A3 (AnxA3) has been recently described as negative regulator of adipocyte differentiation. In RCC, we evidenced that AnxA3 is downregulated and shows a specific pattern of two isoforms of 36 and 33 kDa originated by an alternative splicing event. In the current study, we have investigated the role of AnxA3 isoforms in the modulation of lipid storage responsible of the adipocyte-like morphology of ccRCC cells.

MATERIALS AND METHODS

Primary cell cultures from human ccRCC and normal renal cortex samples and proximal tubular (HK2) and ccRCC (A498 and Caki1) cell lines were used. Adipogenic medium contained 0.1 μM dexamethasone, 10 μg/ml insulin, 100 μM indomethacin and 500 μM IBMX. Intracellular lipid storage was evaluated by Oil Red "O" staining or by FACS and immunofluorescence analysis after Bodipy staining. AnxA3, PLIN2 and PPARγ expression was evaluated by real-time PCR, western blot and immunofluorescence analysis. Cell viability was evaluated by Annexin V/PI FACS analysis.

RESULTS

In ccRCC primary cultures the ratio between 36 and 33 kDa AnxA3 isoform expression (36/33 ratio) negatively correlated with PLIN2, marker of lipid storage, and PPARγ-, master activator of adipogenesis. Even in ccRCC cell lines the increase of lipid storage matched with the decrease of 36/33 kDa ratio. Cell lines were grown for 8 days in adipogenic medium used to induce adipocyte differentiation. Adipogenic treatment affected HK2 viability, whereas in Caki1 cells (low 36/33 kDa ratio) but not in A498 (high 36/33 kDa ratio) induced an increase of lipid storage. Of note, in Caki1 cells this increase matched with a further decrement of 36 kDa AnxA3 and 36/33 kDa ratio and with an increment of PPARγ expression.

CONCLUSION

Our data suggest that AnxA3, particularly the 36-kDa isoform, is involved in lipid storage modulation responsible for the adipocyte-like phenotype of ccRCC cells that may represent an important and targetable component of renal carcinogenesis.

PKHHIGH/CD133+/CD24- RENAL STEM-LIKE CELLS ISOLATED FROM HUMAN NEPHROSPHERES CAN BE COMMITTED TO ENDOTHELIAL PHENOTYPE

S. BOMBELLI[1], M. BOLOGNESI[1], A. BRUNO[2], B. TORSELLO[1], S. DE MARCO[1], C. BIANCHI[1], P. VIGANÒ[1], A. ALBINI[3], G. CATTORETTI[1] and R. PEREGO[1]

[1]School of Medicine and Surgery, Milano-Bicocca University, Monza, Italy; [2]Vascular Biology and Angiogenesis Laboratory, Scientific and Technology Pole, IRCCS MultiMedica, Milano, Italy; [3]Vascular Biology and Angiogenesis Laboratory, Scientific and Technology Pole, IRCCS MultiMedica, Milano, Italy. School of Medicine and Surgery, Milano-Bicocca University, Monza, Italy

Nephrosphere (NS) model permits to culture a heterogeneous population of renal stem-like cells (RSC) and progenitors. RSC are the quiescent (PKH^{high}) cells with a CD133+/CD24- phenotype. We demonstrated that NS cells cultured on decellularized scaffolds repopulated proximal and distal tubular portions, the Bowman's capsule, and vascular portions differentiating into endothelial cells. We now aim to evaluate the capacity of endothelial commitment of RSC isolated from human NS.

MATERIALS AND METHODS

FACS sorted RSC were cultured on decellularized renal scaffolds for 30 days and then immunofluorescence (IF) on FFPE slices was performed, evaluating the expression of epithelial tubular (CK8.18, CK7, AQP1) and endothelial (vWf) markers. RSC were single cell FACS sorted and tested for differentiation in specific endothelial or epithelial media. The expression of epithelial and endothelial markers was tested by FACS, and by IF after cytospin of RSC or after single cell differentiation. CD31- RSC and CD31+ progenitor cells within the NS were FACS sorted and cultured in Matrigel in complete endothelial medium to test for their capacity to form capillary like structures or sprouts.

RESULTS

RSC do not express endothelial markers and they were able to generate clonal secondary NS containing CD31+ and vWf+ cells, indicating an endothelial commitment that coexists with the epithelial one. In fact, RSC were also able to generate vWf+ endothelial structures, in addition to the epithelial structures, when cultured on decellularized scaffolds. Our preliminary data of single RSC differentiation confirmed their capacity of commitment towards different lineages, since the obtained clones contained cells expressing endothelial and epithelial markers. CD31+ endothelial committed cells sorted from RSC-derived NS were able to generate spheroids in Matrigel and after 7 days, we morphologically documented the formation of endothelial-like sprouts, while the sorted RSC could only generate organized structures without sprouts.

CONCLUSION

We have various evidences that RSC isolated from NS exhibited a double commitment: an expected tubular epithelial capacity, and an unexpected endothelial capacity that will be interesting to disclose.

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ROLE OF 1ALCTL AND 1BLCTL ARG ISOFORMS IN THE FIBROBLAST ACTIVATION

B. TORSELLO, S. DE MARCO, S. BOMBELLI, V. CASSINA, E. CHISCI, C. BIANCHI and R. PEREGO

School of Medicine and Surgery, University of Milano-Bicocca

The excessive scar tissue deposition of fibrotic process and the tumor stroma have in the activated fibroblasts (myofibroblasts) the main players. Myofibroblasts remodel the extra cellular matrix (ECM) by specialised cytoskeletal contractile features (stress fibres and focal adhesions) that reorganise the supportive tumour stroma. The non-receptor tyrosine kinase Arg binds directly to the cytoskeleton transducing extracellular signals into cytoskeletal rearrangements. ARG, through alternative splicing events, codes eight Arg isofoms that differently modulate stress fibers and motility. We analysed the role of 1ALCTL and 1BLCTL Arg isoforms in fibroblast activation using Arg KO murine embryonal fibroblast (MEF).

MATERIALS AND METHODS

Wt and Arg KO MEF transfected with empty and 1ALCTL-EGFP, 1BLCTL-EGFP vectors were FACS sorted for EGFP cell positivity. By immunofluorescence, stress fibres, focal adhesions and proliferation were analysed using phalloidin and antibodies against anti-paxillin and anti-PCNA. By Real time PCR and western blot, TGFß1 and α-sma expression were assessed. Wound healing, Boyden chamber, cell adhesion and ECM contraction assays were performed. MEF-derived matrices (DM) were stained by fibronectin and collagen I antibodies and their stiffness measured by Atomic Force Microscopy (AFM). Elliptical factor of 786-O RCC cell line plated on MEF-DM was calculated. One-way ANOVA and post-hoc Tukey's test were used ($P<0.05$).

RESULTS

Transfection of 1ALCTL in Arg KO MEF determined: a) both matrix contraction and adhesion increase; b) fibronectin and collagen I deposition increase. Instead, transfection of 1BLCTL caused: proliferation increase; a) α-sma expression increase; b) migration and invasion improvement; c) only matrix contraction increase d) fibronectin and collagen I deposition increase. Of note, Arg KO determined: a) TGFß1 increase; b) strong decrease in the matrix adhesion and contraction. AFM analysis of MEF-DM showed a decrease in Arg KO MEF-DM stiffness that only 1BLCTL rescued to wt MEF-DM level. Wt and 1BLCTL-DM induced a more elongated morphology in 786-O RCC cell line grown in these matrices.

CONCLUSION

1ALCTL and 1BLCTL Arg isoforms play a different role in fibroblast activation and in ECM secretion and remodelling.

JAGGED1/2 INHIBITION PROMOTES MULTIPLE MYELOMA CELL SENSITIVITY TO BORTEZOMIB *IN VITRO*, *EX VIVO* AND *IN VIVO* IN A NOVEL ZEBRAFISH MODEL

M. COLOMBO[1], M. MAZZOLA[2], N. PLATONOVA[1], S. GARAVELLI[1], M.T. PALANO[1], M. BARBIERI[3], D. GIANNANDREA[1], E. LAZZARI[1], A. BASILE[3], A. PISTOCCHI[2], A. NERI[3] and R. CHIARAMONTE[1]

[1]Department of Health Sciences, Università degli Studi di Milano; [2]Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano; [3]Department of Oncology and Hemato-oncology, Università degli Studi di Milano

Multiple myeloma (MM) is the second most diffuse hematological malignancy and nowadays is still incurable, despite the development of innovative therapies. MM cells accumulate in the bone marrow (BM) and establish vicious interactions with the surrounding normal cells, inducing them to promote tumor progression and the development of drug resistance. In this process, a crucial role is played by the dyregulated Notch ligands Jagged1 and 2, whose overexpression boosts Notch both in MM cells and in the BM cells. Here, we investigated how Jagged1/2 inhibition affects MM cells resistance to the standard-of-care drug Bortezomib.

MATERIALS AND METHODS

Jagged1/2 expression was inhibited in MM cell lines using specific siRNAs and in primary MM cells using a lentiviral vector that encodes two shRNAs. Cells were cultured alone or with BMSCs and treated with 6nM Bortezomib. Apoptosis was evaluated by Annexin V staining in flow cytometry. For *in vivo* experiments cells were stained with the CM-Dil dye, resuspended in PBS+3% polyvinyl pyrrolidone and injected in the yolk of 2dpf (days post fertilization) zebrafish embryos. Injected embryos were treated with 10nM Bortezomib or DMSO for 48h. Tumor burden was evaluated by fluorescence microscopy and tumor area was calculated using ImageJ and normalized on tumor volume at the time of injection.

RESULTS

Jagged1/2 silencing hamper MM cell capacity to trigger Notch activation in BMSCs, reducing in their ability to promote MM resistance to Bortezomib. Results obtained *in vitro* on MM cell lines were further validated on co-cultures of primary BMSCs and CD138+ cells siolated from newly diagnosed patients.

Data on zebrafish xenotransplanted embryos showed that the treatment with 10nM Bortezomib decrease tumor growth of about the 57% in comparison to DMSO-treated controls, with no effect on embryos viability. Jagged1/2 knockdown alone shown a similar effect, while the combination of Jagged1/2 inhibition and Bortezomib causes a stronger decrease in MM growth of approximately 82% in comparison to the controls.

CONCLUSION

Our findings provide the first proof-of-principle that Jagged1/2 withdrawal represents a suitable strategy to increase MM cells response to the commonly-used drug Bortezomib, restoring response to therapy.

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THE MOLECULAR CROSS-TALK AMONG ARG/ABL2, TGF-β1 AND LOX IN CLEAR CELL RENAL CELL CARCINOMA PROGRESSION

S. DE MARCO[1], C. BIANCHI[1], B. TORSELLO[1], S. BOMBELLI[1], N. ZUCCHINI[2], P. VIGANÒ[3] and R.A. PEREGO[1]

[1]Anatomo-Pathology Unit, ASST Monza, San Gerardo Hospital, Monza, Italy ;[2]School of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy; [3]Urology Unit, ASST North Milan, Bassini Hospital, Cinisello Balsamo, Italy

An involvement of TGF-β1 in promoting bone metastases affecting about 30% of clear cell Renal Cell Carcinoma (ccRCC) patients was described. The extracellular matrix enzyme Lysyl oxidase (Lox) through osteoclast activation and osteoblast inhibition induces pre-metastatic bone lesions in breast and colon cancer. We evidenced Lox overexpression in ccRCC and TGF-β1 production modulated by Arg tyrosine kinase in renal tubular cells. Arg modulates invasion and metastasis of breast and prostate cancer through cytoskeleton regulation. These data suggest that Arg, TGF-β1 and Lox might interact to promote tumor invasion and metastasis. Here we analyse the molecular and functional interactions among Arg, TGF-β1 and Lox in ccRCC and their effects on osteoclast and osteoblast pre-metastatic functions.

MATERIALS AND METHODS

Primary cell cultures, obtained from ccRCC and normal renal cortex specimens, and ccRCC (786-O), osteoclast (Raw264) and osteoblast (MC3T3-E1) cell lines used. 786-O cells treated with 5ng/ml TGF-β1 or with Arg siRNA with or without the TGFβ-receptor inhibitor SB431542. Arg, TGFβ1, Lox, phospho-Smad2 and Smad2/3 evaluated by western blot, Real-Time PCR or ELISA. Raw264 activation and MC3T3-E1 proliferation after treatment with Arg-silenced ccRCC cell conditioned media evaluated by TRAP staining and MTT assay, respectively.

RESULTS

Arg, TGF-β1 and Lox transcripts and proteins are upregulated in ccRCC cells. Treatment of 786-O cells with TGF-β1 upregulated Lox expression and secretion and downregulated Arg. Treatment with SB431542 reverted these effects. Moreover, Arg silencing in 786-O cells increased TGF-β1 expression and signalling inducing an increment of Lox secretion reverted by SB431542 treatment. The evaluation of Raw264 cell activation and MC3T3-E1 cell proliferation after treatment with Arg-silenced ccRCC cell conditioned media containing increased Lox level is ongoing.

CONCLUSION

Our data evidenced that in ccRCC cells Arg modulates Lox production through TGF-B1 signalling, which in turn modulates Arg expression. The complex interactions among Arg, TGF-β1 and Lox might be important also for ccRCC bone pre-metastatic niche formation, and treatment of osteoclasts and osteoblasts with Arg-silenced ccRCC cell conditioned media will be instrumental to validate this hypothesis.

OBESITY, ADIPOKINES AND BREAST CANCER: UNRAVELING THE MOLECULAR LINKS

S. CATALANO

University of Calabria

The prevalence of obesity has been increasing at an alarming rate in several developed and developing countries, reaching pandemic proportions over the last two decades. This growing incidence has deep clinical implications, since obesity is a key driver of serious health problems, such as type II diabetes, cardiovascular diseases, hypertension and cancer. Indeed, prospective epidemiological studies have shown that excessive adiposity strongly influences risk, prognosis and progression of multiple malignancies, including breast cancer. Several hypotheses have been proposed to unravel the direct link between obesity and breast cancer and these include hyperinsulinemia, estrogen signaling, inflammation and adipokine expression. Certainly, the revised concept of adipose tissues from an inert depot for body energy to endocrine and immunologically active organs placed particular emphasis on the potential role of adipokines in various biological processes and metabolic pathways. Acting through endocrine, paracrine and autocrine mechanisms, adipokines, that are not only produced by adipocytes but also by stromal cells, macrophages and cancer cells, impact the development and progression of obesity-related cancers. In this talk, I will present an overview of the clinical and experimental evidences highlighting the adipokines leptin and adiponectin, as the most important molecular mediators of obesity-breast cancer axis.

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MMP-9 AS PROGNOSTIC MARKER IN MELANOMA PATIENTS WITH CIRCULATING-FREE DNA BRAF V600E MUTATION

L. FALZONE[1], R. SALEMI[1], S. CANDIDO[1], D. CINÀ[2], P.A. ASCIERTO[3] and M. LIBRA[1]

[1]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy; [2]Garibaldi Hospital - Clinical Pathology Unit - Catania, Italy; [3]Melanoma Research Unit, National Cancer Institute "G Pascale", Naples, Italy

Despite the revolution in the field of melanoma treatment with the introduction of novel therapies (immunotherapy and target therapy), there is still a percentage of melanoma patients that often experiences drug-resistance and therapeutic failure. The early recognition of therapeutic failure, by the identification of new biomarkers of therapeutic response, is the main challenge of medical oncology in order to personalize the therapeutic strategies and avoid the toxicity of ineffective treatments. The circulating-free DNA BRAF^{V600E} mutation was proposed as a marker of therapeutic response. However, it cannot be revealed in all melanoma patients with this mutation, detected in tumour biopsy specimens. Therefore, there is a need to identify new prognostic biomarkers. Matrix Metalloproteinase-9 (MMP-9) could be one of them since its role in tumour invasiveness have been demonstrated.

MATERIALS AND METHODS

In the present study, MMP-9 was analysed in melanoma cells after treatment with dabrafenib. *In vitro* data were confirmed in 26 melanoma patients treated with BRAF and MEK inhibitors by ELISA assay and droplet digital PCR for measuring MMP-9 serum levels and circulating-free DNA BRAF^{V600E} mutation, respectively. Statistical analyses were achieved to calculate the prognostic significance of MMP-9, progression-free survival (PFS) and overall survival (OS) according to the BRAF^{V600E} mutation and MMP-9 levels.

RESULTS

In vitro data indicate that MMP-9 and pEKR1-2 decreased in a dose-dependent manner after treatment with dabrafenib. Circulating-free DNA BRAF^{V600E} mutation was detected in a fraction of melanoma patients showing higher levels of MMP-9 compared to those with undetectable BRAF^{V600E} mutation. Additionally, higher levels of MMP-9 and circulating-free DNA BRAF^{V600E} mutation were associated with reduced PFS and OS.

CONCLUSION

Overall, these results suggest that MMP-9 may represent a prognostic indicator of therapeutic response to targeted therapies in the subset of patients harbouring the circulating-free DNA BRAF^{V600E} mutation.

FLAGELLAR MOTILITY REDUCTION IN LISTERIA MONOCYTOGENES AFTER TREATMENT WITH *CANNABIS SATIVA L.* ESSENTIAL OIL

E. MARINI[1], G. MAGI[1], A. GIULIANI[2], V. DE DOMINICIS[2], M. PAVANI[2], M.R. RIPPO[2], A. PUGNALONI[2] and B. FACINELLI[1]

[1]Department of Biomedical Sciences and Public Health, Unit of Microbiology, Polytechnic University of Marche, Ancona, Italy; [2]Department of Clinical and Molecular Sciences, Unit of Experimental Pathology, Polytechnic University of Marche, Ancona, Italy

A major group of plant antimicrobial compounds is represented by essential oils (EOs), complex mixtures of volatile secondary metabolites belonging to different chemical families. *Cannabis sativa L.* has been grown for thousands of years for a multiplicity of purposes; in recent years, some genotypes containing low cannabinoid concentrations have been selected and used for research purposes. Listeria monocytogenes is a facultative intracellular food pathogen, mobile for the presence of several flagella. In humans, the treatment of listeriosis is hampered by the intracellular location of listeriae and the poor intracellular penetration of some antibiotics. Flagellum-based motility has been implicated in virulence since it is critical for initial surface attachment. The purpose of this study was to investigate the antibacterial and anti-virulence properties of an EO extracted from a legal *C. sativa L.* variety against a clinical isolate of L. monocytogenes.

MATERIALS AND METHODS

L. monocytogenes 80466, a blood isolate collected from a patient with invasive listeriosis in 2016, was studied. The EO was extracted from the *C. sativa L.* variety Futura 75 by steam-distillation. The antibacterial and anti-virulence activity of the EO was determined by susceptibility tests, motility assays, flagella staining, optical and scanning electron microscopy (SEM), and Real-time RT-PCR experiments.

RESULTS

A moderate bactericidal activity of the *C. sativa* EO was detected (Minimum Bactericidal Concentration >2048 mg/mL). In presence of EO at sub-lethal concentrations (256 μg/mL), motility tests demonstrated that listeriae became non-motile, i.e. they did not show the typical umbrella-like growth. L. monocytogenes optical examination after flagella staining showed that the listeriae incubated of the *C. sativa* EO formed aggregates with the flagella trapped inside the cluster. SEM observation confirmed these findings and demonstrated that there were fewer flagella in the extract-incubated strains. Real-time RT-PCR assays demonstrated a significant downregulation of motility genes (flaA, motA, and motB) in L. monocytogenes exposed to the EO.

CONCLUSION

Anti-virulence strategies are being explored among plant products as a novel approach to combat bacterial pathogens. The significant anti-motility properties of *C. sativa L*. variety Futura 75 EO against L. monocytogenes suggests that it could be employed as an agent to control L. monocytogenes.

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EPIMETHEX: AN *IN SILICO* PLATFORM TO EVALUATE THE CORRELATION BETWEEN GENE EXPRESSION AND GENE METHYLATION

S. CANDIDO[1], G. PARASILITI[2], G. RUSSO[2], F. PAPPALARDO[2] and M. LIBRA[1]

[1]Department of Biomedical and Biotechnological Sciences, Section of General Pathology, University of Catania; [2]Department of Drug Sciences, University of Catania

Cancer transformation is a multistep process sustained by several molecular alterations including those of DNA methylation. The high-throughput technology allowed understanding the functional role of global methylation patterns in the regulation of genes involved in cancer development. In order to identify specific methylation patterns associated with gene expression, an R package algorithm able to perform integrated analysis of gene expression and methylation profiles datasets was developed.

MATERIALS AND METHODS

The EpiMethEx R (R Project) package was developed to identify methylation hotspots as well as the extended methylation regions involved in gene regulation performing cyclic correlation analysis between gene expression and methylation levels of each gene. EpiMethEx stratifies the samples in three groups according to the gene expression levels. The algorithm retrieves the methylation data relative to selected genes and performs for each methylation probeset (CG) the differential analysis among the three groups of expression. CG probsets were stratified according to their relative gene region (from TSS1500 to 3'UTR regions) and CpG Island positions (CG methylation group). Finally, EpiMethEx performs differential analysis between the three groups of gene expression according to the CG methylation groups. The Cancer Genome Atlas (TCGA) Cutaneous Melanoma dataset was used to test the EpiMethEx package.

RESULTS

Our results show that the hypomethylation of the promoter regions (TSS1500, TSS200, 5'UTR) leads mainly to the gene upregulation, while the DNA methylation is associated with gene over-expression when the hypermethylation affects the gene body and 3'UTR regions. Furthermore, the frequency of genes, showing a global hypomethylation status, was higher compared to those that were hypermethylated (70% *vs* 30%). The hypomethylation of promoter was frequently identified within CpG island bodies.

CONCLUSION

EpiMethEx allows identification of the methylation patterns involved in the regulation of key genes associated with cancer development. This analysis may contribute to the development of new therapeutic strategies targeting the DNA methylation hotspot in tumor cells. Finally, EpiMethEx may be also used to identify novel biomarkers.

ROLE OF THE TRANSCRIPTION FACTOR YIN YANG 1 IN CANCER

S. VIVARELLI, S. CANDIDO, R. SALEMI, L. FALZONE and M. LIBRA

Department of Biomedical and Biotechnological Sciences, Section of General Pathology, University of Catania

Yin Yang 1 (YY1) is transcription factor ubiquitously expressed, able to either activate or repress transcription through direct or indirect interaction with the chromatin. YY1 is thought to be involved in the modulation of a large number of mammalian genes. The ability of this DNA binding protein to promote or inhibit gene expression depends on its specific target sequence, chromatin structure as well as its specific protein interactors. YY1, found expressed at different levels in many cancer types, has been seen to have the effect of either stimulating or inhibiting cancer growth. The mechanism(s) responsible for such diverging effects needs to be further clarified.

MATERIALS AND METHODS

To assess YY1 protein basal levels, several tumour cell lines were screened, including non-Hodgkin lymphoma, colorectal cancer and melanoma. Moreover, in order to assess *in vitro* if YY1 plays a role in improving the efficacy of chemotherapy, YY1 silenced cancer cell lines were generated and their survival rates compared with the parental wild-type cells, following the drug treatments. Finally, the effects on the apoptotic pathway were measured, after YY1 protein levels modulation (either through YY1 silencing or overexpression).

RESULTS

The results of the current study suggest that YY1 is often overexpressed in cancer, however different YY1 protein levels were detected in diverse tumour types. This divergence was also observed in cancer cells upon chemotherapy treatments. Likewise, the apoptotic pathway response, following YY1 silencing or overexpression was affected differently, according to the tumour type.

CONCLUSION

Altogether, our study suggests that YY1 has a dual role in cancer progression, and that role depends on the nature of the tumour. This study also indicates that YY1 may be a valuable therapeutic target in cancer.

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LOCAL ENDOTHELIAL ACTIVATION AND CONTRIBUTION TO ESTABLISHMENT OF LEISHMANIA SPP INFECTION

S. D'ALESSANDRO[1], S. PARAPINI[1], D. SCACCABAROZZI[2], L. CAVICCHINI[1], D. TARAMELLI[2] and N. BASILICO[1]

[1]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan; [2]Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan

Leishmaniasis comprises a group of neglected vector-borne diseases caused by intracellular protozoa of the genus Leishmania. The promastigotes are deposited into the human skin by the bite of phlebotomine sandflies and internalized by phagocytic cells where they develop into amastigotes, multiply and cause the cutaneous or visceral disease. Neutrophils, immediately recruited to the bite site, phagocytize Leishmania promastigotes, which elude the neutrophils killing mechanisms and infect macrophages. Although endothelial cells represent one of the first cells type encountered by Leishmania promastigotes, little is known on their role in the establishment of the infection. Here, the interaction between human endothelial cells and Leishmania promastigotes was studied.

