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PROCEEDINGS OF THE 31st NATIONAL CONGRESS OF THE ITALIAN SOCIETY OF HISTOCHEMISTRY

Pisa, June 15-17, 2005

Dipartimento di Morfologia Umana e
Biologia Applicata
Facoltà di Medicina e Chirurgia
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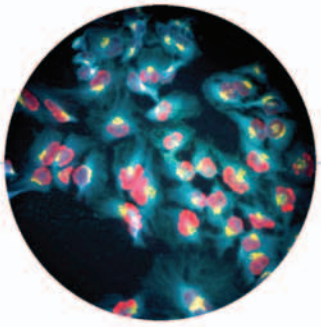

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OPENING LECTURE

Histochemistry of bone tissue: the pathway to creating intelligent surfaces to be used in nanomedicine

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Laboratory for the Study of Calcified Tissues and Biomaterials, Faculty of Dentistry, Université de Montréal, CANADA

Calcified tissues play a crucial role in supporting the body, in mastication and in maintaining the phosphocalcic balance. Without them we could not exist and the body has developed a number of redundant mechanisms to ensure its integrity. The integrity of calcified matrices resides in the physicochemical characteristics of matrix proteins as well as their temporo-spatial distribution, intermolecular interactions and expression levels which together define their physiological role. An exciting recent recognition is that although the processes of biomineralization in different species and tissues have some distinct features, there clearly are common threads. This presentation will review some of our histochemical analyses of bone matrix and how they have led to the creation of biomaterials with 'intelligent' surfaces that can influence cell response and guide tissue repair Such nanostructured surfaces are expected to find application in cardiovascular, dental and orthopedic nanomedicine.

Supported by the Canadian Institutes for Health Research and the Natural Sciences and Engineering Council of Canada

I SYMPOSIUM

The spatial properties of calcium signals modulate contraction in atrial myocytes

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Ca^{2+} is a ubiquitous and versatile intracellular messenger. Cellular Ca^{2+} signals can occur as brief events localised to specific regions within cells, or traverse whole tissues in a regenerative manner. The types of responses that a cell can display are a function of its Ca^{2+} signalling proteome. Such proteomes are cell specific- no two cell types utilise exactly the same combination of Ca^{2+} signalling proteins, and as such display unique responses. Atrial cardiac myocytes are an example of a cell type where Ca^{2+} signals are shaped by multiple interacting homeostatic mechanisms. Alteration of the spatial pattern of Ca^{2+} signalling modulates contraction of these cells. Under control conditions, electrical depolarisation of atrial cardiac myocytes evoked calcium transients that originated in sub-sarcolemmal locations, and gave rise to a sharply-defined ring of elevated calcium around their periphery. Despite functional ryanodine receptors being expressed at regular (~2 μm) intervals throughout the cells, the subsarcolemmal calcium signal did not spread in a regenerative manner to the cellular interior. The averaged subsarcolemmal calcium response was 1656±219 nM and took 38±3ms to reach peak, whilst the calcium rise in the central region of the atrial cells was typically 400±63 nM and was maximal after 91±7 ms (n=5 cells). Positive inotropic agents, such as the β-adrenergic agonist isoproterenol, induced globalisation of action potential-evoked calcium signals. The functional consequence of globalising calcium signals was a significant increase in the contractility of the cells. We suggest that atrial myocytes have two functionally distinct populations of ryanodine receptors. The subsarcolemmal population is recruited during each action potential, but produces only a spatially limited calcium signal that triggers modest contraction. The central non-junctional ryanodine receptors represent an inotropic reserve, but are located behind a calcium ATPase and mitochondrial firewall. Physiological inotropes can activate this second population of ryanodine receptors to enhance the contractility of the cells.

New techniques for the identification of specific markers at the chromosome level

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In the past decade multicolour FISH karyotyping as well as comparative genomic hybridization using arrays (array CGH) have shown to be highly complimentary to detect chromosomal aberrations that occur during tumour development. FISH karyotyping allows detection of balanced as well as unbalanced abnormalities, but highly relies on the success by which tumour representative metaphase cells can be prepared. Array CGH detects unbalanced abnormalities such as amplifications and deletions, often not found by FISH karyotyping, but results are impacted by tumour heterogeneity and sampling problems. We have addressed both issues and evaluated the improvements of both techniques in a clinical context, e.g. the diagnosis of tumours of soft tissues.

A chemical induced PCC (precondensed chromosome condensation) technique was developed to generate pre-mature chromosomes of virtually every type of cycling cell during short culture with Calyculin A[1]. The success rate was found better than 95%. Thus produced chromosomes were stained using p and q arm specific paints and a 48 colour FISH technique called COBRA[2] and analysed automatically. For array CGH we use home-printed genomic arrays consisting of 3500 BACs (kindly provided by The Sanger Institute; Dr. Nigel Carter), which are directly linked to the DNA database. To successfully apply array CGH to heterogeneous tumour samples we optimised protocols for cell selection (optionally, by laser microdissection), DNA isolation, PCR amplification and fluorescent labeling in such as way that samples as low as 50 pg of DNA were successfully analysed. In a combined way these methods were applied to support the diagnosis of soft tissue tumours (malignant versus non malignant), and to support the choice of therapy, e.g. with Glivec (a tyrosine kinase inhibitor) for cases in which specific chromosomal translocations could be demonstrated.

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Cyclin expression during myogenesis

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Mammalian cell cycle is controlled by several cyclin-dependent kinases (cdks), however these complexes are also implicated in muscle differentiation. For example overproduction of cyclins E and A impinge on MyoD function by virtue of their ability to phosphorylate pRb. On the contrary, Cyclin D1 overexpression inhibits myogenic differentiation by promoting the nuclear accumulation of cdk4, which in turn binds the C-terminus of MyoD and prevents MyoD DNA binding and activation of myogenic transcription. Moreover, our group has demonstrated that overexpression of cdk9/cyclin T2a enhances MyoD function and promotes myogenic differentiation, while inhibition of cdk9 kinase activity by a dominant negative form prevents the activation of the myogenic program. The N-terminal region of cdk9 and full-length cyclin T2a directly interact with the bHLH region of MyoD, allowing the formation of a complex that stimulates transcription of specific muscle genes. To further inquire on the role of cyclinT2a in myogenic processes, we performed a two-hybrid screening in yeast using the full length human cyclin as bait. Upon several interesting genes selected for interaction, our attention has been focused on PKN-alpha, a fatty acid- and Rho-activated serine/threonine protein kinase. We performed co-immunoprecipitation and *in vitro* pull-down assays to independently confirm the interaction. Luciferase assays using a MyoD-responsive promoter demonstrated significant increase in luciferase-reporter expression in the presence of PKN-alpha. These results strongly support a functional significance on these genes in muscle differentiation.

PKCe modulates the sensitivity of human CD34-derived erythroblasts to the apoptogenic effect of TRAIL

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Apoptosis plays a central role in the regulation of the size of the hematopoietic stem cell pool and in cell differentiation along the various hematopoietic lineages[1,2] whereas cytokine-activated mature NK and CD8 T cells are protected from TRAIL-induced apoptosis by elevated intracellular levels of

cFLIP.[3] TRAIL interacts with 4 high affinity transmembrane receptors: TRAIL-R1 and R2 transduce apoptotic signals, whereas TRAIL-R3 and R4 lack the intracellular death domain and apoptosis inducing capability. We have demonstrated that PKCe is selectively post-transcriptionally downmodulated in the Epo-dependent murine 32D-Epo.1 cells. The subsequent observation that the pharmacological inhibition of PKCe increased the number of erythroid colonies *in vitro*, strongly suggested a relevant role for this isoform of PKC in erythropoiesis.[4] Given this complex background, we investigated the role of PKCe in the modulation of TRAIL activity during human erythropoiesis. Our results essentially show that the Epo-dependent surface expression of TRAIL death receptors during early erythropoiesis determines the sensitivity of reythroblasts to the apoptogenic effects of TRAIL. However, the Epo-dependent subsequent (>day 10) up-regulation of PKCe blocks the intracellular signalling cascade downstream TRAIL-Rs, thus protecting more mature cells from caspase activation and apoptosis.

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Nuclear expression of DG-kinase isoenzymes: possible involvement in DNA replication

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Several independent laboratories have shown that lipid-dependent signal transduction systems operate in the nucleus and that they are regulated independently from their membrane/cytosolic counterparts. A sizable body of evidence suggests that nuclear lipid signalling controls critical biological functions such as cell proliferation and differentiation. Diacylglycerol (DG) is a fundamental lipid second messenger which is produced in the nucleus. The levels of nuclear DG fluctuate during the cell cycle progression, suggesting that such a molecule has important regulatory roles. Most likely, nuclear DG serves as a chemoattractant for some isoforms of protein kinase C that migrate to the nucleus in response to a variety of agonists. The nucleus also contains DG kinases, i.e. the enzymes that, by converting DG into phosphatidic acid, terminate DG-dependent events. DG kinases are either resident within the nucleus or migrate there in response to a number of agonists. At least two of DG kinase isozymes present in tqhe nucleus (i.e. -θ and -ζ) localizes to nuclear speckles and are components of the nuclear matrix. Available evidence suggests that DG kinase-ζ might be involved in the control of DNA replication. Indeed, overexpression of a green fluorescent protein (GFP)/DG kinase-ζ cDNA which mainly localized to the nucleus of C2C12 cells resulted in inhibition of DNA synthesis as assessed by incorporation of biotinylated 11-dUTP in mildly permeabilized (0.04% Triton X-100) cells. In contrast, overexpression of a GFP/DG kinase-˙ cDNA lacking the nuclear localization signal did not stop DNA replication.

Nuclear envelope proteins, chromatin arrangement, and transcriptional regulation: a pathogenic mechanism for laminopathies

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The involvement of the nuclear envelope NE in the modulation of chromatin organization and transcriptional control is strongly suggested by the increasing number of human diseases due to mutations of NE proteins (emerin, LBR) or lamin A/C. These diseases, named *laminopathies*, share in some instance their clinical features, but each of them is characterized by a phenotype that involves one or few tissues, suggesting tissue-specific defects of lamin A/C functioning.[1] Some pathogenic mechanisms for laminopathies have been advanced, but direct evidence is still lacking.[2] We previously reported that cells from laminopathic patients show an altered nuclear profile, and a loss or detachment of HC from the NE.[3] Similar alterations can be induced by mevinolin, a drug that interferes with lamin A maturation, by blocking the metalloprotease ZMPSTE24. In this case, nuclei show accumulation of pre-lamin A similar to that reported in Hutchison-Gilford progeria.[4] By analyzing cells from MAD patients, we found that, following pre-lamin A accumulation, HC is almost completely lost, as indicated by the de-localization of LBR and HP1, that normally maintain HC linked to the NE. These findings indicate that altered chromatin remodeling is a key event in the cascade of epigenetic events leading to a laminopathic phenotype. Premature aging of different tissues may be due to an altered developmental process in cells that are unable to maintain the silenced HC organization capable to preserve a state of terminal differentiation.

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II SYMPOSIUM

Differentiation and plant cell death as a reaction to cold stress

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Olive tree is a warm-temperature tree with a particular sensitivity to cold. In olive-tree leaf protoplasts a rise in cytosolic calcium has been observed as a primary response after cold shock, and cold acclimation has been shown to reduce calcium signalling.[1] Cold acclimation involves the accumulation of cryoprotectans, which leads to the physical and biochemical restructuring of l membranes and results into anatomical changes. Osmotin is a 26kDa protein which accumulates in defense responses against pathogens.[2] It also acts in defense responses to abiotic stresses, including osmotic stress and low temperatures.[2] The mechanism of action of the protein is unknown, however in the cases tested up to now an interaction with cell membranes has been observed. Programmed cell death (PCD) is a plant suicide program involving sequential changes in the cells, including genomic DNA fragmentation, degradation of organelles, and tonoplast rupture, which lead to cell death. There are many evidences that PCD plays an essential role also in normal plant development, controlling cell number, differentiation, and organ morphogenesis.[3] Up to now, a role for osmotin as PCD inducer has been demonstrated only in yeast.[4] The aim of the present paper was to investigate the relationship between osmotin, calcium signalling and PCD in olive tree, using normal and overexpressing osmotin plants exposed to cold shocks. This research demonstrates that osmotin accumulates in cold stressed leaves and stems, and the cell types in which the protein is observed are the same in which PCD is observed. Under non stressed conditions, osmotin only accumulates in transgenic leaf protoplasts, and this event couples with a high frequence of PCD. Osmotin overexpression also causes arrest of cold-induced cytosolic calcium rise, sustaining the hypothesis that osmotin affects cold-induced PCD programme interfering with calcium signalling.

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Cell death and hypersensitive reaction of the plant

M. Delledonne
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Nitric oxide (NO) is a highly reactive molecule that rapidly diffuses and permeates cell membranes. In animals, NO is implicated in different physiological processes such as neurotransmission, vascular smooth muscle relaxation, and platelet inhibition. It may have beneficial effects, as a messenger in immune responses, but is also potentially toxic when an excess of reactive oxygen species (ROS) accumulates. The increasing number of reports on NO function in plants have implicated this molecule as an important effector of growth, development, and defense. The innate immune system of vertebrates, invertebrates, and plants shows several characteristics similar with respect to the involvement of NO. In the mammalian immune system, NO cooperates with ROS to induce apoptosis of tumor cells and macrophage killing of bacteria. In plants a similar mechanism, the hypersensitive reaction (HR), has evolved to prevent tissue invasion by pathogens. The rapid accumulation of ROS and NO through the activation of enzyme systems similar to neutrophil NADPH oxidase and nitric oxide synthase is one of the earliest events in the HR. Both NO and ROS are necessary to trigger host cell death; they are also components of a highly amplified and integrated defense system that triggers the local expression of resistance genes. NO also functions independently of ROS in the induction of various defence genes including pathogenesis-related proteins and enzymes of phenylpropanoid metabolism and the secondary signal salicylic acid. NO signaling functions depend on its reactivity and ROS are key modulators of NO in triggering cell death, although through mechanisms different from those commonly observed in animals. The talk will focus on the signaling functions of NO when channeled through the cell death pathway by ROS.

