XXI Convegno Annuale ABCD sul Tema

Stress cellulare: sopravvivenza ed apoptosi

Urbino
7-8 maggio 2010

Comitato organizzatore

Piero Sestili
Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino

Lina Ghibelli
Dipartimento di Biologia, Università Tor Vergata, Roma

Pier Giorgio Petronini
Dipartimento di Medicina Sperimentale, Università degli Studi di Parma

Chiara Martinelli
Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino

Evelin Colombo
Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino

Col contributo di

Enzo Life Sciences

In Italia: Vinci-Biochem

M·MEDICAL

Col patrocinio di

Università degli Studi di Urbino "Carlo Bo"

CITTÀ DI URBINO

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PROGRAMMA

Venerdì, 7 maggio

12.00 **Registrazione**

12.30 **pranzo**

14.30 **Saluto delle Autorità – Introduzione**

15.00 **Lettura Magistrale**

Moderatore: Piero Sestili

Come le cellule rispondono alla crisi:
tagli e riorganizzazione strutturale
Paolo Pinton (Ferrara)

Sessione 1: Stress and cell death

Moderatori: Pier Giorgio Petronini e Lorenza Tacchini

15.30 The iron chelator dexrazoxane protects cardiomyocytes from doxorubicin-mediated cell death: role of HIF-1
Rosalin Spagnuolo (Milano)

15.50 Human articular chondrocyte cell death:
from ultrastructure to biochemistry
Michela Battistelli (Urbino)

16.10 Nanoceria antagonizes apoptosis via ROS scavenging
Lina Ghibelli (Roma)

16.30 Mechanisms underlying UVB-induced apoptosis in skeletal muscle tissue
Elisabetta Falcieri (Urbino)

16.50 Magnetic fields promote a pro-survival non-capacitative Ca^{2+} entry via phospholipase C signaling
Emanuele Bruni (Roma)

17.10 Sphingosine, not ceramide, mediates TNF-induced death of rat hepatoma cells
Chiara Ullio (Torino)

17.30 **Pausa caffè**

Sessione 2: Oxidative stress

Moderatori: Paolo Pinton e Lina Ghibelli

17.50 KRIT1 helps cells to prevent oxidative stress
Saverio Francesco Retta (Torino)

18.10 Involvement of protein ERp57 in cellular response to oxidative stress
Daniela Ricci (Roma)

18.30 Mesoangioblast behaviour in oxidative stress condition
Giuseppina Turturici (Palermo)

18.50 RNA as a new target for toxic and protective agents
Carmela Fimognari (Bologna)

19.10 Effects of either ischemic postconditioning or early acidotic reperfusion on cardiac post-ischemic redox-environment
Claudia Penna (Torino)

20.30 **Cena**

Sabato, 8 maggio

Sessione 3: Cellular response to drugs

Moderatori: Paolino Ninfali e Elisabetta Falcieri

9.00 New therapeutic strategies in the treatment of non small cell lung cancer
Roberta Alfieri (Parma)

9.20 Synergistic effect of Epigallocatechine-3-gallate, Xylosylvitexin and Glucoraphasatin isothiocyanate in human carcinoma cell lines
Alessio Papi (Bologna)

9.40 TNFα potentiates apoptotic effect induced by taxol in human hepatoma cells
Valerio Giacomo Minero (Torino)

10.00 Xylosylvitexin: a chemopreventive molecule extracted from *Beta vulgaris cicla* seeds
Lorenzo Gennari (Urbino)

10.20 Doxorubicin enhances protein ubiquitination and exerts synergic cytotoxic effect in combination with Bortezomib
Giorgia Mandili (Torino)

10.40 **Pausa caffè**

Sessione 4: Cell damage

Moderatori: Mariela Roccheri e Roberta Alfieri

11.00 UV-B stress induces 14-3-3 epsilon mRNA increase and delocalisation in *Paracentrotus lividus* sea urchin embryos
Roberta Russo (Palermo)

11.20 Cellular and molecular effects of a 300 mT static magnetic field on HUVECs
Emanuela Polidori (Urbino)

11.40 Evaluation of sperm quality and DNA integrity after freeze/thawing
Liana Bosco (Palermo)

12.00 Role of neutrophils in the Shiga toxin-induced endothelial damage in hemolytic uremic syndrome
Maurizio Brigotti (Bologna)

12.20 Sea urchin embryo as a model system for studying autophagy induced by cadmium stress
Roberto Chiarelli (Palermo)

12.40 Adenosine A_2A receptor but not HIF-1 mediates tyrosine hydroxylase induction in hypoxic PC12 cells
Elena Gammella (Milano)

13.00 Another function of Hsp70 in mesoangioblast stem cells
Fabiana Geraci (Palermo)

13.20 **Conclusioni e prospettive**

13.30 **Pranzo**

Poster

Effect of androgen exposure on inflammatory response of male and female vascular endothelial cell
Giosuè Annibalini (Urbino)

Myoblastic cell apoptotic sensitivity: an *in vitro* study
Sara Salucci (Urbino)

Creatine protects from oxidative stress and improves the morpho-functional differentiation of neuroblasts in spinal cord primary culture
Stefano Sartini (Urbino)

I poster resteranno esposti per l'intera durata del meeting

ABSTRACTS

in ordine alfabetico del relatore

New therapeutic strategies in the treatment of non small cell lung cancer

Roberta Alfieri, Maricla Galetti, Silvia La Monica, Andrea Cavazzoni, Galvani Elena, Pier Giorgio Petroniani

Dip. di Medicina Sperimentale, Università degli Studi di Parma

Epidermal Growth Factor Receptor (EGFR) is an established new target for the treatment of epithelial tumors, including non-small cell lung cancer (NSCLC). Small molecules inhibitors, such as erlotinib and gefitinib, have proven to be a useful addition to standard therapy in advanced NSCLC. However, tumor cells often acquire resistance to these EGFR inhibitors. The mechanisms that mediate resistance to gefitinib treatment include secondary mutations in EGFR i.e. the point mutation T790M, the amplification of MET, constitutive or alternative activation of downstream pathways and efflux of the drug from the cells. In this study we have investigated in a panel of NSCLC cell lines new therapeutic approaches to circumvent acquired gefitinib resistance. Clear evidence exists for the involvement of constitutive activation of the PI3K/AKT signaling pathway in lung carcinogenesis and in resistance to tyrosine-kinase inhibitors. We assessed the effects of combining the mTOR inhibitor everolimus (RAD001) with gefitinib on a panel of NSCLC cell lines characterized by gefitinib-resistance and able to maintain pS6K phosphorylation after gefitinib treatment. Everolimus plus gefitinib induced a significant decrease in the activation of MAPK and mTOR signaling pathways downstream of EGFR and resulted in a growth-inhibitory effect rather than in an enhancement of cell death. A synergistic effect was observed in those cell lines characterized by high proliferative index and low doubling time. Trail receptor targeting agents represent an anti-cancer strategy able to induce apoptosis in tumor cells. We observed that the combined use of Trail with gefitinib increased the anti-proliferative effect and induced apoptosis in many of the cell line tested suggesting that this combined therapy might be an interesting strategy to exert a cytotoxic effect in gefitinib resistant cells. The design and synthesis of new tyrosine kinase inhibitors is another rational approach to treat acquired resistance to gefitinib. In the last few years we focused on the design and synthesis of a new generation of EGFR reversibile and irreversible inhibitors with an improved in vitro potency and selectivity. We identify a novel EGFR tyrosine kinase inhibitor, 5-benzylidene-hydantoin UPR1024, able of both blocking EGFR tyrosine kinase activity and inducing genomic DNA damage. Moreover, we are now extending the chemical diversity of new irreversible EGFR inhibitors. One of the proposed mechanism of resistance to gefitinib and other tyrosine kinase inhibitors is related to low level of intracellular drug concentration. In the last part of our research we have characterized the gefitinib transport system and we have evaluated whether the intracellular concentration of gefitinib may affect the EGFR autophosphorylation.