MATERIALS AND METHODS

Human microvascular endothelial cells (HMEC-1) were exposed to promastigotes of L. infantum, L. tropica and L. braziliensis at different cell/parasite ratios. Morphology and ability of the parasites to infect THP1 macrophages after incubation with HMEC-1 was evaluated by Giemsa stain and microscopy. Production of chemokines was measured by ELISA. Leukocytes migration was evaluated by transwell system using conditioned media from HMEC-1 cultured alone or in the presence of Leishmania promastigotes as chemo-attractants. The number of migrated cells and the percentage of the different cells population were determined by microscopy.

RESULTS

Morphological changes and loss of infectivity of promastigotes were detected after 4 h of co-incubation with HMEC-1. At 2 h and 24 h, dose-dependent production of CXCL-8 by stimulated HMEC-1 was detected. A 4-fold increase in cells migrating toward conditioned medium from HMEC-1 stimulated by promastigotes respect to control conditioned medium was observed. This increase was comparable to that obtained using supernatants from HMEC-1 stimulated with TNFα or LPS. After 2 h of chemotaxis, the migrated cells were mostly neutrophils in all groups, while after 24 h of migration, a higher percentage of monocytes was present.

CONCLUSION

These data indicate that chemokines produced by endothelial cells contribute to the establishment of Leishmania infection, through the neutrophils recruitment to the site of promastigotes deposition by the sandflies.

LUNG CANCER DIAGNOSIS BY USING A HYBRID GAS-LIQUID SENSOR ARRAY. PRELIMINARY DATA

M. BIZZARRI[1], M. SANTONICO[2], G. PENNAZZA[2], R. VERNA[1] and A. D' AMICO[3]

[1]Dept of Experimental Medicine, Sapienza University Rome; [2]Unit of Electronics for Sensor Systems, Department of Engineering, Campus Bio-Medico University of Rome, Italy; [3]Department of Electronic Engineering, University of Rome Tor Vergata, Italy

Sensors performances are highly improved when single sensor arrays are organized in multidimensional systems or networks for particular applications. This result is facilitated by the large improvements in the miniaturization process, power consumption reduction and data analysis techniques nowadays available. Multidimensional sensor systems are conceived to mimic the mechanisms of human senses. Among them, the so-called electronic nose and tongue are becoming more and more popular.

MATERIALS AND METHODS

Anthocyanins are used as chemical interactive materials for both quartz microbalance (QMB) transducers (gas sensors) and for electrodes used as liquid electrochemical sensors. The optical properties of Anthocyanins are well established and widely used, but they have never been exploited as sensing materials for both gas and liquid sensors in non-optical applications. By using the same set of selected Anthocyanins an integrated system has been realized, which includes a gas sensor array based on QMB, and a sensor array for liquids made up of suitable Ion Sensitive Electrodes (ISEs). The arrays are also monitored from an optical point of view. We studied a sample of 48 individuals: 18 healthy volunteers and 26 patients affected by lung cancer.

RESULTS

Exhaled breath has been collected with a simple noninvasive procedure using Pneumopipe, a device allowing exhaled breath catching from a patient normally breathing into it for about three minutes, and exhaled gases have been successively analyzed with a gas sensor array and a liquid-interface approach. Data have been further evaluated by means of Principal Component Analysis, allowing in partitioning samples between control and pathological clusters, respectively. Routine diagnostic assessment (CT scan, X-rays, biopsy and endoscopic examination) was performed to unambiguously recognize presence and features of lung cancers. Data provided by sensor analysis allowed achieving an overall diagnostic accuracy of 85%, with only a limited number of false positive results. The entire procedure, as estimated by both patients and physicians, indicates rather satisfactory performances in terms of comfort, acceptance and usefulness. Enrollment is still running and will ultimately include 80 cancer patients.

CONCLUSION

Analysis of breathing gases through a network sensor system permits to ascertain lung cancer with high accuracy, according to a safe and quick procedure.

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FOLATE DEFICIENCY IN HEPATOCELLULAR CARCINOMA PATIENTS WITH PORTAL VEIN THROMBOSIS

G. MALAGUARNERA[1], S. LATTERI[2], V.E. CATANIA[2], G. BERTINO[3], M. PENNISI[4], F. DRAGO[1] and M. MALAGUARNERA[1]

[1]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy; [2]Department of Experimental and Clinical Medicine, University of Catania, Italy; [3]Department of Medical, Surgical Sciences and Advanced Technologies "G.F. Ingrassia", University of Catania; [4]Research Centre "The Great Senescence", University of Catania, Catania, Italy

Portal vein thrombosis (PVT) is occurring frequently in advanced liver cirrhosis and in Hepatocellular carcinoma and often diagnosed in the course of a routine patient evaluation and surveillance for liver cancer. PVT may relate to metabolic alterations and thrombophilic conditions. The purpose of this study is to investigate the relationship between folate status and portal vein thrombosis.

MATERIALS AND METHODS

In 48 patients HCC with PVT, in 52 HCC patients without PVT and in 50 randomly selected we evaluate serum and red blood cellular folate, homocysteine, alpha-fetoprotein cholesterol, triglycerides, prothrombin time.

RESULTS

HCC patients with PVT and HCC patients without PVT showed lower value of serum folate $p<0.001$ and red blood cells folate $p<0.0001$; the mean value of Homocysteine were higher too $p<0.0001$. The comparison between HCC with PVT and controls showed the mean value of serum folate was lower ($p<0.0001$), like also red blood cells folate ($p<0.0001$), while homocysteine was higher ($p<0.0001$).

CONCLUSION

In our study, we observed a lower serum and blood concentration in HCC patients with and without PVT. It has been suggested that the inactivation of DNA repair pathways, which leads to an increased mutation rate and chromosomal instability, can initiate and accelerate the proliferative process.

PARAMETERS OF EPITHELIAL-MESENCHYMAL TRANSITION AS PREDICTORS OF CANCER AGGRESSIVENESS

S. DINICOLA[1], R. VERNA[1], A. CUCINA[2] and M. BIZZARRI[1]

[1]Dept of Experimental Medicine, Sapienza University Rome; [2]Dept of Surgery Pietro Valdoni, Sapienza University of Rome, Italy

Cancer cell transition from a low-invasive phenotype into a more malignant one constitutes a critical event during tumor progression. The Epithelial-Mesenchymal Transition (EMT) occurring in solid cancers is at the core of such transformation. A recurrent metaphor for the complex developmental path of cell systems across different phenotypic states is given by the Waddington landscape in which cell phenotypes are depicted as stable attractors, while metastable or unstable states represent unstable attractors.

MATERIALS AND METHODS

Our central hypothesis is that the phenotypic transition may be described as a dynamical phase transition by considering only few system parameters and according to a multiscale approach and further mathematical modelling. That model would allow capturing the critical points of the whole process to which further focused investigations are likely to unveil pivotal targets, eventually useful for therapeutically efficient intervention. The ultimate goal is to obtain a physicochemical description of cell transition from which reliable cytological/histological markers predictive of cancer behavior could emerge.

RESULTS

The extensive survey for both biochemical and biophysical factors involved during EMT allowed to identify three main factors predictive of malignant transformation. E-cadherin values, Cytoskeleton coherence and Fractal Dimension of cell shape were found reliable predictors and hence. Development of ordinary equations based on experimentally measured values allowed simulating and depicting the transition from normal to low-malignant or high-malignant breast cancer cell. Furthermore, the simulation enabled the identification of threshold values, corresponding to the bifurcation points of the transition. Investigating such parameters in tissue/cell obtained from patients would identify pre-tumor lesions, as well as low versus potentially, high-metastatic cancers.

CONCLUSION

Mathematical modelling of EMT allows identifying a set of cytological parameters allowing the recognition of low versus potentially high-metastatic tumor. Estimation of such parameters would allow a more tailored adjuvant treatment.

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THE COENZYME Q10 AS COUNTERMEASURE FOR RETINAL DAMAGE ONBOARD THE INTERNATIONAL SPACE STATION: THE CORM PROJECT

M. LULLI[1], F. CIALDAI[2], L. VIGNALI[2], M. MONICI[2], S. LUZZI[3], A. CICCONI[3], S. CACCHIONE[3], A. MAGI[4], M. BALSAMO[5], M. VUKICH[5], G. NERI[5], A. DONATI[5] and S. CAPACCIOLI[1]

[3]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence; [1]ASAcampus Joint Laboratory, ASA Research Division, University of Florence; [2]Department of Biology and Biotechnology «Charles Darwin», Sapienza University of Roma; [4]Department of Experimental and Clinical Medicine, University of Florence; [5]Kayser Italia s.r.l.

Cells, tissues and organs of astronauts aboard the International Space Station (ISS) are exposed to the damaging effects of microgravity and cosmic radiation. Space Agencies are forced to find effective therapeutic countermeasures to safeguard astronauts' health. Since retina is one of the most vulnerable target, we undertook a project entitled The Coenzyme Q10 (CoQ10) as countermeasure for retinal damage onboard the International Space Station: the CORM project, funded by the Italian Space Agency (ASI) and launched in the summer 2017. We selected CoQ10 as promising candidate drug, having previously first demonstrated its direct antiapoptotic property due to its ability to inhibit mitochondrial depolarization.

MATERIALS AND METHODS

Beside apoptosis prevention, the parameters we are measuring to evaluate the therapeutic effectiveness of CoQ10 are attenuation of cytoskeleton modifications, lowering of telomeric DNA damages, and whole transcriptome alterations.

RESULTS

Here, we present preliminary on-ground experiments that have been carried out utilizing human retinal pigment epithelial ARPE-19 cells as a model. We revealed that CoQ10 prevents simulated microgravity-induced apoptosis and lowers the accumulation of DNA damage foci and Telomere dysfunction-Induced Foci (TIF). In addition, we present the experimental design of the CORM experiment aboard the ISS.

CONCLUSION

Since the excess of apoptosis is the main pathogenic mechanism of the most severe retinopathies such as glaucoma, age-related macular degeneration and diabetic retinopathy, results obtained from the CORM project could have an important therapeutic impact on astronauts who are facing long-term missions and on humans on ground.

CLINICAL IMPACT OF EARLY MOLECULAR RESPONSE IN CML PATIENTS TREATED WITH IMATINIB

S. STELLA [1], S.R. VITALE [2], F. STAGNO [3], M.S. PENNISI[1], M. MASSIMINO[1], E. TIRRO'[1], M. LIVIA[1], D.R. FRANCESCO[3] and P. VIGNERI[1]

[1]Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy. Center of Experimental Oncology and Hematology, A.O.U. "Policlinico-Vittorio Emanuele", Catania, Italy; [2]Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy, Center of Experimental Oncology and Hematology, A.O.U. "Policlinico-Vittorio Emanuele", Catania, Italy; [3]Division of Hematology, A.O.U. "Policlinico-Vittorio Emanuele", Catania, Italy

The approval of second-generation tyrosine kinase inhibitors (TKIs) for the first line treatment of Chronic Myeloid Leukemia (CML) has generated a need for early molecular parameters predictive of inadequate responses to the first-generation TKI imatinib mesylate (IM). Several groups have shown that BCR-ABL/ABLIS transcript levels <10% after 3 months (mos) or <1% after 6 mos of IM are strongly associated with superior clinical outcomes. We wanted to investigate which BCR-ABL threshold would be more reliable in predicting treatment outcome in patients (pts) with discordant molecular transcripts at the 3 and 6 mos time points, and also wished to evaluate if early molecular responses predicted the probability of achieving a sustained deep molecular response.

MATERIALS AND METHODS

BCR-ABL transcript levels were measured by Real-Time quantitative RT-PCR in 184 CML pts receiving IM 400 mg/die. Samples were collected at diagnosis, after 3 and 6 months of treatment and every 3/6 months thereafter. Pts were then divided into 4 groups according to their BCR-ABLIS expression at the 3 and 6 months time points:

- group A (BCR-ABLIS at 3 months <10% and at 6 months <1%)
- group B (BCR-ABLIS at 3 months <10% and at 6 months >1%)
- group C (BCR-ABLIS at 3 months >10% and at 6 months <1%)
- group D (BCR-ABLIS at 3 months >10% and at 6 months >1%)

Groups A and D included pts with concordant BCR-ABL expression as both the 3 and the 6 months transcripts were below (group A) or above (group D) the defined thresholds. On the contrary, groups B and C encompassed pts with discordant BCR-ABL levels as either the 3 months (group C) or the 6 months (group B) transcripts were above the requested values. A Kaplan-Meier method was used to calculate the univariate probability of Event Free-Survival (EFS) and the cumulative incidence of deep molecular responses (MR4) for each group.

RESULTS

Using the 2013 European LeukemiaNet recommendations, 67% of pts achieved an optimal response, 9% were classified as "warnings", 21.5% failed IM and 2.5% switched to a different TKI due to drug intolerance. With a median follow-up of 51 months, pts with concordant BCR-ABL transcripts displayed EFS and deep molecular rates that reflected their low (group A) or high BCR-ABLIS values at 3 and 6 months

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(EFS probability 86.1% *vs* 26.6%; p<0.001; MR4 probability 71.5% *vs* 16.1%; p<0.01). Among pts with discordant BCR-ABL expression, those included in group B (<10% at 3 months but >1% at 6 months) displayed significantly lower 6-year EFS as compared to those in group C (>10% at 3 months but <1% at 6 months) (EFS probability 32.7% *vs* 68.2%; p<0.001). Likewise, in group B pts, we found a lower cumulative incidence of MR4 compared to subjects assigned to group C (MR4 probability 18.2% *vs* 75%; p<0.001). Finally, when we correlated BCR-ABL transcripts measured at diagnosis with those detected after 3 or 6 months of treatment, we found that higher BCR-ABL expression was associated with BCR-ABL values >1% at 6 months (p<0.05).

CONCLUSION

Our data suggest that when BCR-ABLIS transcripts measured at 3 and 6 months present discordant values, the 6-month level displays a superior prognostic value if compared to the 3-month measurement. Furthermore, BCR-ABLIS expression >1% at 6 months is associated with inferior rates of deep molecular response. Lastly, higher BCR-ABLIS expression at diagnosis correlates with BCR-ABLIS values >1% at 6 months and, therefore, with inferior outcomes.

ADIPOKINES AFFECT PCSK9 EXPRESSION: *IN VITRO* AND *IN VIVO* EVIDENCE

C. MACCHI[1], R. MASSIMILIANO[1], B. MARGHERITA[1], D. PAOLA[2], V. LUCA[3], C. ARRIGO[4], M. PAOLO[2], F. NICOLA[5] and C. ALBERTO[2]

[1]Università degli Studi di Milano; [2]Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milano; [3]Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti; [4]Dipartimento di Scienze Mediche e Chirurgiche; [5]Università degli Studi di Padova

Adipose tissue is an endocrine organ secreting active molecules, the adipokines, e.g., leptin and resistin). In a condition of dysfunctional visceral fat depots, adipokines are detrimental for cardiovascular system. Aim: To evaluate some of the molecular mechanisms linking the biology of adipokines and PCSK9.

MATERIALS AND METHODS

HepG2 cell line, qPCR, Western blot, silencing RNA, ob/ob mice and mice fed high-fat diet.

RESULTS

In HepG2 cells, leptin and resistin induced PCSK9 gene expression by +95% and +150%, respectively. Leptin and resistin enhanced PCSK9 promoter luciferase activity by +26% and +20%, respectively, through the involvement of Sterol Regulatory Element (SRE) motif and not that of HNF-1. Leptin raised the secretion of PCSK9 in HepG2 medium by +15%. The relationship among leptin, resistin and PCSK9 expression requires the mandatory involvement of STAT3, an important modulator in the inflammatory response. STAT3 knock-down abolished leptin- and resistin-induced hepatic expression of PCSK9 mRNA. Apolipoprotein B and microsomal triglycerides transfer protein mRNA were increased by leptin (+57% and +60%, respectively) and resistin (+50% for both); these effects were dependent on PCSK9 (siRNA anti-PCSK9).

In a clinical setting (80 males, 56±4 y), a positive association between circulating leptin and PCSK9 levels ($p=0.005$) was found only in those with BMI<25 Kg/m^2. Ob/ob mice, lacking leptin, showed significantly lower hepatic PCSK9 gene expression compared to wildtype mice. In mice fed high-fat diet, a positive correlation between leptin, resistin and PCSK9 hepatic expression was found.

CONCLUSION

There is a direct relationship between PCSK9 and adipokines. The molecular basis beyond these findings requires the involvement of STAT3 pathway and the activation of SRE motif.

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MICRORNA NETWORKS INVOLVED IN NEURAL STEM CELLS MAINTENANCE

Z.M. BESHARAT[1], L. ABBALLE[1], M. MORETTI[2], A. VACCA[1], E. DE SMAELE[1], A. PO[2], M. MARODE [2] and E. FERRETTI[1]

[1]Department of Experimental Medicine, Sapienza University, Rome, Italy; [2]Department of Molecular Medicine, Sapienza University, Rome, Italy

Neural Stem Cell (NSC) maintenance is of great interest since NSCs can be used to treat impaired cells and tissues or improve regenerative power of degenerating cells in neurodegenerative diseases or spinal cord injuries. Under maintenance conditions, NSCs require Hedgehog-Gli (Hh-Gli) signalling and express a number of stemness genes (e.g. Nanog, Oct4, Sox2) whose mechanisms of regulation have been investigated. However the interplay between other transcription factors and NSC maintenance is still being charted. Technological advances allow us to use next generation RNA sequencing (RNA-seq), a powerful method for quantifying steady-state mRNA expression levels and detecting alternative splicing events in transcriptomes.

MATERIALS AND METHODS

To identify novel molecular features of the Hh-Gli pathway, we used next-generation sequencing technology to profile mRNA and microRNA expression in cerebellar NSCs, before and after induced differentiation (Diff-NSCs).

RESULTS

The use of two different mapping tools allowed us to identify known and novel isoforms, whereas the use of four different methods of differential expression analysis allowed us to identify transcripts characterizing NSC. Functional analysis of differentially expressed transcripts highlighted genes involved in a number of pathways such as focal adhesion, extracellular matrix (ECM)-receptor interaction, cell cycle, DNA replication, p53, as well as cell stress response. Taking into account that the Hedgehog pathway characterizes our NSC model we investigated transcripts implicated in the pathway. The highest expressed transcript was Forkhead Box m1 (Foxm1), part of the FOX superfamily of transcriptional regulators that play a pivotal role in cell cycle progression, proved to be directly regulated by Gli and Nanog. Foxm1 in turn regulated several microRNAs that were overexpressed in NSCs: miR-130b, miR-301a, and members of the miR-15~16 and miR-17~92 clusters and whose knockdown significantly impaired the neurosphere formation ability.

CONCLUSION

Our results reveal a novel Hh-Gli-Nanog-driven Foxm1-microRNA network that controls the self-renewal capacity of NSCs.

EFFECT OF OXYGEN LOADED NANODROPLETS ON HUMAN ENDOTHELIAL CELLS AND MACROPHAGES CULTURED IN NORMOXIC VERSUS HYPOXIC CONDITIONS

F. PEREGO[1], S. D'ALESSANDRO[2], P. MISIANO[1], S. TURINI[3], A. TROIA[4], M. PRATO[5], D. TARAMELLI[1] and N. BASILICO[2]

[1]Laboratorio di Parassitologia e Immunologia, Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano; [2]Laboratorio di Parassitologia e Immunologia, Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche, Università degli Studi di Milano; [3]Dipartimento di Oncologia, Università degli Studi di Torino; [4]INRIM Istituto Nazionale di Ricerca Metrologica, Torino; [5]Dipartimento di Neuroscienze, Università degli Studi di Torino and Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università degli Studi di Torino

Chronic wounds are an emerging issue in Western countries, but their pathogenetic mechanisms have not been clarified, yet. Oxygen availability is crucial for tissue healing. Thus, hypoxia is detrimental in chronic wounds and new oxygen-based therapies should be investigated. Here, oxygen-loaded nanodroplets (OLND) with an oxygen-binding fluorocarbon (2H, 3H-decafluoropentane) in the inner core and dextran or chitosan in the outer shell are proposed as carriers of oxygen to the hypoxic milieu, to counteract the effects of hypoxia in wound healing.

MATERIALS AND METHODS

In vitro studies were performed on two cell types involved in wound healing: microvascular dermal endothelial cells (HMEC-1) and monocytes (THP-1) differentiated into macrophages. Cytotoxicity of OLND or control formulations (oxygen-free nanodroplets, OFNDs; oxygen-saturated solution) was evaluated by trypan blue staining, MTT assay and LDH release into supernatants. Dextran or chitosan NDs were compared. Analyses were performed upon cell incubation with ND (5, 10 or 20% *vv*) for 24 h, either in normoxia (20% O2) or hypoxia (1% O2). The expression of more than 40 angiogenesis- or hypoxia-related genes in normoxic *vs* hypoxic conditions was evaluated by the RT² Profiler™ PCR Array (QIAGEN).

RESULTS

Dextran-shelled ND were not toxic towards HMEC-1 or differentiated THP-1. On the contrary, chitosan-shelled OLDN and OFDN induced a dose dependent cytotoxicity on THP-1 and HMEC-1. Hypoxia increased the expression of several genes involved in metabolism, angiogenesis and cell survival in both cell types. The down-regulated genes were related only to the angiogenic process. VEGF-A gene expression was validated by RT-PCR. Experiments to check whether NDs are able to revert some of these effects are still ongoing.

CONCLUSION

The research activity will provide new information on the ability of ONLD to counteract hypoxia effects with the aim to establish new tools for oxygen-delivery in chronic wounds.

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PHAGOCYTOSIS OF DIFFERENT STAGES OF PLASMODIUM FALCIPARUM GAMETOCYTES BY BONE MARROW MACROPHAGES

Y. CORBETT[1], S. D' ALESSANDRO[2], S. PARAPINI[2], V. MESSINA[3], F. SILVESTRINI[3], N. BASILICO[2] and D. TARAMELLI[4]

[1]Department of Biosciences and Pediatric Clinical Research Center, Università degli Studi di Milano; [2]Laboratorio di Parassitologia e Immunologia, Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche; [3]Dipartimento di Malattie Infettive, Istituto Superiore di Sanità, Università degli Studi di Milano; [4]Laboratorio di Parassitologia e Immunologia, Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano

Gametocytes (GCT), the sexual stage of malaria parasites, develop in five stages (I-V). Stages I-IV are predominantly sequestered and differentiate in the extravascular compartment of the bone marrow, while stage V are released in the circulation from where they are taken up by a mosquito during a blood meal, causing malaria transmission. Few information are available about the role of the innate immunity against GCT. The aim of this work was to set up a model to study of the ability of P. falciparum (Pf) GCT to activate and be phagocytised by bone marrow macrophages.

MATERIALS AND METHODS

GCT were obtained from the Pf transgenic strain 3D7elo1-pfs16-CBG99 expressing the luciferase CBG99 under the control of the GCT-specific promoter pfs16 (D'Alessandro et al. 2016 JAC 71:1148). Immortalized bone marrow derived macrophages (BMDM) (Hornung V et al.) were incubated with immature (II-III) or mature (V) GCT for 2 or 24h in the presence or not of Cytochalasin D. Non-internalized parasites were removed by a lysis step. The cell permeable substrate luciferin was added and phagocytosis was measured by reading the luminescence using a Synergy4 (Biotek) reader. GCT phagocytosis by BMDM was confirmed by Giemsa staining and confocal microscopy. The activation of macrophages was evaluated by measuring the production of TNF-α (ELISA) and nitric oxide (Griess assay).

RESULTS

Both immature and mature GCT were phagocytized by BMDM. Upon pre-incubation with Cytochalasin D, an inhibitor of cell phagocytosis, the luminescent signal disappeared, indicating that the method is useful for quantifying phagocytosis of GCT by BMDM. This was confirmed by Giemsa staining and confocal microscopy that showed the presence of parasites inside macrophages. When TNF-α and nitric oxide were tested in the supernatants of GCT activated BMDM, it was found that mature GCT were more stimulatory than immature GCT.