Role of transglutaminase in pollen tube growth and in incompatibility-induced cell death

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Higher plant fertilization is a complex process controlled by a variety of interactions between the male and female gametophytes.[1] The growth of a pollen tube has been shown to require an apical tip based Ca^{2+}-gradient, the function of which is not fully understood.[2] We demonstrate that a Ca^{2+}-dependent extracellular pollen tube transglutaminase (TGase) is an essential modulator of pollen tube growth. The extracellular enzyme co-localises with annular structures surrounding the pollen tube. Inhibition of transglutaminase by specific inhibitors or an anti-TGase monoclonal antibody blocked pollen tube growth. The extracellular pollen transglutaminase was also able to crosslink amines and the histidine-tagged green fluorescent protein (His_6-Xpr-GFP)[3] into exogenous proteins, consistent with a possible role for the extracellular pollen transglutaminase in the stabilisation of proteins in the style, to which the pollen tube becomes anchored. As self incompatibility triggers programmed cell death in pollen,[4] the TGase activity has been analyzed during these phenomena.

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III SYMPOSIUM

New horizons in fluorescence microscopy: "back to the origins"

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Fluorescence microscopes are generally operated by means of mercury arc excitation (50 and 100W), in few specific applications replaced by xenon lamp. For advanced applications (i.e. confocal) great benefits had been achieved by the introduction of laser excitation. Light Emitting diodes (LEDs) technology greatly improved in these last few years. From small lighting indicators of few milliWatts, now LEDs are also available in the range of some Watts of power, making them attractive to replace arc lamp excitation sources for both fluorescence microscopy and flow cytometry. Different prototypes of excitation modules (based on the "Luxeon" Lumiled diodes) fitting most of commercially available microscopes and operated by both epiillumination and transmitted light, have been constructed and tested. Instrumental and "visual" comparisons between LEDs and lamp excitations have been performed. Instrumental measurements have been carried out with a power meter located under the microscope objectives, while the visual comparison have been performed by means of various biological samples labeled with several fluorochromes. The results obtained indicate that the excitation performances of 3Watts LEDs and 100W mercury arc lamp are comparable. A significant increase of signal-to-noise ratio is achieved particularly with an original "transmitted excitation" set up, by means of both 2W UV (365nm) and 3W Blue (485nm) Leeds, delivering up to some hundreds milliWatts within narrow spectral bands (15nm). Compared with the standard lamp excitation, FITC labeling observation is consistently improved by blueLED excitation, whereas the optical power emitted by a mercury arc lamp in this spectral region is not very high. Our data indicates that LEDs technology applied to fluorescence microscopy allows improvements both in term of image quality and system efficiency.

Multidimensional light microscopy

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Confocal microscopes traditionally relied and still largely rely on single-point scanning techniques. Instruments in this class are suited to many practical uses, but lasers have to be employed as light sources, often causing specimen photodamage. Costs are relatively high while spectral flexibility is modest. Confocal methods have also been proposed based on multipoint illumination and detection strategies (spinning disk). These present some advantages, such as relatively fast imaging also using conventional lamps, but also significant limitations and high constructional complexity. An alternative technique, Video-Confocal Microscopy (VCM), developed at the CNR in Pisa, offers distinguished advantages over previous confocal methods in a multitude of applications in which not only costs but also spectral flexibility and low specimen photo-invasivity are of concern. In VCM, an arc-lamp is used to scan the specimen with an array of exciting spots. A CCD image sensor collects images during scanning. Hence, the specimen is narrow-field illuminated, but wide-field optical detection is performed. The intensity distribution of corresponding pixels in the set of collected images is computer analyzed emulating narrow-field detection (pinhole). 3D reconstructions and other studies of specimen structure are possible achieving an almost isotropic (XYZ) resolution, in the range of 300 nm or better. The use of conventional excitation sources, covering a wide spectrum of excitation wavelengths, avoids limitations being typical in the use of laser sources. Instrumental simplifications and consequent economies, compared to those of other solutions, are substantial. Results obtained with a ViCo microscope, an industrial incarnation of VCM manufactured by Biomedica Mangoni, demonstrate the performance of the technique in a variety of applications spanning from biomedicine to materials science.

Autofluorescence for an *in situ* biochemical analysis

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Most of the components of the biological material, under excitation at suitable wavelengths, give rise to a fluorescence emission covering the UV – Visible – Near IR spectral range. This emission is called "autofluorescence" or "light-induced fluorescence" (LIF) to be distinguished from the "induced fluorescence" that is obtained upon labelling the structure under investigation by means of exogenous fluorophores. Endogenous fluorophores are associated with various biomolecules that can either be responsible for the structural arrangement or involved in the metabolic and functional processes of cells and tissues.[1] To the former case belong constitutive proteins such as collagen and elastin; the latter include pyridinic coenzymes (NAD(P)H), flavins, porphyrin derivatives, lipopigments and vitamins. Since the overall autofluorescence emission is strictly dependent on the chemical nature, amount, spatial distribution and microenvironment of the fluorophores, changes in morphological and biochemical characteristics of cells and tissues related to physiological state or induced by the occurrence of pathologies, are expected to affect the autofluorescence properties. When the spectral parameters of pure compounds are defined, spectral fitting analysis procedures allow the estimation of each single fluorophore to the whole emission, resembling a biochemical analysis. LIF analysis has been successfully applied to characterize the metabolic engagement at single cell level (e.g.: isolated hepatocytes under different experimental conditions, normal and transformed fibroblasts). The characterization of the autofluorescence properties of the histological components at tissue level provides the basis for the development of an *in vivo*, real time, minimally invasive diagnostic procedure, called "optical biopsy".

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FREE COMMUNICATIONS

Growth factor enhancement of muscle regeneration

A. Musarò, C. Giacinti, G. Dobrowolny, C. Nardis, L. Pelosi, M. Molinaro
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Muscle regeneration and remodeling is an important physiological process which allows skeletal muscle to respond to environmental demands. Satellite cells represent the classical muscle stem cell compartment, localized between the basal lamina and the sarcolemma, which actively participate to muscle regeneration. Recent experimental evidences reported the contribution of other stem cells to muscle repair. However, while it is well documented that circulating stem cells have the capacity to enter skeletal muscle environment, less is known about the potential of the recruited cells to ameliorate the regenerative capacity of damaged muscle tissues. We have previously reported that muscle-specific expression of a local IGF-1 isoform (mIGF-1) promotes and improves regeneration in senescent muscle.[1] In addition, high levels of mIGF-1 transgene expression in the mdx mouse model of muscular dystrophy also preserves muscle function in the absence of dystrophin, inducing significant hypertrophy and hyperplasia at all ages observed, reducing fibrosis and myonecrosis, and elevating signaling pathways associated with muscle survival and regeneration.[2] More recently, we have been demonstrated that the capacity of the transgenic muscle to regenerate is also associate to an increase in the recruitment of circulating stem cells expressing Sca1, CD45, and c-Kit, general markers of stem cells.[3] The recruited uncommitted cells homing the damage muscle, contribute to muscle regeneration and guarantee a reserve of muscle stem cells.

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Differential levels of the neuronal nitric oxide synthase (nNOS) isoform modulate the osteoclastogenetic differentiation of RAW 264.7 murine cell clones

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It has been clearly established that osteoclasts, which play a crucial role in bone resorption, differentiate from hematopoietic cells belonging to the monocyte/macrophage lineage in the presence of macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-kappa B ligand (RANKL).[1] We have here investigated the M-CSF- and RANKL-induced osteoclastic differentiation of two distinct clones of the murine monocytic/macrophagic RAW 264.7 cell line, known as TIB-71 and CRL-2278, the latter cell clone being defective for the expression of the inducible nitric oxide synthase (iNOS) isoform in response to interferon-γ (INF-γ) or lipopolysaccharide (LPS). CRL-2278 cells demonstrated a more rapid osteoclastic differentiation than TIB-71 cells, as documented by morphology, TRAP positivity and bone resorption activity. The enhanced osteoclastic differentiation of CRL-2278 was accompanied by a higher rate of cells in the S/G2M phase of the cell cycle as compared to TIB-71. The analysis of nitric oxide synthase (NOS) isoforms clearly demonstrated that only neuronal nNOS was detectable at high levels in CRL-2278 but not in TIB cells under all tested conditions. Moreover, the broad inhibitor of NOS activity L-NAME significantly inhibited osteoclastic differentiation of CRL-2278 cells. Altogether, these results demonstrate that a basal constitutive nNOS activity positively affects the RANKL/M-CSF-related osteoclastic differentiation.

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Rabphilin colocalizes with cell actin cytoskeleton and stimulates association of granules with F-actin cross-linked by α-actinin

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In endocrine cells, granules accumulate within an F-actin rich region below plasma membrane. The mechanisms controlling this process are largely unknown. Rabphilin is a cytosolic protein expressed in neurons and neuroendocrine cells, and binds with high affinity to members of Rab3 family of GTPases, localized in synaptic vesicles and dense core granules.[1,2] Rabphilin also interacts with α-actinin, a protein cross-linking F-actin into bundles and networks and associating with granule membrane. In this study we investigated whether rabphilin, in addition to its granule localization, also interacts with cell actin cytoskeleton. By using purified components, it was found that association of rabphilin with F-actin is dependent on added α-actinin. In an *in vitro* assay, granules, but not endosomes or mitochondria, associate with F-actin cross-linked by α-actinin, and rabphilin appear to stimulate this process. Rabphilin enhances by approximately 8-fold granule ability to localize within regions at elevated concentration of cross-linked F-actin. These results suggest that rabphilin, by interacting with α-actinin, organizes the cell cytoskeleton to facilitate granule localization within F-actin rich regions.

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Nuclear PLCβ1 affects CD24 expression in murine erythroleukemia cells

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Inositide-specific phospholipase C (PLC) β1 is a key enzyme in nuclear lipid signal transduction affecting cell cycle progression and may be directly involved in regulation of gene expression and hematopoiesis. By microarrays, we compared the effect of nuclear PLCβ1 overexpression with that of PLC M2b cytoplasmatic mutant, which is exclusively located in the cytoplasm, in murine erythroleukemia cells. Out of 9000 genes analyzed, CD24 gene, coding for an antigen involved in differentiation as well as hematopoiesis as well, was up-regulated in cells overexpressing nuclear PLCβ1 as compared to both cells overexpressing the M2b cytoplasmatic mutant and the wild type cells. Here we show that nuclear PLCβ1 up-regulated the expression of CD24. The correlation was strengthened by the

observation that when PLCβ1 expression was silenced by means of siRNA CD24 expression was down-regulated. We also demonstrated that PLCβ1-dependent up-modulation of CD24 was mediated, at least in part, at the transcriptional level, in that PLCβ1 affected the CD24 promoter activity. Moreover the up-regulation of CD24 was higher during erythroid differentiation of murine erythroleukemia cells. Altogether our findings, obtained by combining microarrays, phenotypic analysis and siRNA technology, identify CD24 as an molecular effector of nuclear PLCβ1 signaling pathway in murine erythroleukemia cells and strengthen the contention that nuclear PLCβ1 constitutes a key step in erythroid differentiation *in vitro*.

Aquaporin 4 in rodent spinal cord: a confocal and electron microscopy study

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The distribution of Aquaporin 4 (AQP4), the major water transport channel expressed in the central nervous system (CNS), has been studied in the spinal cords of rats and mice using immunocytochemical techniques at both microscopical and ultrastructural levels. The results showed that AQP4 labeling a) is intense both in the gray and white matter and displays a pattern comparable in the two species and at all levels of the spinal cord, b) in the gray matter is not restricted to perivascular astrocyte profiles, as described in other brain regions, but it is intensely expressed also in the neuropil, c) displays a dorsal to ventral gradient of distribution and regional differences. Confocal microscopy showed that, in the neuropil, AQP4 coexists scarcely with GFAP, a marker of astrocyte intermediate filaments, but extensively with glutamate transporter GLT-1, a marker of astrocyte plasmamembranes. In the white matter, the overlap between AQP4, GLT-1 and GFAP is extended. The ultrastructural examination of sections labeled for AQP4 confirmed the localisation of the protein in astrocyte processes around vessels, neuronal perikarya and dendrites, and both asymmetric and symmetric synapses. Moreover, double labeling experiments showed that the distribution of AQP4 is almost complementary to that of chondroitin sulfate glycosaminoglycans (GAG), the major components of the neural extracellular matrix, as GAG are absent or weakly expressed in AQP4-enriched areas. Since, in the CNS, a role in the control of water and ion distribution has been proposed also for the extracellular matrix, it is possible that different mechanisms may contribute to the regulation of water homeostasis in different spinal cord regions. Supported by MIUR (COFIN 03).

Immunocytochemical localization of Na^+-Ca^{++} exchanger in the central nervous system

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In central nervous system (CNS) Na^+-Ca^{2+} exchanger plays a fundamental role in controlling homeostasis of intracellular Na^+ and Ca^{2+}ions. NCX1 exchanger plays an important role in development of permanent focal ischemia and in sensitizing neurons to NMDA-mediated excitotoxicity. Since detailed information on NCX1 ultrastructural localization are lacking, we investigated NCX1 cellular and subcellular localization in adult rat neocortex and hippocampus using immunocytochemical techniques with a mouse monoclonal antibody (Swant R3F1). Electron microscope analysis revealed an intense positivity in the hippocampus, remarkably in the strata oriens and radiatum of CA1 and CA3 fields, while less intense labelling was detectable throughout the neocortex. NCX1 was localized mainly in dendritic profiles of various size, where clumps of reaction product were scattered within the cytoplasm and associated to microtubules and to the inner part of the plasma membrane, and in dendritic spines. Most NCX1-labeled spines were contacted by unlabeled axon terminals forming asymmetric synapses. Labeling was also observed in few distal astrocytic processes and in rare axon terminals, all forming asymmetric synapses. In distal dendrites, intense rim of labeling was often observed apposed to the (outer) membrane of mitochondria located near the plasma membrane, and this labeling sometimes formed a continuum with the ir apposed to the plasma membrane. Collectively, ultrastructural analyses of NCX1 expression suggests that this exchanger may have a postsynaptic functions in glutamatergic neurotransmission.