Effect of androgen exposure on inflammatory response of male and female vascular endothelial cells

Giosuè Annibalini, Deborah Agostini, Evelin Colombo, Chiara Martinelli, Michele Guescini, Pasquale Tibollo, Cinzia Calcabrini, Marco Paolillo, Vilberto Stocchi, Piero Sestili

Dipartimento di Scienze Biomolecolari, Sezione di Scienze Motorie e della Salute, Università degli Studi di Urbino "Carlo Bo", Italia

Men exhibit higher prevalence of atherosclerosis and coronary heart disease (CAD) compared to age-related pre-menopausal women. Genetic variation within the male-specific region of the Y chromosome and dominance of androgens over estrogens have been proposed as the major factors contributing to the male predisposition to cardiovascular disorders. However, several investigations have shown protective rather than detrimental effects of endogenous testosterone on cardiovascular system. Atherosclerosis is a chronic inflammatory process that begins with the expression of adhesion molecules (CAMs) on endothelial cells surface, which in turn facilitates the monocyte adhesion. In order to clarify the importance of gender on inflammatory endothelial response we analyzed the effect of Testosterone (T), Dihydrotestosterone (DHT) and Estradiol (EST) on cytokine-induced CAM expression and leukocyte adhesion in Human Umbilical Vein Endothelial Cells (HUVECs) from both male and female donors. In our experimental setting both male and female HUVECs expressed low levels of estrogen receptors (ERα and ERβ) and the enzyme aromatase, which irreversibly transforms T into EST. Conversely the androgen receptor (AR) and the enzyme 5α-reductase 1, which converts T in DHT (a non-aromatizable androgen), were strongly expressed both at the mRNA and protein level. These data led us to hypothesize that T could not only have an effect by itself, but could also be converted efficiently to DHT rather than to EST, using mainly -if not exclusively- the AR pathway. Treatment with different concentrations of steroid hormones did not influence the amounts of AR, ERs, aromatase and 5α-reductase. We next analyzed the effect of T, DHT and EST on gene expression of CAMs and on the adhesion of pro-monocytic U937 to TNF-α-stimulated HUVECs. The inflammatory effects of TNF-α were amplified by co-administration of T or DHT (concentrations ranging from 10 to 1000 nM) while EST had no effect. To our knowledge this is the first study demonstrating the same expression pattern of AR, ERs, aromatase and reductase in male and female HUVECs under identical experimental conditions. We also demonstrated, independently of gender, an inflammatory effect of T and DHT in HUVECs treated with TNF-α, without a protective effect of EST. The discrepancy of the results found in literature regarding the endothelial inflammatory response to steroids are likely to be due to different culture conditions rather that to gender.

Human articular chondrocyte cell death: from ultrastructure to biochemistry

Michela Battistelli[1], Sara Salucci[1], Eleonora Olivotto[2], Stefania Pagani[2], Rosi Borzì[2], Andrea Facchini[2], Elisabetta Falcieri[1, 3]

[1]DiSUAN, Università degli Studi di Urbino "Carlo Bo"

[2]Laboratorio di Immunologia e Genetica e [3] Istituto di Genetica Molecolare CNR, Istituti Ortopedici Rizzoli, Bologna, Italy

Chondrocyte death and loss of extracellular matrix are the central features in cartilage degeneration during osteoarthritis (OA) pathogenesis. Cartilage diseases and, in particular, OA, have been widely correlated to apoptosis (Johnson et al. J Surg Orthop Adv 17,2008). Roach et al (Apoptosis 9,2004) proposed the term chondroptosis to evidence this particular type of programmed death, with peculiar features typical of cartilage cells. In this work we investigated condrocyte death in a human experimental model. Micromasses, a tridimensional system of culture chondrocytes, represent a convenient means for studying their biology (Battistelli et al. Microsc Res Tech 67,2005; Olivotto et al. J Cell Physiol 210,2007). Cell death has been induced by different agents, all known to be powerful apoptotic triggers: hyperthermia, 1h, 43°C, followed by 4h recovery, UV-B, 30 min, followed by 4h recovery, 500nM staurosporine, 24h. Conventional electron microscopy (TEM) (Battistelli et al. Microscopie 1,2009) was utilized to analyse morphological changes and TUNEL reaction to investigate DNA fragmentation. Agarose gel DNA electrophoresis and caspases analyses were also performed. A scarce positivity of reaction intensity and stained cell number, appears in control culture. Chondrocyte morphology, in control condition, appears similar to that described in human articular cartilage. After treatments a general increase of TUNEL positivity appears. Hyperthermia treatment shows a diffuse and strong positivity, in agreement with TEM observations, which suggest the presence of both chondroptosis and necrosis. A relevant oligonucleosomic DNA cleavage, in the presence of rare caspase 9, 8 and 3 activation, appears. UV-B incubation shows a strong positivity too, particularly localized at micromass periphery: this could be due to the impaired UV-B capability to fully penetrate micromass. Surface chondrocyte response to in agreement with TEM, showing chromatin condensation and pore clustering, typical of apoptotic nuclei. Occasionally, a characteristic necrotic swelling appears. UV-B induce large scale of chromatin fragmentation, but not oligonucleosomic DNA cleavage. Caspase 9, 8 and 3 activation is present. Staurosporine evidences a diffuse, but significantly reduced, positivity, a behaviour which is, again, supported by TEM, that shows condroptotic features. Staurosporine induces a scarse oligonucleosomic DNA cleavage, again the presence of caspase 9, 8 and 3 activation. Therefore, DNA fragmentation is a common pattern in dying chondrocytes, both in apoptotic and chondroptotic cells.

Evaluation of sperm quality and DNA integrity after freeze/thawing

L. Bosco[1], G. Ruvolo[2], M. Speciale[1], E. Cittadini[2], M.C. Roccheri[1]

[1]Dipartimento di Biologia Cellulare e dello Sviluppo, Palermo Italy

[2]Centro di Biologia della Riproduzione, Palermo Italy

Sperm cryopreservation, as part of an assisted reproduction program, is widely utilized in the case of preservation of male fertility before radio/chemotherapy which may lead to testicular failure or ejaculatory dysfunction. Thus, it is possible to offer couples the option of having children in the future. However, due to the damage induced by freezing, the motility of cryopreserved spermatozoa after thawing is statistically reduced. To date, the problems of cryoprotectant toxicity due to osmotic stress during the addition and removal of cryoprotectants (CPAs) and possible negative effects on the sperm's structures are unsolved. Our interest has been to verify the effect of high cooling rate of vitrification and the toxic effect of higher concentration of CPAs. In this study we investigated sperm quality in terms of motility and DNA integrity, comparing conventional freezing protocol with vitrification protocols with and without CPAs. 18 ejaculates from patients, containing at least 10 millions spermatozoa/ml and showing at least 20% total sperm motility were examined. Each ejaculate was prepared by swim-up and divided into three aliquots for: traditional freezing protocol with the standard used cryoprotectant. Test yolk buffer (TYB), vitrification with (CPAs+) or without (CPAs-) cryoprotectants. DNA fragmentation was investigated using TUNEL assay in situ. We found a significant sperm motility reduction comparing fresh samples with TYB (47% vs 24%, p= 0.01). Absence of sperm motility was found in CPAs-, and lower motility rate was found in CPAs+ if compared with TYB (4.6% vs 24 %, $p < 0.01$). The average of DNA fragmentation rate in the 18 fresh samples was 21.6%. In fresh samples, DNA fragmentation was higher in morphological abnormal sperm than in normal ones (57.8% vs 42%, $p<0.01$). Higher significant DNA fragmentation was found in CPAs-, CPAs+ and TYB compared with fresh samples (respectively 52.2%, 44.8 %, 46.1 and 21.6). No difference in terms of DNA fragmentation was found between the three different freezing protocols. The vitrification of human spermatozoa in presence or in the absence of conventional cryoprotectants is feasible. We found that sperm motility and DNA integrity is affected by freezing procedure. Motility seems to be completely affected in CPAs-vitrification procedure. The DNA integrity of CPAs+/CPAs- vitrified sperm is comparable with that shown by conventional freezing protocol spermatozoa. Vitrification can be used as a quick and simple method. The use of vitrification with CPAa allow to obtain motile sperms after thawing.

Role of neutrophils in the Shiga toxin-induced endothelial damage in hemolytic uremic syndrome

Maurizio Brigotti[1], Pier Luigi Tazzari[2], Elisa Ravanelli[1], Domenica Carnicelli[1], Stefania Barbieri[1], Valentina Arfilli[1], Laura Rocchi[1], Gaia Scavia[3], Fabio Minelli[3], Francesca Ricci[2], Andrea Bontadini[2], Roberta Alfieri[4], Pier Giorgio Petronini[4], Alfonso V. S. Ferretti[5], Carmine Pecoraro[5], Fabio Paglialonga[6], Alberto Edefonti[6], Maria Antonietta Procaccino[7], Alberto E. Tozzi[7], Alfredo Caprioli[3]

[1]Dipartimento di Patologia Sperimentale, Università di Bologna

[2]Servizio di Immunoematologia e Trasfusionale, Ospedale S. Orsola-Malpighi, Bologna

[3]Istituto Superiore di Sanità, Roma

[4]Dipartimento di Medicina Sperimentale, Sezione di Patologia Molecolare e Immunologia, Università di Parma

[5]Ospedale Santobono, Napoli

[6]Clinica Pediatrica De Marchi, Milano

[7]Ospedale Pediatrico Bambino Gesù, Roma, Italy

Human intestinal infections by Shiga toxin-producing *Escherichia coli* (STEC) cause hemolytic uremic syndrome, characterized by thrombocytopenia, hemolytic anemia and renal failure. This syndrome is the most common cause of acute renal failure in early childhood. The toxins released in the intestinal lumen by the non-invasive bacteria, enter the bloodstream, bind to neutrophils and are targeted to renal endothelium. Analyzing the amount of toxins bound to neutrophils of children with STEC-associated hemolytic uremic syndrome, we found that patients with the highest level of Shiga toxins in blood had normal or only slightly impaired renal function, whereas the children with acute renal failure had lower amounts of toxins on their neutrophils. To explain this paradox an experimental model of toxin delivery from neutrophils to endothelial cells was designed to mimic the primary pathogenic event of the syndrome: the toxin-induced renal endothelial injury. Shiga toxins were transferred during the transmigration of neutrophils impairing protein synthesis and triggering production of proinflammatory cytokines in endothelial cells. The released products are involved in the pathogenesis of the syndrome. Transmigration of neutrophils carrying low amounts of toxin was found to induce the release of high amounts of proinflammatory cytokines in viable endothelial cells, whereas in cells challenged with fully-loaded neutrophils the production of cytokines was blocked as a results of an almost total impairment of translation and of the activation of the apoptotic program. In agreement with previous unexplained observations in animal models, the results obtained suggest that a self-amplifying circle triggered by low doses of toxins may lead to the production of proinflammatory mediators of renal damage in hemolytic uremic syndrome.