CONCLUSION

The data indicate that an active interplay occurs between Pf GCT and BMDM suggesting an important, yet unexplored role of the innate immunity against the transmission stages of malaria. Moreover, the new method to quantify phagocytosis dosing the intracellular luminescence is reliable and can be adapted to different cell models.

THE PATHOPHYSIOLOGY OF THE ENDOTHELIUM: MOVING FROM 2D TO 3D CULTURE AND BEYOND

L. LOCATELLI, A. CAZZANIGA, S. CASTIGLIONI, R. SCRIMIERI and J.A.M. MAIER

University of Milan, Department of Biomedical and Clinical Sciences L. Sacco

Vascular endothelial cells (ECs) line the entire circulatory tree. In spite of their well known heterogeneity, ECs display a myriad of fundamental homeostatic functions such as keeping blood fluid, regulating blood flow, regulating macromolecule and fluid exchange with the tissues, preventing leukocyte and platelet activation, and participating in immune surveillance. Most of present knowledge has been achieved using 2D monolayer cell culture systems. However, they do not accurately recapitulate the structure and function of living tissues. To bridge the gap between classical 2D cell culture and *in vivo* models, 3D culture techniques have been generated.

MATERIALS AND METHODS

Human umbilical vein and human microvascular ECs were cultured in perfused 3D microchannels, which mimic blood vessels, and exposed to mechanical or metabolic stress. Cell shape, cytoskeletal alignment, levels of endothelial markers and apoptosis were evaluated by confocal microscopy.

RESULTS

We show quantifiable differences between cells cultured under disturbed flow conditions as well as after metabolic stress. Actin microfilaments are randomly oriented and partially disorganized, and this correlates with cell dysfunction.

CONCLUSION

These microfluidic devices represent the gold standard to study endothelial biology and pathophysiology. In addition, they might be crucial in the maintenance of complex and multilayered organoids. Indeed, delivering nutrients and oxygen as well as removing waste will render organoids amenable for long-term culture.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

TRPM7 AND MAGT1 AS NOVEL PLAYERS IN THE OSTEOGENIC DIFFERENTIATION OF HUMAN BMSC

V. ROMEO[1], L. LOCATELLI[1], A. CAZZANIGA[1], R. SCRIMIERI[1], S. ZECCHINI[2] and S. CASTIGLIONI[1]

[1]University of Milan, Department of Biomedical and Clinical Sciences L. Sacco; [2]Department of Biomedical and Clinical Sciences, Unit of Clinical Pharmacology, University Hospital "L. Sacco"-ASST Fatebenefratelli Sacco, National Research Council-Institute of Neurosciences, University of Milan

Mesenchymal stem cell (MSCs) are crucial in bone repair and regeneration, since they differentiate into osteoblasts in response to specific stimuli from the microenvironment. It is known that magnesium is important for the osteogenic differentiation of MSCs. Since the kinase and cation channel TRPM7 and the magnesium transporter MagT1 regulate intracellular Mg homeostasis, we investigated the role of TRPM7 and MagT1 in the osteogenic differentiation of MSCs.

MATERIALS AND METHODS

MSCs were isolated from adult human bone marrow withdrawn from healthy volunteers. To induce osteogenic differentiation of MSCs, an osteogenic induction cocktail (containing 1α,25-Dihydroxyvitamin D3, β-glycerolphosphate and ascorbic acid), was added to culture medium (osteogenic medium, OM). The cells were transiently transfected with specific siRNAs for TRPM7 and MagT1.

The downregulation of TRPM7 e MagT1 after exposure to siRNA and the expression of runt-related transcription factor-2 (RUNX2) and collagen 1A1 (COL-1A1) were evaluated by Real-time PCR. Western blot was used to study the levels of the autophagic markers LC3B and beclin-1. The Tandem fluorescent-tagged LC3 assay coupled with confocal microscopy was utilized to monitor autophagy flux.

RESULTS

Real Time PCR showed that siRNAs against TRPM7 or MagT1 in MSCs boost the expression of RUNX2 and COL1A1 after cultured in OM for 3 days and accelerate the deposition of extracellular calcium deposits.

It is interesting to note that the onset of osteogenic differentiation is associated with the enhancement of autophagy as demonstrated by the induction of LC3B and beclin-1 and by the increase of autophagolysosomes in cells silenced *vs* controls. Accordingly, the inhibition of autophagy with Bafilomycin A1 prevented the overexpression RUNX2 in siRNA treated cells.

CONCLUSION

Our results indicate that MagT1 and TRPM7 contribute in coordinating the osteogenic differentiation of MSCs by preventing excessive or uncontrolled osteogenesis through the inhibition of autophagy.

AMORPHOUS SILICA NANOPARTICLES INTERFERE WITH LPS-DEPENDENT TRANSDUCTION OF INFLAMMATORY SIGNALS

M.G. BIANCHI[1], M. CHIU[1], G. TAURINO[1], E. BERGAMASCHI[2] and O. BUSSOLATI[1]

[1]Laboratory of General Pathology, Dept. of Medicine and Surgery, University of Parma, Parma; [2]Dept. of Public Health and Pediatrics, University of Turin, Turin

Besides the epithelial barriers, macrophages represent the first cells that interact with engineered nanomaterials (ENM) upon exposure. Many studies report that several ENM are able to trigger macrophage activation. However, possible interference of ENM with the effects of typical macrophage activators, such as PAMPs, has been much less investigated thus far. In this study, we have used two well characterized, food-grade nanoparticles of amorphous SiO_2 (ASNP), the precipitated NM200 and the pyrogenic NM203, to assess their effects on LPS-dependent activation of human macrophage-like cells.

MATERIALS AND METHODS

THP-1 cells were treated with low doses of ASNP (2.5-10 μg/cm^2) and after 24 h, activated with 100 ng/ml of LPS. Cytokine secretion was assessed, along with the expression of pro-inflammatory genes, autophagy markers and transduction pathway components. Metabolic effects of LPS, such as the phosphorylation of the α-subunit of the initiation factor 2 (eIF2α), mTORC1 activation and Glutamine Synthetase (GS) induction, were also monitored.

RESULTS

At the doses used, ASNP had modest effects on macrophage viability but differentially modulated LPS-induced changes. NF-κB- and p38-MAPK-dependent induction of pro-inflammatory genes, such as PTGS2, ILα and CXCL8, were not affected by ASNP. In contrast, eIF2α phosphorylation and GS induction were inhibited by both nanosilica preparations, with a greater effect of NM203. ASNP exposure also led to caspase-1 suppression and to a decrease in the LPS-dependent stimulation fold of IL-1α secretion. Conversely, ASNP did not inhibit LPS-stimulated mTORC1 activity. Moreover, ASNP produced a block of autophagy with a concomitant increase of the autophagy markers LC3II and p62, an effect partially inhibited by LPS.

CONCLUSION

These results demonstrate that ASNP interfere with some of the LPS-dependent changes associated with macrophage activation. Confirming previous results obtained in different models (Di Cristo et al., Toxicol. Sci. 150, 40–53, 2016), pyrogenic ASNP had larger effects than precipitated nanosilica. Given the use of ASNP in widely diffuse products, such as food additives, possible changes in the inflammatory response of the exposed tissues (such as the intestinal mucosa) deserve further investigations.

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FOXO3 RESTORES THE NORMAL METABOLIC FUNCTIONS THAT ARE ALTERED IN TAMOXIFEN RESISTANT BREAST CANCER CELLS

E. RICCI[1], M. FIORILLO[2], S. AQUILA[1], A. NIGRO[1], P. RIZZA[1], M. PELLEGRINO[1], M. LANZINO[1], D. SISCI[1], F. SOTGIA[2], M. LISANTI[2] and C. MORELLI[1]

[1]Department of Pharmacy and Health and Nutritional Sciences, University of Calabria, Rende, Cosenza, Italy; [2]Translational Medicine, School of Environment and Life Sciences, Biomedical Research Centre (BRC), University of Salford, Greater Manchester, United Kingdom

The potential mechanisms underlying the evolution toward an anti-estrogen resistant phenotype have been attributed to various causes, including the alteration of normal metabolism. In this context, the involvement of FoxO3 was evaluated.

MATERIALS AND METHODS

We evaluated the role of FoxO3 in the acquisition and reversion of the metabolic alterations induced by the acquired resistance to tamoxifen treatment in the ER+ breast cancer cells (BCC), MCF-7 and in its derived Tamoxifen resistant phenotype (TamR), and in a Tet-inducible TamR/FoxO3 stable clones. We performed a proteomic analysis, the evaluation of energetic activity (seahorse) and the activity of LDH and G6PDH on TamR/FoxO3 stable clones and in TamR/MCF-7 cells expressing FoxO3.

RESULTS

TamR BCC are characterized by an enhanced metabolic phenotype highlighted by a significant increase of both basal and maximal respiratory capacity. The expression of a constitutively active form of FoxO3 counteracts the increased oxygen consumption rate and extracellular acidification rate observed in TamR BCC, causing a reduction of the energetic activity and of the glycolytic rate. Interestingly, active FoxO3 increases mitochondrial biogenesis but reduces mitochondrial functionality increasing ROS production.

In addition, FoxO3a provokes a lowering effect on the biosynthetic needs of TamR breast cancer cell, evidenced by a reduction of the G6PDH activity that is associated to a reduction of of 6-phosphogluconolactonase (PGLS) and 6-Phosphogluconate dehydrogenase (PGD) expression involved respectively in step 2 and 3 of the oxidative branch of pentose phosphate pathway.

CONCLUSION

FoxO3 interferes on TamR BCC metabolism at various levels and induces a restore of the metabolic functions of TamR BCC associated to a resensitization to Tamoxifen treatment.

RETINOIC ACID INDUCED DIFFERENTIATION MODIFIES HO-1 DEPENDENT NEUROBLASTOMA RESPONSE TO OXIDATIVE STRESS

L. BRONDOLO

Department of Experimental Medicine, University of Genoa

The ability to counteract oxidative stress (OS) plays a pivotal role in neuronal survival. The differentiation of neuroblastoma (NB) cells with retinoic acid (RA) generates a small amount of ROS involved in neurite elongation (Nitti, 2010). However, differentiated cells are more sensitive to the exposure to OS than undifferentiated ones (Piras, 2017). In the present study, we analyzed the behavior of two RA-differentiated NB cell lines, SH-SY5Y and SK-N-BE(2C) in response to OS. Furthermore, the regulation of HO-1 expression has been analyzed evaluating Nrf2 as the main activator and Bach1 as negative regulator.

MATERIALS AND METHODS

SH-SY5Y and SK-N-BE(2C) NB cells were differentiated with 10□M RA for 4 days. The expression of MAP2 was evaluated by RT-PCR. Undifferentiated and differentiated cells were exposed to 500μM H2O2 or specific HO-1 inducers (10μM Hemin or 50μM tBHQ) for 6-24h. HO-1, Nrf2 and Bach1 levels were evaluated by Western Blot (WB) analysis.

RESULTS

The two NB cell lines showed different sensitivity to the treatment with RA. In differentiated SH-SY5Y cells, MAP2, a marker of neurite elongation, was up-regulated (n=3, *p<0.05 *vs* undifferentiated) while no changes were observed in SK-N-BE (2C) cells.

In both cell lines, however, the exposure to H2O2 was able to decrease cell viability only after RA-induced differentiation.

Moreover, after exposure to H2O2, Bach1 expression decreased in undifferentiated SH-SY5Y cells but not in differentiated ones (n=3, *p<0.05 *vs* ctr). Preliminary data revealed a similar trend in SK-N-BE (2C) cells.

WB analysis showed that HO-1 expression was significantly lower in differentiated SH-SY5Y cells exposed to H2O2 in comparison to undifferentiated ones (n=3, *p<0.05 *vs* H2O2 undifferentiated). The same results need to be confirmed in SK-N-BE (2C) cells.

CONCLUSION

The treatment with RA modifies HO-1-dependent response to OS by modifying Bach1-dependent regulation of HO-1.

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ROLE OF FUSOBACTERIUM NUCLEATUM IN INTESTINAL TUMORIGENESIS

P. PIGNATELLI[1], P. RAIMONDI[1], C. CELLINI[2], A. CICHELLA[2], R. GRANDE[3], A. RICCI[1], A. GENTILE[1], R. COTELLESE[1], A. PIATTELLI[1] and M.C. CURIA[1]

[1]Department of Medical, Oral and Biotechnological Sciences, 'G. d'Annunzio' University, Chieti, Italy; [2]Private Hospital "Villa Serena", Città S. Angelo, Pescara, Italy; [3]Department of Pharmacy, 'G. d'Annunzio' University, Chieti, Italy

Colorectal cancer is one of the most common malignant tumors and an important associated-factor is intestinal microbiota. This may enhance the carcinogenicity by proliferation and differentiation of epithelial cells and by decreasing immune response to pathogenic organisms. The aim of the study is to analyze the link between the presence of Fusobacterium nucleatum (FN) in a series of matched oral brushing, colon adenomas/carcinomas and adjacent mucosa, and finally to elucidate the FN dissemination mechanism from oral cavity to colon. FN is detected in saliva, especially in smokers, with its quantities increased in patients with gingivitis and periodontitis, compared to the healthy controls.

MATERIALS AND METHODS

Patients are recruited at endoscopy or surgery in private Hospital "Villa Serena. Three questionnaires are administered to patients: about lifestyle, food and oral health.

Intestinal colon adenomas or carcinomas biopsies are obtained from patients undergoing to colonscopy or surgery. At same time adjacent colonic mucosa was collected. As negative control we recruited normal colon mucosa from individuals undergoing to control colonscopy. The day before endoscopy or surgery a brushing of the oral cavity from patients is carried out. Genomic DNAs from brushing and tissue are extracted using a DNA kit (Zymo). PCR amplifications of bacterial 16S rDNA and FN fadA gene, a region identified to bind host cells and considered the best-characterized virulence factor identified, are performed.

RESULTS

The molecular analysis revealed FN presence in 50% of colon adenomas/carcinomas analyzed (6/12); similarly in adjacent mucosa. FN was also detected in 2 out of 5 oral swabs analyzed, but at first analysis in a more abundant way compared to tissues. Intriguingly these 2 patients are smokers and affected by perodontal disease. The quantitative analysis of the abundance of FN by Real-Time PCR is in progress. Normal colonic mucosa analyzed showed FN absence.

CONCLUSION

These results show FN presence in patients' oral cavity and colon tumor tissue, and absence in control individuals' normal colonic mucosa. The progress of patient recruitment will allow shedding light on fadA levels and their field effect. The final data may lead to the identification of biomarkers for the early diagnosis and prevention of CRC.

EFFECT OF RESVERATROL ON WNT/ β-CATENIN SIGNALLING IN PANCREATIC CANCER CELL LINES

M.C. CURIA[1], S. VESCHI[2], G. FABIETTI[1], L. IEZZI[1], R. FLORIO[2], L. DE LELLIS[2], G. ACETO[1] and A. CAMA[2]

[1]Department of Medical, Oral and Biotechnological Sciences, G. d'Annunzio University, Chieti, Italy; [2]Department of Pharmacy, G. d'Annunzio University, Chieti, Italy

Pancreatic cancer is a very aggressive malignant disease due to lack of early diagnosis and chemotherapeutic resistance of the tumor cells. There is distinct evidence that food derived polyphenols possess chemopreventive effects in the development of several cancers including pancreatic carcinoma. Resveratrol, a phenolic compound found in grape skins and other fruits, is a nutraceutical with several therapeutic effects and known anticancer activity. The aim of the study is to analyze the expression of Wnt/ β-catenin signaling pathway genes after treatment with resveratrol.

MATERIALS AND METHODS

Human pancreatic cancer (PC) cell lines AsPC-1 and Capan-2 were purchased from Cell Lines Service (Germany), BxPC-3 from ATCC (VA, USA). AsPC-1 and Capan-2 carry KRAS mutations, while BxPC-3 and AsPC-1 are TP53 mutated. Resveratrol was obtained from Sigma (MO, USA).

Cells were incubated for 72h with resveratrol at the indicated concentrations (50 e 100 μM) or with 0.11% DMSO vehicle (control). Cell viability was assessed by MTT assay. The mRNA expression of genes related to the Wnt/β-catenin pathway were detected by quantitative Real-Time PCR in triplicate.

RESULTS

We analyzed by MTT the effect of resveratrol on the viability of three PC cell lines. The drug significantly affected cell viability in a dose-dependent manner, with distinct sensitivities for the three cell lines. In AsPC-1, BxPC-3 and Capan-2 resveratrol showed IC50 values of 28.65 μM, 153.98 μM and 252.56 μM respectively. We also analyzed by Real-Time PCR the expression of Wnt/β-catenin signaling receptors (ROR and LPR6), trascription factor (LEF) and target (cyclin D1) genes in resveratrol-treated pancreatic cancer cell lines. Resveratrol inhibits Wnt/β-catenin signaling genes differently in the three cell lines. In particular, the expression of LEF and cyclin D1 genes was considerably downregulated in AsPC-1 cell line compared to control.

CONCLUSION

These results demonstrate that resveratrol significantly inhibited cell viability in the PC cell lines in a dose-dependent manner. Furthermore, we showed that resveratrol treatment reduces the expression of important and crucial genes of Wnt/β-catenin pathway. The two results together provides useful considerations to establish a new chemotherapeutic regimen for pancreatic cancer.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

EFFECT OF A NUTRACEUTICAL COMBINATION CONTAINING THE PROBIOTIC BIFIDOBACTERIUM LONGUM BB536 AND RED YEAST RICE EXTRACT ON CARDIOVASCULAR RISK BIOMARKERS - A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY

M. RUSCICA[1], C. PAVANELLO[2], S. GANDINI[3], C. MACCHI[1], M. BOTTA[1], D. DELL'ORTO[1], M. DEL PUPPO[4], M. BERTOLOTTI[5], R. BOSISIO[6], G. MOMBELLI[6], C.R. SIRTORI[6], L. CALABRESI[2] and P. MAGNI[7]

[1]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy; [2]Dipartimento di Scienze Farmacologiche e Biomolecolari, Centro E. Grossi Paoletti, Università degli Studi di Milano, Milan, Italy; [3]Division of Epidemiology and Biostatistics, European Institute of Oncology, Milan, Italy; [4]Dipartimento di Medicina e Chirurgia, Università degli Studi di Milano Bicocca, Monza, Italy; [5]Dipartimento di Scienze Biomediche, Metaboliche e Neuroscienze, Università degli Studi di Modena e Reggio Emilia, Modena, Italy; [6]Centro Dislipidemie, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy; [7]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy and IRCCS MultiMedica, Sesto S. Giovanni, Milan, Italy

In subjects with moderate hypercholesterolemia, probiotics incorporated into dairy products can reduce circulating total (TC) and LDL cholesterol (LDL-C). Probiotics with high biliary salt hydrolase activity, e.g. Bifidobacterium longum BB536, decrease TC and LDL-C by lowering intestinal cholesterol reabsorption and, combined with other nutraceuticals, may be useful to manage hypercholesterolemia in subjects with low cardiovascular (CV) risk. The objective of the study was to evaluate efficacy and safety of a nutraceutical combination containing Bifidobacterium longum BB536, red yeast rice (RYR) extract (10 mg/day monacolin K), niacin, coenzyme Q10 (Lactoflorene Colesterolo®). The end-points were changes of lipid CV risk markers (LDL-C, TC, non-HDL-cholesterol (HDL-C), triglycerides (TG), apolipoprotein B (ApoB), HDL-C, apolipoprotein AI (ApoAI), lipoprotein (a) [Lp(a)], proprotein convertase subtilisin/kexin type 9 (PCSK9) and of markers of cholesterol synthesis and absorption.

MATERIALS AND METHODS

The study had a randomized, parallel, double blind, placebo-controlled design. Intervention duration: 12 weeks. ClinicalTrials.gov n°NCT02689934. Thirty-three subjects (18-70 years) in primary CV prevention/low CV risk (SCORE: 0-1% in 24 and 2-4% in 9 subjects; LDL-C: 130-200 mg/dL) were randomly allocated to nutraceutical (N=16) or placebo (N=17). The nutraceutical combination contained 1 bn UFC Bifidobacterium longum BB536, RYR extract (10 mg monacolin K), 16 mg niacin, 20 mg coenzyme Q10.

RESULTS

A 12-week treatment with the nutraceutical combination, compared to placebo, significantly reduced TC (-16.7%), LDL-C (-25.7%), non-HDL-C (-24%) (all $p<0.0001$), oxidized LDL ($p<0.05$), apoB (-17%, $p=0.003$). TG, HDL-C, apoAI, Lp(a), PCSK9 were unchanged. Lathosterol:TC ratio (a marker of

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choelsterol synthesis inhibition) was significantly reduced by the nutraceutical combination; campesterol:TC ratio and sitosterol:TC ratio (markers of intestinal cholesterol absorption) did not change. No adverse effects and a 97% compliance were observed.

CONCLUSION

A 12-week treatment with a nutraceutical combination containing the probiotic Bifidobacterium longum BB536 and RYR extract was well tolerated by low CV risk subjects and significantly improved the atherogenic lipid profile.

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THE ANTIBIOFILM EFFECT OF A MEDICAL DEVICE (MD) CONTAINING TIAB ON MICROORGANISMS ASSOCIATED WITH SURGICAL SITE INFECTIONS (SSIS)

V. PUCA[1], T. TRAINI[2], S. GUARNIERI[3], R. GRANDE[4], S. CARRADORI[5], F. SISTO[6], N. MACCHIONE[7], G. MINCIONE[8] and R. MURARO[8]

[1]Department of Medical, Oral, and Biotechnological Sciences, "G. d'Annunzio" University of Chieti-Pescara Chieti, Italy. Center of Excellence on Aging and Translational Medicine (CeSI-MeT), Chieti, Italy; [2]Department of Medical, Oral, and Biotechnological Sciences, "G. d'Annunzio" University of Chieti-Pescara Chieti, Italy. Center of Excellence on Aging and Translational Medicine (CeSI-MeT), Chieti, Italy; [3]Department of Neuroscience, Imaging and Clinical Sciences, "G. d'Annunzio" University of Chieti-Pescara Chieti, Italy. Center of Excellence on Aging and Translational Medicine (CeSI-MeT), Chieti, Italy; [4]Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Chieti, Italy. Center of Excellence on Aging and Translational Medicine (CeSI-MeT), Chieti, Italy; [5]Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Chieti, Italy; [6]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; [7]Department of Urology, University of Milan, ASST Santi Paolo e Carlo, Milan, Italy; [8]Department of Medical, Oral, and Biotechnological Sciences, "G. d'Annunzio" University of Chieti-Pescara Chieti, Italy

Surgical Site Infections (SSIs) are the most common nosocomial infections. Surgical sutures are optimal surfaces for bacterial adhesion and biofilm formation. The most commonly isolated microorganisms in SSIs are Staphyloccocus aureus (Sa), coagulase-negative Staphylococcus spp., Enterococcus spp., Escherichia coli (Ec) and other Gram-negative bacilli. The aim of this study was to evaluate the antibiofilm activity of a Medical Device (MD) containing TIAB, which is titanium dioxide linked with monovalent silver ions.

MATERIALS AND METHODS

Sa ATCC 29213, Enterococcus faecalis (Ef) ATCC 29212, and Ec ATCC 25922 were the micro-organisms used in the study. The antibacterial effect was evaluated by determining the Minimum Inhibitory Concentration (MIC) using the broth microdilution method and the Alamar Blue (AB) assay. The antibiofilm effect was determined by the evaluation of Minimum Biofilm Inibitory Concentration (MBIC). Biofilm inhibition was confirmed by the Live/Dead Cell Viability Staining and Fluorescence Microscopy Analysis. The Colony Forming Unit (CFU) count was performed. The MD was applied on different surgical sutures exposed to the bacterial species. The antimicrobial effect was evaluated by the Agar Diffusion Test (ADT) method, while, the antibiofilm activity was evaluated by Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM).