α-tocopherol affects neuronal plasticity in adult rat dentate gyrus by inhibiting PKCα

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PSA-NCAM plays a key role during nervous system development; in adult brain, it is expressed in regions undergoing neuronal plasticity. Previously, we demonstrated that in dentate gyrus (DG) α-tocopherol administration induced an increase in PSA-NCAM-positive granule cells. Recently, it has been proposed that PSA-NCAM expression may be regulated by PKCδ; moreover, it is known that α-tocopherol inhibits PKC activity *in vitro*. Therefore, we hypothesized that α-tocopherol may be able to affect PSA-NCAM expression by inhibiting PKCδ. In order to demonstrate this hypothesis, we performed a quantitative analysis of phospho-PKC and phospho-PKCδ by western blotting in α-tocopherol-treated and control rats. Moreover, both total PKCδ and phospho-PKC‰ were detected by immunoperoxidase and the resulting specimens were analyzed by densitometric analysis. PSA-NCAM expression in DG was detected by immunoperoxidase. In treated rats, an increase of PSA-NCAM-positive granule cells and of the dendrite mean length per PSA-NCAM-positive neuron occurred. Western blotting showed in treated rats both a decrease of phospho-PKC, demonstrating that α-tocopherol inhibits PKC activity also *in vivo*, and especially a decrease of phospho-PKCδ. The densitometry showed that no differences in total PKCδ staining were present between treated and control rats and confirmed in treated rat DG a decrease of phospho-PKCδ. This finding reveals that α-tocopherol does not change the expression of PKCδ, but inhibits its activity. Therefore, our results confirm the hypothesis that PKCδ regulates PSA-NCAM expression and suggest that α-tocopherol may be able to affect neuronal plasticity in adult rat DG through PKCδ inhibition.

Chromium effects on kiwifruit pollen

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Chromium is a highly toxic metal which has became a serious pollutant in diverse environmental settings. It occurs in several oxidation states ranging from Cr^{2-} to Cr^{6+}, with the trivalent and hexavalent states being the most stable and common in terrestrial environments.[1] Both forms produce serious

damages to plant tissues and organs, although at different concentrations, probably due to their extremely different solubility.[1] Moreover, Cr(VI) is highly toxic, especially, for humans. Pollen is known to be a highly suitable model system for detecting basal cell toxicity to a wide range of chemicals due to its high growth rate and sensitivity to external or internal factors.[2] Here we report an ultrastructural analysis using TEM[3] of kiwifruit pollen treated with Cr(III) and Cr(VI), supplied respectively as $CrCl_3$ and CrO_3 at the beginning of germination *in vitro*. Pollen cell wall consists of outer exine and inner intine. In both Cr(III)- and Cr(VI)- treated pollen, the cell wall appeared thicker than the control, and a massive cytoplasmic vacuolization was observed. A certain chromatin condensation, generally, in generative cell nucleus, but also in vegetative nucleus was also detected. The TUNEL reaction[4] demonstrated the induction of DNA fragmentation. Taken together, our data seem to indicate, in the treated cells, particular death patterns, which appear suggestive of apoptosis.

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Effects of cisplatin on myogenic differentiation

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Cisplatin is a potent chemoimmunotherapeutic drug, as well as a genotoxic agent. In skeletal muscle cells it induces cell death, cell cycle arrest and a reversible inhibition of myogenic differentiation if applied before differentiation induction, but not when the differentiation program is established.[1] DNA damage by cytotoxic agents, determinates the inhibition of MyoD activity, a checkpoint of differentiation, and, consequently, myogenesis block.[2] Mouse C2C12 myoblasts were grown, and induced to differentiate as previously described.[3] They were treated with various cisplatin concentrations in undifferentiated condition and during differentiation. The observations with reverted microscope, transmission electron microscopy and Giemsa staining were performed as previously described.[4] The analysis of cell death was determined using the MTT assay.[2] In undifferentiated cells, the MTT assay shows that viability decreases in treated cells, in time and dependently on used concentrations. Furthermore, Giemsa staining and TEM analysis show a low induction of cells death. At 6 days of differentiation after treatment, most myotubes detach from the monolayer and appear floating, showing apoptotic and necrotic features.[5] From these preliminary results, an interruption of myogenic differentiation and some negative effects of cisplatin on C2C12 cells are evident. Further studies are in progress to inquire the modified expression of myogenic regulatory factors.

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Immunocytochemical localization of proteasome subunits in mouse spinal cord

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The ubiquitin-proteasome pathway is the major proteolytic system in the cytosol and nucleus of eukaryotic cells, where it catalyzes the hydrolysis of ubiquitin-conjugated proteins. The critical enzyme in this pathway is the 26S proteasome, formed by the combination of the 20S core containing the catalytic sites, and the 19S and 11S regulatory complexes. Alterations of this pathway have been recently suggested to play a role in several neurodegenerative disorders, including those affecting the spinal cord, but the exact distribution of proteasome subunits in the nervous system is scarcely known. We have studied, with immunocytochemical methods, the distribution of proteasome subunits in the spinal cord of mice to provide a background for further investigations on pathological samples. The results show that in the spinal cord of adult mice the 20S proteasome, identified by an antiserum against alpha and beta constitutive subunits, is extensively expressed, with a similar pattern, at cervical, thoracic and lumbar levels. Motor neurons in lamina IX are always the most intensely labeled cells, but 20S labeling is present also in neurons located in dorsal and ventral horn and in the intermediate gray. Double labeling experiments show colocalization of 20S proteasome with calcium binding proteins that are markers of GABAergic interneurons. The labeling for the three beta inducible subunits of 20S is instead very weak throughout the spinal cord. The 19S proteasome has a distribution similar to that of 20S, whereas the 11S proteasome is generally weakly expressed. Besides neurons, also astrocytes are immunoreactive for 20S, 19S and 11S proteasomes, particularly in the white matter, as shown by the colocalization of the proteasomes with the cytoskeletal protein GFAP. In spinal cord samples of mice at 2 and 4 weeks of age the 20S, 19S and 11S proteasomes are expressed in a pattern similar to that of the adults but with a lower intensity.

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Sarcoglycans in normal human smooth muscle: a CLSM and molecular study

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The sarcoglycans are transmembrane components of the dystrophin-glycoprotein complex which links the cytoskeleton to the extracellular matrix in adult muscle fibers. In the skeletal and cardiac muscle, the sarcoglycan subcomplex is a heterotetrameric unit composed of the transmembrane glycoproteins α-, β-, γ- and δ-sarcoglycan. A fifth sarcoglycan with significant homology to α-sarcoglycan, ε-sarcoglycan, has been identified; this sarcoglycan is expressed in both muscle and non-muscle cells. It is hypothesized that e-sarcoglycan might replace a-sarcoglycan in smooth muscle, forming a novel sarcoglycan subcomplex consisting of ε-, β-, γ-, and δ-sarcoglycan. Recently, a novel sarcoglycan, ζ-sarcoglycan, highly related to g-sarcoglycan and d-sarcoglycan, has been identified. On this basis, growing evidence suggest that there are two types of sarcoglycan complexes; one, in skeletal and cardiac muscle, consisting of α-, β-, γ- and d-sarcoglycan, and the other, in smooth muscle, containing β-, δ-, ζ- and e-sarcoglycan. ε-sarcoglycan may substitute for α-sarcoglycan in a subset of striated muscle complexes. Our results, obtained with immunofluorescence semi-quantitative analysis and molecular methods on

smooth muscle biopsies of human adult gastroenteric tract, vessels, uterus and bladder, show, for the first time, that in smooth muscle also α-sarcoglycan fluorescence is always detectable, although its staining pattern is lower than ε-sarcoglycan. Sometimes α-sarcoglycan staining was normally detected, whereas there was reduced but clearly detectable staining for ε-sarcoglycan. In our opinion, on the basis of our results, we would be able to hypothesize the existence of a pentameric or, also considering ζ-sarcolgycan, hexameric arrangement of sarcoglycan subcomplex. The hexameric sarcoglycan subcomplex, in conformity with larger or lower expression of single sarcoglycans could characterize skeletal, cardiac or smooth muscle.

The periodontal ligament during orthodontic movements: immunohistochemical study using a confocal laser scanning microscope

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It's known that collagen and fibronectin molecules play a key role in tissue's remodelling processes, following orthodontic movement. The aim of this study was to evaluate periodontal ligament (PDL) collagen I and IV and on the fibronectin modification induced by application of a precalibrated and constant orthodontic strength. A NiTi 50 gr. coil-spring was applied on maxillary and mandibular premolars of orthodontic patients; teeth were subsequently extracted 3 and 72 hour, and 7 and 14 day after the application of the strength. Results were compared with those related to homologous teeth of the opposite side not submitted to any strength. PDL samples were examined with immunofluorescence technique using the following antibodies: anti-fibronectin, anti-collagen type I, type IV and type VII. Results show type I collagen increase in the side of pressure in comparison to the tension one, and evenmore towards control elements; such an increase is particularly evident after 72 hours and 14 days rather than after 3 hours. Fibronectin likewise, shows a consistent increase in pressure side after 72 hours, 7 and 14 days. Type IV collagen instead decreased both in pressure and tension side versus controls. Collagen I and fibronectin increase above all on pressure side, authorize to believe that stimulation induced by the application of the strength could have caused an increase of PDL metabolic activity. Differently than other studies conduced in alive experiment animals and *in vitro*, this contribution was been carried on a human pattern without using enrichment methods. Authors suppose that an increase of fibrillary component and PDL matrix could shape a defensive mechanism towards mechanical stress. Type IV collagen reduction could be related to PDL vascular component decrement.

Functional expression of TRAIL and TRAIL-R2 during human megakaryocytic development

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The aim of this study was to investigate the expression and function of surface TRAIL and TRAIL-receptors in primary megakaryocytic cells, generated in serum-free liquid cultures from human $CD34^+$ cells purified from peripheral blood. The surface expression of both TRAIL and "death receptor" TRAIL-R2 became detectable starting from the early phase of megakaryocytic differentiation (day 6 of culture), and persisted at later (days 10-14) culture times. On the other hand, "death receptor" TRAIL-R1 and "decoy receptors" TRAIL-R3 and TRAIL-R4 were barely detectable or undetectable at any time point examined. The effect of the addition of recombinant TRAIL at day 6 of culture was to increase the rate of spontaneous apoptosis of $CD34^+/CD41^{dim}$ megakaryoblasts and to significantly decrease the total output of mature megakaryocytic cells after additional 4-8 days of culture. Conversely, addition in culture of TRAIL-R2-Fc chimera, which blocked the interaction between endogenous TRAIL and TRAIL-R2 on the surface of the cells, increased the total megakaryocytic cell count. Moreover, recombinant TRAIL promoted a small but reproducible increase of maturation in the surviving megakaryocytic cell population, evaluated by both phenotypic and morphological analysis. A similar pro-maturation effect was observed when TRAIL was added to bone marrow-derived $CD61^+$ megakaryocytic cells. Thus, our data suggest a role of TRAIL as a regulator of megakaryocytopoiesis.

Immunohistochemical characterization of astroglial cells in the central nervous system of the lizard *Anolis sagrei*

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Glial fibrillary acidic protein (GFAP) and vimentin are molecular markers of the gliofilaments belonging to the intermediate filament class. Despite the fact that reptiles are phylogenetically the earlier amniotes, the studies on the reptilian glial cells are still relatively scarce. This immunohistochemical study on the central nervous system (CNS) of the lizard *Anolis sagrei* can provide new findings about the comparative analysis of the glial cytoarchitecture in saurians with regard to brain evolution. The indirect immunoperoxidase technique was applied to transverse sections of the CNS. In *A. sagrei*, GFAP-positive structures were found in both the gray and the white matters throughout the brain and the spinal cord. The general pattern of immunopositivity fundamentally consisted of long and thick fibers extending from cell bodies lining the ventricular system to the meningeal surface. In the brain regions which have a thicker neural wall, the radial orientation was not so evident as in the thinner areas. These radially oriented fibers formed adjacent and variously extended endfeet constituting the submeningeal glial layer of the CNS. They also formed the perivascular glial coating. These ependymal cells showed regional specialization resulting from different size and immunohistochemical staining intensities of their cell bodies and emerging processes. Consequently, all brain regions did not show the same GFAP-staining intensity. Star-shaped astrocytes could be detected next to the meningeal layer in the septum, striatum, optic tectum, tegmentum, medulla oblongata and spinal cord. In the anole CNS, vimentin-positive glial structures were not found. The immunohistochemical response appears typical of mature astroglial cells since vimentin is not expressed. The star-shaped astrocytes show a different distribution in saurians. The glial pattern of *A. sagrei* is less evolved compared with crocodilians, birds and mammals.

Immunolocalization of the circadian Clock protein in hepatocytes of adult and old rats

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Many biochemical, physiological and behavioural processes

exhibit circadian rhythms, defined as cyclic oscillations of about 24 h. The oscillatory mechanism of the circadian clock is cell-autonomous, and Clock protein is a transcription factor essential for normal circadian rhythms. Clock protein undergoes intranuclear redistribution in rodent hepatocytes along both the circadian and the circannual cycle. However, aging leads to a progressive deterioration of the circadian rhythm at the behavioural, physiological and cellular levels. In the present study, we carried out ultrastructural immunocytochemical analyses on hepatocytes of adult and old rats, in order to investigate possible quali-quantitative modifications of the Clock protein related to the aging process. Female Wistar rats of 9 (adult) and 28 (old) months of age were exposed to a cycle of 12 hours of light and 12 hours of dark (12L:12D). Clock protein distribution was analysed in the morning, at ZT4 (ZT, zeitgeber time; ZT0 = light on, ZT12 = light off), in the afternoon, at ZT12, and in the night, at ZT 22. Our observations demonstrated that: a) most Clock protein was always located in the cell nucleus, where it accumulated on perichromatin fibrils (the sites of pre-mRNA transcription and early splicing); b) Clock protein showed circadian oscillations in the different nuclear compartments; c) these oscillations differed significantly between adult and old animals. The prolonged diurnal activity of old animals and/or altered nuclear pathways could explain the unusual intranuclear distribution of Clock protein during ageing.

Ultrastructural analyses on tissues from mice fed on genetically modified soybean

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We carried out ultrastructural morphometrical and immunocytochemical studies on different tissues -liver and exocrine pancreas for their involvement in food processing and testis as a bioindicator- from mice fed on wild type (control) and GM soybean. Our observations, made on animals from weaning to 8 months of age, demonstrated significant modifications in hepatocytes of GM fed mice, where cell nuclei showed changes suggestive of increased metabolic rate. In pancreatic acinar cells of mice fed on GM soybean, the nuclear features indicated reduced post-transcriptional hnRNA processing and/or nuclear export, and modifications were found in zymogen synthesis/processing. In the testis, a lower transcription and splicing factor content was observed in spermatogonia, spermatocytes and Sertoli cells of young GM fed mice, together with RER enlargements in Sertoli cells. We do not know yet which could be the factors present in the GM soybean capable of inducing such modifications. Among these, it should be taken in consideration the possible presence of traces of glyphosate, the herbicide to which the GM soybean has been rendered resistant. Alternatively, it should be mentioned that GM soybean contains lower amounts of phytoestrogens, nonsteroidal plant compounds able to influence cell activity by binding to oestrogen receptors. Interestingly, recent experiments, made on adult mice fed on GM soybean since their weaning and then submitted to a diet containing wild type soybean only, revealed that the modifications related to GM soybean are potentially reversible.