Magnetic fields promote a pro-survival non-capacitative Ca^{2+} entry via phospholipase C signaling

Caludia Cerella[1,2], Emanuele Bruni[1], Sonia Cordisco[1], Maria Cristina Albertini[3], Augusto Accorsi[3], Marc Diederich[2], Lina Ghibelli[1]

[1]Dipartimento di Biologia, Universita' di Roma "Tor Vergata", Roma, Italy

[2]Dipartimento di Chimica Biologica "G. Fornaini", Università di Urbino, Urbino, Italy

[3]Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, Luxembourg

The ability of magnetic fields to promote/increase Ca^{2+} influx into cells is widely recognized, but the underlying mechanisms are obscure. Here we analyze how 6mT static magnetic fields (MFs) modulate thapsigargin (THG)-induced Ca^{2+} movements in non-excitable U937 monocytes, and how this relates to the anti-apoptotic effect of MFs. We already reported that MFs do not affect THG-induced Ca^{2+} mobilization from endoplasmic reticulum, but increase the ensuing Ca^{2+} influx [Fanelli et al, 1999]; here we show that this increase requires signalling events including phospholipase C, G protein and nitric oxide production, and occurs through plasma membrane Ca^{2+} channels different from those responsible for the capacitative Ca^{2+} entry (CCE), rather resembling those involved in non-capacitative Ca^{2+} entry (NCCE). All treatments abrogating the extra Ca^{2+} influx also abrogate MFs anti-apoptotic effect, showing that an NCCE induced by MFs, possibly as a response to apoptotic ER Ca^{2+} emptying, is responsible for the anti-apoptotic effect.

Sea urchin embryo as a model system for studying autophagy induced by cadmium stress

Roberto Chiarelli, Maria Agnello, Elisa Filippi, Maria Carmela Roccheri
Dip. di Biologia Cellulare e dello Sviluppo, Univ. di Palermo, Italia

It is well known that sea urchin embryos are able to activate different defense strategies against stress. It has been demonstrated that cadmium treatment triggers the accumulation of metal in embryo cells and the activation of defence mechanisms, depending on concentration and exposure time, by synthesis of hsps and/or activation of apoptosis [Roccheri et al., 2004; Agnello et al., 2007; Agnello and Roccheri 2010]. Here we show that P. lividus embryos adopt autophagy as an additional strategy to safeguard the developmental program. Autophagy is a highly regulated mechanism that enhances cell eukaryotic survival under various environmental and cellular stresses, by breakdown and recycling of macromolecules and organelles. We found that in P. lividus embryos autophagic processes occur, at basal levels during physiological development and at greater levels after Cd treatment. In this study we propose different methods to detect this process during development. Using Neutral Red vital dye (NR), specific for acid compartments including lysosomes, we found that embryos exposed to Cd display a massive punctiform red staining indicative of acidic vacuoles, probably autophagolysosomes. We obtained similar results using Acridine Orange vital staining (AO): embryonic cells exposed to Cd display green fluorescence in cytoplasm and nucleus and show a considerable number of red fluorescent dots in cytoplasm. The metachromatic properties of AO allows the simultaneous evaluation of monomeric (green fluorescence) and aggregated (red fluorescence) state of the dye. The results obtained by Confocal Laser Scanning Microscopy (CLSM) showed important spatial features in the localization of AO granules near nuclei. These data have been sustained by detection of LC3 protein, a specific marker of autophagy, both through western blotting and immunofluorescence analysis, using an antibody against LC3. Quantitative analysis showed an increase of autophagosomes in embryos exposed to Cd. By immunofluorescence and CLSM we studied the LC3 protein distribution in whole embryos, finding an increase in LC3 dots related to elevated levels of LC3-II. The induction of autophagy in sea urchin embryos following Cd stress, has been confirmed using bafilomycin A1, a specific inhibitor of this process. On the basis of these results we hypothesize that autophagy can be adopted in this model system as a cell survival defence strategy for the safeguard of development program or, alternatively, for the clearance of apoptotic bodies.

Mechanisms underlying UVB-induced apoptosis in skeletal muscle tissue

Sabrina Burattini[1], Michela Battistelli[1], Sara Salucci[1], Barbara Canonico[1,2], Camilla Evangelisti[3], Paola Ferri[1], Stefano Papa[1,2], Alberto Maria Martelli[3,4], Elisabetta Falcieri[1,4]

[1] DiSUAN, Università degli Studi di Urbino "Carlo Bo"

[2]Centro di Citometria e Citomorfologia, Università degli Studi di Urbino "Carlo Bo"

[3]Dipartimento di Scienze Anatomiche Umane e fisiopatologia dell'Apparato Locomotore, Università di Bologna

[4] Istituto di Genetica Molecolare, CNR, Istituti Ortopedici Rizzoli, Bologna, Italia

Apoptosis has an important role in skeletal muscle biology, where it has been proposed as a normal developmental event both in proliferating myoblasts and in postmitotic muscle fibers by eliminating defective cells in the first case or cells undergoing damage during differentiation, in the last case (Shiokawa et al., J Biochem Chem 277,2002). Therefore, its deregulation is involved in several muscle disorders such as Duchenne Muscular Dystrophy, Becker and Ulrich myopathies (Tiepolo et al., Br J Pharmacol 157,2009) and others. A significant numerical decrease of myofibrils, an increase in the number of nuclei with typical apoptotic chromatin changes, the reduction of their number per fiber (Adhihetty et al., J Appl Physiol 102,2007), and an increased DNA fragmentation, as assessed by TUNEL staining (Davats et al., Int J Morphol 25,2007), have been indeed described. In addition, chronic heart failure, motor neuron disorders, spinal cord injury and chronic muscle disuse, including hind limb suspension, microgravity, limb immobilization or denervation, have been all associated to an increase of apoptosis, as revealed by TUNEL or by DNA fragmentation analysis in gel electrophoresis (Siu, Med Sci Sports Exerc 41,2009), both suggesting apoptotic DNA cleavage. In this study we have analysed muscle cell death "in vitro" in C2C12 cells at both myoblast and myotube stage. Apoptosis, induced by UV-B irradiation, was investigated by scanning and electron microscopy as well as by TUNEL reaction. DNA behavior was studied by means of gel electrophoresis and flow cytometry. Confocal analysis was used to investigate mitochondrial behavior. Finally, caspase activity was analysed. Surface blebs and the characteristic chromatin condensation were observed at SEM and TEM analysis. Apoptotic nuclei were identified after TUNEL reaction. DNA fragmentation never appeared, while caspase cascade activation was evidenced by Westen blot. In addition, a decrease of apoptotic cell number, after caspase-9 and -3 inhibitor treatment, appeared. Mitochondrial functionality decrease was also evidenced after UV-B exposure, assessed by the specific fluorescent probes. Our results demonstrated that UV-B irradiation is able to induce apoptosis both in differentiated and undifferentiated conditions. C2C12 cells seem most sensitive to oxidative stress, generated after UV-B treatment, suggesting that both myoblasts and myotubes undergo cell death probably following, if not exclusively, the intrinsic pathway.

RNA as a new target for toxic and protective agents

Monia Lenzi[1], Piero Sestili[2], Lorenzo Ferruzzi[1], Patrizia Hrelia[1], Carmela Fimognari[1]

[1]Dip. Farmacologia, Univ. Di Bologna, Italia

[2]Dip. Scienze Biomolecolari, Univ. di Urbino "Carlo Bo", Italia

DNA damage plays a well-established role in cancer initiation and other chronic degenerative diseases. In contrast to damage of genomic DNA and despite its potential to affect cell physiology, RNA damage is a poorly examined field in biomedical research. Potential triggers of RNA damage as well as its pathophysiological implications remain largely unknown. This is somewhat surprising since RNA possesses some characteristics (largely single-stranded, more abundant than DNA, subcellular distribution in close proximity of mitochondria, etc) making it theoretically more susceptible to chemical insults than DNA. A significant loss of RNA integrity has been demonstrated in advanced human atherosclerotic plaques. Oxidative RNA damage has been described in several neurodegenerative diseases. Oxidative RNA damage is also a feature in vulnerable neurons at the earliest stages of these disorders, suggesting that RNA oxidation may actively contribute to the onset or to the development of disease. Therefore, further advances in studies on RNA damage and its surveillance may have a significant impact on the understanding of the pathophysiology of currently unresolved complex diseases. We investigated whether RNA damage could be related to the exposure of particular xenobiotics by testing the RNA-damaging activity of a series of chemicals with different mechanisms of action. Cultured human T-lymphoblasts were treated with ethyl methanesulfonate (EMS), an alkylating agent; H2O2, an oxidizing agent that also increases the levels of PtdIns(3,4,5)P3 and activates downstream signaling; doxorubicin, which acts as both an intercalating and an oxidizing agent; spermine, one of the few NO-donors releasing authentic NO and reactive oxygen species; S-nitroso-N-acetylpenicillamine (SNAP), a donor of NO. Furthermore, we studied the potential protective activity of different agents previously shown to interfere with tumor and atherosclerotic lesion development, and able to counteract inflammation and oxidative stress. We demonstrated that RNA is susceptible to chemical attack. A degradation of RNA could be accomplished with doxorubicin, H2O2, spermine and SNAP. However, EMS, a well-known DNA-damaging agent, was devoid of RNA-damaging properties, while spermine and SNAP, although lacking of DNA-damaging properties, were able to damage RNA. Pronounced differences were also observed for protective agents. In conclusion, our data unequivocally show that cellular RNA is a sensitive target for different xenobiotics. This may present serious ramifications for cellular biochemical processes, particularly involving de novo protein synthesis, thus contributing to cytotoxic events. The observation may also further support efforts to establish the assay of RNA integrity in studying the toxicological profile of a xenobiotic.