RESULTS

The MIC was determined for Sa and Ef at 2 mg/mL while the MBIC was determined at 1.5 mg/mL for

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Sa and 1 mg/mL for Ef. The formation of an inhibition zone around the treated sutures on agar plates confirmed the antimicrobial activity of MD. The morphological analysis in SEM underlined the presence of a few adhesive cells on the sutures coated with the MD in respect to the control. The functional analysis in Live/Dead Staining and CLSM showed that in treated samples, the majority of adhesive cells were dead.

CONCLUSION

The MD containing TIAB, showed antimicrobial and antibiofilm activities versus Sa and Ef. The MD showed less efficacy against Ec. Surgical sutures coated with the MD have the potential to reduce SSIs as well as the risk of biofilm formation in post-surgery.

ANALYSIS OF ESTROGEN–MEDIATED SIGNALING REVEALS A NOVEL EPIGENETIC TARGET FOR ENDOCRINE THERAPY-RESISTANT BREAST CANCER TREATMENT

G. GIURATO[1], G. NASSA[1], A. SALVATI[1], R. TARALLO[1], F. RIZZO[1], V. GIGANTINO[1], L. MAUTONE[1], T.A. NYMAN [2] and A. WEISZ [1]

[1]Laboratory of Molecular Medicine and Genomics, Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno, Baronissi (SA), Italy; [2]Proteomics Core Facility, Institute of Clinical Medicine, University of Oslo, Oslo, Norway

Estrogen Receptor alpha (ERα) is the master regulator of estrogen signaling in hormone-responsive breast cancer (BC) and it is the primary target of specific anticancer therapies. Nevertheless, the development of resistance to treatment represents the key problem in clinical management of patients affected by this disease. We reported that the epigenetic writer DOT1L (DOT1-Like Histone Lysine Methyltransferase) associates with ERα [1] as part of a multiprotein chromatin regulatory complex. We investigated here the role of this enzyme in estrogen signaling to the genome in luminal-like BC cells.

MATERIALS AND METHODS

The involvement of DOT1L in mediating ERα actions on the genome and evaluation of its functional impact in hormone-responsive and antiestrogen-resistant BC cells was investigated by a combination of chromatin immunoprecipitation coupled to Mass-Spectometry (ChIP-MS) or sequencing (ChIP-Seq), gene silencing and selective pharmacological inhibition of DOT1L, followed by steady-state (RNA-Seq) and nascent (Nascent-Seq) transcriptome profiling, in human BC cells.

RESULTS

ChIP-MS revealed co-recruitment of the two proteins within a chromatin multiprotein complex comprising several other transcriptional regulators, while global analysis of ERα and DOT1L binding to the genome showed co-recruitment of both proteins on several (>2.000) chromatin sites. Gene expression profiling before and after DOT1L pharmacological inhibition confirmed direct involvement of the enzyme in ERα-mediated transcriptional regulation of estrogen responsive genes. DOT1L silencing by RNAi resulted in BC cell growth inhibition and apoptotic death, and integration of ChIP-Seq and transcriptome data provided a genetic explanation for control of BC cell proliferation and survival by the ERα-DOT1L complex. Interestingly, antiestrogen-resistant cell lines modeling endocrine therapy-resistant BC were found to be affected by DOT1L pharmacological inhibition both *in vitro* and *in vivo*.

CONCLUSION

These results reveal for the first time how the physical and functional interplay between ERα and DOT1L represents a key molecular event in estrogen signaling controlling BC cell functions, and suggest that this epienzyme represents a potential therapeutic target against endocrine therapy-resistant breast tumors.

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EVALUATION OF DNA BASE EXCISION REPAIR AND ERBB SIGNALING IN EUTHYROID GOITERS

C. MOSCATELLO[1], M.C. DI MARCANTONIO[1], A. CICHELLA[2], G. SPACCO[3], G. MINCIONE[3], A. MARCHETTI[2], R. COTELLESE[1], R. MURARO[1] and G.M. ACETO[1]

[1]Department of Medical, Oral and Biotechnological Sciences, University 'G. d'Annunzio-Chieti-Pescara', Italy; [2]Postgraduate School of General Surgery; [3]Center of Predictive Molecular Medicine, Center for Excellence on Ageing and Translational Medicine (CeSI-MeT), University of Chieti-Pescara, Chieti, Italy

The onset of thyroid gland nodules (TN) is determined by loss of homeostasis regulation by interaction of endogenous/genetic and environmental factors. The nature of the oxidative processes that lead to physiologic synthesis of thyroid hormones together with environmental factors may be the root causes of thyroid nodular transformation due to an increase in DNA damages. A link between EGF and DNA damage repair signalling has been documented in cancer, but their cross-regulation is poorly understood in TN. In order to define the role of Base Excision Repair (BER) and ErbB signaling in TN, we analysed euthyroid goitres tissues that are not theoretically affect by any hormonal dysfunction.

MATERIALS AND METHODS

We analysed euthyroid goiter tissues obtained from patients undergoing thyroidectomy at Unit of General and Laparoscopic Surgery, SS. Annunziata Hospital. Gene and protein expression of ErbB and BER pathways were evaluated by qRT-PCR and Western Blot. The results were subjected to T-test analysis; statistical significance was set at $p < 0.05$. Gene expression data were evaluated by bioinformatics tools.

RESULTS

In this study, euthyroid goiters cases were divided into two groups based on patient clinical data report with and without inflammatory features (IG *vs* NIG). Real-time analyses revealed a strong expression of NRF2, ERBB2 and JUN/AP1 in all 28 euthyroid goiters tissues although it was lower in IG *vs* NIG. The average expression of the HO-1, IL-8 and MUTYH genes, was significantly higher in IG *vs* NIG. Whereas, OGG1 and PPARγ gene expression of did not vary between the two groups. Cluster analysis showed a correlation between IL-8 and JUN/AP1 in IG but not in NIG where JUN/AP1 resulted related to ErbB2 and MUTYH. WB analyses revealed that OGG1 high weight isoform in all NIG but low weight isoform only in IG. The EGFR receptor resulted expressed in NIG/IG and was activated in 50% of them.

CONCLUSION

From this study emerges the need to deepen the aspects concerning the physiological and pathological crosstalk between the BER and ErbB pathways in the better understanding of the mechanisms of onset and development of thyroid diseases on the light of c-JUN-AP1 pivotal role in maintenance of the self-renewal and tumorigenicity.

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TRIGGERING OF TOLL-LIKE RECEPTORS IN THE ELDERLY. A PILOT STUDY RELEVANT FOR VACCINATION

C.M. GAMBINO[1], G. ACCARDI[1], A. AIELLO[1], K. IOANNOU[2], F. FARZANEH[2], S. VASTO[3], C. CARUSO[1], F. GERVASI[4] and G. CANDORE[1]

[1]Department of Pathobiology and Medical Biotechnologies (Di.Bi.Med.), University of Palermo, Palermo, Italy; [2]Department of Haematological Medicine, King's College London, Rayne Institute, London,UK; [3]Department of Biological Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Italy; [4]Specialistic Oncology Laboratory Unit, ARNAS Hospital, Palermo, Italy

Immunosenescence impacts on the immune system of older people leading to an inefficient reaction to pathogens and reduced ability to develop a powerful immune response after vaccination. Recently, research in immunological ageing have suggested that stimulation of Toll-like receptors (TLRs) is a promising strategy to enhance vaccine efficacy in the elderly by activation of innate immune cells and production of inflammatory cytokines. In this scenario, great importance assume dendritic cells (DCs), the most potent antigen presenting cells, specialized for the uptake, processing, transport and presentation of antigens to T cells.

MATERIALS AND METHODS

We stimulated total PBMCs isolated from 15 10-year-olds, and 10 healthy centenarian's offspring (CO) donors with two complementary TLR agonists, R848 and MPLA. The nature and magnitude of IL-6, TNF-α and IL-12/p40 pro-inflammatory cytokine production was assessed using multi-parameter flow cytometry. Statistical analyses were performed using GraphPad Prism software; the significant level of p value was determined using Student t-test ($p<0.05$).

RESULTS

Our preliminary *in vitro* experiments suggest that the stimulation of cells with the combination of agonists for TLR7/8 and TLR4 (R848 and MPLA respectively), is *de facto* an excellent inducer of TNF-α, IL-6, and IL-12/p40 cytokines in myeloid, as well as plasmacytoid DCs from young, old and CO subjects, with significantly high levels of cytokines compared to unstimulated samples.

CONCLUSION

Our preliminary results demonstrate that TLR activation by both agonist combination can significantly enhance the activation of DCs in PBMCs isolated from healthy elderly donors despite their immunosenescent phenotype. This data suggest that the inclusion of appropriate combination of TLR agonists may enhance the efficacy of vaccination in the elderly, and point towards the potential use of TLR agonists in future vaccination approaches for several therapeutic strategies.

ROLE OF KIR AND HLA INTERACTION IN HIV-INFECTED PATIENTS WITH LOW-LEVEL VIREMIA

M.E. LIGOTTI[1], C.M. GAMBINO[1], A. AIELLO[1], G. ACCARDI[1], C. CARUSO[1], C. COLOMBA[2], D. DI BONA[3] and G. CANDORE[1]

[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Palermo, Italy; [2]Department of Health and Maternal-Infant Promotion Sciences "G. D'Alessandro", Università di Palermo, Palermo, Italy; [3]Department of Emergency and Organ Transplants, University of Bari Aldo Moro, Bari, Italy

The impact of host genetic variation on outcome of HIV infection in the absence of combination antiretroviral therapy (cART) has been well documented. Growing evidence reports relationship between specific killer-cells immunoglobulin-like receptor (KIR) polymorphisms and different HIV progression in the absence of treatment. The goal of the present study is to determine the association of KIR genes and their human leukocytes antigen (HLA) ligands with viral load and HIV progression in patients on cART.

MATERIALS AND METHODS

To date, genomic DNA from 11 HIV-infected patients on cART with residual viremia (case group) and 10 HIV-infected patients on cART with virologic suppression (control group) were genotyped by SSP-PCR. Preliminary comparison of genotype frequencies between both groups were analysed using 2×2 contingency tables.

RESULTS

Statistical analysis does not show any significant results and it is probably due to the actually small sample size. Nevertheless, in our preliminary observation it would seem that subjects with virologic suppression have higher frequency of 2DS1, 2DS5, and 3DS1 KIR genes in comparison to subjects with residual viremia.

CONCLUSION

Achieving undetectable viral load is the primary goal of cART. To date, up to 30% of patients on cART fail to achieve a satisfactory immunological recovery and have transient episodes of detectable plasma viremia or persistent viral rebound. Unfortunately, due to low number of subject analysed, in this preliminary study we actually did not observe significant statistical association between KIR/HLA genetic variation and HIV outcome. However, taken together recent data from literature, it is plausible to speculate that KIR/HLA interactions might play a key role in different virologic responses to cART. We hope that the completion of the study will allow us to confirm this hypothesis.

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A CROSSTALK BETWEEN PROGESTERONE RECEPTOR B AND P53 ALTERS THE METABOLIC REPROGRAMMING IN BREAST CANCER CELL LINES

M. SANTORO, D. DE ROSE, F. DE AMICIS, V. RAGO, R. MALIVINDI and S. AQUILA

Department of Pharmacy and Sciences of Health and Nutrition - University of Calabria, Arcavacata di Rende (CS) Italy

Progesterone-Receptor (PR) is good prognostic marker for breast cancer patients. The effects of Progesterone on its target tissues are mediated by the Progesterone Receptors (PR-A and PR-B). In breast tumors from patients with poor prognosis, a loss of PR-B occurs suggesting a protective role of PR-B in breast cancer. The molecular mechanisms of PR-B–protective effects are still to be defined. Recently, we reported in breast cancer cells a novel functional interplay between PR-B and PTEN in modulating autophagy, which is retained a way to reprogram cellular metabolism activating oncogenes and inactivating tumor suppressors. Among them, the p53 tumor suppressor gene has emerged as important mediator of energy metabolism. To deepen the PR-B anticancer role and given the relationship between autophagy and cellular metabolism, we investigated a role for OHPg/PR-B signaling through p53 in altering metabolic reprogramming of breast cancer cells.

MATERIALS AND METHODS

Enzymatic activities were studied with kits using spectrophotometric method. Key proteins of different metabolic branches were studied by Western Blotting Analysis. Silencing of PR-B and p53 by specific siRNA were used to evaluate their link in the alteration of breast cancer metabolic reprogramming.

RESULTS

Using MCF7 and T47D human breast-cancer cell lines and increasing OHPg concentrations, we disclosed a PR-B/p53-action on different metabolic ways. OHPg/PR-B collapsed glucose metabolism significantly affecting glycolysis and the PPP, while LDH activity significantly increased. AMPK expression increased upon OHPg while ATP content and OXPHOS complexes expression were significantly reduced. Glutaminolysis refueled a 'truncated' TCA cycle, ACLY expression increased while ACC and FAS were reduced indicating a poor de novo lipid synthesis. Presumable the ACLY-induced expression diverts versus the OAA formation explaining at least in part, the LDH activity increase. Lipase activity and FAO increased concomitantly to the reduction of triglycerides level and this may represent a compensatory mechanism to obtain energy by lipid degradation.

CONCLUSION

Here we reported, for the first time, that OHPg/PR-B hardly disturbs breast cancer cell metabolism. Intriguingly, OHPg/PR-B concertize with p53 in perturbing breast cancer cells metabolic reprogramming.

D-DIMER PLASMATIC LEVELS INCREASE IN HEPATOCELLULAR CARCINOMA WITH PORTAL VEIN THROMBOSIS

M. MALAGUARNERA[1], S. LATTERI[2], G. BERTINO[3], R. MADEDDU[4], M. PENNISI[5], V.E. CATANIA[2], G. CURRÒ[6], A.M. BORZÌ[5], F. DRAGO[7] and G. MALAGUARNERA[7]

[1]Department of Biomedical and Biotechnological Science, University of Catania and the Research Center "The Great Senescence", University of Catania; [2]Department of Medical, Surgical Sciences and Advanced Technologies "G.F. Ingrassia", University of Catania; [3]Department of Experimental and Clinical Medicine, University of Catania; [4]Department of Biomedical Sciences, University of Sassari; [5]Research Center "The Great Senescence", University of Catania; [6]Department of Human Pathology, University of Messina; [7]Department of Biomedical and Biotechnological Science, University of Catania

Portal vein thrombosis (PVT) is one of the severe complications of Hepatocellular Carcinoma (HCC). PVT deteriorates the liver and its dysfunction increases the risk of bleeding, influencing the prognosis of patients with liver cirrhosis and HCC. The aim of our study was to investigate whether D-dimer testing could be a sensitive marker for the diagnosis and prognosis of HCC patients with PVT.

MATERIALS AND METHODS

Between June 2010 and December 2015 118 HCC patients were admitted to Cannizzaro Hospital, Catania, and 50 controls were recruited from the relatives for health examinations. All enrolled patients were diagnosed and pathologically confirmed as HCC. D-dimer was measured with an enzyme linked non-immunosorbent assay using two monoclonal antibodies against non-overlapping determinants of D-dimer.

RESULTS

D-dimer in HCC patients with PVT were significant higher versus HCC patients without PVT ($p<0.002$) and versus controls ($p<0.001$).

CONCLUSION

Plasma D-dimer is a sensitive marker of fibrin turnover and allows the recognition of activated coagulation, which may be manifest in HCC with PVT.

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γ-GLUTAMYLTRANSFERASE MODULATES L-γ-GLUTAMYL-P-NITROANILIDE (GPNA) CYTOTOXICITY: THE POOR SPECIFICITY OF AN OLD INHIBITOR OF GLUTAMINE TRANSPORT

A. CORTI[1], S. DOMINICI[1], S. PIAGGI[1], E. BELCASTRO[2], M. CHIU[3], G. TAURINO[3], S. PACINI[4], O. BUSSOLATI[3] and A. POMPELLA[1]

[1]Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Italy; [2]Dep. of Translational Research NTMS, Univ. of Pisa, Italy; INSERM (French National Institute of Health and Medical Research), UMR 1260, Regenerative Nanomedicine, FMTS, Strasbourg, France; Université de Strasbourg, Faculté de Pharmacie, Strasbourg, France; [3]Department of Medicine and Surgery, University of Parma; [4]Department of Clinical and Experimental Medicine, University of Pisa, Italy

Glutamine (Gln) plays a critical role in supporting cell growth and proliferation of different cancer cell types. Several studies have focused on the sodium-dependent Gln transporter ASCT2 as a potential therapeutic target and different approaches, including its inhibition or silencing, are commonly used. L-γ-glutamyl-p-nitroanilide (GPNA) is a widely used ASCT2 inhibitor, even if several concerns about its specificity have recently emerged since GPNA has a limited selectivity for ASCT2, being able to inhibit many other transporters for amino acids.. Moreover, it is rarely considered that GPNA is also a well-known substrate of the enzyme gamma-glutamyltransferase (GGT). The aim of this study was thus to evaluate the effect of GPNA catabolism by GGT on its efficacy as an inhibitor of cell proliferation.

MATERIALS AND METHODS

The human lung cancer cell line A549 expressing sizable levels of both ASCT2 and GGT was used. The effect of GPNA on Gln transport, cell viability and apoptosis were analyzed.

RESULTS

The GGT-catalyzed hydrolysis of GPNA produced cytotoxic effects in A549 cells resulting from the release of the chromogen p-nitroaniline (PNA) rather than the inhibition of Gln uptake. These effects were completely prevented by GGT inhibition. Interestingly, compounds like valproic acid, verapamil and reversan were able to increase the cytotoxicity of GPNA and PNA, thus suggesting a key role of intracellular detoxification mechanisms in the effects observed.

CONCLUSION

Our data indicate that the mechanism of action of GPNA is more complex than expected and further confirm the poor specificity of GPNA as an inhibitor of Gln transport. We propose that other factors could contribute to the final effects of GPNA, including GGT expression and the levels of intracellular defences against xenobiotics. Other strategies, such as a genetic suppression of ASCT2 or the identification of new specific inhibitors, should be preferred when ASCT2 has to be inhibited. However, in selected cancer cell types expressing high GGT activities, GPNA may effectively exert a significant, GGT-dependent toxicity.

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XCT/CD44 MODULATION INDUCES THE SENSITIZATION OF NEUROBLASTOMA-STEM CELLS TO ETOPOSIDE

A. SPECIALE[1], B. MARENGO[1], N. TRAVERSO[1], L. MONTELEONE[1], C. CANTONI[1], S. RAVERA[2], O. GARBARINO[1], M.A. PRONZATO[1] and C. DOMENICOTTI[1]

[1]Department of Experimental Medicine, University of Genoa, Italy; [2]Department of Pharmacy, University of Genoa, Italy

The current anticancer therapies for human neuroblastoma (NB) include the use of etoposide, a drug that exerts its cytotoxic effect by inducing oxidative stress. Although the therapeutic approach with etoposide is initially efficacious, subsequently, it selects a chemoresistant cell population, which is able to adapt to the oxidative environment induced by the drug, increasing the levels of intracellular glutathione (GSH). The chemoresistance is also due to the presence of cancer stem cells (CSCs), which have a low proliferative rate and are less susceptible to therapies acting on highly proliferating cells. Moreover, CSCs expressing CD44, a PKC-alpha-modulated staminality marker, are able to influence GSH levels by stabilizing xCT, a transporter promoting the influx of cystine, essential for GSH synthesis. Based on this evidence, our aim was to investigate whether targeting CD44/xCT might be a useful strategy to increase neuroblastoma sensitivity to etoposide.

MATERIALS AND METHODS

CSCs selected from HTLA-230, a malignant human NB cell line, and from etoposide-resistant HTLA (HTLA-ER), were treated once a week with 1.25μM etoposide alone or in combination with 0.5μM PKC-alpha peptidic inhibitor (C2-4) or 5μM sulfasalazine (SSZ), an xCT inhibitor. The treatments were carried out for 2 and 5 weeks. CSC propagation, staminality marker expression (FACS analysis), GSH levels (HPLC analysis) and metabolic parameters were analyzed.

RESULTS

The single treatment with etoposide is able to deplete GSH and to reduce proliferation in both sensitive and drug-resistant CSC populations. Notably, sensitive CSCs respond to etoposide already after 2 weeks while the resistant CSCs after 5 weeks. Interestingly, co-treatments with SSZ or C 2-4, exerted a drug-sensitizing effect in resistant CSCs by anticipating GSH depletion and anti-proliferative action and inducing a switch towards an anaerobic metabolism with the consequent increase of lactic acid and the induction of lipid peroxidation, two events responsible for the death of cancer cells.

CONCLUSION

Collectively, our data demonstrates that the modulation of CD44/xCT in association with etoposide, by reducing GSH levels and increasing lipid peroxidation, could be an effective strategy to sensitize CSCs to etoposide.

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A 47-YEAR-OLD MAN WITH A 2-YEAR HISTORY OF MISDIAGNOSED DYSPHAGIA

C. SITZIA[1], V. SANSONE[2], R. RIGOLINI[3], P. GIUBBILINI[3], B. RAMPOLDI[3], C. FULCERI[3], E. COSTA[3] and M.M. CORSI ROMANELLI[4]

[1]Scuola di Specializzazione di Patologia Clinica e Biochimica Clinica, Università degli Studi di Milano; [2]Centro Clinico Nemo, Milan, Italy; Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; [3]Service of Laboratory Medicine1-Clinical Pathology, I.R.C.C.S. Policlinico San Donato, San Donato Milanese, Milan, Italy; [4]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; Service of Laboratory Medicine1-Clinical Pathology, I.R.C.C.S. Policlinico San Donato, San Donato Milanese, Milan, Italy

Here we present a clinical case of 2 year-misdiagnosed polymyositis (PM), where patient underwent unnecessary invasive procedure while he developed progressive muscular weakness. Erroneous diagnosis could have been correctly addressed by evaluation of serum Creatine kinase (CK), a cheap and easy to perform lab exam.

MATERIALS AND METHODS

A 47-year-old man had a Medical history of 2-year of progressive inability to swallow solid foods and liquids and unexplained 15kg weight lost. EGD excluded Gastritis and reflux esophagitis; lab tests showed increased transaminase levels. Patient was screened for different causes of hepatopathy including infection, autoimmunity, deficit of α1-antitrypsin and metabolism disorder: all tests resulted negative. Two abdominal ultrasound excluded primary hepatic disease, but patient progressively developed muscular weakness and hypophonia. Finally, he arrived to our ED presenting general weakness, hyposthenia of cervical flexor and limb girdles, elevation of transaminase levels and serum hyperCKemia of 23741 U/L - a value 100-fold higher than normal that led to muscular biopsy. EMG analysis and RNS excluded Myasthenia Gravis.

RESULTS

Patient's biopsy of left brachial biceps showed the presence of atrophic and necrotic fibers, myofibers HLA over-expression and CD4+ and CD8+ infiltrating cells thus PM was diagnosed and steroid therapy started (Deltacortene 75mg/die). A tumor markers screening excluded paraneoplastic polymyositis: elevation of NSE was observed but Ct scan of thorax, abdomen and brain did not show any neoplastic mass. Immunoglobulin IG adjuvant treatment (0.4mg/kg/die) was necessary to observe a mild improvement of muscular symptoms. CK at discharge was 4210 U/L. After 2 months, general muscular strength greatly improved and after two years patient's CK were behind normal threshold and he presented full functional recovery of muscular strength.

CONCLUSION

PM is a rare disease showing a typical sub-acute onset of proximal muscular weakness that progress to a chronic state. Diagnosis is based on clinical presentation and supported by Lab investigations, including serum CK, myositis antibodies and muscular biopsy. Esophageal or pharyngeal

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involvement determines dysphagia in one third of patients. In this sense, unexplained oropharyngeal dysphagia should always prompt the suspicion of inflammatory myopathy. Lab tests as CK could be detrimental for the diagnosis that could took over 55 months. In this patient CK have never been evaluated whereas unspecific transaminase elevation addressed clinical investigation towards hepatic disease.

The 100-fold CK increase could have helped to reach the correct diagnosis over 12 months before patient's hospitalization. In this sense, we suggest that CK evaluation should be included in first line assessment of any unexplained condition of muscular weakness or dysphagia thus avoiding prolonged chronic inflammation that could alter muscular architecture and require additional treatments as Immunoglobulin.