Antisense down-regulation of carrot TOP1α and TOP1β genes coding for topoisomerases I leads to delayed entry into PCD induced by reactive oxygen species

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In plants, the production of ROS is enhanced by various forms of biotic and abiotic stresses such as pathogen attack, UV-light, ionizing radiations, drought, salt and temperature conditions. ROS accumulation generates oxidative DNA lesions such as the production of 7,8-dihydro-8-oxoguanine (8-oxoG), a base modification of guanine. The conversion of guanine to 8-oxoG causes a mis-incorporation of adenine opposite to 8-oxoG and then G-T transversions in the DNA. Eukaryotic DNA topoisomerase I is a IB topoisomerase which play an essential role in cellular processes such as replication, transcription and recombination. Under oxidative stress conditions, the reversible Topo I-DNA cleavage complexes are converted into potentially lethal DNA lesions. Depending on the severity of oxidative damage, the cell can activate the apoptotic pathway. Differently from yeast and mammalian cells, the presence of two *top1* genes for two distinct nuclear topoisomerases I has been demonstrated in carrot and *A.thaliana* cells. We have used the antisense RNA technology to downregulate the expression of the *top1*α and β genes encoding the two isoforms of DNA topoisomerase I in *Daucus carota*. The transgenic antisense *top1* carrot lines, characterized by reduced growth rates and resistance to camptothecin, have been investigated for their response to oxidative stress: cells were exposed to different H_2O_2 doses and the activation of programmed cell death was monitored using molecular and cellular techniques. Preliminary results suggest a correlation between the reduced expression of carrot *top1* genes and resistance to oxidative damage induced by hydrogen peroxide.

Antigenic expression and morpho-functional properties of human dental pulp stem cells for applied therapies

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In this study we examined the kinetics of dental pulp stem cell antigen expression and the morpho-functional properties of both stem and their osteoblast-derived cells. We found that stem cells change their antigen pattern many times during their differentiation process into osteoblasts, as assessed by immunofluorescence and confirmed by RT-PCR assay. In fact, by day 30 of culture onwards, stem cells become negative for stem cell markers (CD117 and CD34) but were still positive for STRO-1, contemporarily showing a 100% positivity for CD44. Later, at day 40, STRO-1$^+$/CD44+ cells started to differentiate towards two cytotypes: about 30% of them were endothelial progenitor cells, + and the remaining 70% were osteogenic progenitor cells. At day 45 of culture, within the latter population, OC$^+$ cells were detected. This positivity, as well as that for BAP and the ultrastructural appearance, confirms that these cells differentiated toward osteoblasts. Confocal and transmission electron microscopy revealed that differentiated osteoblasts were cuboidal in shape and showed basal nuclei

and apical accumulation of matrix vesicles. In addition, adhesion to substrates, although already observable in stem cells, is considerably improved with differentiation due to an intimate adherence of osteoblasts with the substrate, when compared to that shown by control fibroblasts. Therefore, we can address that dental pulp stem cells and their osteoblast-derived cells are good candidates in applied therapies thanks to their morpho-functional properties.

Expression of CD1a by Barrett's metaplasia epithelial cells

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In this study we performed an immunohistochemical research of CD1a on 113 formalin-fixed paraffin-embedded esophageal biopsies of Barrett's metaplasia, with follow-up at 1 year. We found CD1a protein to be expressed in metaplastic epithelial cells of Barrett's esophagus, both gastric and intestinal types, but not in normal epithelium of gastric and intestinal mucosae.[1] We showed, for the first time, that metaplastic esophageal specimens from Barrett's esophagus may show a consistent number of $CD1a^+$ epithelial cells. Moreover, at the follow-up analysis, almost all cases expressing CD1a did not progress towards the development of a dysplasia or cancer. These data suggested that in Barrett's esophagus, the presence of CD1a, typically expressed by DCs, on epithelial cells could prevent metaplastic cells to undergo the following steps of neoplastic transformation.[2,3] We are now investigating about the mechanism leading to this phenomenon, since the hypothesis derived from these results is in agreement with other studies that showed that DCs associated to cancer of various sites may correlate with a better prognosis. Therefore, epithelial expression of CD1a, in particular by transformed cells of Barrett's metaplasia and potentially by other types of cancer cells, may result in improved recognition of these cells by the immune system, leading to a better prognosis for the patients.

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Interleukin-1β and interleukin-8 and interferon-γ gene expression in probiotic therapy

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Probiotic therapy has been demonstrated to prevent the onset of pouchitis and to improve quality of life in ulcerative colitis patients who have required ileal pouch anal anastomosis.[1] Pouchitis has been associated with elevated levels of pro-inflammatory cytokines and chemokines. In this retrospective analysis of archived endoscopic samples from responding patients enrolled in the above mentioned trial, we were interested to study mucosal gene expression of the pleiotropic pro-inflammatory cytokines (interleukin-1β, interleukin-6), Th1 cytokines (interferon-γ, tumour necrosis factor-α, interleukin-12), regulatory cytokines (interleukin-10, transforming growth factor-β) and the chemokine interleukin-8. In addition to assessment of cytokine gene expression, the presence of polymorphonuclear cells in the mucosal tissue was evaluated. Data show that patients who were treated with probiotics had significant lower mucosal mRNA expression levels of interleukin-1β, interleukin-8 and interferon-γ compared to placebo-treated patients. In addition, a lower number of polymorphonuclear cells was present in the tissue of patients within the probiotic group compared to the number of polymorphonuclear cells in the tissue of patients receiving placebo and patients having an episode of pouchitis.

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Degeneration and regeneration of the olfactory epithelium of fish, *Tilapia mariae*, after exposure to copper ions

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In the aquatic environment a lot of pollutants are diluted. They are commonly present in very low concentrations, far from being lethal, but all the same toxic. Copper is one of the most studied heavy metals in fresh water. Even exiguous amounts of this element can damage fish olfactory epithelium, decreasing the efficiency of a sense that play an important role in various behaviours. This study investigated the effects of 4 days exposure to low level (30 μg/L) of copper ions on the olfactory mucosa of the teleost fish, *Tilapia mariae*. Transmission electron microscopy and Fluoro Jade-B staining permitted to observe, at the end of the exposure, a selective damage circumscribed to the population of receptor cells, subject to necrosis. Fishes sacrificed at 0, 3, and 10 days of recovering in dechlorinated tap water showed the regeneration process in the olfactory tissue. Immunostaining with PCNA described a massive mitotic activity in the basal region of the mucosa immediately after the end of the exposure. It has been revealed that these new differentiating elements were immature neurons since they expressed the neural growth-associated phosphoprotein GAP-43. After 3 days of recovery the nuclei had already finished their migration to the upper portion of the epithelium, and cellular division was much less intensive. At this stage the GAP-43, initially localized around the nuclei of the receptors and in the proximal part of the emerging dendrites, appeared particularly expressed in the apical portion of the dendrites, called terminal knobs, and in some cases new axonal fibres were detectable. After 10 days the olfactory tissue did not present differences with the control one, even if a weak signal of GAP-43 expression was still visible. The present results suggest that after 10 days the regeneration seems to be completed and the integrity of the tissue restored.

Apelin and its receptor APJ are modulated in normal and pre-eclamptic human placentae

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Apelin is synthesized as a pre-proprotein of 77 amino acids that undergoes the enzymatic cleavage of C-terminus producing the active form of 36 aa. Apelin receptor, APJ, is a 380 amino acid seven-transmembrane domain Gi-coupled receptor originally isolated by PCR from human genomic DNA. Despite to a large number of reports describing expression and distribution of APJ receptor and its ligand in different organs and

tissues, little is known about their physiological roles. Several studies have demonstrated an apelin/APJ involvement in the regulation of blood pressure and blood flow in different organs. Vasculogenesis and blood regulation are mainly important in human placenta to allow a normal fetal development and growth and disorders of these systems lead to preeclampsia. So, we decided to investigated the expression pattern of apelin and APJ in normal and preeclamptic human placentae. We showed that in normal placenta apelin expression decreased from the first to the third trimester whereas APJ receptor increased from the first trimester to the third trimester of gestation. In preeclamptic placentae, we showed an increase of apelin/APJ expression in both compartments of villous trophoblasts and in endothelial cells. These results suggest that the disturbed balance between apelin and APJ may lead to reduction of blood flow and placental hypoperfusion that characterize preeclampsia.

Effects of fungicide methyl tiophanate on adrenal gland physiology of lizard *Podarcis sicula*

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Many pesticides belong to Endocrine Disrupting Contaminants (EDCs) since are able to change, mimic or antagonize the normal functioning of the endocrine system by interfering with the synthesis, metabolism, receptor binding and cellular responses of endogenous hormones. Among these, methyl tiophanate (MT), is widely used to control important fungal diseases of crops since it possesses a broader range of activity than most other available fungicides. The aim of this study was to evaluate the effects of MT exposure on adrenal glands of lizard Podarcis sicula by biochemical and histochemical approaches during winter stasis. Reptiles are particularly suitable as contaminant biomonitors due to their persistence in a variety of habitats, wide geographic distribution, longevity and, in many cases, site fidelity and to their ability to bioaccumulate. We have demonstrated that prolonged exposure to MT induced a significant increase of corticosterone and adrenaline serum levels compared with the control group. On the contrary, ACTH and noradrenaline serum levels decreased after MT exposure. From a morphological point of view, we found an intense hypertrophy of steroidogenic cells together with a great increase of vasculogenesis of the whole gland. In addition, we also found an increase of adrenaline cell number distributed in dorsal chromaffin ribbon of lizard adrenal gland. This study showed that MT caused histopathological alterations of lizard adrenal gland and that this fungicide was able to influence adrenal activity.

Pattern of expression of Notch in placenta throughout the pregnancy

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Notch belongs to the family of EGF 1-like homeotic genes, which encode transmembrane proteins with a variable number of cysteine-rich EGF-like repeats in the extracellular region. Notch signaling regulates proliferation, differentiation and cell death. During gestation all processes described above are particularly relevant to the physiology of human placenta. Drawing from this background, we have decided to further investigate the immunohistochemical distribution of Notch-1 and Jagged-1 in human placenta during gestation using human placental samples obtained with informed consent from patients from first and third trimester of gestation. In the first trimester, Notch-1 shows a moderate positivity in cytoplasm of cytotrophoblast (CT) cells and an intense membrane positivity in sincytiotrophoblast (ST) cells. On the contrary, Jagged showed an intense cytoplasmic immunostaining in both placental compartments. In the third trimester of gestation, the immunopositivity for Notch-1 appeared most uniform in both placental compartments. Jagged, instead, showed a weak and granular immunopositivity in ST and stromal cells. These results suggest that in human placenta the modulation of Notch pathway is important in consideration of its involvement in both proliferation during the first trimester of gestation and in differentiation during the third trimester.

Human adult stem cells: an emerging reality

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In the past recent years, basic research work and initial clinical trials have provided starting evidence that stem cells are of potential value for treatment of certain human diseases, where they could help to regenerate tissues which are defective because of either genetic or acquired diseases. This area represents an emerging field of biomedicine based on a series of new discoveries in the field of stem cell biology and developmental biology that have made it possible to isolate and expand stem cells from many human tissues. While the usage of human stem cells of embryonic origin has been limited in several countries on the ground of ethical concerns, the usage of adult human stem cells has provided an alternative for studies aimed at providing a reliable supply of cells of potential use for treatment of human diseases. We have recently started experiments aimed at defining conditions for isolation, *in vitro* expansion and characterization of adult human stem cells. Cells with apparent properties of stem cells have been isolated from cord and peripheral blood and from different tissues. A major effort has been devoted to establishing conditions to optimise *in vitro* expansion and differentiation of these cells. Phenotypic characterization of these cells is currently being performed aiming to define markers of stemness which are common to different cell lineages and markers that are more lineage specific.

Immunolocalization studies of a non-conventional receptor for vasostatin-1 on three-dimensional primary cultures of adult rat myocardiocytes

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Cardiomyocytes are highly differentiated and immobile cells. 3D cultures of these differentiated cells may resemble cardiac tissue and may help understanding the cellular target of molecules inducing negative inotropism as Vasostatin-1 (VS-1), a Chromogranin A (CGA) derived peptide. The enzyme eNOS is targeted to signal-transducing caveolae. In cardiac myocytes, eNOS associates with the muscle-specific caveolin-3 isoform.[1] The heat shock protein HSP90 associates with eNOS nearby caveolae, and modulates its enzymatic activity both in myocardium[2] and in endothelial cells.[3] In particular, inactive eNOS is bound to caveolin-3, and it is activated through phosphorylation by Akt/PKB kinase, coupled to eNOS by HSP90

binding.[3] Myocardiocytes and fibroblasts, from the same myocardium, have been obtained treating adult rat ventricles with collagenase II. From the cells' suspension we have obtained adherent cells and cardiomyocytes. For monolayer cultures of fibroblasts and cardiomyocytes, cells have been cultivated over fibronectin 5 mg/mL or Matrigel or Collagen I or a monolayer of fibroblasts. For 3D cultures, cells have been cultivated in PET inserts embedded in a thick layer of Matrigel. Using different extracellular matrix (ECM) components to maintain in culture adult differentiated myocardiocytes, we have demonstrated that the cellular localization of HSP90 depends on interactions cell-ECM. VS-1 seems to interfere with cell-ECM interactions and, in this way, to induce a different cellular localization of HSP90.

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Expression of HSP, TNF-α, TRAIL and DR5 in rat kidneys after cisplatin treatment

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Platinum compounds are used in treatment of human malignancies. Cis-diamminedichloroplatinum (CDDP) seems to react as chelate agent, alkylating DNA and inhibiting mitosis in malignant cells. Letterature data shown an high cisplatin nephrotoxicity even if the pathogenesis of renal injury is not still completely known. The purpose of this report was to analyse morphologic and structural modifications in rat kidneys, treated with cisplatin, by using histochemical and immunohistochemical techniques. Wistar albino rats were used. The control received physiological saline solution, i.p., instead of CDDP (7.5 mg/kg i.p.). Animals, at 7 and 15 days after treatment, were killed by cervical dislocation and the kidneys were quickly isolated. Tissues were fixed with the paraformaldehyde 4% buffer solution, included in paraffin. Histopathological examinations were conduced using Masson Trichromic Goldner staining, Silver Impregnation, PAS reaction and Cation Iron Colloid histochemical preparation. Immunohistochemical stainings for Heat Shock Protein (HSP), Tumor necrosis factor-alpha (TNF-α), member of the tumor necrosis factor family (TRAIL) and its death receptor (DR5) were performed. Focal regressive glomerular and proximal tubule changes were found with PAS reaction in glomerular basal membranes and mesangium. Cellular degenerative alterations, including cytoplasmic vacuolization of the proximal tubular cells and cellular degeneration, were observed. Amorphous PAS^+ contents in tubular lumen was also demonstrated. HSP expression, at 7 days, indicate the response to the stress induced by membrane peroxidation while TRAIL, DR5 and TNF-α overexpressions, at 15 days, could represent the apoptotic response to cisplatin nephrotoxicity.