Adenosine A_2A receptor but not HIF-1 mediates tyrosine hydroxylase induction in hypoxic PC12 cells

Elena Gammella, Gaetano Cairo, Lorenza Tacchini

Dept of Human Morphology and Biomedical Sciences Città Studi, Università degli Studi di Milano, Milan, Italy

Individual cells within a multicellular organism respond to low oxygen levels by the activation of hypoxia inducible factor (HIF-1), which helps the cell to adapt to oxygen deprivation. The O_2-sensitive cells (type I) of the carotid bodies (CB) closely monitor O_2 tension in the arterial blood and, through release of various neurotransmitters, deliver information to the respiratory and cardiovascular networks in the brain stem, thereby triggering hyperventilation and increased sympathetic nerve activity. A key role in this response is played by tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, which are the predominant neurotransmitters produced by CB type I cells. Adenosine, the final metabolite in the dephosphorylation of ATP, is produced and released in response to ischemia and hypoxia also in the central nervous system. It has been demonstrated that adenosine modulates the response to hypoxia via the A_2A receptor and plays an essential role in neuroprotection against hypoxia. Moreover, a variety of stimuli including CGS21680, an adenosine A_2A receptor agonist, enhanced the expression of the TH gene in PC12 cells. Based on our previous results showing HIF-1 activation in adenosine-treated macrophages and hepatocytes, we hypothesized that adenosine may induce TH through HIF-1-dependent pathways. Exposure to adenosine or CGS21680 increased TH mRNA and protein levels in PC12 cells. The transcription of a reporter gene under the control of the wild type TH promoter was induced 3.5-fold in CGS21680-treated PC12 cells, but both the mutation of the hypoxia responsive element in the TH promoter and the co-transfection of a dominant negative of the HIF-1b subunit did not prevent the increase in transcription. Furthermore, CGS21680 increased CREB binding activity but did not induce HIF-1 DNA binding activity and protein levels. These findings demonstrate that HIF-1 plays a very limited role in TH induction in response to adenosine. To investigate whether HIF-1 was involved in hypoxia-mediated TH induction, PC12 cells were exposed to hypoxia in the presence of the A_2A receptor antagonist ZM241385, which, despite HIF-1 activation, prevented hypoxia-dependent TH induction. These results indicate that under hypoxic conditions the typical hypoxia-regulated TH gene which is key in the production of catecholamines released by the nervous system to permit the body's adaptation to reduced oxygen availability is not regulated by HIF-1, the primary modulator of the transcriptional response to hypoxia, but by binding of adenosine to the A_2A receptor and activation of a PKA-dependent signalling cascade leading to CREB-mediated TH transcription.

Xylosylvitexin: a chemopreventive molecule extracted from *Beta vulgaris cicla* seeds

Lorenzo Gennari[1], Marcella Felletti[2], Manuela Blasa[1], DonatoO Angelino[1], Claudio Celeghini[3], Alfredo Corallini[2], Paolino Ninfali[1]

[1]University of Urbino "Carlo Bo", Department of Biomolecular Sciences, Urbino (PU), Italy

[2]University of Ferrara, Department of Experimental and Diagnostics Medicine, Ferrara, Italy

[3]University of Trieste, Department of Biomedicine, Trieste, Italy

Beta vulgaris subsp. *cicla* (BV) seeds represent a stable and all year long available biomass for purification of phytochemicals, which are able to exploit anticancer activity. In this study, we demonstrated that the ethyl-acetate (EA) extract of BV seeds showed a remarkable antiproliferative activity on human colon cancer (RKO) cells. A fraction of this extract, labeled as P4, obtained by mild pressure liquid chromatography, exploited antiproliferative activity on RKO cells, not significantly different from that of the EA extract. The components of P4 fraction were purified and identified by HPLC-ESI-MS as: 2,4,5-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, vanillic acid, xylosylvitexin, glucopyranosyl-glucopyrasylrhamnetin and glucopyranosyl-xylosylrhamnetin. Among the P4 components, xylosylvitexin exhibited the strongest antiproliferative activity on RKO cells. The effects of xylosylvitexin on RKO cell cycle phases and apoptosis were evaluated in comparison with the EA extract and P4 fraction. The EA extract enhanced nearly three-fold the number of apoptotic cells with respect to the control, the P4 fraction increased twofold the apoptosis percentage, whereas xylosylvitexin about 1.5 fold. This decrease may accounts for some synergistic effect among the components of EA extract and P4 fraction. Concerning to the influence on the cell cycle phases, EA extract, P4 and xylosylvitexin showed a reduction of S phase, due to a specific arrest of the cell cycle in G1. The induction of apoptosis and the block of G1, show analogy with treatment of human dermal fibroblasts with the vitexin aglycone, apigenin. This compound produced a G1 cell-cycle arrest by inhibiting cdk2 kinase activity through the formation of the cdk inhibitor p21/WAF. Since xylosylvitexin is an apigenin diglycoside, it is possible that, also in our experiments with RKO cells, apigenin is the responsible agent of apoptosis stimulation and block in G1. In conclusion, xylosylvitexin is an interesting molecule to be used individually or in combination with other compounds, to inhibit all growth and to induce apoptosis in human colon cancers.

Another function of Hsp70 in mesoangioblast stem cells

Fabiana Geraci, Giuseppina Turturici, Maria Magdalena Barreca, Mariano Di Trapani, Gabriella Sconzo

Dipartimento di Biologia Cellulare e dello Sviluppo, Univ. di Palermo, Italia

In recent years, it has been demonstrated that Hsp70 is released in the extracellular space under normal cell culture conditions, and this release is mediated through exosomes. We have demonstrated that A6 cells, a clone of mouse mesoangioblasts, produce and release in the extracellular space membrane vesicles, independently of culture growth conditions. These vesicles contains both structural proteins and biological active molecules, such as FGF-2 and the metalloproteinases MMP 2 and 9. We have also demonstrated that A6 vesicles contain HspP70 and its release is highly regulated. Some of the intracellular Hsp70 is localized on lipid rafts and its concentration in unsoluble fraction increases after heat shock. Vesicle shedding is influenced by lipid raft integrity, but not so Hsp70 release. Recent experiments have demonstrated that A6 vesicles also contain urokinase and tissue-type plasminogen activators (uPA and tPA), both responsible of plasmin activation. These two proteins could be responsible for MMP activation, as reported in other cell lines. Recently it has been demonstrated that MMP9 expression depends on Hsp70, through NF-kB and AP1 activation. Our preliminary data demonstrated that NM3 cells, a clone with a 55% Hsp70 knockdown, release an increased level of MMP 2/9 in the milieu. Therefore, it is possible to hypothesize a negative role of Hsp70 in MMP expression, in addition with its previously demonstrated role in cell proliferation. The data we have obtained on A6 vesicles also demonstrated that they contain RNA, both mRNA and miRNA, and they are able to be phagocytated by target cells.

Nanoceria antagonizes apoptosis via ROS scavenging

Ivana Celardo[1], Milena De Nicola[1], Corrado Mandoli[2,3], Enrico Traversa[2,3], Lina Ghibelli[1]
[1]Dipartimento di Biologia, [2]Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Roma, Italia; [3]World Premier International Research Center for Materials Nanoarchitectonics and National Institute for Materials Science, Tsukuba, Japan

Cerium oxide, when in form of nanoparticles (nanoceria), contains oxygen vacancies or defects in the lattice structure, that cause a change in the local electronic and valence arrangement that stabilizes the trivalent oxidation state. Moreover, the mixed Ce^{3+}/Ce^{4+} valence states on the surface of nanoceria confer anti-oxidant ability allowing free radicals scavenging. These peculiar features allowed proposing nanoceria as a biologically active agent, possibly neutralizing oxygen in O2-donors enzymes such as those involved in the inflammatory response on the one side, and on the other, by scavenging excess oxidation in biological processes, with the goal of contrasting the many pathologies and disturbances caused by deregulated or excessive oxidation. Since apoptosis is a cellular process implicated in many of the oxidation-dependent pathologies, and some forms of apoptosis do depend on deregulated intracellular oxidative processes, we analyzed the effects of nanoceria on apoptosis induced by the chemotherapic agent etoposide on the human leukocytes cell lines U937 and Jurkat. The experiments were performed adding cerium oxide nanoparticles, with average particles sizes of 5 nm in diameter, to the culture medium. We found that nanoceria reduces the extent of etoposide-induced apoptosis by about 30%. Nanoceria also inhibits apoptotic rise of ROS and superoxide, demonstrating in vivo anti-oxidant ability. To understand whether the anti-apoptotic ability of nanoceria was due to its oxidant scavenger ability, we evaluated the effect that nanoceria exerts on each of the two apoptotic subtypes, the oxidation-independent budding and the oxidation-dependent cleavage. Nanoceria only and strongly affects the ROS-dependent "cleavage" pathway, in the same guise as known anti-oxidant do, demonstrating that nanoceria antagonizes apoptosis by its radical scavenging ability.