HYPOMETHYLATING AGENT 5-AZA-2'-DEOXYCYTIDINE (DAC) AMELIORATES THE CLINICAL COURSE OF RHEUMATOID ARTHRITIS IN A MOUSE MODEL

P. FAGONE[1], K. MANGANO[1], S. MAMMANA[2], E. CAVALLI[2], M.S. BASILE[1] and F. NICOLETTI[1]

[1]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy; [2]IRCCS Centro Neurolesi Bonino Pulejo, Messina, Italy

Increasing body of data suggest a key role for epigenetics in the etiopathogenesis/development of autoimmune disorders. Indeed, epigenetic dysregulations have already been described in rheumatoid arthritis (RA), lupus erythematosus (SLE), and multiple sclerosis (MS) patients. Decitabine (5-aza-2'-deoxycytidine, Dacogen, (DAC) is a hypomethylating agent used for the treatment of myelodysplastic syndrome. We have previously shown that DAC has both prophylactic and therapeutic disease-modifying properties in two mouse models of MS.

MATERIALS AND METHODS

In the present study, we have first predicted in silico the potential applications of DAC in autoimmune diseases, by performing a gene enrichment analysis on data obtained from the Enrichr database (http://amp.pharm.mssm.edu/Enrichr/). Based on the results of the in silico analysis, we have evaluated the effect of DAC in a mouse model of RA, the collagen-induced arthritis (CIA) model in DBA1 mice.

RESULTS

DAC administration significantly improved the clinical and histopathological signs of the disease.

CONCLUSION

Our data support the development of DAC as a therapeutic strategy in RA patients.

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LIGAND ACTIVATED ESTROGEN RECEPTOR BETA DISRUPTS METABOLIC REPROGRAMMING IN HUMAN SEMINOMA TCAM-2 CELLS

D. DE ROSE, S. PANZA, M. SANTORO, V. RAGO, R. MALIVINDI and S. AQUILA

Department of Pharmacy and Sciences of Health and Nutrition - University of Calabria 87030 Arcavacata di Rende (CS) Italy

Testicular germ cell tumors (TGCTs) represent the most common invasive malignant tumors in young male. The most widely accepted model of TGCTs development proposes a tumorigenic event in utero followed by a dormancy period until puberty when TGCTs emerge, suggesting hormonal involvement and the estrogen-dependence of these tumors has been reported. Estrogens act through estrogen receptors and human testis mainly express ERβ. ERβ loss is associated with advanced tumor stage, however the molecular mechanisms of the ERβ–protective effects are not defined yet. Recently, we showed that estradiol (E2) induced death in human testicular seminoma cells TCAM2 by a crosstalk between E2/ERα and the tumor suppressor gene PTEN, inducing autophagy and necroptosis. Cell survival is closely coupled to the cellular metabolism and tumor metabolism is considered a cancer hallmark and a novel target for cancer therapy. To further elucidate the role of ERβ in human seminoma we studied its effect on glucose and lipid metabolism in TCAM2 cells.

MATERIALS AND METHODS

Our cells were treated with increasing E2. ERβ silencing by a specific ERβ-siRNA was used to evaluate the role of this receptor in mediating E2 action. Enzymatic activities were analyzed by kits using spectrophotometric method. Key proteins of different metabolic ways were studied by Western Blotting.

RESULTS

The effect of E2/ERβ on glucose metabolism through glycolysis was evaluated by assaying glucose cellular content, PFK1, aldolase, PKM2 and LDHA enzymes; LDH activity was also tested. The glucose utilization through the PPP was examined by the G6PDH activity. Our data showed that E2 through ERβ significantly reduced glucose utilization in both ways. Focusing on bioenergetic requirements, AMPK expression significantly increased upon E2 while ATP content and OXPHOS complexes expression were not significantly increased. As it concerns lipid metabolism, lipase activity significantly increased while the triglycerides content was significantly reduced, indicating a lipid lowering effect probably due to an alternative method to recover energy and explaining at least in part the increase of the ATP amount. Further, ACLY, ACC and FAS expression were significantly decreased, evidencing a reduced lipid synthesis ex novo. Collectively, these data indicate that E2/ERβ lower the TCAM2 metabolic rate inducing their death.

CONCLUSION

Our findings showed another important tumor-suppressive action of E2/ERβ against seminoma in perturbing their metabolic reprogramming. Future studies may lead to additional strategies for the current therapy of seminoma.

HEME OXYGENASE 1 DOWN-REGULATION FAVORS NATURAL KILLER RECOGNITION OF PRIMARY MELANOMA CELLS TREATED WITH BRAFV600E INHIBITOR

A.L. FURFARO[1], G. LOI[1], I. COSSU[2], S. OTTONELLO[3], U.M. MARINARI[1], L. MORETTA[4], M.A. PRONZATO[1], M.C. MINGARI[5], G. PIETRA[3] and M. NITTI[1]

[1]Department of Experimental Medicine, University of Genoa, Italy; [2]UOC Immunologia, IRCCS Policlinico San Martino, Genoa, Italy; [3]Department of Experimental Medicine, University of Genoa, Italy; UOC Immunologia, IRCCS Policlinico San Martino, Genoa, Italy; [4]IRCCS Bambino Gesù Children Hospital, Rome, Italy; [5]Department of Experimental Medicine, University of Genoa, Italy; UOC Immunologia, IRCCS Policlinico San Martino, Genoa, Italy and CEBR, Center of Excellence for Biomedica Research, University of Genoa, Italy

Cell redox balance is crucially maintained by heme oxygenase 1 (HO-1) activity through its metabolic products which exert antioxidant, anti-apoptotic and anti-inflammatory effects. HO-1 up-regulation has been correlated with the gain of resistance to therapy in different types of cancers and its involvement in cancer immune-escape has been hypothesized. In this work, we have investigated the involvement of HO-1 in BRAFV600E mutated melanoma cell resistance to Vemurafenib/PLX4032 (PLX) and in Natural Killer (NK)-dependent killing.

MATERIALS AND METHODS

Primary BRAFV600E mutant melanoma cell line MeOV-1 derived from skin metastasis was exposed to 1-10 μM PLX for 24h. Cell viability was checked by MTT and Trypan Blue assays. HO-1 expression was evaluated by RT-PCR and Western Blot analysis. HO-1 silencing was performed by using a specific pool of oligonucleotides against human HO-1. The degranulation ability and the killing efficiency of activated NK cells was measured by FACS analysis of CD107a expression and by Cr51 release, respectively. The expression of B7H6 and ULBP3 ligands was measured by FACS analysis and RT-PCR.

RESULTS

PLX treatment was able to strongly reduce ERK phosphorylation but only a slight decrease of cell viability was measured. HO-1 expression was increased with regard to both mRNA and protein level. HO-1 silencing efficiently prevented HO-1 up-regulation in cell exposed to PLX and was able to induce a mild but significant reduction of PLX-treated MeOV-1 viability. Moreover, NK degranulation and killing activity was decreased upon interaction with MeOV-1 cells exposed to PLX and HO-1 silencing was able to completely restore NK ability to degranulate and their cytolytic activity. We provided also evidence that HO-1 up-regulation induced by PLX down-regulated B7H6 and ULBP3 expression on MeOV-1 cell surface since HO-1 silencing was able to restore their expression.

CONCLUSION

Our results indicate that HO-1 inhibition increases cytotoxic/cytostatic activity of PLX in MeOV-1 cell line, favors the degranulation and increases the cytolytic activity of activated NK cells. Thus, HO-1 inhibition can effectively improve the efficacy of BRAF mutant inhibitors and favor NK-dependent killing.

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EFFECT OF BIOCOMPATIBLE EXTRACTS OF *OLEA EUROPAEA L.* ON OVARIAN CANCER CELLS MODEL

C. MOSCATELLO[1], E. D'AMICO[1], E. TONIATO[1], B. LANZA[2] and G.M. ACETO[1]

[1]Department of Medical, Oral and Biotechnological Sciences, University 'G. d'Annunzio-Chieti-Pescara', Italy; [2]Council for Agricultural Research and Economics (CREA), Research Centre for Engineering and Agro-Food Processing (CREA-IT), Cepagatti (PE), Italy

Ovarian cancer is characterized by a high mortality rate among gynaecological malignancies worldwide. Accumulating evidence indicates that impairments of DNA repair and energy metabolism are implicated in the initiation and progression of ovarian cancer (OC). However, the relationships among these aspects are not extensively studied in ovarian cancer physiopathology. The Extracts of *Olea europaea L.* may be used as a potential source of both antioxidant and anticancer effects, although the concentration of the phenolic fraction is several times higher in olive leaf than in olive oil and varies depending on the cultivar and climate. This study investigates the effect, on OC cell model, of phenolic bioactive compounds of *Olea europaea L*: Verbascoside (VB) and Oleuropein (OL), the two major biocompatible extracts from olive leaf and extra-virgin olive oil.

MATERIALS AND METHODS

Estrogen and antiestrogen resistant OC SKOV3 cells were treated with OL or VB at different concentration and exposure times. Cell viability were assayed by MTS (Promega) at 37°C for 1 h and then evaluated at 490 nm using the GloMax-Multi Detection System (Promega). Total RNA was extracted using the Trizol® Reagent (Invitrogen Life Technologies). Gene expression levels were evaluated by SYBR Green quantitative real-time PCR (qRT-PCR) analysis using StepOne™ 2.0 (Applied Biosystems) The results were subjected to T-test analysis; statistical significance was set at $p < 0.05$.

RESULTS

The inhibition rate of cell proliferation was different with MTS assay for the two compounds. A significant reduction in mRNA levels of BRCA1, IL8 and SLUG was observed after treatment with VB 50 μM and OL 100 μM. E-caderin gene expression appears to be increased after treatment with VB, whereas OL in this case shows no effect. OL treatment enhanced the expression of BRCA2, P65-NfkB and ERbB2, whereas, HIFα, PPARγ, FOXO3 and OGG1 showed a significant reduction.

CONCLUSION

Our observations present the first evidence that VB and OL regulate gene expression of EMT, inflammation and DNA repair in estrogen and antiestrogen resistant ovarian cancer model SKOV3, through different pathways. Further studies are needed to explore molecular pathways of tumor suppressive effect by of other tree products of Olea europaea L.

CIRCADIAN CONTROL OF CANCER CELL PROLIFERATION AND RESPONSE TO ANTICANCER TREATMENTS

M.M. BELLET, M.A. DELLA FAZIA, M. CASTELLI, S. PIERONI, D. PIOBBICO, L. ROMANI and G. SERVILLO

Section of Pathology, Department of Experimental Medicine, University of Perugia

Circadian clocks are intrinsic, coordinated systems that operates in all cells and enable organisms to anticipate environmental changes, thereby adapting their behaviour, physiology and metabolism to the appropriate time of day, thus contributing to maintain body homeostasis. Disruption of these rhythms has a profound influence to human health and has been linked to many diseases, from depression to metabolic disorders to cancer. Indeed, the circadian timing system controls rhythmic events in cell cycle, DNA repair and apoptosis in both normal tissue and cancer and drives daily rhythmic changes in drug metabolism. Consequently, the toxicity and anticancer activity of common anticancer drugs can be significantly modified by the time of administration in both experimental models and in cancer patients. The molecular mechanisms at the base of this event are still poorly understood and this is one of the factors that limit the establishment of a successful cancer chronotherapy in clinical practice. Our research is focused on identifying the molecular mechanisms linking circadian rhythm to cancer cell proliferation, explore the mechanisms responsible for the beneficial effects of circadian administration of anticancer drugs, and deciphering the direct effect of specific clock components on different cellular functions in response to anticancer treatment.

MATERIALS AND METHODS

We stably overexpressed circadian clock proteins in human cancer cell lines and analyzed the effects in term of cell proliferation, gene and protein expression and ability to induce tumors in immunocompromised mice. We compared tumor growths and investigated the pathogenesis and molecular mechanisms involved using multidisciplinary approaches.

RESULTS

We obtained promising results showing a role of the circadian protein PER1 in controlling tumor cells proliferation and chemotherapy-induced DNA damage response in cells and mice, in a p53-dependent manner. We also demonstrated that PER1 directly interacts with the tumor suppressor p53 and appears to modulate its activity.

CONCLUSION

Results indicates that the circadian clock is directly involved in the regulation of tumor cell proliferation and response to anticancer treatments. Deciphering the molecular mechanisms that link circadian rhythm, cancer progression and treatment will provide valuable insights towards innovative strategies of therapeutic intervention.

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DETECTION AND QUANTIFICATION OF OUTER MEMBRANE VESICLES (OM*VS*) PRODUCED BY HELICOBACTER PYLORI IN THE PLANKTONIC AND BIOFILM PHENOTYPES

P. LANUTI[1], V. PUCA[2], M.C. DI MARCANTONIO[3], G. BOLOGNA[1], R. MURARO[3], B. BADODI[4], R. GRANDE[4] and G. MINCIONE[3]

[1]Department of Medicine and Aging Science, University G. d'Annunzio Chieti-Pescara, Chieti, Italy. Center of Excellence on Aging and Translational Medicine (CeSI-MeT), Chieti, Italy; [2]Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio Chieti-Pescara, Chieti, Italy. Center of Excellence on Aging and Translational Medicine (CeSI-MeT), Chieti, Italy; [3]Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio Chieti-Pescara, Chieti, Italy; [4]Department of Pharmacy, University G. d'Annunzio Chieti-Pescara, Chieti, Italy. Center of Excellence on Aging and Translational Medicine (CeSI-MeT), Chieti, Italy

Helicobacter pylori (Hp) is a microorganism capable of adapting itself in both the human host and natural environment. Hp persistence and resistance may be associated to both its broad genetic variability as well as its capability of developing a biofilm. Outer Membrane Vesicles (OM*Vs*) associated with extracellular DNA (eDNA) are a component of the Hp biofilm. OM*Vs* are involved in several mechanisms such as pathogenesis, biofilm formation, cell-cell communication, bacterial-host interactions and nutrient supply. OM*Vs* are able to spread and transport virulence factors into the host. The aim of this study was to detect and quantify the OM*Vs* secreted from Hp ATCC 43629 and Hp NCTC 11637 in the biofilm (bOM*Vs*) and planktonic (pOM*Vs*) phenotypes as well as being able to detect the eDNA-OM*Vs* association with the use of Flow Cytometry.

MATERIALS AND METHODS

H. pylori biofilm formation was evaluated by using live/dead staining and fluorescence microscopy at 48 h of growth. The count of OM*Vs* and the detection of eDNA, were carried out by flow cytometry on the total samples (cells plus vesicles) and on the OM*Vs* isolated by ultracentrifugation. The Flow Cytometry analysis was performed by using a known size beaded system and two fluorescent dyes: PKH26 which selectively stain the lipids and PicoGreen that selectively stain the DNA.

RESULTS

Fluorescence microscopy showed the formation of biofilm, characterized by the presence of well-structured towers. The Flow Cytometry analysis showed a higher production of OM*Vs* in the biofilm phenotype compared to the planktonic one. Moreover, the PicoGreen staining showed that most of the generated vesicles were associated with eDNA.

CONCLUSION

Flow Cytometry is a useful tool to analyze OM*Vs* produced by Hp in both biofilm and planktonic phenotypes. Further studies are needed for the application of Flow Cytometry in the detection and quantification of Hp-OM*Vs* directly on clinical samples.

DOXORUBICIN REMEDY OR HARM? CARDIOTOXICITY AND NOT ONLY

B. PALA[1], M. PECORARO[2], M.C. DI MARCANTONIO[1], L. CENTURIONE[3], M.C. BUDANI[4], G.M. TIBONI[5], R. MURARO[1], A. PINTO[2], A. POPOLO[2] and G. MINCIONE[1]

[1]Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio Chieti-Pescara, Chieti, Italy; [2]Department of Pharmacy, University of Salerno, Salerno, Italy; [3]Department of Medicine and Ageing Sciences, University G. d'Annunzio Chieti-Pescara, Chieti, Italy; [4]Unit of Assisted Reproductive Technology, Ortona General Hospital, Ortona, Chieti, Italy; [5]Department of Medicine and Ageing Sciences, University G. d'Annunzio, Chieti-Pescara, Chieti, Italy. Unit of Assisted Reproductive Technology, Ortona General Hospital, Ortona, Chieti, Italy

Doxorubicin (Doxo) is a chemotherapeutic agent whose clinical use is hampered by the serious dose-dependent cardiotoxicity. The accumulation of Reactive Oxygen Species (ROS) is widely accepted as a key factor of cardiotoxic effects. Mitochondrial Connexin 43 (Cx43) conferred cardioprotection by reducing cytosolic and mitochondrial ROS production. Topic of this work was the identification of antioxidant enzymes and molecules involved in Doxo-induced damage, in absence and in presence of Radicicol (Rad), an inhibitor of Cx43 translocation to mitochondria. Due to increasing numbers of young cancer survivors and the raising concerns for their fertility state, elucidating the biological mechanisms of chemotherapy risk is highly relevant. Moreover, it is known that Doxo-induced ovarian toxicity is associated with apoptosis of mouse granulosa cells.

MATERIALS AND METHODS

Rat cardiac cells H9c2 were treated with Doxo and Rad. Changes in expression level of SOD, iNOS, CAT and HO were evaluated by FACS and qReal Time-PCR. The expression of molecules involved in apoptosis and NFkB pathway was analysed by FACS and Western blot. Doxo effects was also investigated in Human Granulosa Cells (HGC).

RESULTS

In H9c2 cells, FACS analysis showed that cotreatment with Doxo and Rad increased SOD, CAT, HO and the apoptotic response, as shown by hypodiploid nuclei. The induction of apoptosis was confirmed by Western blot analysis that showed how the combined treatment with Doxo and Rad increased caspase-9 expression and reduced procaspase-3 levels. Moreover, after 3h of Doxo treatment a significant increase in IKKα expression was observed, more evident after 6h of co-treatment with Doxo and Rad. These results suggested a rapid activation of pathway involved in inflammatory response mostly in the presence of Cx43 inhibitor. Our preliminary results obtained treating HGC with Doxo showed toxic effects, as determined by MTS assay.

CONCLUSION

In the model of cardiomyocytes, the understanding of the basic mechanisms underlying the cellular insult induced by Doxo will result in developing new applications for preventing cardiotoxicity. Antioxidant enzymes and molecules involved in inflammatory pathways are mostly increased in presence of Rad. Therefore, our findings help to strengthen the role of Cx43 in cardioprotection mechanisms.

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CHARACTERIZATION OF HEPATITIS B VIRUS INTEGRATION LANDSCAPE IN PATIENTS WITH HEPATOCELLULAR CARCINOMA AND IN PLC/PRF/5 CELL LINES

D. GIOSA[1], D. D'ALIBERTI[1], F. CASUSCELLI DI TOCCO[1], G. RAFFA[1], C. MUSOLINO[1], G. TRIPODI[1], D. LOMBARDO[1], C. SAITTA[2], R. AIESE CIGLIANO[3], O. ROMEO[4], G. NAVARRA[5], G. RAIMONDO[1] and T. POLLICINO[5]

[1]Unit of Clinical and Molecular Hepatology, Department of Clinical and Experimental Medicine, University of Messina; [2]Unit of Clinical and Molecular Hepatology, Department of Internal Medicine, University of Messina; [3]Sequentia Biotech Sl, Barcelona, Spain; [4]Department of ChiBioFarAm, University of Messina; [5]Unit of Clinical and Molecular Hepatology, Department of Human Pathology, University of Messina

HBV DNA integration into the host hepatocyte genome is an early event in HBV infection and it is found in about 85% of HCC. Most of the data on HBV integration in HCC have been generated by specific restriction enzymes and cloning methods that may favor preferential amplification and bias identification of unique integration sites. On the other hand, a limitation of the more recent next-generation sequencing (NGSs) approaches is the low coverage of HBV reads. Aims. To conduct a high-throughput viral integration detection analysis on tumor (T) and non-tumor (NT) liver tissues, and on PLC/PRF/5 cells, using NGS of enriched HBV integrants. To characterize the HBV DNA integration events and enumerate clones of expanded hepatocytes that bear identical integrations. To identify HBV junctures at the whole-transcriptome level and define the transcribed viral-human fusions.

MATERIALS AND METHODS

A total of 225 integration libraries were constructed from liver genomes of 4 HCC patients and PLC/PRF/5 cells. Each experiment was performed on fragmented genomic DNA by sonication. DNA was blunted and adenosine-tailed. A-tailed DNA fragments were ligated to Linkers. Virus integration sites were recovered by PCR using 24 different HBV- specific primers covering the whole viral genome and primer-Linkers. PCR products were paired-end sequenced on Illumina MiSeq. RNA-Seq was performed on an Illumina HiSeq 2500 platform, generating paired-end sequence reads (2x150bp, 60M reads/sample). High-quality reads were mapped with BWA software against hybrid reference including the human genome reference GRCh38.p10 and HBV genome (NC_003977; designated as a separate pseudo-chromosome; chrVir).

RESULTS

We detected a total of 1.661 HBV integration breakpoints (1,326 in Ts, 322 in NTs, and 13 in cells) and a total of 61,769 chimeras (44,410 in Ts, 17,228 in NTs and 131 in cells). Among the viral integration breakpoints, 1,211 mapped to genic and intergeninc regions [(980 in Ts, 219 in NTs, and 12 in cells). In particular, 116 mapped to exons (107 in Ts, 8 in NTs, and 1 in cells)], 667 to introns (530 in Ts, 144 in NTs, and 3 in cells), and 227 in lncRNAs (171 in Ts, 34 in NTs, and 2 in cells). A total of 1.071 integration breakpoints mapped to repetitive DNA sequences

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[SINE (29%), simple repeats (27.2%), LINE (24.5%) and LTR (12.5%)]. An enrichment of microhomology (MH) sequences between host DNA and HBV integrants was found in 69.5% of the chimeras. All T and NT samples analyzed showed both HBV integrants containing pre-S/S sequences and integrants containing ENHI/X promoter/X (X) sequences, with pre-S/S integrants showing a higher frequency than X integrants. Preliminary RNA-Seq results confirmed the transcription of viral/human chimeras (i.e. HBs-CCDC57 and HBs-MVK in cells, HBx-LINE/L1 and HBs-SLC22A in T and paired NT, respectively).

CONCLUSION

We developed a high-throughput HBV-integration detection method that allows characterizing thoroughly the landscape of HBV integration events. MH seems to be a major mechanism implicated in HBV integration that preferentially occurs into genic and regulatory regions. HBV integration in exons and lncRNAs occurs more frequently in T than NT tissues. A favored integration of pre-S/S sequences was found in all the samples.

P27KIP1 IN PSORIASIS. A PRELIMINARY IMMUNOHISTOCHEMICAL STUDY

M. GRANATA[1], E. SKARMOUTSOU[1], P. GANGEMI[2], M.C. MAZZARINO[1] and F. D'AMICO[1]

[1]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy; [2]Servizio di Anatomia Patologica, Azienda Ospedaliera Universitaria Policlinico-Vittorio Emanuele, Catania, Italy

Psoriasis, a chronic immune-inflammatory skin disease, is characterized by intense proliferation and abnormal differentiation of keratinocytes. It is a complex multifactorial disease and the exact pathogenesis remains to be elucidated. The cyclin dependent kinase inhibitor $p27^{Kip1}$ is known to play a key role in cell cycle regulation by controlling the G1 to S phase transition. It is a negative regulator of cell cycle progression, as well as an inducer of apoptosis. The aim of the present study was to investigate $p27^{Kip1}$ expression in skin samples from healthy subjects and psoriatic patients.

MATERIALS AND METHODS

The expression of $p27^{Kip1}$ was examined by immunohistochemistry in formalin-fixed and paraffin-embedded archival skin samples from healthy subjects and plaque psoriatic patients. Skin samples from patients with a Psoriasis Area Severity Index (PASI) score >15 were selected.

RESULTS

In skin samples from healthy subjects, $p27^{Kip1}$ was mainly immunolocalized in keratinocyte cytoplasm. Several nuclei of these cells were also immunostained for this protein. In both unlesional and lesional psoriatic samples, $p27^{Kip1}$ immunostaining was dramatically reduced compared to control specimens. Moreover, some fields of psoriatic samples showed a unique immunostaining pattern, where nuclear $p27^{Kip1}$ immunostaining intensity gradually increased outwards. This pattern could reflect the different cell proliferation degrees among skin layers.