Aleurone PCD in naturally aged wheat seeds during imbibition

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During cereal germination, aleurone cells undergo a process of PCD[1] after taking part in the degradation of seed storage material. The process was studied analysing PCD related nucleases, eventual DNA laddering, hystochemical data in aleurone layers obtained from germinating viable seeds of *Triticum durum*; this death pattern was compared to that revealed in aged seeds. The recorded laddering of DNA coincided with the increase in Zn^{2+}-dependent nucleases in viable seeds. The later germination was characterised by a shift to smaller DNA fragments and by a general reduction in DNA content. During imbibition the number of Feulgen stained nuclei decreased, chromatin gradually became more condensed and undetectable until they completely disappeared, in parallel with a manifest cell wall degradation. Prolonged storage of seeds is accompanied by a progressive reduction in the rate of germination and seedling growth till the loss of viability.[2] Seed ageing was associated with a lower activity of nucleases, a slight DNA laddering during imbibition and a more precocious nuclei degeneration, in particular in not viable seeds. This may be the result of previous degradation of DNA during seed storage, as highlighted by the decrease in genomic DNA and confirmed by the hystochemical results: low frequency and slight Feulgen stainability of nuclei in not viable material accompanied by precocious cell degeneration. Although the DNA content in aged seeds was low, seed ageing does not seem to influence the fate of aleurone cells, with the exception of PCD timing.

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Antioxidant effect of creatine in cultured mammalian cells

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Creatine (Cr) is the most popular supplement proposed to be an ergogenic aid. However, the potential mechanisms underlying this effect have not been fully clarified. Since oxidative stress can affect muscle fatigue, it has been recently proposed that Cr might exert antioxidant activity.[1] However, to the best of our knowlege, no research has been aimed to directly test the antioxidant effect of Cr on cultured cells. In the present research the effects of Cr were studied using cultured human promonocytic (U937) and endothelial (HUVE) cells and murine muscular (C2C12) cells exposed to H_2O_2. Cr, at pharmacologically attainable concentrations, markedly attenuated the cytotoxic effects induced by H_2O_2 (as assessed with the trypan blue exclusion assay and morphologic analysis) in all cell lines. Cr-cytoprotection was independent of chelation of transition metals; Cr did not affect cellular antioxidant status; cytoprotection was invariably associated with elevation of the intracellular fractions of free and phosphorylated Cr; mass spectrometry experiments showed that a 136 MW molecule, which is likely to represent an oxidation by-product of creatine, formed in reaction buffers containing Cr and H_2O_2, as well as in cellular extracts from H_2O_2/Cr-treated U937 cells. On the basis of these findings it is suggested that Cr exerts significant antioxidant activity in cultured cells via a pleiotropic mechanism involving direct scavenging of oxidative species and amelioration of the energy status of the cells.

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Analysis of the extracellular signal–regulated kinase 1 and 2 activated neurons distribution in the rat periacqueductal gray matter after noxious stimulation: an immunohistochemical study

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The periacqueductal gray matter (PAG), the midbrain region made up of neuronal columns encircling the cerebral acqueduct, plays a key role in nociception.[1,2] As the extracellular signal-regulated kinases (ERKs) 1 and 2 are activated after noxious stimulation, [3,4] we analyzed the distribution of ERK-activated neurons in the PAG after visceral noxious stimulation. Ether and urethane anesthetized rats received an intraperitoneal injection of acetic acid or were left untreated and were perfused after two hours. Serial sections immunoreacted with an antibody selective for the activated ERKs. Significant ERK activation occurred only in the ether-anesthetized noxious stimulated rats. In these rats we evaluated the number of ERK-activated neurons, and their density as the ratio of the number of immunolabeled neurons to the extension of the region where they were located. ERK-activated neurons were more numerous in the lateral (LPAG) and ventrolateral (VLPAG) columns, but without significant differences. No ERK activation was seen in neurons of the most rostral PAG. The ERK-activated neurons were significantly denser at the intermediate level of the PAG. At the caudal level they were denser in the LPAG and VLPAG columns, and in the DPAG column at the intermediate and rostral level. These findings suggest that noxious stimulation activates ERKs in neurons involved in the different functional activities related to nociception, overlapping in the PAG columns, and strengthens the role of PAG in integration.

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Morphological and molecular evaluation of ochratoxin A effect on rat kidney cortex

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Ochratoxin A (OTA), a mycotoxin produced by several species of fungi, is a frequent contaminant of a great variety of food, including cereal and grain products. It has been implicated as one of the aetiological agents involved in the development of Balkan endemic nephropathy, a chronic renal disease characterized by progressive renal fibrosis and impaired renal function. Aim of this study was to characterize by morphological and molecular approach in an *in vivo* experimental model the effect of a chronic OTA-treatment on rat kidney, with particular attention to the analysis of collagen content and collagen turnover. For this study, Wistar rats were treated for 3 months with OTA (289 µg/Kg /48h) by gastric gavage. Untreated rats served as controls (CT). Collagen content was determined by computerized analysis of Sirius red stained paraffin embedded rat kidney sections and expressed as fibrotic index. In kidney cortex homogenates collagen type I and III (COL-I and COL-III), transforming growth factor-β1 (TGF-β1) mRNA levels were assessed by RT-PCR, and matrix metalloproteinases (MMP) were analyzed by western blot and SDS-zymography. Computerized analysis of Sirius red stained kidney sections revealed an increased collagen content in the kidney cortex of rats treated with OTA, compared to CT. The mRNA levels of COL-I, COL-III and TGF-β1 were similar in the two experimental groups. MMP-1 and MMP-9 levels in kidney homogenates were not modified by OTA treatment. Considered as a whole, our data suggest that after a chronic administration of OTA this toxin induces evident tubulointerstitial kidney fibrosis. Conversely, at the molecular level the genes related to fibrosis are similarly expressed in CT and OTA-treated rats. Since MMP-1 levels are similar in CT and OTA-treated rats, we suggest that post-translational mechanisms, such as increased collagen crosslinks, may be relevant in the development of OTA-induced kidney fibrosis.

P-glycoprotein is cleaved during apoptosis of human T-lymphoblastoid CEM cells

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P-glycoprotein, the product of the multidrug resistance-1 (MDR-1) gene, is a broad-specificity transmembrane efflux pump molecule that decreases intracellular drug concentration by effluxing them from the intracellular space. Chemotherapy-induced up-regulation of P-glycoprotein is considered a major event in establishment of MDR in human leukemia cells that were sensitive before drug exposure with a consequent dramatic decrease in the sensitivity to chemotherapeutic drugs leading to therapeutic failure. We have investigated the behaviour of P-glycoprotein during apoptosis of MDR human T-lymphoblastoid CEM cells. In comparison to wild type cells, MDR CEM cells expressed high levels of P-glycoprotein, which was assessed by western blot and a flow cytometric functional efflux assay based on the extrusion of Rhodamine 123 and DiOC2. Apoptosis was induced by either LY294002 (an inhibitor of phosphoinositide 3-kinase whose activity is required for CEM cell survival) or H_2O_2. Flow cytometric analysis of propidium iodide staining indicated that after 16 h incubation with the apoptosis inducers, at least 50% of cells were apoptotic. Western blot analysis indicated cleavage of poly(ADP-ribose) polymerase into the characteristic 85-kDa apoptotic fragment. As to P-glycoprotein, it was cleaved into a 83-kDa fragment. Therefore, our results identify P-glycoprotein as a protein which undergoes proteolytic cleavage during apoptosis.

Extramedullary hemopoiesis and erythrophagocytosis in tumor stroma in MMTN-neu (erbB-2) transgenic mice

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Iron (Fe) is indispensable for cell metabolism and proliferation. It is normally recycled by phagocytosis of senescent erythrocytes in liver and spleen. MMTV-neu mice are transgenic models of human mammary cancer. We reported that their liver undergoes hemopoiesis, that later regresses, followed by several features of early hepatocarcinogenesis. Pigment-laden macrophages were absent from liver, scarce in the red pulp of the spleen (mostly occupied by hemopoietic foci) but abundant in the tumor stroma. This suggested Fe utilization in the tumor. We thus investigated Fe with Perls' reaction, relating it to the

presence of heme-related products which were identified by their red autofluorescence when excited by light around 400 nm. Spectrofluorimetric analysis was performed with a microspectrograph equipped with an OMA system (excitation with a 405 nm interference filter). Red autofluorescence, ascribed to bacterial invasion of necrotic tissue was often reported in tumors. Iron was detected in macrophages in the spleen and in the capsule and angiogenic areas of the tumor, but not in the liver. In the liver, red fluorescence emission peaking at ~630 nm, ascribed to protoporphyrin IX, was detected in hemopoietic foci. The red emission of the spleen and tumor had two peaks ~630 and ~675 nm; the latter may correspond to a protoporphyrin IX photoproduct. Our preliminary findings of heme degradation products in tumor invasion and angiogenesis regions suggest that Fe is necessary for these processes. They are in keeping with the rationale for antitumor therapy with Fe chelating agents.

Improvement of liver function by melatonin after cold storage and reoxygenation

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In liver transplantation restoring function after cold storage is a key issue dependent on ischemia-reperfusion injury. Melatonin (MLT) is a potent antioxidant which also improves mitochondria function. We studied isolated rat liver, preserved for 20h at 4°C with either University of Wisconsin or Celsior solutions and reperfused without or with 100 μM MLT with oxygenated KHB at 37° for 2h. Biochemical assays revealed a MLT-induced increase in bile production and ATP levels for both solutions. Lipid peroxidation (TBARS) and tissue GSH/GSSG ratio were unaltered by MLT. The improvement thus appeared related to enhancement of mitochondria function rather than to an antioxidant effect. To test this hypothesis *in situ*, we used the DAB-Mn^{2+}-Co^{2+} method to demonstrate superoxide. In control liver the reaction was present in periportal and mid-zonal hepatocytes and in sinusoidal cells. Livers submitted to cold IR were edematous (especially for Celsior preservation), and staining occurred only in a narrow hepatocyte layer, suggesting poor metabolic activity. The absence of reaction in sinusoidal cells was in keeping with the low TBARS reaction in the perfusate. By contrast, when reperfusion was made with MLT, the morphology was markedly improved and the stained hepatocyte area wider for both solutions. In conclusion, the histochemical data confirm that MLT improves electron transfer in mitochondria and support the usefulness of the indole in liver transplantation.

Human cutaneous malignant melanoma: prognostic value of immune system infiltrating cells

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To establish the prognostic value of immune system cells infiltrating melanoma we evaluated the distribution and density of T lymphocyte subsets, macrophages and dendritic cells in primary cutaneous melanoma from 47 patients (American Joint Committee on Cancer Stage I and II). Immunohistochemical demonstration of CD8 and CD4 lymphocytes, CD68 macrophages, HLA-DR cells, Protein S100 and melanoma-associated antigens Melan A and HMB45 was performed. The low, moderate and high density groups of cells infiltrating the base of the tumor vertical growth phase were compared to the overall survival rate, by the Kaplan-Meier method, and the log-rank test. Clinical variables (gender, age, tumor location, Clark's level, vascular/lymphatic invasion, and thickness) were also analyzed. The CD8 lymphocytes exhibited independent statistically positive significance in survival (Log-Rank test 8.49, p=0.01) between patients with different lymphocytes density. There was a difference in 5-year survival among high (78.8%), moderate (44.4%) and low (25%) density groups. The CD4 lymphocytes, less numerous than CD8 cells, had similar distribution. Also, there was a correlation of HLA-DR cells with survival overall (Log-Rank test 5.29, p= *0.02*). CD68 cells density was not correlated with survival. The presence and number of infiltrating CD8 lymphocytes could be considered an independent favourable prognostic factor in melanoma, as well as the overall occurrence of HLA-DR cells. Our results could be important to indicate further prognostic factors in evaluation of the disease progression and for the development of immune therapies.

Basal lamina and apoptosis in human aorta aneurysm

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Aortic dilation is accompanied by the basal lamina (BL) remodeling. We examined main components of BL and apoptosis rate in the vessel wall using surgical fragments from 27 patients with aortic root aneurysm and 5 normal aortic specimens. Samples of the aortic convexity and concavity collected from each patient were processed for molecular biology and immunohistochemistry. The DNA strand breaks generated upon DNA fragmentation during apoptosis were detected by enzymatically labeling of the free 3'-OH termini with modified nucleotides (TUNEL). The increased number of apoptotic nuclei in dilated vessels appears clearly related to the loss of the regular tridimensional arrangement in BL major components in pathological specimens. Quantitative modifications involve decrease in Collagen I, Laminin α2 chain and Fibronectin and type IV Collagen and Tenascin increase. Ascending aorta dilations expand asymmetrically, with prevalent involvement of the vessel convexity. We found it to be poor in type I and III Collagens, traditionally associated with mechanical and trophic properties of BL, and more rich in proteins involved in tissue remodeling as type IV Collagen and Tenascins. The role of α2 and β1 chains of Laminins in tissue remodeling is intriguing; Laminin 2 isoform (merosin) in the BL of dilated aorta is significantly decreased, as in some muscle pathologies characterized by weakness. This suggests that mechanical properties of aortic wall could be strongly modified, decreasing wall resistance to blood flow with consequent dilation. We conclude that the pre-existing intrinsic wall weakness may predispose the arterial wall to degeneration when locally subjected to high flow and shear stress, leading to the development of aorta dilation.