Doxorubicin enhances protein ubiquitination and exerts synergic cytotoxic effect in combination with Bortezomib

Giorgia Mandili[1,2], Amina Khadjavi[1,2], Valentina Gallo[2], Valerio G. Minero[3], Franco Carta[4], Giuliana Giribaldi[2], Franco Turrini[2]

[1]Research Center for Experimental Medicine (CeRMS), Turin, Italy

[2]Department of Genetics, Biology and Biochemistry, University of Turin, Italy

[3]Department of Experimental Medicine and Oncology, University of Turin, Italy

[4]Nurex s.r.l., Sassari, Italy

Neuroblastoma (NB) is a neoplasm that originates in the neural crest and is the most common extracranial solid tumor in children. Advanced NB is still frequently fatal despite aggressive management. Doxorubicin, an anti-tumour agent of the anthracycline family widely used in the treatment of solid tumors, is commonly considered to exert its anti-tumor activity leading to the apoptosis of cancer cells through the damage of DNA but our recent results evidenced that Doxorubicin also induces Heat Shock Proteins (HSP) expression, normally triggered by protein damage, and that HSP inhibition or their silencing enhance Doxorubicin apoptotic effect in Neuroblastoma (NB) cells. A combination of Doxorubicin and Bortezomib, a proteasome inhibitor, has been proposed in the treatment of a variety of tumour. In different NB cell lines it has been demonstrated a cooperation of the two drugs. Those data suggest that Doxorubicin may exert its anti-tumor effect also through a mechanism triggered by protein damage. AIMS Aim of the present work, is to explore the effect of Doxorubicin on protein modification. RESULTS The neuroblastoma cell line SJNKP was treated with different concentration of Doxorubicin and Bortezomib at different times. We observed that after Doxorubicin treatment HSP27 decreased its solubility possibly due to its binding to denatured proteins. We also observed a Doxorubicin dose dependent protein over-ubiquitination. Following the identification of the ubiquitinated proteins and their ubiquitination sites by mass spectrometry, we noticed a remarkable similarity between the ubiquitinated protein patterns induced by Doxorubicin or Bortezomib treatments. We did not detect any inhibitory effect of Doxorubicin on the proteasome activity indicating that Doxorubicin induces protein over-ubiquitination because of protein damage. We also confirm a synergic effect of Doxorubicin and Bortezomib on cell death. CONCLUSIONS Our results suggest that Doxorubicin may exert its cytotoxic effect through cellular protein damage and their accumulation. This finding may help the comprehension of the cooperative effects of Doxorubicin and Bortezomib suggesting a rationale for the design of novel therapeutic associations.

TNFα potentiates apoptotic effect induced by taxol in human hepatoma cells

Valerio Giacomo Minero, Daniela De Stefanis, Paola Costelli, Gabriella Bonelli, Francesco Maria Baccino

Department of Experimental Medicine & Oncology, University of Torino, Italy

Background: Paclitaxel (TAX), a chemotherapeutic drug used in the treatment of many tumors, promotes tubulin polymerization, arresting the cell cycle in the G2/M phase and eventually leading to apoptosis. TAX is cytotoxic for hepatoma (HCC) cell lines, but its effectiveness in HCC patients is poor. However, in nude mice bearing solid tumors, the antineoplastic effect of TAX is significantly enhanced by TNFα. Therefore, we investigated if TNFα potentiates the effect of TAX on the human hepatoma cell lines Huh7 and HepG2.

Methods: Huh7 and HepG2 cells were exposed to TAX (0,1 μM) or other microtubular poisons followed or not by TNFα (15 ng/ml). Where indicated, cells were pretreated with the pan-caspase inhibitor z-VAD-fmk, or the caspase-3, -8 or -9 inhibitors (Ac-DEVD-cmk, z-IETD-fmk, Ac-LEHD-cmk; 20 μM), cyclosporine A (CsA, 5 μM) or ETANERCEPT (10 μg/ml). Cell cycle distribution and subG1 cells were analysed by flow cytometry; caspase activation by western blotting and enzyme activity; cell surface TNF-R1 expression by flow cytometry and immunofluorescence.

Results: Apoptosis was significantly increased in hepatoma cells treated with TAX combined with TNFα for 24 h, as compared to TAX alone, and was significantly prevented by pre-treatment with z-VAD-fmk or with the caspase-8 inhibitor z-IETD-fmk. Furthermore, combined treatment with TNFα+TAX induced caspase-3 and caspase-8 activation. Apoptotic death in cultures treated with TNFα+TAX was not dependent on mitochondria, and was not reduced by Ac-DEVD-cmk. These data indicate that TNFα and TAX act sinercistically to activate apoptosis in HCC cell lines. This synergism, that occurred also when cells were treated with other microtubular poisons, was dependent on treatment sequence since it only occurred when TNFα was added to Huh7 cells previously treated with TAX for 24h. Moreover, it is worth remarking that membrane TNF-R1 expression was increased in TAX-treated cells.

Conclusions: These results show that in HCC cell lines TAX treatment induced arrest in the G2/M phase of the cell cycle, followed by caspase-dependent apoptosis. Cell death was enhanced by subsequent treatment with TNFα and was associated with an increased expression of membrane TNF-R1. Apoptotic death induced by TAX+TNFα treatment was partially, but significantly, prevented by z-VAD-fmk and z-IETD-fmk, suggesting a role for caspase 8 in TAX+TNFα-induced apoptosis.

Synergistic effect of Epigallocatechine-3-gallate, Xylosylvitexin and Glucoraphasatin isothiocyanate in human carcinoma cell lines

Papi Alessio[1], Orlandi Marina[1], Farabegoli Fulvia[2], De Nicola Gina Rosalinda[3], Bagatta Manuela[3], Donato Angelino[4], Lorenzo Gennari[4], Manuela Blasa[4], Ninfali Paolino[4]

[1]Dept of Experimental Evolutive Biology, University of Bologna, Bologna, Italy

[2]Dept of Experimental Pathology, University of Bologna, Bologna, Italy

[3]Agricultural Research Council- Research Center for Industrial Crops, (CRA-CIN), Bologna, Italy

[4]Dept of Biomolecular Sciences, University of Urbino, Urbino (PU), Italy

In phytotherapy, the combination of many active components of plant extracts is considered a useful strategy, which is able to increase effectiveness with lower dosages of each single component and reduction of adverse reactions. To investigate the synergism of phytochemical combination, we compared the cytotoxic effects of three compounds, naturally present in dietary supplements and food, on 4 human cancer cell lines: LoVo and CaCo2 (colorectal carcinoma) and MDA-MB-231 and MCF-7 (breast carcinoma) after single treatment or in combination. Epigallocatechine-3-gallate (EGCG), present in green tea, Xylosylvitexin (XVX), a naturally occurring plant flavone, and 4-methylthio-3-butenyl isothiocyanate (GRH-ITC), obtained from myrosinase-catalyzed hydrolysis of Glucoraphasatin (GRH), a glucosinolate mainly found in Raphanus sativus roots and sprouts, were used. Sulphoradamine B (SRB), Hoescht staining and Annexin V/Propidium Iodide dual staining assay were carried out to detect the cytostatic/cytotoxic effect. The combination of very low concentration of EGCG (10 μg/ml), XVX (60 μg/ml) and GRH-ITC (10 μM) was more effective in inhibiting cell growth and induce cell cytotoxicity than the three phytochemicals used independently in LoVo and CaCo-2 cells. In agreement with SFR data, we also detected a significant increase in apoptosis when using EGCG, XVX and GRH-ITC together. After 24 h treatment, apoptosis increased from 4% (after single treatment with EGCG, XVX or GRH-ITC) to 14% (combined treatment) in LoVo cells and from 10% to 25% in CaCo-2 cells. The results were confirmed by Annexin V/PI dual staining assay. In contrast, MCF-7 and MDA-MB-231 cell growth was inhibited by high concentrations of the phytochemicals individually administrated (120-150 μg/ml EGCG and XVX and 30 μM GRH-ITC). The inhibitory effect was greater after the combined treatment: cell growth was reduced to 26% in MDA-MB-231 cells and to 37% in MCF-7 cells. Apoptosis increased from 2-30% (after single treatment) to 33% (after combined treatment) in MDA-MB-231cells and from 3-11% to 85% in MCF-7 cells. The present results support the systematic exploitation of synergy effects in drug development as a tool to improve the use of phytochemicals for cancer chemoprevention and/or therapy.