CONCLUSION

These findings suggest that decrease of $p27^{Kip1}$ expression levels represents a common event in the maintaining phase of psoriasis. Downregulation of $p27^{Kip1}$ may impair cell proliferation and apoptosis, indicating an important and contributing role of this protein in the pathophysiology of psoriasis. Further research is necessary to elucidate the function of $p27^{Kip1}$ in psoriasis in order to uncover possible novel therapeutic strategies in this disease.

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EVALUATION OF ENDOCRINE DISRAPTORS, TPHP AND DPHP, EFFECT ON DNA REPAIR DAMAGE PATHWAIS IN A NORMAL HUMAN THYROID CELL MODEL

E. D'AMICO[1], M.C. DI MARCANTONIO[1], C. MOSCATELLO[1], L. MARINELLI[2], M. CIULLA[2], L. CENTURIONE[3], G. MINCIONE[1], A. DI STEFANO[2], R. COTELLESE[1], R. MURARO[1], and G.M. ACETO[1]

[1]Department of Medical, Oral and Biotechnological Sciences, University 'G. d'Annunzio-Chieti-Pescara', Italy; [2]Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Italy; [3]Department of Medicine and Ageing Sciences, G. d'Annunzio University of Chieti-Pescara, Chieti, Italy

Thyroid diseases affect about 20% of the Italian Population and thyroid cancer (TC) is the most common endocrine neoplasm. TC incidence showed an increase over the past few years, especially among women. The recent attention of researchers has turned to the possible role of environmental factors able to interfere as Endocrine Disrupting Chemicals (EDCs) may affect thyroid function. Some EDCs may play a role in the etiology of thyroid disorders and increase the risk of TC, based on interactions between metabolic homeostasis and DNA repair systems. Triphenyl phosphate (TPHP) is a commonly used flame retardant and plasticizer the exposure of them were been recently suggested to alter thyroid function. This study investigates the effect of TPhP and its metabolite, diphenyl phosphate (DPhP), on DNA repair and metabolism in a normal human thyroid cells model, Nthy-ori3-1.

MATERIALS AND METHODS

Nthy-ori3-1 were treated with vehicle alone and with 2, 5, 10, 50μM of TPhP and DPhP, for 48, 72, 120h. Cell viability were assayed by MTS; DNA repairs and metabolic pathways were evaluated by qRT-PCR. HPLC was used to measure the level of TPhP and DPhP internalization in the cells. Superoxide production was assayed by MitoSox. The results were subjected to T-test analysis. Statistical significance was set at $p<0.05$.

RESULTS

TPhP and DPhP, concentrations ranging from 2 to 50μM did not induce any significant changes in cell viability. After 48 h of exposure (2μM) we detected a higher DPhP concentration in cell lysate compared to the supernatant, this could be indicative of a cellular internalization. MitoSox detected the metabolic involvement of mitochondria and oxidative effect cells treated by DPhP 2μM. In proliferative cells, we observed a significant reduction in expression of the DNA repair genes: BRCA1, OGG1, MUTYH and hMSH2; TPO increased at 120h but reduced in quiescent cells, while ZEB1 showed an opposite trend. Transcription factors NRF2, LEF1 and FOXO3 increased their expression after 48 h in exposed quiescent cells. A reduction in the sensitivity to metabolize glucose may associate with PPARg low gene expression.

CONCLUSION

The present study provides the first evidences that low concentrations of flame retards TPhP and its metabolite DPhP affect DNA repair and metabolic homeostasis in thyroid epithelial cells.

THE ONCOLYTIC ADENOVIRUS DL922-947: A POSSIBLE THERAPEUTIC STRATEGY AGAINST MALIGNANT PLEURAL MESOTHELIOMA

S. DI SOMMA[1], A.M. MALFITANO[1], R. IANNONE[1], C. PASSARO[2], C.A. IANNUZZI[3], F. PENTIMALLI[3] and G. PORTELLA[1]

[1]Department of Translational Medicine, University of Naples Federico II, Italy; [2]Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, USA; [3]National Cancer Institute of Naples, CROM, Italy

Malignant Pleural Mesothelioma (MPM) is a rare cancer type mainly caused by asbestos exposure. MPM has limited treatment options and a poor outcome. New therapeutic approaches are urgently needed. Promising anticancer therapeutics are Oncolytic viruses (O*Vs*) that selectively replicate in and kill cancer cells, leading to the tumor lysis.

MATERIALS AND METHODS

In this study, we evaluated the anticancer activity of the oncolytic adenovirus dl922-947, *in vitro* and *in vivo* model of MPM. *In vitro*, we evaluated the cytotoxic activity of dl922-947, by sulforhodamine B (SRB) assays, on a panel of human MPM cell lines representative of the main different histotypes: the epithelioid NCI-H28 and NCI-H2452, the biphasic MSTO-211H, the sarcomathoid NCI-H2052. We assessed the replicative potential of dl922-947, by Real Time PCR. We characterized cell death mechanisms activated in MPM cell lines treated with dl922-947, firstly assessing apoptosis induction by Annexin V/Propidium Iodide staining. Then, we investigated induction of immunogenic cell death (ICD), evaluating the translocation of Calreticulin on the plasma membrane by flow cytometry, quantifying ATP extracellular levels on supernatants of MPM infected cells, by a commercially available kit. Moreover, we have assessed the effect of dl922-947 on cell cycle regulation by flow cytometry. Finally, we have evaluated *in vivo*, in MPM xenograft mice, the efficacy of the treatment with dl922-947.

RESULTS

We demonstrated that dl922-947 is cytotoxic and able to replicate in two human MPM cell lines, NCI-H28 and MSTO-211H. The treatment with dl922-947 leads to activation of cell death pathway such as apoptosis and ICD, and induces cell cycle alterations, resulting in an increased subG1 cell fraction (apoptosis induction) and hyperdiploid (4N) population (mitotic defect). Our *in vivo* studies showed that treatment with dl922-947 suppresses tumor growth in MPM xenograft model. Moreover, we have observed a complete tumor regression in 33% of dl922-947 treated mice.

CONCLUSION

Our results indicate that dl922-947 exerts an oncolytic effect on MPM cells and intra-tumor administration of dl922-947 might represent a promising therapeutic strategy for MPM.

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ASPECTS OF MELANOMA CELLS ADAPTED TO A CHRONIC ACIDOSIS RELEVANT FOR THERAPY

S. PEPPICELLI, F. BIANCHINI, E. ANDREUCCI, J. RUZZOLINI and L. CALORINI

Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence

The chaotic and incomplete vasculature of tumor stroma is frequently responsible for a transient and/or persistent state of hypoxia, and tumor cells adapt their metabolism to anaerobic glycolysis. Therefore, lactate and protons are produced in large excess and transported outside by redundant families of lactate and H+ transporters in order to maintain an intracellular pH compatible with survival and/or proliferation. Therefore, the extracellular pH of tumors turns to acidic for a different period of time, conferring new adaptive aspects to cancer cells, characterizing the transient-exposed and chronically-exposed tumor cell subpopulations.

MATERIALS AND METHODS

Protein expression was assessed via Western blot analysis (N/E-Cadherin, MCT1, MCT4, P-p70S6k, p70S6k, P-AKT, AKT, ßactin and ßtubilin); Invasiveness of acute and chronic acidic and non-acidic melanoma cells was performed using 8.0μ cell culture inserts; Cellular apoptosis of acute and chronic acidic and non-acidic melanoma cells was determined using PI and Annexin V-FITC staining by FACS analysis; Proliferation index was evaluated by CFSE staining; Quantitative real-time PCR for glucose transporters GLUT1 and GLUT3 expression, glycolytic enzymes HK2 and LDHA, glutaminase 1 and 2 (GLS1, GLS2) and glutamine transporter ASCT2; Lactate extrusion was determined using a colorimetric assay.

RESULTS

We exposed melanoma cells either for a short period (24 hours) to a pH 6.7 acidic medium (transient exposure) or during a three-month period in the same low pH medium (chronic exposure). Both transient and chronic-exposure did not modify the viability of melanoma cells. Both types of acidic cells showed traits compatible with an epithelial-mesenchymal transition, a higher invasiveness through Matrigel-coated filters and resistance to apoptosis. Unlike the transiently exposed cells, which expressed a much-reduced rate of proliferation, chronic adapted cells showed a rate of proliferation almost similar to that of control cells. When we investigated the metabolic profile, we highlighted a different metabolic behavior between the two types of acidic cells. Cells exposed to acidosis for a short period changed their metabolic profile from glycolysis to oxidative phosphorylation, reducing glucose transporters, monocarboxylate transporter (MCT)-4 and lactate production. Interestingly, chronic acidic cells recovered a glycolytic profile, identified by high glucose transporters and lactate production. Moreover, at the variant with the transient acidic cells, chronic acidic adapted cells expressed high level of glutamine metabolism, suggested by the increased expression of glutaminases (GLS1, GLS2) and glutamine transporters. Metformin, a synthetic biguanide widely used as antidiabetic drug, was effective in kill acute acidic cells but his effect was less evident on chronically exposed acidic cells. On the other hands, both type of cells expressed high level of AKT/mTOR activation which lead to a high sensitivity to everolimus treatment.

CONCLUSION

Overall, our results underline the possibility that acidic adapted cells, both transiently or chronically exposed, might be targeted with a mTOR inhibitor.

MULTIPOTENCY OF EPICARDIAL ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS IS IMPAIRED IN TYPE 2 DIABETES

S. CABARO[1], G. PERRUOLO[1], V. D'ESPOSITO[2], V. PARISI[1], M. LECCE[1], I. CIMMINO[1], A. CURCI[1], F. ORIENTE[1], D. LEOSCO[1], F. BEGUINOT[1] and P. FORMISANO[1]

[1]Department of Translational Medical Sciences, University of Naples "Federico II", Naples, Italy; [2]URT GDD-IEOS, National Council of Research (CNR), Naples, Italy

Excess of visceral fat is a major culprit in the development of type 2 diabetes and related disorders. A growing body of evidence indicates that epicardial adipose tissue (EAT), the visceral fat of the heart, may play an active role in dysregulation of cardiac function. Indeed, EAT thickness positively correlates with the release of inflammatory molecules and with the severity of heart pathologies. In patients with diabetes, prolonged hyperglycemia damages several organs, including heart. Thus, potent pro-inflammatory activation of EAT suggests a direct involvement of cardiac visceral fat in inflammatory phenomena occurring in patients with cardiovascular diseases. This study aims at investigating whether different glucose concentrations may impact on EAT functions.

MATERIALS AND METHODS

EAT biopsies were collected from diabetic (n=7) and non-diabetic (n=7) individuals with CAD. EAT-derived mesenchymal stem cells (MSCs) were isolated (n=5) and cultured in high glucose (HG 25 mmol/l) or normal glucose (NG 5.5 mmol/l) concentration, as control. Cell surface markers were assessed by FACS analysis and gene expression levels by real time RT-PCR.

RESULTS

EAT-MSCs cultured in NG and HG exhibited similar proliferation rate. Additionally, the expression of cell surface markers, characteristic of adipose tissue-derived MSCs ($CD90^+$, $CD31^-$, $CD45^-$, $CD73^+$), was similar in both conditions. Interestingly, HG-treated EAT-MSCs displayed a 50% decreased expression of the multipotent markers Oct-4 and Nanog. Moreover, mRNA levels of the senescence marker $p21^{CIP1/WAF1}$ were significantly increased in HG-cultured cells. HG-treated EAT-MSCs maintained their adipogenic and osteogenic potential, after the application of appropriate induction media, however either lipid or calcium accumulation seemed to be lower compared with NG-cultured cells. Consistent with these data, in EAT biopsies from diabetic patients there was a 1.7- and 2.2-fold reduction of mRNA levels of PPAR□ and BMP1, respectively master gene regulators of adipogenic and osteogenic differentiation.

CONCLUSION

Diabetic-like environment directly influences EAT-derived MSC features and significantly affects their stemness and differentiation potential, driving towards accelerated senescence.

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ERα DRIVES LKB1 FUNCTIONAL ROLE IN ADIPONECTIN-TREATED BREAST CANCER CELLS

L. MAURO, G.D. NAIMO, L. GELSOMINO, A. PAOLÌ, F. GIORDANO, M.L. PANNO and S. ANDÒ

Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Cosenza - Italy

Adipose microenvironment is involved in signaling pathways that influence breast cancer development and progression, through the secretion of different adipocytokines. Among these, adiponectin plays a pivotal role in the pathogenesis of breast cancer. An inverse correlation is reported between obesity and adiponectin, suggesting that low levels represent a risk factor for mammary cancer. In the recent years, our research group has identified ERα as an important regulator of adiponectin action in breast cancer, clarifying that the role of this adipocytokine seems to be dependent on cell phenotypes. LKB1 is an important target of adiponectin action, being involved in multiple cellular functions, such as cell metabolism, cell cycle arrest, apoptosis, motility and cell polarity. Here, we investigated the effect of adiponectin on expression and function of LKB1 in ERα-positive (MCF-7) and negative (MDA-MB-231) breast cancer cells.

MATERIALS AND METHODS

RNA levels were assessed by RT-PCR. Protein expressions were evaluated by immunoblotting in the presence or absence of ERα siRNA. To study promoter activity, transient transfection assays were done. The recruitment of ERα on LKB1 promoter was evaluated by ChIP and DAPA assays.

RESULTS

We demonstrated that in MCF-7 cells low adiponectin doses increased LKB1 expression in term of RNA and protein levels, whereas no significant changes were observed in ERα-negative MDA-MB-231 cells.

For instance, in MCF-7 cells, transiently transfected with plasmids containing a series of 5' deleted segments of human LKB1 promoter, we identified a sequence (-1626 to -1598) containing Estrogen Responsive Elements (ERE). This led us to investigate the recruitment of ERα on LKB1 promoter by ChIP and DAPA assays, evidencing that adiponectin treatment increased the association of ERα on ERE-containing sequence. The crucial role of ERα in the upregulation of LKB1 expression upon adiponectin exposure was demonstrated by the evidence that the latter effect was no longer noticeable in the presence of ERα siRNA. It is extremely intriguing to remark how the increasing intracellular pool of LKB1 upon adiponectin was utilized in the nuclear compartment as coactivator of ERα, which in such way uncoupled it from AMPK interaction, generally leading to the inhibition of cellular energy expenditure.

CONCLUSION

On the basis of our results we propose that ERα may negatively interfere with the action of adiponectin on the LKB1/AMPK signaling, thus influencing its antitumor action.

GLUCOSE MODIFIES THE CROSSTALK BETWEEN CANCER CELLS AND ADIPOSE-DERIVED MESENCHYMAL STEM CELLS CONTRIBUTING TO CANCER PROGRESSION

V. D'ESPOSITO[1], T. MIGLIACCIO[2], G. MOSCA[2], M.R. AMBROSIO[2], G. PERRUOLO[2], E. MOLINARI[2], N. PREVETE[2], G. NELE[3], F. D'ANDREA[3], F. BEGUINOT[2] and P. FORMISANO[2]

[1]URT GDD-IEOS, National Council of Research (CNR), Naples, Italy; [2]Department of Translational Medical Sciences, University of Naples "Federico II", Naples, Italy; [3]Department of Public Health, University of Naples "Federico II", Naples, Italy

Hyperglycaemia increases breast cancer (BC) incidence and progression. Glucose may exert its effects on both BC cells and tumour microenvironment. Here, we analysed whether glucose could interfere on the crosstalk between mammary adipose tissue derived- mesenchymal stem cells (MAT-MSCs) and oestrogen positive-MCF7 BC cells, thereby modifying MSC phenotype and affecting tumour progression.

MATERIALS AND METHODS

MAT-MSCs were isolated (n=8) and characterized for MSC markers ($CD90^+$, $CD29^+$, $CD106^-$, $CD45^-$) by FACS analysis. Adipocyte differentiation was tested by Oil Red O staining. MAT-MSCs were co-cultured with MCF7 in 25mM glucose (high glucose, HG) or in 5.5mM glucose (low glucose, LG) by using transwell systems. Upon 4 days, gene expression levels were analysed by real time RT-PCR. 3D co-cultures (spheroids) of MSC -MCF7 (1:5 ratio) were established in ultra-low attachment plates in HG or LG. Upon 10 days, spheroids were analyzed. Proteins levels were determined by FACS analysis.

RESULTS

MAT-MSCs stained positive for CD90 and CD29 (99.2 % and 99.9% of cells respectively) and negative for CD106 and CD45 markers (99.9% and 98.1% of cells). Moreover, they were able to differentiate through the adipogenic lineage. When treated with HG for 4 days, the cells did not modify the expression of multipotency genes OCT4, SOX2 and NANOG and of the fibrosis marker a-SMA, as compared with LG. However, when co-cultured with MCF7 in HG, MAT-MSCs displayed a down-regulation of OCT4, SOX2 and NANOG (1.4, 1.6 and 1.4 fold, respectively) and a 1.4-fold increase of a-SMA. In HG 3D co-cultures, MAT-MSCs significantly increased the number of MCF7-derived spheroids either compared to MCF7 alone either to MCF7 – MSC co-cultured in LG. Spheroid dissociation showed a reduction of OCT4 and an increase of a-SMA proteins in MAT-MSCs and, interestingly, an increase of OCT4 protein in MCF7 in HG.

CONCLUSION

Glucose modifies the complex relationship between BC cells and MSCs contributing to loss of multipotency and acquisition of fibroblast-like features in MSCs (cancer-associated fibroblasts). Interestingly, by acting through MSCs, glucose increases the stemness potential of BC cells, thus suggesting a reduction of drug sensitivity and an increase of metastatic potential.

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EFFICACY OF ABL001 - ALONE OR IN COMBINATION - ON CD34+ PROGENITORS ISOLATED FROM CML PATIENTS DISPLAYING HIGH OR LOW BCR-ABL TRANSCRIPTS AT DIAGNOSIS

L. MANZELLA, C. ROMANO, E. TIRRÒ, M. MASSIMINO and P. VIGNERI

Department of Clinical and Experimental Medicine, University of Catania, Catania

ABL001 (Asciminib) is a selective allosteric inhibitor of the BCR-ABL oncoprotein that binds to the myristoyl pocket of ABL favouring the formation of an inactive kinase conformation. We evaluated the sensitivity to ABL001 - alone or in combination with the tyrosine kinase inhibitors (TKIs) Imatinib (IM) and Nilotinib (NIL) - of CD34+ leukemic progenitors isolated from 30 patients displaying either high (n=15) or low (n=15) BCR-ABL/GUSIS levels at diagnosis.

MATERIALS AND METHODS

To study cell proliferation and the phosphorylation status of BCR-ABL per se or of several of its downstream targets, CD34+ cells were incubated with escalating doses (ranging from 0.1 nM and 10.000 nM) of either IM, NIL or ABL001. We than assessed the number of colonies grown in methylcellulose after incubating CD34+ cells with IM, NIL or ABL001.

RESULTS

We detected a significant reduction in the proliferation rate of CD34+ cells after incubation with the two TKIs while the effect of ABL001 was considerably lower.

Regarding phosphorylation experiments, we noticed that while reduction of the oncoprotein's phosphorylation was more evident in cells with low BCR-ABL, the three drugs generally displayed a comparable efficacy. Interestingly, the combination of ABL001 with the two TKIs did not potentiate the effect of ABL001 on BCR-ABL auto-phosphorylation. This was also the case when we measured CRKL and ERK1/2 phosphorylation. The only appreciable difference that we detected was observed in phospho-AKT levels in patients with high BCR-ABL/GUSIS transcripts exposed to the combination of NIL and ABL001 (but not to IM plus ABL001).

Initial results point to a similar efficacy of the two TKIs in reducing the colony numbers of progenitors with high or low BCR-ABL/GUSIS. Interestingly, ABL001 employed alone does not significantly affect total colony number as compared to the untreated condition. Likewise, we have yet to detect significant differences in the colony-forming potential of CD34+ cells treated with ABL001 in combination with IM or NIL as compared to those receiving each TKI alone. LTC-IC experiments evaluating the effect of each drug on both the frequency and absolute number of the primitive hematopoietic population are currently on going.

CONCLUSION

These results, while preliminary, suggest a limited short-term response to ABL001 of CD34+ progenitors isolated from CML patients regardless of their BCR-ABL/GUSIS transcripts at diagnosis. Moreover, with few exceptions, the combination of ABL001 with IM or NIL fails to increase the effect of each TKI used individually.

CELL-TARGETED RGD-SUNITINIB MOLECULAR CONJUGATES IMPAIR TUMOR GROWTH OF MELANOMA

F. BIANCHINI[1], S. PEPPICELLI[1], E. ANDREUCCI[1], E. PORTIOLI[2], M. LULLI[1], A. SARTORI[2], L. BATTISTINI[2], J. RUZZOLINI[1], F. ZANARDI[2] and L. CALORINI[1]

[1]Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence; [2]Department of Food and Drug, University of Parma

Despite progress in the clinical management of advanced-stage melanoma with different treatment options available, mainly based on targeted therapy and immunotherapy, many concerns still exist dealing with the observed overall toxicity. Effective dosing regimens and the insurgence of resistance mechanisms lead to tumor relapse and progression to a metastatic disease with incredibly aggressive features. Given the involvement of multiple, yet strictly related biological targets and signaling cascades in the metastatic melanoma disease, the combination therapy has become the standard-of-care treatment, in both small molecule-based (e.g. vemurafenib+cobinetinib in BRAFV600E/K-mutant disease) and antibody-based therapies (e.g. ipilimumab+nivolumab), with the primary goal to improve clinical benefit while overcoming the insurgence of drug resistance and compensating mechanisms often observed using targeted monotherapy. In these cases, however, off-organ (and possible synergistic) toxicity remains a still unsolved issue. Thus, the creation of new molecular conjugates which combine the ability to target melanoma cells using recognition of specific surface-exposed receptors, enter melanoma cells via receptor-mediated endocytosis, and modulate key intracellular targets and signaling pathways, is an appealing approach toward enhanced drug efficacy at lowered drug dosage and increased safety window.

MATERIALS AND METHODS

Recently, we launched a new class of molecular conjugates, where the anti-αVß3 integrin peptidomimetic cyclic (AminoprolineRGD) was connected to the antiangiogenic and antitumor multikinase inhibitor sunitinib through robust, and covalently bonded linkers. The efficacy and cell selectivity of RGD-conjugated compounds were evaluated using different *in vitro* and *in vivo* assays: Inhibition of cell adhesion to vitronectin; Clonogenic assay; Cell proliferation assay; Cytofluorimetric assay for cellular internalization; Western blot assay; Annexin V/PI binding staining assay; Inhibition of tumor growth in xenografted mice and biodistribution studies.

RESULTS

We found that low concentrations of the three conjugates 1-3 significantly reduced the growth and clonogenic activity of M21 melanoma cells and that the selective uptake of the RGD-sunitinib conjugates by melanoma cells was mainly due to the binding to the αVß3-integrin receptor, which is overexpressed in the melanoma cells. We found that the RGD-conjugated compounds drastically reduced the growth of melanoma xenografts in tumor-bearing mice, showing a selective localization in the tumor tissue, compared to free sunitinib.

CONCLUSION

The observed melanoma cell-selectivity of the presented RGD-conjugated compounds is a good premise for targeted therapy with consequent dosage reduction and decreased adverse and toxic effects on healthy tissues.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

BRASSICA EXOSOME-MICRORNAS: NEW THERAPEUTIC APPROACH IN CANCER TREATMENT?

F.M. PEZZINO[1], A. RUSSO[1], L. LO CICERO[1], D. CATALANO[1], M. PULVIRENTI[1], B. FICILI[1], M.C. DI BELLA[1], L. AMODEO[1], M.N. TERZO[2] and F. BRANCA[3]

[1]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy; [2]Department of Biomedical and Biotechnological Sciences and Di3A, University of Catania, Catania, Italy; [3]Department of Agriculture, Alimentation and Environment (Di3A) University of Catania, Catania, Italy

Natural medicine has been practiced for centuries and, today, has receiving attentions in both pharmaceutical industry and academia. Although, many mechanisms of action of plant products remain unknown, new possible mechanisms are becoming evident: exosomes and their content, miRNA included, might regulate gene expression with potential therapeutic activities in human chronic diseases. Plant miRNAs are found in sera and tissues of mammals after plant ingestion, supporting an effective communication between the two kingdoms. In our lab, we have utilized exosomes isolated from Brassica oleracea sprouts to inhibit the growth of tumor cell lines, as assayed by *in vitro* wound scratch test and by 3D cultures. To better understand the molecular mechanisms, we performed an in silico study using the Brassica miRNA database to predict the human target transcripts. Among others, MIR414 exhibited a perfect base paring to Spindlin 1 (SPIN1), a human gene upregulated in melanoma cancer.