Contractile proteins expression in developing human myocardium

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The mechanisms involved in the precardiomyocytes "committement" to form the heart tube are not completely understood. Proliferating, not-terminally differentiated myocytes and their progenitors are present in the adult heart, indicating that the process of new myocytes formation takes place throughout the life. We used human fetal heart tissue and *in vitro* obtained cells from biopsies of adult hearts to examine the expression of contractile proteins during myocytes formation and to compare the cardiomyocytes differentiation. In the cultured primitive cells from adult hearts we observed a progressive increase of α-sarcomeric actin and myosin light chain in the subsequent passages. In the paraffin embedded sections of fetal hearts the amount of α-sarcomeric actin increased between XII and XXXIX week, while the expression of mRNA of α-sarcomeric actin measured by RT-PCR increased with the progression of the fetal development, with a certain variability between the samples of the same estimated gestational age. The expression of α-sarcomeric actin examined by western blot was increasing slowly during the heart development. We observed the lack of miosin heavy chain mRNA expression between XV and XX week and its appearance at XXIV, followed by the progressive increase with age. This level of expression was always significantly lower when compared with that of the adult tissue. The myosin light chain protein did not change with the gestational age until XXIX week, when it reached the highest level of expression. On the other hand, the expression of both α- and β-isoforms of myosin heavy chain increased progressively with the gestational age, reflecting the general increase of the heart size and the formation of ventricular chambers and their walls during fetal development.

Sarcoglycan subcomplex and integrins in human skeletal muscle: fluorescence near-field optical microscopy (SNOM) study

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The sarcoglycan subcomplex and the integrins are two protein systems with structural and signaling functions, allowing interaction between muscle fibers and extracellular matrix. Although several studies have been conducted on these systems, their localization and distribution patterns along the non-junctional sarcolemma are not clear.[1] In this paper we first apply SNOM[2] to study this problem on human muscle samples, taking advantage of the enhanced spatial resolution. Four samples were obtained during orthopedic surgery from 10 subjects not affected by neuromuscular diseases. Samples were studied by fluorescence confocal laser scanning microscopy (CLSM) and showed that all tested proteins of the two systems have a costameric spatial distribution. Samples were later studied by SNOM, in reflection mode, and the results compared to CLSM. Tapered, metal coated optical fiber probes were used in illumination mode (aperture diameter ~100nm). Our results show respectively the topography and the immunofluorescence patterns of the TRITC, indicating the presence of the sarcoglycans and provide the same information for FITC related to integrins. The costameric band structure is clearly visible, with a period of ~2μm. The sample immunofluorescence distribution, shows a protein pattern slightly different than CLSM to SNOM resulting in a wide and better separated bands.

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Presence of different isoforms of Laminin in IUGR and normal human placentae

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Our study evaluate the modifications of the regular tridimensional arrangement in Basal Lamina (BL) major components in normal placentae and in pathological specimens obtained from pregnancies affected by Intra Uterine Growth Retard (IUGR). Tissue samples were collected at delivery and submitted to morphological as well as to biomolecular techniques (RT-PCR and Western Blot). Quantitative modifications involve decrease in type IV Collagen, while Laminin 5 increase in IUGR placentae. As regards β1 and β2 chains of Laminin, it is intriguing that both them increase in the fetal side of IUGR placentae while decrease in the maternal one, so suggesting that in IUGR there are deep differences between them. Type C Tenascin synthesis is dramatically increased in IUGR placentae, as we previously demonstrated by immunohistochemistry. These data clarify our morphological observations on the different distribution of Extra Cellular Matrix proteins between normal and IUGR placental specimens. As different types of Laminins and Tenascins are involved in tissue morphogenesis already at the time of embryonic implantation, with a role in the induction and maintenance of cell polarity, the establishment of tissue compartments organization and the regulation of adhesion, we hypotesize that their alterated distribution may directly influence the development of IUGR pathology. In addition, Laminins and Tenascins are important components of the BL structure, so influencing the exchange of blood and nutrients between placental vessels and tissue. The growth retard of IUGR babies might be due also to the changes in BL proteins we evidentiated.

Increased mast cell number and activity in the hypertrophied heart

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Mast cell (MC) are multifunctional cells that can be found in most connective tissue and that can play an important role in many process of tissue repair and inflammation. They act through the extracellular release of many mediator such as natural proteases in particular chymase and tryptase, profibrotic cytochines (IL-4, IL-2 and IL-6), and growth factor including TNF-α ,TGF-β and bFGFα Aim of the present study was to quantify the degree of myocardial infiltration by MC in a model of pressure-overload hypertrophy (POH). Moreover, since chronic sympathectomy (Sx) has been shown to have favourable effects on the left ventricular (LV) function and on survival in this setting, the effects of chronic abrogation of the sympathetic activity on MC density were also assessed. Sprague-Dawley rats were subjected to aortic banding (B) or sham operation (S), to subsequently undergo chronic sympatectomy (Sx, 6- hydroxydopamine, 150 mg.kg-1 i.p. twice weekly) or vehicle (Vh) treatment for 10 week. LV and lung weight were measured at sacrifice. Collagen abundance (Fraction%) and mast cell density and degranulation (cell/mm^2) were quantitatively assessed by computer-aided analysis in myocardial tissue sample, after Sirius Red and Toluidine blue technique respective. POH was associated with marked interstitial fibro-

sis, lung congestion, as well as with a clear cut increase in activated MC density. In sympathectomized animals degranulating MC density was significantly increased, independently of the presence of POH. However in these animals, POH was not associated with an increase in degranulating MC density and in myocardial fibrosis. Interstitial fibrosis caused by POH is associated with an increased activation of myocardial mast cells. Chronic abrogation of sympathetic nerve activity inhibits pressure-overload related myocardial fibrosis and mast cell activation, indicating a role of adrenergic hyperactivity in mast cell biological activity and extracellular matrix remodelling.

ANA or ANNA? A new method to characterize atypical anti-neuronal nuclear antibodies present in patients with paraneoplastic neurological syndromes

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Paraneoplastic neurological syndromes (PNS) are remote complication of systemic malignancy, characterised clinically by neurological disability and pathologically by destruction of neurons. In recent years an important finding has been that many affected patients have serum and cerebrospinal fluid antibodies that are reactive with internal neuronal antigens. Thus detection of anti-neuronal autoantibodies in a patient with neurological disorders may allow the diagnosis of a paraneoplastic syndromes and also provide information about the underlying neoplasm. Today we know 12 well characterized anti-neuronal antibodies and their correlations between PNS and tumours. The first step to detect the presence of an anti-neuronal antibody in the serum of patient with neurological disorders is the immunohistochemistry analysis on rat, human, primate cerebellum. Then a positive resulted is confirmed at the immuno dot blot with recombinant proteins. In 90% of cases studied the positivity to the immunohistochemical reaction showed ether nuclear or cytoplasmic staining. 80% of these are negative at the dot blot with recombinant onconeuronal proteins and so they are possible new atypic anti-neuronal antibodies. We describe a new immunohistochemical method on no-neuronal tissues (kidney, spleen and liver), to distinguish new possible atypical anti-neuronal nuclear antibodies (ANNA) from systemic anti-nuclear antibodies (ANA). Our aim was suggested a new immunohistochemical method to allow a completely characterization of unknown paraneoplastic anti-neuronal antibodies.

Redistribution of the nucleolar proteins, fibrillarin, phosphorylated c-Myc and Ki-67 during tumor cell apoptosis

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Besides chromatin, other nuclear components are affected during apoptosis, including the nucleolus whose complex structure is disrupted; its dense-fibrillar and granular components segregate and may form, in late apoptosis, heterogeneous aggregates with other RNP-containing structures which migrate to the cytoplasm. Proteolysis is not a constant feature for nucleolar proteins, during apoptosis: in fact, PARP-1 and UBF are cleaved whereas fibrillarin (Fbr), topoisomerase I, B23 and C23 are not. In the present investigation the fate of two other nucleolar proteins, phosphorylated c-Myc (P-c-Myc) and Ki-67, during apoptosis was monitored, using conventional and confocal microscopy after fluorescence immunolabeling in order to elucidate whether these nucleolar proteins are differently redistributed and/or degraded in apoptotic HeLa cells; Fbr was simultaneously labeled as a nucleolar protein which redistributes ectopically during apoptosis although being uncleaved. In interphase control cells, Fbr and P-c-Myc colocalize in the nucleolus, whereas Ki-67 does not, consistent with its cycle-related redistribution. Ki-67 is degraded during apoptosis and can be immunodetected only in early apoptotic cells; on the contrary, Fbr and P-c-Myc move into the cytoplasm and are found in apoptotic blebs, where they often do not colocalize. This indicates that protein components which are all found in the nucleolus of non-apoptotic cells may undergo distinct fates during apoptosis. It is worth noting that also during mitosis, when the nucleolus disassembles, P-c-Myc, Ki-67 and Fbr differently redistribute and never colocalize.

Irradiation-induced organelle photodamage in Rose-Bengal-acetate-treated cells

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Photosensitizers (PS) are energy transducing molecules used in photodynamic therapy, since they exert a cytotoxic effect when excited by light at appropriate wavelengths. By the addition of chemical groups (e.g., acetate), PS may be modified so as to improve their intracellular accumulation, although quenching their fluorescence emission and photosensitizing properties; once inside the cells, however, these compounds may behave as fluorogenic substrates, the added groups being enzymatically removed with restoration of the native chemical structure as well as of the original photophysical characteristics. The aim of this investigation was to describe the organelle photodamage induced on cells previously exposed to the fluorogenic substrate, Rose Bengal Acetate (RB-Ac, 10^{-5}M for 60 min). RB-Ac-treated HeLa cells were irradiated at 545 nm (light dose, 1.6 J/cm^2) and observed after 24 to 72hr recovery times in drug-free medium. Spectrofluorometry, conventional and confocal fluorescence microscopy, and electron microscopy were used to investigate the organelle damage, after cytochemical labeling of the endoplasmic reticulum (ER), Golgi apparatus (GA), microtubules and mitochondria. In irradiated cells, the ER and the GA became enlarged and swollen, with dilated cisternae and large scattered vescicles, especially at longer post-irradiation times. Microtubules aggregated into thick bundles, which then became more irregularly dispersed than in control cells. The mitochondria redistributed in the cytoplasm, often in a polarized form, and became functionally inactive. As a consequence of this organelle photodamage, apoptosis was observed to take place.

Cytochemical properties of *Botryllus schlosseri* haemocytes: indications for morpho-functional characterisation

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In the present study, we carried out a detailed light microscopy investigation of the cytochemical properties of the haemocytes of the colonial ascidian *Botryllus schlosseri*, using new cytochemical stains and enzymatic markers, a panel of antibodies and lectins as probes to characterise Botryllus blood cells further. Results indicate that lymphocyte-like cells are circulating stem cells recognised by anti-CD34 antibody and there are at least two defined haemocyte differentiation pathways: i) phagocytes, represented by hyaline amoebocytes and macrophage-like cells, which share similar staining properties,

the same hydrolytic enzyme content as well as the presence of detectable cytochrome-c-oxidase activity, recognition by anti-CD39 and *Narcissus pseudonarcissus* agglutinin; ii) cytotoxic cell line, represented by granular amoebocytes and morula cells which have vacuoles stained by Ehrlich's stain and Neutral Red; DOPA-containing protein are present inside morula cell vacuoles. Pigment cells and nephrocytes are involved in catabolite storage but their relationships with other cell types are less clear.

PKC inhibitors induced apoptosis in Morris hepatoma cell line

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Protein Kinase C (PKC) is a key enzyme involved in many cellular activities, including proliferation and neoplastic transformation. Recently we have observed that in Morris hepatoma cell lines only some of the eleven PKCs up today known are present, i.e. alfa, beta 1, delta, epsilon, eta, teta and zeta. The main differences in the expression of these isoforms between low and fast proliferating hepatoma cells are due to the conventional PKC alfa, the novel PKCs epsilon, eta, teta and the atypical PKC zeta. In the aim to investigate the possible correlation between cellular growth and the expression of specific PKC isoforms we have analysed by flow cytometry the cell cycle of Morris hepatoma cells treated with specific inhibitors of the conventional PKC alfa, the novel PKC eta and the atypical PKC zeta. By using increasing concentrations of the single or associated inhibitors at different times of incubation we did not observe significant modifications of the phases of the cell cycle On the contrary serum starvation for 24 hours before the inhibitors treatment produces a significant increase of apoptosis, even if the absence of calf serum by its own does not alter cell cycle progress. We suggest that combined treatment (i.e. serum starvation and PKC inhibitors) reduce the threshold for the onset of apoptosis or for inhibition of cell proliferation.

Estrogen receptor and aromatase expression in pig spermatozoa

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Estrogen receptors and cytochrome P450 aromatase, an enzyme catalysing androgen aromatization into estrogens, have been detected in testicular and/or epididymal germ cells of different species, as well as in human ejaculated spermatozoa. This suggested a role of estrogens in sperm maturation and in functional properties of mature cells. Conversely, no data have been reported on the possible interaction between these hormones and sperm of pig. Therefore, this work has investigated estrogen receptor and aromatase expression in ejaculated spermatozoa of *Sus scrofa domestica*. Ejaculated pig spermatozoa were treated for immunofluorescence assays. Immunolabelling was performed using monoclonal mouse anti-human ERα, polyclonal rabbit anti-human ERβ and monoclonal mouse anti-human P450aromatase as primary antibodies, while horse anti-mouse IgG Texas-red conjugated and goat anti-rabbit IgG FITC coniugated were applied as secondary antibodies. Then lysates of sperm pellets were submitted to Western blot analysis. A red fluorescence localised ERα in the sperm midpiece region, while a green fluorescence revealed ERβ in all the tail. Furthermore, a red intense staining detected P450arom in the sperm tail. Western blot analysis confirmed the protein detection. This study has demonstrated, for the first time, estrogen receptor expression in pig ejaculated sperm, with a differential ERα/ERβ localization similar to that one recently detected in human ejaculated spermatozoa. Furthermore, aromatase was also expressed in pig sperm, remainding to the enzyme localization in rat, mouse and rooster testicular spermatozoa. Therefore, our results have indicated the sperm of pig as estrogen targets and sites of estrogen biosynthesis, representing a basic foundation for future functional studies.

Immunohistochemistry of doppel protein as a new marker in astrocytic tumour progression

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A novel prion-like gene, doppel (Dpl), reported aberrant expression profiles in human astrocytic tumors. In the present study we investigated by immunohistochemical and in situ hybridization techniques the cellular localization of Dpl gene and its coded protein in frozen and paraffin embedded sections of 45 astrocytic tumours speciments, classified as WHO. Negative and positive controls were human adult brain and testis biopsies, respectively. Several astrocytoma-derived cell lines were employed: IPDDC A-2 (astrocytoma),POR (GBL), GBM-HU12 (GBL), PRT-HU2 (GBL), MAC (GBL) and D384 (astrocytoma).The following anti-Dpl antibodies were used: pDDC39 (raised against a peptide mapping at N-ter), pG-20 (C-ter), pN-20 (N-ter), pDpl (entire rDpl protein); polyclonal antibodies GFAP and S100 were assayed as typical astrocytic markers. Dpl mRNA *in situ* hybridization was performed using a cRNA digossigenin-conjugated probe. Dpl transcripts were localized in the above mentioned cell lines, showing a peculiar and predominant nuclear localization, suggestive of a nuclear retention phenomenon. At the protein level, immunohistochemistry results in cell lines and in the tumor specimens showed fine cytoplasmic granules. A weak cytoplasmatic localization of Dpl protein was detected in low grade of astrocytic tumours, while a stronger localization was detected in high grade of astrocytic tumours. These evidences are suggestive of an association between Dpl expression and tumor grade progression. This study documents a novel astrocytic tumor progression marker that has the potential to help in histological classification of the tumors.