Effects of either ischemic postconditioning or early acidotic reperfusion on cardiac post-ischemic redox-environment

Claudia Penna, Francesca Moro, Maria-Giulia Perrelli, Francesca Tullio, Pasquale Pagliaro
Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Italy

The mechanisms by which Postconditioning (PostC) alters the pathophysiology of reperfusion injury is complex, and involves physiological mechanisms (e.g. delaying realkalinization of tissue pH, triggering release of autacoids and reactive oxygen specie, opening and closing of various channels) and molecular mechanisms (activation inactivation of of enzymes) that impact on cellular and subcellular targets or effectors. PostC may delay the recovery of intracellular pH during initial reperfusion and its ability to limit infarct size critically depends on this effect. In fact, prolongation of postischemic intracellular acidosis inhibits hypercontracture, mitochondrial permeability transition, calpain-medited proteolysis and gap junction-mediated spread of injury during the first minutes of reflow. This role of prolonged acidosis does not exclude the participation of other pathways in PostC induced cardioprotection. To study how either PostC or acidosis modify post-ischemic redox-environment we tested a) the role of endogenous-and exogenous-catalase and superoxide-dismutase (SOD) and b) the levels of 3-nitrotyrosine and S-nitrosylated proteins. Isolated rat hearts underwent 30-min ischemia/120-min reperfusion (I/R) only or postconditioning (5 cycles of 10-s at the beginning of 120-min reperfusion) either with or without exogenous-catalase or -SOD given during the early reperfusion. The experiments were also performed with three min acidosis reperfusion instead of PostC. The activity and levels of endogenous-SOD and -catalase,3-nitrotyrosine and nitrosylated proteins were studied during reperfusion. Infarct-size and lactate-dehydrogenase release were measured. Activity of endogenous-catalase decreased and that of -SOD increased after I/R. Postconditioning or acidosis inverted these effects on enzyme activities, reduced 3-nitrotyrosine and increased S-nitrosylated proteins in early reperfusion. Subsequently, Cu-SOD protein levels are decreased by I/R, but kept to control levels by postconditioning. Mn-SOD and catalase levels are not affected by both I/R and postconditioning. Three min exogenous-SOD, but not exogenous-catalase infusion abolished postconditioning-cardioprotection (infarct-size and lactate-dehydrogenase release reduction). Conclusions: Here we show that either postconditioning or acidosis down-regulate endogenous-SOD and preserves/up-regulate endogenous-catalase activity. Hence, exogenous-SOD, but not exogenous-catalase, may interfere with postconditioning-triggering. Reduction of 3-nitrotyrosine levels by postconditioning suggests the involvement of nitric-oxide and derivatives in favoring S-nitrosylation, thus preventing further cellular oxidative/nitrosative stress.

Come le cellule rispondono alla crisi: tagli e riorganizzazione strutturale

Chiara Agnoletto, Claudio Baldini, Angela Bononi, Massimo Bonora, Carlotta Giorgi, Saverio Marchi, Sonia Missiroli, Simone Patergnani, Federica Poletti, Alessandro Rimessi, Paolo Pinton

Signal Transduction Lab, Department of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI) and Emilia Romagna Laboratory BioPharmaNet, University of Ferrara, Ferrara, Italy

Scriveva Charles Darwin: “Ogni creatura vivente deve essere considerata un microcosmo: un piccolo universo costituito da una moltitudine di organismi che si riproducono incomparabilmente piccoli e non meno numerosi delle stelle del cielo”. Poiché ciascuno di noi e’ un microcosmo, una popolazione eterogenea di decine di migliaia di miliardi di cellule, ogni domanda sulla nostra vita e la nostra morte ci riporta a una domanda sulla vita e la morte delle cellule che ci compongono. La stretta relazione tra vita e morte è una decisione ricorrente che deve prendere in ogni momento il macchinario all'interno delle nostre cellule. Nel complesso interscambio che permette ai differenti segnali di essere codificati all'interno dell'attivazione della morte cellulare, il calcio (Ca^{2+}) gioca un ruolo significativo. In tutte le cellule eucariotiche, la concentrazione citosolica degli ioni Ca^{2+} è fortemente controllata da trasportatori, pompe, canali e proteine di legame. I cambiamenti di concentrazione di Ca^{2+} [Ca^{2+}] finemente controllati modulano una varietà di funzioni intracellulari che spaziano dalla contrazione muscolare alla proliferazione. Sebbene il ruolo del Ca^{2+} come controllore di una varietà di funzioni fisiologiche fosse gradualmente emerso già dalla fine del diciannovesimo secolo, la teoria generale del Ca^{2+} come secondo messaggero universale è stata proposta solo a metà del secolo scorso per opera di Lewis Victor Heilbrunn. Dall’analisi di dati sperimentali, Heilbrunn nel 1956 concludeva che "la reazione di questo Ca^{2+} con il protoplasma all'interno della cellula è la più basilare di tutte le reazioni protoplasmiche". Questa teoria, nonostante fosse stata completamente ignorata al tempo della sua pubblicazione, ha resistito in maniera brillante al test del tempo e agli sforzi sperimentali: oggi il segnale Ca^{2+} è generalmente considerato come il sistema maggiormente ubiquitario e pluripotente, coinvolto nella regolazione di quasi tutti i processi cellulari conosciuti. Come conseguenza di questa complessità di segnale, lo ione Ca^{2+} solitamente produce effetti opposti anche all'interno della stessa cellula. Non sorprende quindi che la versatilità del segnale Ca^{2+} giochi un ruolo importante non solo in condizioni normali ma anche in reazioni patologiche cellulari.

Cellular and molecular effects of a 300 mT static magnetic field on HUVECs

Emanuela Polidori, Sabrina Zeppa, Lucia Potenza, Chiara Martinelli, Cinzia Calcabrini, Evelin Colombo, Piero Sestili, Vilberto Stocchi

Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino "Carlo Bo", Urbino, Italia

In recent years a substantial evidence has emerged indicating that static magnetic fields (SMFs) of moderate intensity are capable of influencing a number of biological systems. In this study we describe the effects of 300 mT SMF on cell growth and nuclear and mitochondrial DNA integrity of human umbilical vein endothelial cells (HUVECs). Fast halo assay was used to investigate nuclear damage; PCR approaches were used to evaluate mitochondrial DNA integrity, content and gene expression. HUVEC were continually exposed to SMF for 4, 24, 48 and 72 h. As compared to control samples, the SMF-exposed cells did not show a statistically significant change in their viability. Conversely, the exposure to 4 h static magnetic field induced damage both at the nuclear and the mitochondrial level, reduced mitochondrial DNA content and increased reactive oxygen species. Twenty-four hours exposure increased mitochondrial DNA content, as well as expression of one of the main genes related to mitochondrial biogenesis. No significant differences between exposed and sham cultures were found after 48 and 72 h of exposure. Considering the complexity of the biological systems and the largely unknown SMF targets, the use of a technique that is capable of simultaneously assaying a large number of gene or proteins would provide clues to analyze as human cells respond to SMF at the molecular level. In order to investigate the global transcriptional response of HUVEC exposed to SMF for 4 h, a high-quality suppressive subtractive cDNA library, representative of the genes induced in exposed cells, was constructed. Up to 300 clones were selected from the subtracted cDNA library and the clones showing a more intensive hybridisation signal by reverse northern experiments, were subsequently sequenced and further analyzed by Real-time PCR. Our preliminary results highlighted an up regulation at 4 and 24 h of exposure of clones encoding for the NUDT 3, belonging to the Nudix protein family and glutathione peroxidase. The increased expression of these genes represents a cell response to the increment of reactive oxygen species previously found, which in turn could also result in an increased level of 8-OHdG, one of the most common products of oxidative DNA damage. In order to detect the nuclear and mitochondrial 8-OHdG in SMF exposed cells Real-time PCR has been set up. Significant levels of this modified base have been found in mitochondrial DNA after 4 and 24 hours of exposure. The results herein reported suggest that a 300 mT SMF does not cause permanent DNA damage in HUVEC and stimulates a transient mitochondrial biogenesis. The characterization of further clones will provide a wider knowledge of the 300 mT SMF effects on living organisms.