MATERIALS AND METHODS

The presence of MIR414 was evaluated by stem-loop real time RT-PCR in total miRNAs isolated from Brassica sprouts exosomes-like. Gene expression of SPIN1 was studied by RT-PCR, using PGK1 as housekeeping gene.

RESULTS

First, we confirmed that that MIR414 was present in exosomes-like vesicles isolated from Brassica oleracea sprouts. RT-PCR of SPIN1, a gene whose expression is upregulated in many cancer cells, melanoma included, promoting cell proliferation via activation of the Wnt signaling pathway, demonstrated a significant mRNA reduction (~70 %) in A2058 cells treated with brassica exosomes, containing MIR414, compared to untreated controls.

CONCLUSION

In the present study, we showed that brassica exosomes, vehicolating a plant miRNA (MIR414), were able to significantly decrease levels of target mRNAs (SPIN1). To definitively confirm that SPIN1 is directly targeted by MIR414, we will design a specific mimic miRNA.

Although further studies with other miRNA/mRNA targets are required, our data showed that miRNAs, packaged in exosomes, could represent new functional molecules mediating intercellular communications, opening to the possibility to develop new target anticancer strategies.

Plant kingdom, with the capability to crosstalk with mammalian cell, could constitute a giant low-cost factory for these novel drugs.

PHOSPHODIESTERASE 5 (PDE5) EXPRESSION AND FUNCTION IN BREAST CANCER STROMA: AN ATTRACTIVE OPPORTUNITY FOR TARGETED THERAPY

I. BARONE[1], S. PANZA[1], C. GIORDANO[2], B. GYORFFY[3], L. GELSOMINO[1], D. BONOFIGLIO[1], S. ANDO'[1] and S. CATALANO[1]

[1]Department of Pharmacy and Health and Nutritional Sciences, University of Calabria; [2]Centro Sanitario, University of Calabria; [3]MTA TTK Lendulet Cancer Biomarker Research Group, 2nd Department of Pediatrics, Semmelweis University, Budapest, Hungary

By catalyzing cGMP hydrolysis, phosphodiesterase (PDE) type 5 acts as a critical regulator of its concentration and effects in different (patho) physiologic processes, including cancers. We have previously shown that PDE5 overexpression greatly enhances the invasive potential of breast cancer cells and reduces survival in patients, highlighting the benefit of therapeutic targeting PDE5. To extend these observations, we propose to assess PDE5 function in tumor microenvironment, which has increasingly known as an active contributor to breast cancer growth and progression.

MATERIALS AND METHODS

The expression and role of stromal PDE5 was first investigated in human breast tumor stroma microarrays (GSE9014 database). Human cancer-associated fibroblasts (CAFs) were isolated from biopsies of primary breast tumors and PDE5 expression was evaluated by realtime RT-PCR and immunoblotting analyses. PDE5 functional role in CAFs was tested by evaluating the effects of selective PDE5 inhibitors in proliferation, motility and invasion assays. In order to examine the significance of PDE5 expression in stromal fibroblasts, PDE5 was also stably integrated into mouse embryonic fibroblasts (MEFs) and cell proliferation, motility and invasion were investigated. Acquisition of CAF hallmarks was evaluated by assessing α-SMA, N-cadherin and RhoGTPase expression as well as by stress fiber formation. The impact of PDE5 overexpressing MEFs on breast cancer epithelial cell proliferation and motility was examined in coculture experiments.

RESULTS

PDE5 expression is increased in the stroma of human breast cancer compared with normal breast stroma and was correlated to shorter overall survival in patients. Increased PDE5 mRNA and protein expression was detected in CAFs isolated from primary breast tumors and treatment with PDE5 inhibitors significantly decreased CAF proliferation, motility and invasiveness. PDE5 transformed MEFs towards a cancer-associated fibroblast phenotype, as evidenced by increased expression of CAF markers along with enhanced proliferative, migratory and invasive capabilities. Coculture experiments showed that proliferation and motility of human breast cancer cells was increased by PDE5-overexpressing MEFs compared to vector-expressing MEFs, suggesting that stromal PDE5 participates in heterocellular signals to breast cancer cells.

CONCLUSION

Being PDE5 a known druggable target, the present and our previous studies may offer promising insights into future cancer treatments by providing the rationale to implement safer and more efficacious drugs in the adjuvant therapy for breast cancer.

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A POSSIBLE ROLE OF A CMV-LIKE VIRUS IN HEAD AND NECK PARAGANGLIOMA PATHOGENESIS

F. VERGINELLI[1], D.L. ESPOSITO[2], M.R. PANTALONE[3], S. PERCONTI[1], S. VESPA[4], F. SCHIAVI[5], S. SOLIMAN[2], S. DE FABRITIIS[1], V. TARANTINI[1], S. VALENTINUZZI[1], M. DI MARCO[2], A. RAMASSONE[2], R. FLORIO[6], L. DE LELLIS[6], A. SHEU[2], M. SERLUCA[2], A. CAMA[6], S.C. PRASAD[7], A. VERONESE[2], R. VISONE[2], C. PATIES[8], C. SÖDERBERG-NAUCLÉR[3], L.V. LOTTI[4], M. SANNA[7] and R. MARIANI-COSTANTINI[2]

[1]Department of Pharmacy and Laboratory of General Pathology, Center of Aging Science and Translational Medicine (CeSI-MeT), "G. d'Annunzio" University, Chieti, Italy; [2]Laboratory of General Pathology, Center of Aging Science and Translational Medicine (CeSI-MeT), 'G. d'Annunzio' University, Chieti, Italy; [3]Cell and Molecular Immunology, Experimental Cardiovascular Unit, Departments of Medicine and Neurology, Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; [4]Laboratory of Ultrastructural Biopathology, Department of Experimental Medicine, "Sapienza" University of Rome, Italy; [5]Familial Cancer Clinic and Oncoendocrinology, Veneto Institute of Oncology, IRCCS, Padua, Italy; [6]Department of Pharmacy, 'G. d'Annunzio' University, Chieti, Italy; [7]Otology and Skull Base Unit, Gruppo Otologico, Piacenza, Italy; [8]Department of Oncology-Hematology, Service of Anatomic Pathology, Guglielmo da Saliceto Hospital, Piacenza, Italy

Paragangliomas (PGLs) are neurovascular autonomic nervous system tumors, still incurable when radical surgery is not possible. They are frequently associated with susceptibility mutations in genes encoding the mitochondrial SDH complex components. However, the etiology of PGLs remains unclear as the penetrance of SDHx mutations is incomplete (21% at age 50). Studying a large set of freshly-collected head and neck PGLs (HNPGLs) by electron microscopy we found herpesvirus-like particles in all tumors. These were similar to particles attributed to cytomegalovirus (HCMV) reported in 1971 by Heine et al. in a retroperitoneal PGL.

MATERIALS AND METHODS

The HNPGL series of over 140 collected cases was characterized for SDHx mutational status and HCMV virus infection. The germline point mutations and large deletion/rearrangements of the SDHx genes were analysed by PCR, sequencing and MLPA. Blood and tumor DNAs were analysed for HCMV sequences using PCR. Tumor tissues were analysed by RISH, EM, IF, IHC and WB. Viral infection using HNPGL tumor extracts was assessed *in vitro* using a modified protocol of Peyton-Rous (1911). HNPGL derived cells were treated by the antiviral Ganciclovir *in vitro* and *in vivo*.

RESULTS

SDHx mutational analysis showed a mutation frequency of 34.3%. Viral particles were observed in all analysed cases by EM. The HCMV reactive proteins (IE, gB, pp65, vMIA) and their interacting host proteins (PDGFRA, viperin) were detected by IF, IHC and WB. In our attempts to amplify the viral

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DNA using HCMV-specific primers, we detected viral glycoprotein B for only 68.2% and 82.3% in tumors and in blood samples respectively. Viral infection using HNPGL in HEK293T and MRC5 cell lines induced cell fusion and cell death. Moreover, cell-derived xenografts presented the same viral particles and HCMV sequences were retrievable from the blood of xenografted mice. Ganciclovir inhibited HNPGL cell growth and prevented tumor xenograft formation (P=0.005).

CONCLUSION

Infection with this virus appears to be a constitutive feature of PGL, found in all cases, both associated, or not with germline SDHx mutations. Coherently with emerging evidence concerning other neural cancers, our data suggest that HCMV-like virus plays a role in the PGL pathogenesis, possibly favoured by germline mutations predisposing to oncogenic infection.

EFFECTS OF HDAC INHIBITORS ON GLIOBLASTOMA CELLS

O. PASTORINO[1], M.T. GENTILE[1], A. BAJETTO[2], A. DI COSTANZO[3], A. MANCINI[4], L. ALTUCCI[3], T. FLORIO[2], M.P. STOPPELLI[5] and G.L. COLUCCI-D'AMATO[1]

[1]Laboratory of Molecular and Cellular NeuroPathology, Di.S.T.A.Bi.F. University of Campania "L. Vanvitelli", Caserta; [2]Sezione di Farmacologia, Dipartimento di Medicina Interna & Centro di Eccellenza per la Ricerca Biomedica (CEBR), Università di Genova, Genova, Italy; [3]Dipartimento di Medicina di Precisione,University of Campania "L. Vanvitelli", Caserta; [4]Dipartimento di Scienze Mediche Traslazionali, University of Campania "L. Vanvitelli", Caserta; BIOUP Sagl Lugano (Switzerland); [5]Istituto di Genetica e Biofisica "AB-T", CNR, Naples

Glioblastoma Multiforme (GBM), a high-grade glioma (WHO grade IV), is the most aggressive form of brain cancer. Treatment options for GBM involving a combination of surgery, chemotherapy and radiation resulted in a poor survival outcome. Epigenetic mechanisms are increasingly implicated in GBM pathogenesis. Unlike genetic mutations, epigenetic changes are reversible and can be targeted by drugs. We evaluated whether different Histone Deacetylase Inhibitors (HDACis) are able to affect migration, invasion and vasculogenic mimicry in GBM cells.

MATERIALS AND METHODS

We tested Varinostat (SAHA) and Trichostatin A (TSA) as inhibitors of class I and II HDACs, Entinostat (MS275) as selective inhibitor of class I HDACs (specifically of HDAC 1 and 3) and MC1568 as selective HDAC class II inhibitor. Tube Assay was used to evaluate the ability of HDACis to interfere with vessels formation by human GBM U87MG and rat glioma C6 cell lines and by cancer stem cells (CSCs) isolated from human GBMs. U87MG directional cell migration and cell invasion have been assessed by Boyden chamber assay and using xCELLigence Real-Time Cell Analyzer. Trypan Blue exclusion test and MTT assays were employed to assess cell viability and cell proliferation.

RESULTS

Our data show that sublethal doses of HDACis significantly decrease U87MG directional cell migration and that MS275 HDACi is able to impair U87MG matrigel invasion. Finally, we observed that HDACis inhibit significantly vasculogenic mimicry in U87MG, C6 and CSCs cells.

CONCLUSION

GBM is a hypervascular neoplasia and depends on vascular networks to supply blood, oxygen, and nutrients. Tumor blood vessels can either be formed from pre-existing blood vessels (neo-angiogenesis) or from tumor cells (vasculogenic mimicry,VM); VM provides a mechanism whereby GBM could escape anti-angiogenic therapies. Our results suggest that HDACis may be promising candidates for blocking vascular mimicry.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

INTERPLAY BETWEEN CANNABINOIDS AND INFLAMMATORY CYTOKINES IN HUMAN BREAST CANCER

C. PISCOPO[1], M.C. PROTO[1], A.M. CASCONE[2], P. ZEPPA[2], D. FIORE[1], B.M. FUSCO[1], M. BIFULCO[3] and P. GAZZERRO[1]

[1]Department of Pharmacy, University of Salerno, Fisciano, Italy; [2]Department of Pathology, Azienda Ospedaliera Universitaria AOU San Giovanni di Dio e Ruggi d'Aragona, University of Salerno, Salerno, Italy; [3]Department of Molecular Medicine and Medical Biotechnologies, University of Naples "Federico II", Naples, Italy

Breast cancer is a highly heterogeneous disease and one of the few tumour types molecularly characterized. While luminal and human epidermal growth factor receptor 2 positive (HER2+) tumours can be controlled by hormonal and anti-HER2 interventions, the triple-negative breast cancers (TNBC) are resistant to standard treatments. Since the tumour microenvironment is an important player in breast tumour progression, the stromal components could represent a source of new prognostic biomarkers and targets for potential therapeutic strategies mainly in tumors lacking molecular sites of intervention. We previously found that Met-F-AEA, agonist at cannabinoid receptor 1 (CB1) inhibited cell migration and epithelial-mesenchymal transition of the TNBC cell line MDA-MB-231, through modulation of MMP2 and Wnt/ß-catenin signaling, respectively.

MATERIALS AND METHODS

Through western blot analysis, CB1 receptor, the mesenchymal marker Vimentin and CD44, a surface receptor involved in ECM remodelling, have been analysed in 16 breast cancer tissues. Through MTT and BrdU assays, the effects of cannabinoid compounds (SR141716 and CBD), TGF-ß1 and estradiol have been tested in breast cancer cell lines (MCF7 and MDA-MB-231), in normal fibroblast cell line (HDFa) and in primary fibroblast cell lines purified from tumors. The level of TGF-ß1 and pro-inflammatory cytokines have been quantified in cell culture supernatants by ELISA assays.

RESULTS

Both SR141716 and CBD were able to reduce TGF-ß1 levels in MCF7 and, interestingly, CBD reduced TGF-ß1 levels also in the TNBC MDA-MB-231 cell line with a more marked effect. In primary normal fibroblasts HDFa, TGF-ß1 induced, as expected, a significant secretion of IL-6 in cell culture supernatants, while SR141716 and CBD did not affect IL-6 production, even at a concentration of 10 μM, and partially counteracted the effects of exogenous administered TGF-ß1. Results obtained in primary fibroblast cell lines purified from tumors seem support a potential role of cannabinoid compounds in the secretion of pro-inflammatory cytokines.

CONCLUSION

Although further investigations are needed, these preliminary results suggested a possible interplay between the endocannabinoid system and the breast tumor microenvironment.

EFFECT OF OXIDATIVE STRESS ON CANONICAL/NON-CANONICAL WNT PATHWAYS IN COLON CANCER CELL LINES

T. CATALANO[1], C. MOSCATELLO[2], M.C. DI MARCANTONIO[2], R. CORSARO[1], G. FERLAZZO[3], M.C. CURIA[2] and G. ACETO[2]

[1]Department of Clinical and Experimental Medicine, University of Messina, Italy; [2]Department of Medical, Oral and Biotechnological Sciences, 'G. d'Annunzio' University, Chieti, Italy; [3]Department of Human Pathology, University of Messina, Italy

The importance of aberrant regulation of Wnt/β-Catenin signaling in the pathogenesis and colorectal cancer progression has long been recognised. Recent studies have shown that reactive oxygen species (ROS) production activates the Wnt/β-Catenin pathways, but the mechanisms involved remain unclear. Excess in ROS production is linked to chronic inflammation and promotes DNA damages and repair systems. We aim to evaluate the relationship among oxidative stress response and canonical/non-canonical Wnt pathways in colorectal cancer cell line models with different Wnt signaling behaviour.

MATERIALS AND METHODS

The HCT116 (MSI) and SW480 (MSS) cells were treated with H2O2 at different concentration (0.05, 05, 1, 2, 5, 10 μM) and timing. Cell viability were assayed by MTS (Promega) at 37°C for 1h and then evaluated at 490 nm using the GloMax-Multi Detection System (Promega). Total RNA was extracted using the Trizol® Reagent. Gene expression levels were evaluated by SYBR Green quantitative real-time PCR (qRT-PCR) analysis. The results were subjected to *T*-test analysis; statistical significance was set at $p < 0.05$.

RESULTS

The inhibition rate of cell proliferation was different with MTS assay for the H2O2 concentrations. To evaluate the ROS acute stress we chose H2O2 (2 μM and 10 μM) for 15' and 30'.

After H2O2 (2μM) treatment we observed gene expression up regulation of canonical/non-canonical molecules (LRP6 and LEF1; ROR2 and JUN/AP1) in SW480, while in HCT116 the expression of co-receptors, ROR2 and LRP6, decreased. In SW480, at H2O2 (10μM), we observed a dose dependent increase for both ways. Whereas in HCT116, APC, LRP6, LEF1, P65-NFkB gene expression showed an opposite behaviour depending on time of treatment, 15'-30' in opposition to non-canonical ROR2 receptor. A significant increase in expression was detected for MUTYH, OGG1, NRF2, COX2 and JUN/AP1.

CONCLUSION

Our observations present the evidence that a H2O2 stress miming ROS may induce differently WNT canonical/non-canonical pathways in colon cancer cells with and without microsatellite instability.

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PHENFORMIN INHIBITS TUMOR GROWTH THROUGH A COMPLEX I-INDEPENDENT REDOX/COREPRESSOR MODULE

L. DI MAGNO[1], S. MANNI[2], F. DI PASTENA[3], S. CONI[2], M. MARODER[3] and G. CANETTIERI[2]

[1]Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy; [3]Department of Molecular Medicine, Sapienza University of Rome, 00161, Rome, Italy; [2]Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Rome, Italy

The antidiabetic drug phenformin displays potent anticancer activity in different tumors but its mechanism of action remains elusive. In this study we have investigated the mechanism of cancer growth inhibition of phenformin, using as a model SHH Medulloblastoma, a cerebellar brain tumor characterized by an inappropriate activation of the Sonic Hedgehog signaling.

MATERIALS AND METHODS

Functional, biological and biochemical analyses were performed in murine Medulloblastoma cells (Med1-MB and primary culture of SHH tumors) treated with clinical doses of phenformin. The knockdown of mGPD and CtBP2 was obtained by lentiviral-mediated shRNA approaches. The relevance of *in vitro* findings has been evaluated *in vivo* on mouse models of SHH Medulloblastoma.

RESULTS

We show that at the clinically relevant concentrations phenformin elicits a significant therapeutic effect through a redox-dependent, but complex I-independent mechanism. Phenformin inhibits mitochondrial glycerol-phosphate dehydrogenase (mGPD), a component of the glycerophosphate shuttle, and causes elevations of intracellular NADH content. Inhibition of mGPD mimics phenformin action and promotes an association between the corepressor CtBP2 and Gli1, thereby inhibiting Hh transcriptional output and tumor growth. Since ablation of CtBP2 abrogates the therapeutic effect of phenformin in mice, these data illustrate a biguanide-mediated redox/corepressor interplay, which may represent a relevant target for tumor therapy.

CONCLUSION

These findings reveal a rationale for the antineoplastic action of a clinically tested drug, indicating that mGPD and CtBP2 are potential targets for therapeutic intervention.

HEME OXYGENASE-1 AND BRAIN OXYSTEROLS METABOLISM ARE LINKED TO EGR-1 EXPRESSION IN AGED MICE CORTEX, BUT NOT IN HIPPOCAMPUS

P. ROSA[1], C. ZERBINATI[2], A. CRESTINI[3], A. CANUDAS[4], M. MARODER[1], R. MURARO[5], A. CONFALONI[3], L. IULIANO[2] and A. CALOGERO[2]

[1]Department of Medical-Surgical Sciences and Biotechnologies, University of Rome "Sapienza", Polo Pontino, Latina, Italy; [2]Department of Medical-Surgical Sciences and Biotechnologies, University of Rome "Sapienza", Polo Pontino, Latina, Italy - Istituto Chirurgico Ortopedico Traumatologico, ICOT, Latina, Italy; [3]Department of Neuroscience, Istituto Superiore di Sanità, Rome, Italy; [4]Department of Pharmacology, Toxicology and Therapeutic Chemistry (Pharmacology Section), Institute of Neuroscience, CIBERNED, University of Barcelona, Barcelona, Spain; [5]Department of Medical, Oral and Biotechnological Sciences, University "G. D'Annunzio" Chieti-Pescara, Italy

Age is the main risk factor for many pathologies, neurodegenerative disorders included. With the world population getting older, aging will represent the disease of the future leaving an increasing need to understand the molecular basis of this process in normal and pathological conditions. Egr-1 (early growth response 1) is a transcriptional factor rapidly induced by many stress stimuli and it is involved in cell survival, proliferation and differentiation, as well as in memory, cognition and synaptic plasticity. Another molecule with neuroprotective properties is HO-1 (heme oxygenase-1), which converts heme to iron, carbon monoxide and biliverdin and it was shown to regulate the metabolism of oxysterols (derivatives of cholesterol oxidation) which represent new markers of oxidative stress. The only evidence of a link between Egr-1 and HO-1 was described in mouse lung cells exposed to cigarettes smoke. The aim of this study was to investigate whether Egr-1 can be implicated in oxysterol metabolism during brain aging.

MATERIALS AND METHODS

The study was conducted on young, adult and old wild-type and Egr-1 KO mice brain cortices and hippocampi (n=6 per group). The expression of Egr-1 and HO-1 proteins were observed in adult mice brains by immunohistochemical analysis. The quantification of these proteins was performed by western blot and oxysterols quantifications were obtained by isotope dilution mass spectrometry.

RESULTS

Our results show that the levels of Egr-1 in the mouse brain cortex increase in an age-dependent manner and correlate with those of HO-1 and oxysterols. Interestingly, this is a situation not observed in the hippocampal area. Our data are supported by the observations on the Egr-1 KO mouse model that confirms the dependence of HO-1 induction for oxysterols metabolism on Egr-1, exclusively in the aged brain cortex. It is important to notice that the main oxysterols involved in this process are those generally induced by oxidative stress, which would represent the triggering factor for this mechanism.

CONCLUSION

Our study offers new evidence for a fundamental role of Egr-1 in the metabolism of brain oxysterols as age advances, providing precious insights for any interventions aimed to slow down the aging process that may lead to cognitive decline and neurodegeneration.

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EFFECTS OF THE REPURPOSED DRUG CANDIDATE PARBENDAZOLE IN PANCREATIC CANCER CELLS

L. DE LELLIS[1], R. FLORIO[1], F. VERGINELLI[2], S. VESCHI[1], T. DI GENNI[1], V. DI GIACOMO[1], S. PAGOTTO[3], S. CARRADORI[1], A. CATALDI[1] and A. CAMA[1]

[1]Department of Pharmacy, "G. d'Annunzio" University Chieti-Pescara, 66100 Chieti, Italy; [2]Department of Pharmacy, "G. d'Annunzio" University Chieti-Pescara, 66100 Chieti, Italy and Aging Research Center and Translational Medicine-CeSI-MeT, Chieti, 66100, Italy; [3]Department of Medical, Oral and Biotechnological Sciences, "G. d'Annunzio" University Chieti-Pescara, Chieti, 66100, Italy and Aging Research Center and Translational Medicine-CeSI-MeT, Chieti, 66100, Italy

Pancreatic cancer (PC) is the fourth most common cause of cancer death. Classical chemotherapeutic drugs, used alone or in combination, have a limited advantage in term of 5-year survival, at the cost of a relevant toxicity. Thus, novel therapeutic options with minimal side effects are urgently needed for this lethal disease. In this regard, the use of several non-toxic repurposed drug candidates has been explored in preclinical models of several tumors. In the present study, we investigated the potential repurposing in PC of the FDA-approved anthelmintic drug parbendazole.

MATERIALS AND METHODS

The effects of parbendazole on the viability of PC cell lines AsPC-1 and Capan-2 were tested by MTT. The effect of the drug on microtubular network was examined by confocal microscopy. The effects of treatment on cell cycle and apoptosis were analyzed by flow cytometry and immunoblot analyses. Self-renewal capacity of PC cell lines after drug treatment was evaluated using a clonogenic assay. The effects of the drug on PC cell migration were assessed by wound-healing assay.