Formation of *Elodea densa* stem aerenchyma involves programmed cell death

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Aerenchyma is formed in the roots and shoots in adverse conditions, either constitutively or because of abiotic stress and occurs as one of two basic types: lysigenous and schizogenous.[1] Lysigeneous formation involves cell death for necrosis or for programmed cell death (PCD). The aim of this study was to characterize the differentiation of the constitutive aerenchyma of *Elodea densa* stem and to verify if a forme of cell death might be involved. Results performed by cytohistochemical and molecular analyses evidenced: a) a basipetally and centripetally pattern of aerenchyma development; b) a schizogenous pathway of aerenchyma formation until 0.8 mm from apex, followed by a widening of intercellular spaces by a lysigenous pathway; c) some hallmarks of PCD: TUNEL positivity, chromatin condensation, persistence of organelles, PAS positivity,

apoptotic-like bodies, partial cell wall lysis. Our results, showing a coupling of schizogenic and PCD dependent lysigenic formation of the same tissue in the same organ, are, at our knowledge, the first data in literature. The aerenchyma PCD is an asincronous process and evidences peculiar features, according to hypothesis[2] that a specialized cell-death pathway might have evolved in plants for each distinct purpose. It might be[3] that the loading of distinct sets of hydrolytic enzymes into the vacuole, as induced by cell-death-triggering signals, could define the various morphotypes of plant PCD. However, whether vacuole functions as a key executioner, or whether it has a supporting role as a provider of the necessary enzymes for recycling of the contents of the dying cell, it is not clear at present.[2]

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Quantitative digital analysis as a novel tool to evaluate the biocompatibility of new candidate microcapsules for use in islet transplantation

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Transplantation of encapsulated pancreatic islets is a promising approach for the treatment of type 1 diabetes. Large-scale application of this technique, however, is hampered by insufficient biocompatibility of the capsules. We have evaluated the biocompatibility of a new synthetic material with 6 different chemical groups on their surface used for the fabrication of the microcapsules. A suspension of empty capsules made of each candidate materials was implanted in the retroperitoneal ileopsoas muscle and renal subcapsular space. Two lewis rats were inoculated with each candidate material. Kidney and muscle containing all the capsules were explanted after 4 week for histological analysis. The bioptic samples were paraffin embedded, sectioned and stained with histochemical specific staining for the identification of collagen. The samples were then subjected to digitalized quantitative analysis using specific software to determine the degree of fibrotic overgrowth. The quantification of collagen deposition, calculated in proximity of the microcapsules, is expressed as a percentage of the total area and can be considered a good index for the biocompatibility. Our results show that there is a difference in fibrotic overgrowth depending on implantation site (subcapsular renal space rather than ileopsoas muscle) and on specific chemical properties of different investigated microcapsules.

Immunohistochemical expression of Orexin A and Orexin Type 2 receptors in the rat gastrointestinal apparatus and endocrine pancreas

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Orexins A and B (hypocretins), are a novel family of neuropeptides synthesized in lateral and posterior hypothalamus, a feeding-controlling center. Intracerebroventricular injection of Orexin A or B was shown to stimulate food intake in rat. There as been accumulating evidences that Orexins, in addition the appetite regulation, are implicated in various other functions both central and peripheral. Their possible implication in insulin and gastric acid secretion has stimulated us to examine immunohistochemical distribution of Orexin A and Orexin type 2 receptor in the rat gastrointestinal apparatus and endocrine pancreas. We have studied immunohistochemical distribution of Orexin A and Orexin type 2 receptor in 10 rats. All specimens of stomach, gut and pancreas are fixed in Bouin's mixture and processed with Orexin A antibody (Chemicon AB3098) and Orexin type2 receptor antibody (Chemicon AB3094). In the stomach moderate orexinergic immunoreactivity is detectable both in mucosal epithelium and in medio basal portion of glands in the different gastric regions with discriminated intensity in various cell types. In the small duodenal and jejunual intestine discrete reactivity is detected in the adsorbent epithelium and an intense immunopositivity of type 2 receptor in staggered cells in villi connectival axis and in the interstice among glands. Enteric neurons show weak immunopositivity. In the endocrine pancreas immunopositivity is detected in all citotypes of Langherans islets in uniform way. These data provide a morphological evidence to understand the physiological role of Orexins as paracrine modulator in digestive secretory and adsorbent functions and as neuromodulator in motor activity of gastrointestinal apparatus. These results, in addition, show endocrine-paracrine influence of Orexins on pancreatic secretions.

Immunohistochemical signals of apoptotic pathways in several types of meningioma

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Meningiomas are slow-growing intracranial tumor, derived from meningothelial cells. Histopathologically they are separated into three grades: benign meningiomas (further subdivided in according to histological aspect in several types), atypical meningiomas and anaplastic (malignant proper) meningiomas in according to clinical behaviour of the tumors. Apoptotic pathways activation is an important findings related to growing and clinical outcome of tumors. Benign (meningotheliomatous, transitional, fibrous, angiomatous), atypical and anaplastic meningiomas were immunohistochemically analysed for the expression of active form of caspase-8, caspase-9, caspase-6, caspase-3, expression of bax, bcl-2 and TUNEL signals. Heterogeneous expression and quantitatively different profiles of apoptotic steps activation were found, in several type of meningiomas. The presence of TUNEL positive signals and picnotic cell nuclear morphology were present in all type of meningiomas analysed, with exception of meningotheliomatous. In this one only caspase-3 activation was observed, accompanied with heterochromatic cell nuclear morphology, not compatible with cell apoptotic profile. Besides caspase-8 and caspase-9 active form were observed only in meningiomas type which express a bax / bcl-2 ratio ≤1: angiomatous and atypical type. Our findings reveal a substantial differences of apoptotic activation pathway between meningotheliomatous type, not affected by apoptosis, and the other benign meningiomas types, where apoptosis signals were widely found. An other interesting findings is the association between caspase -8 and -9 active form with high bcl-2 positive signal, known to act as surviving signals for the cells. A better comprehension of pro- and anti-apoptotic profile in several types of meningiomas may promote a more focused pharmacological and medical intervention in these types of tumor.

Mitochondrial protection by melatonin in U937 cells triggered by UV-B irradiation

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UV-B irradiation alters mitochondrial function causing release of pro-apoptotic molecules, like cytochrome c, which acts as a trigger for the formation of a complex including APAF-1 and procaspase-9.[1] This mechanism is mediated by reactive oxygen species (ROS) that modify mitochondrial membrane permeability.[2] Being well known that melatonin (Mel) is a scavenger of ROS,[3] we have investigated the potential effect of Mel in UV-B irradiated U937 cells. In our model, the treatment with 1 mM of Mel before UV-B irradiation showed a significant protection vs apoptotic cell death respect to that found in UV-B apoptotic U937 cells; on the other hand, the treatment with Mel after UV-B trigger showed a not significant weak protective effect, mostly consisting in an apoptosis delaying.[4] In particular, mitochondrial structure and function were preserved by apoptotic pathways when U937 cells were incubated with Mel before UV-B exposure, as demonstrated by peculiar fluorescence of both Mitotracker Green FM and JC-1, (intensity and distribution close to those found in control cells) markers of mitochondrial mass and function, respectively. Our observations suggest that, other than the well known role of Mel in basic physiology and pathophysiology,[5] this neuro-hormone may play a key role in inhibiting apoptosis through protection of mitochondrial function and structure.

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RhoA activation in renal proximal tubules: its role in cellular pathogenetic dynamics of IgA nephropathy fibrosis

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The Rho/transforming growth factor-beta (TGF-β) system plays a crucial role in the progression of renal damage due to stimulation of extracellular matrix molecule deposition. In fact the *in vitro* TGF-β-mediated production of fibronectin, one of the major TGF-β-regulated extracellular component, has recently been correlated with Rho protein signalling molecules. Although a relationship between increased renal tissue levels of TGF-β1 and fibronectin has been reported, no data are available on renal tissue expression of Rho proteins in IgA nephropathy. This study was designed to assess the kidney tissue immunohistochemical expression of RhoA, TGF-β1, and fibronectin in IgA nephropathy patients. An increase in RhoA, TGF-β1, and fibronectin expression was detected in tubulointerstitium and in glomeruli of IgA nephropathy compared to normal kidneys; in particular, RhoA was found also in proximal tubules, unlike control kidneys and mainly at the cell boundary level. The image analysis confirmed that the kidney tissue levels of RhoA, TGF-β1, and fibronectin were significantly enhanced in IgA patients. RhoA is expressed at higher levels and mainly at membrane level in its active form in all the renal tubules compared with control kidneys that showed inactive RhoA only in distal tubular cells. The high levels of activated RhoA, TGF-β1 and fibronectin detected in proximal renal tubules might be induced by the detected inflammatory interstitial monocytes able to activate renal epithelial cells by direct cell contact and to stimulate their TGF-β1 transcription and protein synthesis as recently demonstrated *in vitro*. These findings suggest that the proximal tubules deserve to be taken into consideration within the histological alterations in IgA nephropathy.

Nuclear protein kinase C-δ: involvement in cell cycle regulation

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PKC isozymes are phospholipid-dependent serine/threonine kinases, consisting of closely related molecules grouped into three categories: Ca^{++}/diacylglycerol dependent conventional (α, βI, βII, γ), diacylglycerol and phorbol esters dependent, Ca^{++} independent novel (δ, ε,ι, θ) and Ca^{++}, diacylglycerol and phorbol esters independent atypical (ζ, ι, λ, η).[1] These PKC isozymes are involved in a wide range of physiological processes including cell growth,[2] apoptosis[3] and differentiation.[4] It is known that lipid second messengers generated at nuclear level from either inositol lipids or phosphatidylcholine may capture and/or activate nuclear PKC isozymes,[5,6] but, despite such evidences, the knowledge about the precise role of the different PKC isoforms in the nuclear events related to cell cycle progression is very poor. Here we report a transient and evident detection of phosphorylated PKC-δ in the nucleus of physiologically cycling cells during S-G2/M phase transition, reaching the highest level in concomitance with chromosome organization and alignment on metaphase plate, then decreasing at basal levels as soon as the cell division occurred. This was evidenced by a novel flow cytometry method, developed in our laboratories, consisting of a biparametric analysis of PKC-δ and p-PKC-δ expression versus cell cycle progression.

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Preliminary observations on the immunohistochemical distribution of nNOS in the development of human foetus eye

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The ocular apparatus of human foetus 12-week-old (length Crown-Rump, CR =77-79 mm) shows after standard staining E.E. an evident differentiation both crystalline lens and vitreous body. The vitreous body, enveloped by not nervous part of retina and by nervous pluristratified part (neuroépithelium), it is still bording with the intraretinic space, and it is bounded by retinic pigmented epithelium. It is known that many substances are responsible of morphogenesys of this apparatus and our immunohistochemical study represents a preliminary observation of nNOS distribution in this area. We used 12 week-old human foetus. We investigated the immunohistochemical distribution of constitutive NOS (nNOS) using polyclonal antibody against nNOS (transduction laboratories). The section were processed for immunohistochemistry by the standard streptavidin-biotin complex method (Chemicon). The immuno-

histochemical reaction shows the nNOS presence with a brown-dark staining. The section observations evidence the crystalline with its fibers. It also evident that these structures are completely nNOS immunonegative. The vitreus body, that came of nervous portion of retina is negative to the reaction as well. The stratified epithelium of retina alone appears strongly positive. These data are included in our program of nNOS immunohistochemical topographic study in many foetal areas. It is showed that the NANC mediator has a inductive role, and our work in progress aim to clarify the appearance of nNOS and its possible role in development of human eye.

Presence of ANP in the suprachiasmatic nucleus of the developing rat

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ANP, observed in SO in the adult rat, was, by us, observed at 18th day of foetal life in developing rat. Therefore in present work we extend the immunohistochemical research for ANP to the suprachiasmatic nucleus (SCh) in developing-rat. Indeed, the SCh synthesize VP which plays a role on the electrolyte balance of the body fluids and since ANP plays the same activity, we think that the SCh might be site of ANP synthesis. Six embryos at 16th and 18th day and six neonates at 0 and 3 old day neonates of Wistar rats were anaesthesized, fixed in Bouin's fluid, dehydrated and embedded in paraffin. The sections were immunostained with polyclonal ANP antibody. The ANP-immunostained sections of the SCh ANP evidence: in embryos at 16th immunonegative neurons; in embryos at 18th day numerous immunopositive neurons; the granules of ANP are visible in perinuclear area and in the fibers; in the neonates at 0 day, the nucleus is well defined and are visible numerous immunopositive neurons (>50%); in the neonates at 3rd day, from birth, the nucleus is voluminous and more immunopositive neurons are visible. In the SCh the ANP, which was observed in the adult rat, is synthesized from 16th day of foetal life. In consideration of presence of VP and ANP in the developing rat, it deduces that the VP of the SO and PV and ANP of the SCh appear at the same developmental stage and this contemporaneity is probably to be related to their action on the balance of body fluids. By contrast, the Ox- presence in SO and PV only after the birth can suggest that Ox depends on the ANP influence, unlikely in the heart.

Immunohistochemical expression of Orphanin FQ in the rat male genital tract

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Orphanin FQ (Nociceptin) is a recently discovered endogenous neuropeptide that structurally resembles on opioide peptide. In the central nervous system this peptide is implicated in the nociception. More recently its documented distribution in other structures suggests a possible role fro OFQ in regulation of other physiological functions. On this subject the mouse vas deferens is proposed as avaluable bioassay for nociceptin. It seems to us interesting to evaluate immunohistochemical expression of this neuropeptide in distinct segments of rat male genital tract. We have studied the immunohistochemical distribution of orphanin in male genital apparatus of 10 Wistar rats. Specimens of penis, bulbourethral gland, vas deferens and seminal vesicle are fixed in Bouin's mixture, processed with Orphanine FQ antibody (Chemicon AB5515) and revealed with a visualizing system based on streptavidin biotin complex method. In bulbourethral gland intense Orphanin FQ immunoreactivity is detectable in glandular epithelium, whereas more weak immunopositivity is present in nervous fibers that come side by side with the gland. In seminal vesicle the surface and glandular epithelia are strongly immunopositive. In the vas deferens the surface epithelium is discretely immunoreactive. The results obtained supply the morphological basis to demonstrate that Orphanin plays a neurocrine control on motility and a paracrine control on secretion in examined genital apparatus segments.