KRIT1 helps cells to prevent oxidative stress

Luca Goitre[1], Fiorella Balzac[1], Simona Degani[1], Paolo Degan[2], Saverio Francesco Retta[1]

[1]Molecular Biotechnology Centre, Department of Genetics, Biology and Biochemistry, University of Turin, Italy

[2]Dept of Translational Oncology, National Cancer Research Institute, IST, Genoa, Italy

KRIT1 is a gene responsible for Cerebral Cavernous Malformations (CCM), a major cerebrovascular disease characterized by abnormally enlarged and leaky capillaries that predispose to seizures, focal neurological deficits, and fatal intracerebral hemorrhage. Comprehensive analysis of the KRIT1 gene in CCM patients has suggested that KRIT1 functions need to be severely impaired for pathogenesis. However, the molecular and cellular functions of KRIT1 as well as CCM pathogenesis mechanisms are still research challenges. We found that KRIT1 plays an important role in molecular mechanisms involved in the maintenance of the intracellular Reactive Oxygen Species (ROS) homeostasis to prevent oxidative cellular damage. In particular, we demonstrate that KRIT1 loss/down-regulation is associated with a significant increase in intracellular ROS levels. Conversely, ROS levels in KRIT1$^{-/-}$ cells are significantly and dose-dependently reduced after restoration of KRIT1 expression. Moreover, we show that the modulation of intracellular ROS levels by KRIT1 loss/restoration is strictly correlated with the modulation of the expression of the antioxidant protein SOD2 as well as of the transcriptional factor FoxO1, a master regulator of cell responses to oxidative stress and a modulator of SOD2 levels. Furthermore, we show that the KRIT1-dependent maintenance of low ROS levels facilitates the downregulation of cyclin D1 expression required for cell transition from proliferative growth to quiescence. Finally, we demonstrate that the enhanced ROS levels in KRIT1$^{-/-}$ cells are associated with an increased cell susceptibility to oxidative DNA damage and a marked induction of the DNA damage sensor and repair gene Gadd45α. Taken together, our results point to a new model where KRIT1 limits the accumulation of intracellular oxidants and prevent oxidative stress-mediated cellular dysfunction and DNA damage by enhancing the cell capacity to scavenge intracellular ROS through an antioxidant pathway involving FoxO1 and SOD2, thus providing novel and useful insights into the understanding of KRIT1 molecular and cellular functions, and suggesting a novel mechanism for CCM pathogenesis.

Involvement of protein ERp57 in cellular response to oxidative stress

Caterina Grillo, Daniela Ricci, Rossana Cocchiola, Margherita Eufemi, Fabio Altieri
Dip. Scienze Biochimiche "A. Rossi Fanelli", "Sapienza" Università di Roma

ERp57, also known as PDIA3, ERp60, ERp61 or GRP57, consists of four domains named a, b, b' and a', with a and a' containing the thioredoxin-like active site sequence WCGHCK, while domains b and b' are redox inactive. The two thioredoxin-like active sites confer the protein a redox activity toward cysteine residues of target peptides, catalyzing the oxidation, reduction and/or isomerization of disulfides. On the other side ERp57 has an intrinsic propensity to associate with other polypeptides and can exert a role as molecular chaperone. Although abundant in the endoplasmic reticulum, ERp57 has also been found in lesser amounts in other cellular locations, i.e. in the cytosol and nucleus. In the cytosol, ERp57 is associated with STAT3 in a multiprotein complex named statosome, and in HepB2 cells ERp57 is present in nuclear STAT3-containing complexes associated with DNA. Recently, ERp57 has been identified as the cell surface receptor for the metabolite 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], that may play a pivotal role in many cell functions regulated by the vitamin D endocrine system and is involved in important disease states, in differentiation of certain cancer cells and in muscle function. ERp57 has been found in nuclei of rat spermatidis and spermatozoa, and in the nuclear matrix of chicken hepatocytes and various mammalian cell lines. We observed that ERp57 has DNA binding capabilities and this property, which is dependent on protein redox state, has been detected both in vitro and in vivo. We also reported that ERp57 displays affinity for Ref-1, a protein involved in DNA repair, reduction/activation of transcription factors, and more directly in transcriptional regulation through binding to gene promoters. Thus, the identification of proteins associating with ERp57 might help to disclose functional processes in which ERp57 is actually involved. To address these questions we designed a new cellular model, consisting of HeLa cells stably transfected with an expression vector coding for a GFP-tagged ERp57, in which a study of the cellular response to oxidative stress can be carried out. Cellular model has been characterized monitoring variations in cell functions, redox state, morphology and protein composition, focusing the attention on ERp57 expression levels, chemical modifications, cellular distribution and associated proteins.

UV-B stress induces 14-3-3 epsilon mRNA increase and delocalisation in *Paracentrotus lividus* sea urchin embryos

Roberta Russo, Francesca Zito, Caterina Costa, Rosa Bonaventura, Valeria Matranga
Consiglio Nazionale delle Ricerche, Istituto di Biomedicina e Immunologia Molecolare "Alberto Monroy", Palermo, Italy

Members of the 14-3-3 protein family are involved in many important cellular events regulating the equilibrium state between cell survival and apoptosis. They take part in complex mechanisms involved in signal transduction, transcription, mitochondrial pathways and others (Morrison 2008). The 14-3-3 genes are highly conserved, from plants to humans, and some are responsive to UV radiation. We have previously shown that *Paracentrotus lividus (Pl)* sea urchin embryos exposed at cleavage stages to different doses of UV-B, ranging from 10 to 800 J/m2, develop with dose-dependent morphological abnormalities, including major defects in skeleton and archenteron (primitive intestine) elongation, and increased hsp70 protein levels (Bonaventura et al. 2006). It was of interest to investigate the expression levels and spatial localization of the 14-3-3 gene product (*Pl*14-3-3 epsilon) in response to UV-B radiation in sea urchin embryos. To this purpose we isolated the complete cDNA encoding for the 14-3-3 epsilon isoform from embryos exposed to UV-B at the dose of 800 J/m2. By QPCR we analyzed the expression levels of the *Pl*14-3-3 epsilon mRNA during development and found a stage-dependent proportional increase of the transcript from mesenchyme blastula to prism. When cleavage stage embryos (32 cells) were exposed to UV-B, we found a 2.3-fold (400 J/m2) and 2.7-fold (800 J/m2) increase in *Pl*14-3-3 epsilon mRNA levels. In addition, we examined the spatial expression of 14-3-3 epsilon mRNA by in situ hybridization in both control and UV-B exposed embryos harvested at late developmental stages (pluteus). The transcripts were found restricted to the archenteron in gastrula stage control embryos, whereas they were widely delocalised in all germ layers in UV-B exposed embryo. These results paralleled the failure of archenteron elongation, observed microscopically, and the abnormal tissue differentiation, detected by specific endoderm markers. In conclusion, this is the first report describing the complete cDNA coding for the 14-3-3 epsilon isoform in *P. lividus* sea urchin embryos and the first evidence of a transcriptional regulation of the gene in response to UV-B exposure. This work has been supported in part by: EU – UV-TOX Project, Contract N. EVK3-CT-1999-00005, ASI MoMA Project Contract N?1/014/06/0, EU- ITN Biomintec Project, Contract N.215507.

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Myoblastic cell apoptotic sensitivity: an *in vitro* study

Sara Salucci[1], Michela Battistelli[1], Sabrina Burattini[1], Cesare Squillace[1], Davide Curzi[1], Barbara Canonico[1,2], Pietro Gobbi[1], Stefano Papa [1,2], Elisabetta Falcieri[1,3]

[1]DiSUAN and [2] Centro di Citometria e Citomorfologia, Università degli Studi di Urbino "Carlo Bo", Urbino

[3]IGM, CNR, Istituti Ortopedici Rizzoli, Bologna, Italy

Apoptosis plays a pivotal role in maintaining skeletal muscle homeostasis, both in proliferating and differentiated conditions. Its deregulation is involved in several muscular disorders, including neuromuscolar diseases, muscle disuse, denervation and sarcopenia (Siu et al., Med Sci Sport Exer 9, 1876-80, 2009). In all these conditions muscle mass decrease, associated with fiber loss and reduction in myonuclei number, appears. In addition, nuclear changes, characterized by chromatin condensation and DNA fragmentation increase, assed by TUNEL staining and by gel electrophoresis (Davats et al., Int J Morphol 25, 529-536, 2007; Braga et al., Apoptosis. 13: 822–32, 2008.), have been documented. In this work we have studied skeletal muscle apoptosis, by means of flow cytometry and electron microscopic analyses, induced by a variety of chemical triggers, acting through different mechanisms of action. For this purpose, C2C12 myoblasts were exposed to 0.5 μM hydrogen peroxide (H_2O_2), 0.1 μM staurosporine, 30 μM cisplatin and 30 μM etoposide for 24 h. Flow cytometry analysis evidenced a certain DNA cleavage, quantified as 2% for control cells, 17% for H_2O_2, 27% for staurosporine, 28% for cisplatin and 60% for etoposide. Ultrastructural analyses revealed evident cellular alterations in all experimental conditions, respect to the control ones. In general, treated myoblasts appeared detached from underlying substrate and lost their fusiform or star-shaped aspect, becoming rounding. In addition, chromatin margination and condensation, general vacuolisation and surface bleb formation were observed at microscopic level. In particular, H_2O_2 and staurosporine treatments were characterized by last apoptotic stages, such as apoptotic bodies and cells in secondary necrosis. After cisplatin incubation, early apoptotic features and myolasts with an initial chromatin margination were observed. A significant apoptotic cell number increase was detected after etoposide treatment. These findings demonstrated a certain myoblast sensitivity to apoptosis. All triggers used, even if acting through different mechanisms of action, seem to follow mitochondrial pathway to initiate cell death. Therefore, mitochondria could be play a central role in skeletal muscle apoptosis, in agreement with the demonstration that some muscular diseases and chronic muscle disuse are characterized by an increase in mitochondrial apoptotic susceptibility (Adhihetty et al., Exerc Sport Sci Rev 36, 116-121, 2007).