RESULTS

Parbendazole significantly and markedly inhibited viability and growth of the two PC cell lines through mechanisms involving the alteration of tubulin distribution and the formation of irregular mitotic spindles. Parbendazole also caused a drastic cell cycle arrest in G2/M phase in both PC cell lines, with alteration of the expression of cell cycle regulators such as cyclin D3 and cyclin B1. In addition, parbendazole promoted PC cell apoptosis, as indicated by flow cytometry evaluation of Annexin-V staining and PARP cleavage. Finally, parbendazole drastically impaired clonogenic activity and migration of the two PC cell lines.

CONCLUSION

Taken together, our results show an interesting antitumor potential of parbendazole in PC cells. This should be further investigated in preclinical models of PC and eventually in clinical trials to evaluate the potential repurposing of parbendazole in PC treatment.

STUDY OF THE TRANSCRIPTIONAL REGULATION OF THE ONCOSUPPRESSOR KCASH2

A. ANGRISANI[1], C.A. DI TRANI[1], M. MORETTI[2], M. IZZO[3], A. CIOCIA[1], S. FONTE[1] and E. DE SMAELE[1]

[1]Department of Experimental Medicine, Sapienza University of Rome, Italy; [2]Department of Molecular Medicine, Sapienza University of Rome, Italy; [3]Department of Molecular Medicine, Sapienza University of Rome, Italy

KCTD containing Cullin3 adaptor, suppressor of Hedgehog (KCASH2) belongs to the KCASH family of proteins, which are involved in the negative regulation of the Sonic Hedgehog (Hh) signaling pathway. All members of KCASH family have shown to be downregulated in Hh dependent Medulloblastoma (Mb), by either allelic loss or promoter methylation. Particularly, KCASH2 expression is reduced also in other tumors, not directly connected with Hh pathway modulation; moreover, chromosome 11q, where KCASH2 is localized, is lost in several sporadic tumors. In turn, correlation studies and data of laboratory show a role of KCASH2 in differentiation and cell cycle regulation, suggesting its fine regulation during development.

MATERIALS AND METHODS

We performed bioinformatics analysis to study KCASH2 proximal promoter and identify putative transcriptional factors (TFs) that could be responsible of gene regulation. We cloned the human sequence of KCASH2 promoter into a luciferase vector to create a broad-spectrum system that allows us to test the transcriptional modulation of KCASH2. At the same time, we analyzed the beta-galactosidase activity from mouse embrional fibroblasts (MEF) generated by our KCASH2-KO mouse model.

RESULTS

By informatics screening, on KCASH2 promoter we identified several Sp1 binding sites, a well-characterized transcriptional activator. To understand the regulation of KCASH2 by Sp1, we analyzed the transcriptional activity of full-length and mutated forms of Sp1 core sequences of the binding sites on promoter. Interestingly, full-length KCASH2 promoter showed a strong basal activity, compared with the empty luciferase vector, suggesting that Sp1 TF is necessary for the basal transcriptional activity of KCASH2 TATA-less promoter. Moreover, the treatment of D283, a medulloblastoma cell line, with EGF increases the KCASH2 transcriptional activity. In turn, we have assayed STAT1/STAT3, TFs downstream the EGFR signaling and other TFs identified by informatics analysis, such as p53, Gli3, GliS3, relA/p65.

CONCLUSION

The knowledge of TFs involved in the physiologic and pathological regulation of KCASH2 transcription could be important to develop therapeutic approaches in order to re-establish the onco-suppressor role of KCASH2.

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ITCH/α-ARRESTIN2-DEPENDENT NON-PROTEOLYTIC UBIQUITYLATION OF SUFU CONTROLS HEDGEHOG SIGNALLING AND MEDULLOBLASTOMA TUMORIGENESIS

L. LOSPINOSO SEVERINI[1], F. BERNARDI[1], R. FAEDDA[1], P. INFANTE[2], F. BUFALIERI[1], R. ALFONSI, D. MAZZÀ[1], M. SILER[1], S. CONI[1], A. PO[1], M. PETRONI[2], E. FERRETTI[3], M. MORI[2], E. DE SMAELE[3], G. CANETTIERI[1], C. CAPALBO[1], M. MARODER[4], I. SCREPANTI[1], M. KOOL[5], S.M. PFISTER[5], D. GUARDAVACCARO[6], A. GULINO[1],L. DI MARCOTULLIO[1]

[1]Department of Molecular Medicine, University La Sapienza, Rome, Italy; [2]Center for Life NanoScience@ Sapienza, Istituto Italiano di Tecnologia, Rome, Italy; [3]Department of Experimental Medicine, University La Sapienza, Rome, Italy; [4]Department of Medico-Surgical Sciences and Biotechnologies, University La Sapienza, Rome, Italy; [5]Hopp Children's Cancer Center at the NCT (KiTZ), Heidelberg, Germany; [6]Hubrecht Institute-KNAW and University Medical Center Utrecht, Utrecht, The Netherlands

Suppressor of Fused (SuFu), a tumour suppressor mutated in medulloblastoma (MB), is a central player of Hh signalling, a crucial pathway for development and deregulated in tumours. Although the control of Gli transcription factors by SuFu is critical in Hh pathway, our understanding of the mechanism regulating this key event remains limited. To this end, the main objective of this study is to investigate the role of the ubiquitylation processes in the regulation of SuFu/Gli interaction.

MATERIALS AND METHODS

We evaluated the effect of several E3 ubiquitin ligases and adaptor proteins on SuFu ubiquitylation by *in vivo* ubiquitylation assay. Subsequently, we investigated the ability of the HECT E3 ligase Itch and the adaptor α-arrestin2 to bind and ubiquitylate SuFu in MEF cells and during cerebellar development in order to gain insight into the biological role of this post-translational modification. We also analysed the mechanisms by which Itch-dependent SuFu ubiquitylation regulates SuFu/Gli interaction through NanoBiT technology and co-immunoprecipitation assays. Finally, proof-of-concept *in vivo* studies (xenograft/orthotopic models and PET/SPECT/CT analysis) were performed in human MB Daoy cells infected with lentiviral vectors expressing SuFu WT or SuFu mutant insensitive to Itch activity.

RESULTS

In the present study, we demonstrate that Itch/α-arrestin2 complex ubiquitylates SuFu through K63-mediated linkages, without affecting SuFu stability, characterizing a regulatory rather than a degradative pathway. In this regard, we observed that this process increases the association of SuFu with Gli3, promoting its conversion into Gli3R and keeping the Hh pathway off. Notably, SuFu mutant, missing lysines interested in Itch-dependent ubiquitylation, enhances MB cell proliferation *in vivo*, highlighting the relevance of this mechanism in tumour growth.

CONCLUSION

Our findings identify a novel molecular mechanism in the negative control of Hh signalling and unveil, for the first time, that Itch-dependent K63-polyubiquitylation of SuFu may play an important role in the MB onset.

THE EFFECT OF THE HYPOXIC MICROENVIRONMENT ON GLIOBLASTOMA CELLS: BK CHANNELS BLOCKADE AS A NOVEL STRATEGY TO LIMIT DRUG RESISTANCE

P. ROSA[1], G. MANGINO[1], F. FAZI[2], S. MASCIARELLI[2], S. SCIARRETTA[1], G. MINCIONE[3], V. PETROZZA[4], G. RAGONA[4] and A. CALOGERO[4]

[1]Department of Medical-Surgical Sciences and Biotechnologies, University of Rome "Sapienza", Polo Pontino, Latina, Italy; [2]Department of Anatomical, Histological, Forensic & Orthopedic Sciences, Section of Histology & Medical Embryology, Sapienza University of Rome; [3]Department of Medical, Oral and Biotechnological Sciences, University "G. D'Annunzio" Chieti-Pescara, Italy; [4]Department of Medical-Surgical Sciences and Biotechnologies, University of Rome "Sapienza", Polo Pontino, Latina, Italy - Istituto Chirurgico Ortopedico Traumatologico, ICOT, Latina, Italy

Glioblastomas (GBMs) are brain tumors of glial origin characterized by a heavy hypoxic microenvironment, which correlates with tumor aggressiveness. GBM cells abundantly express large-conductance, calcium-activated potassium (BK) channels and, since hypoxia modulates their activity in GBM cells, the aim of the present work was to explore their role in the hypoxia-induced chemoresistance and to better understand the molecular mechanisms underlying the effect of the hypoxic microenvironment on BK channels-dependent drug resistance of GBM cells.

MATERIALS AND METHODS

All the experiments were carried out on U87-MG cells grown under normoxia and hypoxia, treated or not with cisplatin and in presence or not of the BK channel blocker, paxilline. Cell viability was assessed by MTS and trypan blue assays. Cell cycle analysis was performed by propidium iodide incorporation and analized with a cytofluorimeter. Autophagy was assessed by western blot analysis of the markers LC3A-B and p62. Intracellular ROS concentration was evaluated by DCF-DA and analyzed by cytofluorimetric analysis.

RESULTS

Our results show that hypoxia is able to induce chemoresistance to cisplatin in U87-MG cells and that the concomitant inhibition of BK channels with paxilline confers resistance when added to Cisplatin in U87-MG under normoxia, while is able to chemosensitize the same cells under hypoxia. These data are supported by the cell cycle analysis of U87-MG cells, by the evaluation of autophagy and by intracellular ROS generation. In particular, BK channels blocker paxilline induces an accumulation of cells in the S phase when added to cisplatin in normoxia, augments autophagy (upregulation of LC3B) and downregulates ROS levels. On the contrary, when we repeated the same experiments under hypoxia by adding paxilline to cisplatin, we observed the arrest in the G2/M phase of an appreciable subpopulation of U87-MG cells, the downregulation of autophagy (lower levels of LC3B) and an upregulation of intracellular ROS.

CONCLUSION

In this study, we have shown the role of BK channels in the hypoxia-induced resistance to cisplatin. The blockade of this channel may be a promising strategy to preferentially target a subpopulation of cells which resides in the hypoxic areas of the tumor and are more difficult to eradicate.

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SYNERGISTIC INHIBITION OF THE HEDGEHOG PATHWAY BY NEWLY DESIGNED SMO AND GLI ANTAGONISTS BEARING THE ISOFLAVONE SCAFFOLD

M. CAIMANO[1], S. BERARDOZZI[2], F. BERNARDI[1], P. INFANTE[3], C. INGALLINA[4], S. TOSCANO[5], E. DE PAOLIS[5], R. ALFONSI[1], B. BOTTA[4], M. MORI[3], F. GHIRGA[3] and L. DI MARCOTULLIO[1]

[1]Department of Molecular Medicine, Sapienza University of Rome, Italy; [2]Department of Chemistry and Technology of Drugs, Sapienza University of Rome, Rome, Italy and Department of Chemistry and Technology of Drugs, Sapienza University of Rome, Rome, Italy; [3]Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy; [4]Department of Chemistry and Technology of Drugs, Sapienza University of Rome, Rome, Italy; [5]Department of Chemistry and Technology of Drugs, Sapienza University of Rome, Rome, Italy

The Hedgehog (Hh) signalling pathway is evolutionarily conserved and its fine regulation is essential for proper development and tissues homeostasis. Aberrant activation of the Hh pathway is responsible for the onset of several malignancies, including medulloblastoma (MB), the most frequent pediatric brain tumor. Small molecules able to block the pathway at the upstream receptor Smoothened (Smo) or the downstream effector Gli1 have thus emerged recently as valuable anticancer agents. The aim of this proposal is to discover novel powerful Smo and Gli inhibitors targeting Hh signaling at both upstream and downstream level, and to investigate their pharmacological effects on MB.

MATERIALS AND METHODS

A multidisciplinary research established mixing chemistry and molecular biology expertise allowed to identify innovative and more effective anti-cancer therapies for the treatment of MB. Computational studies, screening in silico, functional and proliferation assays have been used in this work.

RESULTS

We have designed, synthesized, and tested new Hh inhibitors taking advantage by the highly versatile and privileged isoflavone scaffold. The introduction of specific substitutions on the isoflavone's ring B allowed the identification of molecules targeting preferentially Smo or Gli1. Biological assays coupled with molecular modeling corroborated the design strategy, and provided new insights into the mechanism of action of these molecules.

CONCLUSION

The combined administration of two different isoflavones behaving as Smo and Gli antagonists, respectively, in primary MB cells highlighted the synergistic effects of these agents, thus paving the way to further and innovative strategies for the pharmacological inhibition of Hh signaling.

PD-L1 LEVEL IS UP-REGULATED IN NSCLC CELLS AFTER PEMETREXED TREATMENT

A. CAVAZZONI[1], G. DIGIACOMO[1], S. LA MONICA[1], R. ALFIERI[1], M. FIORENTINO[2], A. ARDIZZONI[2] and P.G. PETRONINI[1]

[1]Department of Medicine and Surgery, University of Parma, Parma, Italy; [2]Medical Oncology Unit, Policlinico S. Orsola-Malpighi, University of Bologna, Bologna, Italy

Immune checkpoint inhibitors, such as PD-1/PD-L1 targeting monoclonal antibodies, have shown efficacy in the treatment of non-small cell lung cancer (NSCLC) in the adjuvant, first- and subsequent-line settings, in monotherapy or combined to standard chemotherapy regimens in both adeno and squamous histologies. Recent results from KEYNOTE-189 trial indicates that the addition of pembrolizumab to pemetrexed and platinum-based drug results in longer OS and PFS than chemotherapy alone even in originally PD-L1 negative patients with lung adenocarcinoma (Gandhi L. et al. NEJM 2018). Nevertheless, the success of this therapy is limited and there is an urgent need for developing strategies to sensitize NSCLC to immune-checkpoint inhibitors. In this study, we evaluated whether chemotherapeutic agents may alter PD-L1 expression in order to guide optimal combination/sequencing with Immune checkpoint inhibitors in the clinic.

MATERIALS AND METHODS

Membrane PD-L1 (mPD-L1) expression was detected by flow cytometry, western blotting and real time PCR. Soluble PD-L1 (sPD-L1) was detected by Elisa Assay. Activation of intracellular signaling pathways was detected by western blotting. Cytokine expression was quantified by RT PCR.

RESULTS

Firstly, we evaluated mPD-L1 level in NSCLC cell lines with adenocarcinoma histotype. mPD-L1 was expressed at different levels in all the cancer cells irrespectively of EGFR or ALK mutational status. We then tested the effect of gemcitabine, pemetrexed, paclitaxel, vinorelbine and cisplatin on PD-L1 expression. Cells were treated with each compound at the corresponding IC50 value and only pemetrexed up-regulated both mPD-L1 and sPD-L1 expression levels in all the cell lines tested. We also demonstrated that after pemetrexed removal, PD-L1 was maintained at high level, indicating that PD-L1 once expressed is a stable protein.

Moreover, cancer cells treated with pemetrexed showed up-regulation of STAT3 and AKT/mTOR signaling pathways and pharmacological inhibition of these axis reduced mPD-L1 expression.

Finally, we observed that pemetrexed significantly reduced T-lymphocyte viability during the activation and in this condition increased levels of both IFN-ϒ (a cytokine known to upregulate mPD-L1) and IL-2 mRNAs were detected.

CONCLUSION

In conclusion, our data indicates that pemetrexed induces the expression of mPD-L1 and sPD-L1 in NSCLC cell lines and cytokine production in PBMC, suggesting a possible reading of the positive results obtained in the phase III clinical trial KEYNOTE-189, where the addition of pembrolizumab to pemetrexed and platinum-based drug results in longer OS and PFS than chemotherapy alone, even in originally PD-L1 negative patients.

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DIAGNOSI DI LABORATORIO EMOMETRICA DI MIELODISPLASIA (MDS)

V. LATELLA, B. MODAFFERI and C. LAGANÀ

U.O.C. Laboratorio Analisi- G.O.M. "Bianchi-Melacrino-Morelli", Reggio Calabria

The aim of this study is to attention the important role that laboratory medicine plays today in the evaluation of hematological diseases. The utility of the collaboration between the laboratory staff and the clinicians has emerged as fundamental to diagnose complex diseases in a short time interval.

MATERIALS AND METHODS

Materials and Methods: In April, 2018, a male patient (age 88 years) was admitted to the Intensive Care Unit of our hospital following major orthopaedic surgery. The complete blood count (CBC) performed during the afternoon shift of the general laboratory directed a suspicion towards a septic state with WBC 34.690/mmc PLT: 65.000/mmc HGB: 7.2 gr/dl MCV: 88.6.fl. 24 h later, the CBC was performed in the specialized hematological Laboratory.

RESULTS

An accurate analysis of the output and graphs with position indexes (SSC:123) placed attention on the hypothesis of a possible diagnosis of Myelodysplasia. Morphological evaluation of the peripheral blood smear showed: dysplasia of the granulocyte lineage, some Pfelger cells, hypogranulated and bilobated neutrophils, and moderate anisocytosis.

CONCLUSION

Probable myelodysplastic syndrome. The data obtained were referred to the attention of the Hematology Unito of our Hospital. The bone marrow morphology confirmed showed: Normocellular marrow, erythroblastic lineage with a maturation block and >10% dysplasia. Hyperplasia of the granulocytic lineage with maturation. Normal megacaryocytes.

The use of new generation analysers in the hands of a laboratory expert provides adequate information to identify cases to refer to clinicians to formulate diagnoses even for complex and specialistic diseases.

DIAGNOSIS OF LYMPHOMA FROM COMPLETE BLOOD COUNT

V. LATELLA, B. MODAFFERI, B. OLIVA, C. GARREFFA and C. LAGANÀ

U.O.C. Laboratorio Analisi- G.O.M. "Bianchi-Melacrino-Morelli"- Reggio Calabria

The aim of the study is to attention the important role that laboratori medicine has in the evaluation of hematological diseases. The utility of the collaboration between the laboratory staff and the clinicians has emerged as fundamental to diagnose complex and specialistic diseases in a short time interval.

MATERIALS AND METHODS

In January 2018, a female patient (aged 76) underwent a complete blood count (CBC) at the hematological Laboratory after having been tested in an external laboratory. She was referred with a suspected diagnosis of myeloproliferative neoplasm. The CBC output and graph were accurately interpreted.

RESULTS

The CBC directed the suspicion towards a lymphoproliferative neoplasm, confirmed by morphological evaluation of the peripheral blood smear: presence of lymphoma cells; compact cromatin and large nuceoli; slightly basophil cytoplasm with irregular membrane. The blood sample was then transfered to the biologists of the Flow Cytometry laboratori who confirmed and better defined the case: immunophenotype compatible with a lymphoproliferative neoplasm B cell non Hodgkin Lymphoma CD19+ CD20+ (a) FMC7+ CD5- CD200- CD23- CD10- CD103- and clonal restriction for immunoglogulin light chain sK.

CONCLUSION

The use of new generation analyzers in the hands of a laboratory expert provides clinicians with adequate information to formulate diagnoses, even of complex and specialistic nature. The use of the most advanced and innovative technologies for laboratory analysis in hematology and the continuous progress in research and development guarantee the choice of evermore adequate methods to ensure accurate testing to generate useful information in the diagnostics in hematology.

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GLYCINE/SARCOSINE RATIO AS NOVEL BIOMARKER FOR ALCOHOL-INDUCED LIVER FIBROSIS UNDER SUMOYLATION CONTROL

C. COSSU[1], R.M. PASCALE[1] and M.L. TOMASI[2]

[1]University of Sassari; [2]Cedars-Sinai Medical Center

Alcohol-induced liver fibrosis/disease (ALD) is characterized by excessive deposition of extracellular matrix (ECM) components in response to chronic abuse that could lead to cirrhosis and hepatocellular carcinoma development. Sarcosine is a derivative of the amino acid glycine, formed by the enzymes glycine N-methyl transferase (GNMT) or dimethylglycine dehydrogenase (DMGDH) and converted back into glycine via sarcosine dehydrogenase (SARDH). GNMT is silenced in human alcohol-induced cirrhosis. In addition, GNMT knockout mice develop oxidative stress, liver injury, fibrosis, and HCC. SUMOylation is a post-translational modification that requires an essential E2-conjugating enzyme 9 (UBC9) to covalently bind of small ubiquitin modifier (SUMO) and plays an important role in a wide range of cellular processes. We previously demonstrated that UBC9 level is induced in intragastric ethanol-infusion (EI) treated mouse liver. We performed SUMO-proteomics of alcohol-fed mouse liver and identified altered sumoylation of GNMT and SARDH. The goal of this work is to examine whether the dysregulated SUMOylation could regulate GNMT and SARDH enzymatic function in ethanol-induced liver fibrosis and elucidate the molecular mechanism(s).

MATERIALS AND METHODS

Studies were performed using mouse hepatocytes (HEP), kupffer cells (KCs) and stellate cells (HSCs) from *in vivo* acute and chronic ethanol-fed mouse models and primary human HSCs. Oxidative stress and metabolic markers were measured using commercial kits.

RESULTS

We found that ethanol treatment *in vivo* induced UBC9 expression in HSCs and HEP and inhibited its expression in KCs. This was associated with increased GNMT sumoylation and total levels, specifically in HSCs. In contrast, SARDH sumoylation fell in HSCs despite an increase in its total level. Ethanol feeding increased glycine and lowered sarcosine levels in mouse plasma, suggesting a potential regulation of GNMT/SARDH enzyme activity. Using ethanol-treated coculture model (HEP, HSCs and KCs), we found increase in ROS production in all liver cells and glycine/sarcosine ratio in HSCs but not in HEP and KCs compared to control. Ubc9 silencing in HSCs inhibited ethanol-mediated ROS production and induction of HSC activation.

CONCLUSION

This data strongly suggests that oxidative stress-induced sumoylation plays a key role in HSC activation and this may influence the transmethylation machinery. Alterations of glycine/sarcosine ratio as a consequence of enzyme sumoylation could be considered as novel biomarker for ALD.

LONGEVITY ASSOCIATED VARIANT OF BPIFB4 TUNES THE MACROPHAGES POLARIZATION AND MITIGATE MONOCYTE MEDIATED ACQUIRED IMMUNE RESPONSE: A POSSIBLE EXPLANATION OF HOW CENTENARIANS ESCAPE AGING DISEASES

E. CIAGLIA, F. MONTELLA and A.A. PUCA

Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno

Reaching exceptional longevity is a consequence of aging delay driven by genetic and epigenetic factors, which are either inherited or acquired. It has been postulated that aging is driven by imbalance between inflammatory and anti-inflammatory networks that results in the low grade chronic pro-inflammatory status causing most of the physiopathological changes triggered by aging. In this respect, the activation of monocyte-macrophages plays a major roles in finely tune the immune responses as they cover a continuum of functional states which oscillate from a patrolling protective effect toward an inflammatory ones. We previously identified a four-SNPs haplotype, the Longevity-associated variant (LAV) of bactericidal/ permeabilityincreasing fold-containing-family-B-member-4 (BPIFB4) able to activate calcium, PKC-α, eNOS, rescuing endothelial dysfunction in aged mice and inducing revascularization through homing of progenitor cells. BPIFB4 abundance in serum of healthy centenarians as compared to non-healthy ones, the therapeutic potentials LAV-BPIFB4 in improving vascular homeostasis, at least in part mediated by Ly6Chigh monocytes, and BPIFB4 expression in bone marrow myeloid cells induced us to evaluate if LAV-BPIFB4 may improve immune regulation.

MATERIALS AND METHODS

Through FACS analysis of healthy PBMC and functional immune assays, we characterized traditional monocyte subsets and their differentiation program toward macrophage and dendritic cell (DCs) lineage, a key process in the initiation of primary adaptive immune responses and their maintenance.

RESULTS

Interestingly, monocytes exposed *in vitro* to LAV-BPIFB4 protein are rapidly polarized toward M2 macrophages (CD45+ CD11b+ CD86− CD206+). Moreover, human MoDC, generated in presence of LAV-BPIFB4 protein, acquire a peculiar immune regulatory profile characterized by a sustained expression of co-stimulatory molecules in resting state and the reduction of pro-inflammatory cytokine IL-1α and TNF-α along with an increase of anti-inflammatory TGF-α and IL-10 production after pro-inflammatory stimuli. Accordingly, mature LAV-DCs fail to prime T cell proliferation.

CONCLUSION

Overall LAV-BPIFB4 effects, that are not observed using WT-BPIFB4, on myeloid compartment could explain how nature have worked in favor of a genetic asset that protect from immunological dysfunction.

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.