Creatine protects from oxidative stress and improves the morpho-functional differentiation of neuroblasts in spinal cord primary cultures

Stefano Sartini[1], Fanny Bartolini[1], Evelin Colombo[2], Davide Lattanzi[1], Stefano Ciuffoli[1], Piero Sestili[2], Chiara Martinelli[2], Riccardo Cuppini[1]

[1]Dip. Scienze dell'Uomo, dell'Ambiente e della Natura, Univ. di Urbino "Carlo Bo", Italia

[2]Dip. Scienze Biomolecolari, Univ. di Urbino "Carlo Bo", Italia

Aim: Creatine (Cr) is an essential component of Cr/Cr-phosphate system. Recent studies on different cell lines showed that Cr supplementation protects from cell death following radical insult, suggesting direct antioxidant properties of Cr in addition to its well known energetic role. During pregnancy endogenous Cr synthesis in the developing brain is very poor. As a consequence, developing SNC is seriously exposed to the risk of Cr deficiency in case of premature birth or placental insufficiency, suggesting the importance of Cr supplementation in pregnancy. We studied the effects of Cr supplementation on primary cell cultures obtained from chick spinal cord embryo exposed to oxidative stress. **Methods**: Neuroblast cultures were evaluated for viability (MTT test), morphological differentiation using Neuron J software, and functional differentiation by whole-cell patch-clamp, in control conditions and following a treatment with hydrogen peroxide. **Results and conclusions**: Hydrogen peroxide (20 μM) caused a significant decrease of viability and of neurite extension. Both actions were fully reversed by preincubation with Cr 10 mM. Protective effect of Cr was absent if Cr membrane transporters were blocked by β-guanidine propionic acid, thus demonstrating an intracellular action of Cr. Neuroblast NPSH antioxidant pool was preserved if Cr or Trolox was introduced before peroxide treatment in cultures, leaving to suppose a direct antioxidant action of Cr. These findings confirm a protective role against peroxidative insult in differentiating neuronal cells. Moreover, electrophysiological studies demonstrated that Cr supplementation increases Na^+ e K^+ voltage-gated currents with respect to controls with a dose-dependence relation, showing a pro-differentiative effect of Cr on neuronal cells. A possible role of Cr supplementation in preventing of radical-induced damage in developing CNS is suggested by our results.

The iron chelator dexrazoxane protects cardiomyocytes from doxorubicin-mediated cell death: role of HIF-1

Rosalin Spagnuolo, Lorenza Tacchini, Stefania Recalcati, Gaetano Cairo

Dept of Human Morphology and Biomedical Sciences Città Studi, Università degli Studi di Milano, Milan, Italy

Iron aggravates the cardiotoxicity of doxorubicin (DOX), a widely used anticancer anthracycline. Indeed dexrazoxane (DRZ) is the only agent able to protect the myocardium from anthracycline-induced toxicity both in experimental and clinical settings. Iron has been proposed to catalyse ROS formation in reactions primed by DOX. However, the oxidative nature of the role of iron in cardiotoxicity is challanged by results showing that antioxidants do not always protect against cardiotoxicity. The mechanisms of DOX-mediated cardiotoxicity, and the protective role of DRZ, remain to be estabilished. The hypoxia inducile factor (HIF-1) is a transcription factor which regulates the expression of several genes mediating adaptive responses to lack of oxygen. Iron is required for HIF-1 degradation and therefore decreased iron availability activates HIF-1 in normoxic cells. In consideration of the antiapoptotic and protective role of some HIF-1-induced genes, in this study we tested the hypothesis that DRZ-dependent HIF-1 activation may mediate the cardioprotective effect of DRZ. Treatment with DRZ dose-dependently induced HIF-1 protein levels and its capacity to activate the transcription of a reporter gene under the control of hypoxia responsive elements in the H9c2 cardiomyocyte cell line. The transactivation capacity of HIF-1 was abolished by a dominant negative of the constitutive HIF-1β subunit (DARNT) or by HIF-1 knockdown by siRNA. DRZ also prevented the induction of cell death and apoptosis caused by the exposure of H9c2 cells to clinically-relevant concentrations of DOX. Suppression of HIF-1 activity by means of DARNT showed that the protective effect of DRZ was dependent on HIF-1 activity. Conversely, HIF-1 overexpression was able to provide cytoprotection from DOX-mediated cell death and apoptosis also in cells not exposed to iron chelators. Finally, we examined the expression of HIF-1 target genes with a possible role in cell survival in DRZ-treated H9c2 cells and we found a strong increase in the levels of survivin and Mcl1, an antiapoptotic member of the BCL2 gene family. In summary, our result showing HIF-1-dependent prevention of DOX toxicity in DRZ-treated H9c2 cardiomyocytes suggest that HIF-1 activation may be a mechanism contributing to the protective effect of DRZ against anthracycline cardiotoxicity.

Mesoangioblast behaviour in oxidative stress condition

Giuseppina Turturici[1], Fabiana Geraci[1], Alba Cavallaro[1], Maria Magdalena Barreca[1], Gabriella Sconzo[1]

[1]Dipartimento di Biologia Cellulare e dello Sviluppo, Univ. di Palermo, Italia

Mesoangioblasts are stem cells capable of differentiating in various mesodermal tissues and are excellent candidates for cell therapy in muscle degenerative diseases, as well as in myocardial infarction. When mesoangioblasts are injected into the circulation they localize to damaged tissues reducing the severity of experimental muscular dystrophy in mouse and dog and in infarcted mouse hearts. These tissues are in an inflammation state, which induces the release of cytokines and ROS, that could damage the injected mesoangioblasts. The knowledge about the mesoangioblast behaviour in unfavourable environment could start new strategies to enhance their proliferation and survival after injection and improve their ability to repopulate damaged tissues. To study mesoangioblast behaviour in stress conditions we analyze in vitro the effects of different H_2O_2 concentrations. We choose concentrations close to those that could occur in chronic inflamed tissues. After treatment or subsequent recovery we have estimate survival/cell death, cell cycle phase distribution, apoptosis, and autophagy. Viability assays indicate that cells showed a slowed proliferation depending on the treatment concentrations and a part died. Flow cytometric analysis indicated that all H_2O_2 concentrations induced G_2/M cell cycle arrest during the 24h of treatment. Apoptotic pathway is already activated during the first 24h of treatments. On the contrary, the autophagic pathway is activated during the treatment and it is still evident during the first days of recovery. In conclusion, we can hypothesize that H_2O_2 accumulates in the cells in the first hours of treatments and the negative effects of this accumulation are observed even after treatment.

Sphingosine, not ceramide, mediates TNF-induced death of rat hepatoma cells

Chiara Ullio[1], Josefina Casas[2], Ulf T. Brunk[3], Giuseppina Sala[4], Gemma Fabrias[2], Riccardo Ghidoni[4], Gabriella Bonelli[1], Francesco M. Baccino[1], Riccardo Autelli[1]

[1]Dept of Experimental Medicine and Oncology, University of Turin, Italy

[2]Research Unit on Bioactive Molecule (RUBAM), Department of Biomedicinal Biochemistry, IQAC-CSIC, Barcelona, Spain

[3]Division of Pharmacology, Faculty of Health Sciences, University of Linköping, Sweden

[4]Laboratory of Biochemistry and Molecular Biology, San Paolo University Hospital, University of Milan, Italy

We previously described that treatment of the rat hepatoma cells HTC with TNF and cycloheximide (CHX) triggers an apoptotic-like, caspase-independent death mediated by the lysosomal compartment. Lysosomal intervention in apoptosis has been associated to various mechanisms, including the synthesis of ceramide, which, in turn, activates ceramide-dependent mechanisms eventually leading to cell death. In the present research we investigated whether ceramide production is affected and, in case, how this change relates to death of HTC cells. TNF/CHX time-dependently increased intracellular ceramide to rather high levels over 6 hours of treatment, as preliminarily evidenced by the DGK technique. Liquid-chromatography/mass spectrometry analysis of total sphingolipids confirmed that TNF/CHX elevated by about three-fold the content of all the ceramide species and significantly increased sphingosine levels. The latter has been demonstrated to act as a detergent on the lysosomal membrane, determining relocation of lysosomal content to the cytoplasm and subsequent death in liver-derived or other kinds of cells. These data thus support the possibility that this sphingolipid plays a role in death processes involving the lysosomal compartment. In addition, we previously reported that the death pathways triggered by TNF or ceramide substantially differ from each other in the present hepatoma cells. Hence, we further investigated the possibility that, rather than ceramide itself, the sphingosine generated from ceramide mediates TNF toxicity. In fact, in HTC cells exogenous sphingosine triggered apoptosis in a dose-dependent manner, which was accompanied by lysosomal membrane permeabilization (LMP), as demonstrated by the acridine orange uptake test. We evidenced that also TNF/CHX, but not C_2-ceramide, induced LMP and that the latter was prevented by inhibitors of sphingolipid metabolism such as desipramine (Dpm), a known inhibitor of acid sphingomyelinase. In HTC cells, however, Dpm mostly behaved as an acid ceramidase inhibitor; accordingly, it completely abrogated TNF/CHX-induced sphingosine generation, while further increasing ceramide accumulation. In addition to LMP, this drug efficiently reduced death, further demonstrating that sphingosine, and not ceramide, actively mediates TNF/CHX toxicity in HTC cells. Thus, our results strongly support the view that lysosomal ceramide induced by TNF/CHX is rapidly converted to sphingosine, which subsequently kills HTC cells by a mechanisms involving LMP and alteration of physiological functions of the lysosomal compartment.

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