GPIIbIIIa modulates Ca^{2+}-induced δ-granule release from human platelets

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GPIIbIIIa is the more abundant receptor at the membrane of normal platelets. Platelet agonists, that commonly induce an increase of $[Ca^{2+}]_i$, lead to the conversion of GPIIbIIIa from a low affinity/avidity to a high affinity/avidity receptor state, a phenomenon commonly indicated as inside-out signalling, that implies conformational changes of GPIIbIIIa. Release of platelet granule content activates more platelets, while efficient plasmatic fibrinogen binding bridges platelets to each other into the forming thrombus. By interaction of GPIIbIIIa with cytoskeleton, tense forces are generated on platelet pseudopodia, causing clot retraction that facilitates wound closure. Fibrinogen binding to its receptor initiates a cascade of events that leads to release of procoagulant vescicles and platelet cytoskeleton reorganization. A large body of evidence supports a general role for Ca^{2+} in granule secretion, although the Ca^{2+}-binding proteins that mediate this effect have not been fully elucidated yet, as well as the functional role of GPIIbIIIa in the modulation of serotonin-containing δ-granule secretion.

We have recently developed a flow cytometric method to detect platelet δ-granule release in association with platelet surface phenotype. We demonstrate here that, upon $[Ca^{2+}]_i$ elevation, the functional expression of GPIIbIIIa is necessary for a fully efficient platelet δ-granule release; receptor occupancy by RGDS or GPIIbIIIa defects, like in Glanzmann Thrombasthenia (GT) patients, decreases platelet δ-granule release. In GT patients we found a defect of platelet δ-granule release upon activation that is proportional to the expression of platelet surface GPIIbIIIa.

Immunodetection of protein kinase C isoforms in osteoblasts from patients affected by osteoarthritis or rheumatoid arthritis

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Protein kinase C isoforms (PKCs) play a central role in signal transduction networks which modulate various physiological cell responses, such as cell proliferation, differentiation, release of hormones and neurotransmitters, as well as cytokine production. Alteration of these pathways is common to many diseases. In chondro-osseous tissues, PKCs have been reported to play an important role in cartilage metabolism,[1] and pro- and anti-inflammatory cytokines were differently regulated by PKCs in macrophages/monocytes,[2] the major source of cytokines in chronic inflammatory diseases such as rheumatoid

arthritis (RA). These cytokines have been reported to induce changes in the expression of PLC β1 and PIP_2 in normal osteoblasts similar to those occurring in patients with RA[3]. In this study we analyze by Western blotting the presence of PKCs in primary osteoblast cultures from osteoarthritis (OA) and RA with respect to post-traumatic after fall (PT) patients. PKCα, βI, βII, δ, ε, θ, ζ, μ were detected in these cells. In pathological conditions, PKC θ and μ were more expressed whereas PKC ε and ζ, as demonstrated also by quantitative immunoelectron microscopy, decreased suggesting that these isozymes may be involved in the pathogenetic mechanism of arthritis. Studies are in progress to determine whether these alterations could be induced in PT osteoblasts by pro-inflammatory cytokines.

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UV-B irradiation induces both biochemical and morphological alterations in U937 cells

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Ultraviolet (UV) irradiation induces oxidative stress in mammalian cells through increased production of reactive oxygen species;[1] UV generates dysregulation of intra- and extra-cellular homeostasis by altered gene function and protein modification, even though UV response can be initiated in the cytoplasm or at the cell membrane independently of a nuclear event.[2] Although in skin tissue UV light affects cell membrane regulating pathways of matrix metalloproteinases (MMPs)[3] (key effectors in physiologic and tumour processes),[4] the UV-triggered alteration of MMP expression and modification of cytoskeletal protein profile is unknown to-date in leukemia cell lines. Through biochemical, ultrastructural and cytometric approaches we have investigated the UV-induced aberrations in U937 monocytic leukemia cells. UV-B treatment induced apoptosis as revealed by TEM and quantified by AnnexinV-PI cytometric evaluation; in particular, apoptotic cells showed a consistent bleb formation, suggesting a cytoskeletal alteration, as demonstrated by electrophoretic profile of membrane-bound proteins. Furthermore, UV-B exposure induced in U937 a time- and dose-dependent modification of MMP secretion. Our data suggest that UV-B triggers both cytoskeletal protein rearrangement and reduced MMPs activity, leading to "aberrant" leukemia cells with reduced invasive potential.

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Expression of matrix metalloproteinase isoforms in human mesenchymal cells of extraembryonic tissues

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Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into various tissues;[1] while MSCs are considered suitable candidates for cell-based strategies owing to their capacity to self-renew/differentiate,[2] little information is available about molecular mechanisms governing their stem cell potential. Although matrix metalloproteinases (MMPs) (key effectors/mediators of physio-pathological processes) are crucially involved in human mesenchymal differentiation,[3] the basal expression and MMP isoform distribution in human extraembyonic MSCs is unknown to-date. Through biochemical and cytometric approaches we have investigated the expression and characterization of MMP isoforms, the cell morphology and the immunophenotypical characterization of MSCs from placenta, amniotic and chorionic membrane of healthy newborns. Adherent cells isolated from different sources were homogeneously positive to typical mesenchymal markers (CD105, CD73, CD29 and CD166) whereas they do not express hematopoietic markers CD14, CD34 and CD45. Gelatinolytic bands found in MSCs are MMPs (biochemically characterized as Ca/Zn-dependent proteinases and immunologically recognized as MMP-2 and -9), circulating as latent proenzymes in zymogenic activable isoforms; extraembryonic MSCs differently produce and secrete MMP isoforms, suggesting potential regulatory functions on MSC differentiation.

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Immunolocalization of lactoferrin in human sporadic renal cell carcinomas: an investigation by mono- and polyclonal antisera

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Lactoferrin (Lf) has been immunohistochemically revealed in many neoplastic human conditions, although its biological meaning remains unexplained. We have investigated the Lf immunopattern in 35 formalin-fixed paraffin-embedded renal cell neoplastic (RCC) samples (22 left and 13 right kidneys), obtained at surgery from an equal number of patients (21 male, 14 female; age range 33-80 years); the morphology and the stage of tumours were available. On 4Ìm thick parallel sections, Lf immunostaining was revealed either by a polyclonal rabbit anti-human Lf (Dako, Denmark; w.d. 1:300; overnight at 4°C) and by a monoclonal mouse anti-human Lf (Biodesign International, USA; w.d. 1:75; 60 min. at room temperature). Quantification of immunoreactions was performed using the intensity-distribution (ID) score based on both the percentage and the staining intensity of positive neoplastic cells. A cytoplasmic immunoreactivity for Lf was encountered in 24/35 RCC with a variable ID score, while a nuclear immunolocalization was occasionally found. The pattern of positivity was different in clear cell or cromophobe variants; in fact, Lf was mainly evident at the periphery of neoplastic clear cells, mainly by monoclonal antibody, while intensely stained positive cromophobe elements were found in direct contact with negative ones with both antisera. Normal tubular structures, present in renal parenchyma adjacent to RCC, as well as polymorphonu-

clear neutrophils or monocytes were positive for Lf. Finally, no differences in Lf immunostaining were found in relation to the grade of RCC, site and stage of the disease; moreover, no relationships between immunohistochemical data and sex or age of patients were noted. We suggest that Lf immunoexpression may represent the result of an endogenous synthesis by neoplastic cells in order to have greater availability of iron for their metabolism or, alternatively, to modulate an unspecific anti-oxidant response against tumour in itself.

Altered lamin A/C phosphorylation in Emery-Dreifuss muscle

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Lamin A/C is a nuclear lamina constituent mutated in a number of inherited diseases collectively referred to as laminopathies. Skeletal muscle disorders associated with mutations of lamin A/C gene include autosomal Emery-Dreifuss muscular dystrophy[1] and limb-girdle muscular dystrophy 1 B. The pathogenic mechanism underlying these diseases is unknown, though recent data suggest an impairment of signaling mechanisms as a possible cause of muscle mis-functioning. We previously identified a molecular complex in muscle cells formed by lamin A/C, emerin and nuclear actin.[2,3] The stability of this protein complex appears to be related to phosphorylation mechanisms. In the present study, we analyzed lamin A/C phosphorylation in control and laminopathic muscle cells. Lamin A/C N-terminal phosphorylation was determined in mouse myoblasts using a specific antibody. We observed phosphorylation of lamin A during myoblast differentiation or proliferation, and reduced lamin A/C phosphorylation in quiescent myoblasts. Lamin A/C was hyperphosphorylated in mature muscle, mostly in regenerating fibers. Lamin A/C phosphorylation was strikingly reduced in laminopathic myoblasts and muscle fibers, while it was preserved in interstitial fibroblasts. We suggest that altered lamin A/C interplay with a muscle-specific phosphorylation partner might be involved in the pathogenic mechanism of Emery-Dreifuss muscular dystrophy.

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Cytological and functional features of spermatogonial stem cells

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The aim of this study was to characterize the molecular phenotype of spermatogonial stem cells (SSC) in the mouse. We first investigated the "Side Population" (SP) marker on the basis of cell ability to efflux the fluorescent dye, Hoechst 33342. When a testicular cell suspension obtained from both young and adult mice was analyzed by flow cytometry, a cell population (SP cells) was detected that exhibited low Hoechst staining and that disappeared from fluorescence profile by dye efflux inhibition with verapamil. Taking advantage of the differential staining of another fluorescent dye, Rhodamine 123 (Rho) and by double staining adult testis cells, we identified a subpopulation of Hoechst SP cells which were Rho dull and verapamil sensitive. To determine the germ cell identity of the SP cells and Rho dull cells we analyzed the expression of some markers of spermatogonia and other surface antigens by FACS and immunohistochemistry. Interestingly, SP cells appeared to express EE2, Ep-CAM, TRA-98, c-Kit, Plzf, but not CD45 and CD34, indicating that they were indeed spermatogonia. We then addressed the question of whether SP cells have a complete stem cell activity, by sorting SP cells obtained from 20-day-old ROSA 26 mice and transplanting them into adult recipients testes of busulfan-treated mice. Our results show that, two months after transplantation, the SP cells were able to give rise to colonies displaying a complete seminiferous epithelium wave, whereas colonies derived from total unsorted cells consisted of only one or two germ cell associations. Experiments are in progress to evaluate SSC activity in Hoechst SP, Rho dull, verapamil sensitive cells. Overall, these results indicate new possibilities to further purify spermatogonial stem cells.

In vivo and *in vitro* PAR-1 upregulation in rat astrocytes following trimethyltin treatment

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Protease-activated receptors (PARs) represent a novel G-protein coupled receptors family that mediate most cellular effects of thrombin and related-proteases.[1,2] By RT-PCR and immunohistochemistry we demonstrated that various PAR isoforms, mainly PAR-1, were upregulated in reactive astrocytes of rat hippocampus following i.p. administration of trimethyltin (TMT), a neurotoxin inducing neuronal degeneration and reactive gliosis. Western blot, RT-PCR and immunohistochemical analysis revealed that this PAR-1 upregulation was also mimicked in primary cultures of neonatal rat cortex astrocytes after exposure to TMT. This result suggests that the PAR-1 increase we have observed *in vivo* may represent a direct effect of TMT on astrocytes rather than a consequence of a complex astrocytic reaction following neuronal death. Furthermore, an evident upregulation of PAR-1 in cultured primary astrocytes also occurred following exposure to lipopolysaccharide (LPS) (a well known inductor of glial cells activation) whereas other neurotoxic agents, such as staurosporine, hydrogen peroxide and sodium azide, were unable to determine any PAR-1 variation. Similarly to astrocytes, both TMT and LPS induced an upregulation of PAR-1 in the rat astrocytoma cell line C6, thus indicating that this phenomenon was independent from microglial cells eventually contaminating astrocyte primary cultures. Furthermore after exposure to TMT and LPS, the levels of tumor necrosis factor-α and interleukin-1, were also increased in astrocyte cultures, suggesting that PAR-1 upregulation we have detected may be involved in glial inflammatory response rather than in cell death.

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ERK1/2 expression in glioblastoma multiforme

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Glioblastoma multiforme (GBM) is particularly known for the rapid invasion of neighbouring brain structures. Molecular genetic and biochemical studies have revealed, in GBMs, an over-expression of some growth factors whose transduction is

mediated via mitogen activated protein kinase family. The extracellular signal-regulated kinase (ERK1/2) belongs to this family and is generally involved in cell proliferation and differentiation. The aim of the present work was to evaluate by immunohistochemistry the expression of total (t) and phosphorylated (p) ERK1/2 in the enhanced lesion of GBM and in relative peripheral areas up to 3.5 cm from the tumour margin. Formalin fixed and paraffin embedded samples from 26 patients were analysed. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex method. The proportion of t and p ERK 1/2-labeled cells was evaluated from randomly selected fields. tERK1/2 was uniformly expressed in each area and preferentially localised in the cytoplasm. A variable percentage of pERK1/2 positive cells with immunoreactivity mostly located in the nuclei was present, even if cytoplasmic expression occurred. In the enhanced lesion, the highest percentage of pERK1/2 positive cells was often present in the region surrounding vessels and necrotic areas. In the peripheral areas pERK1/2 staining was also found in perinuclear regions and in glial cell processes. The expression of pERK1/2 was not linked to the presence of neoplastic cells. ERK activation in the tumour may be due to the stimulation induced by growth factors, while ERK activation in areas that do not contain neoplastic cells may be linked to a reactive status and merits further investigation.

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Markers of pericytes in the human brain angiogenesis

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Pericytes are microvessel wall encircling cells that consist of a prominent nuclear region and thin processes, both embedded in a basement membrane. The brain vasculature develops by angiogenesis, which is outgrowth of new vessels from pre-existing ones, and during angiogenesis pericytes interact with endothelial cells to form capillaries. Both cells are essential in vessel growth and maturation. Endothelial cells are well characterized, whereas pericytes, which are now coming into focus in each angiogenesis steps, are difficult to study owing to their heterogeneity and the scarcity of proven markers, mainly as concerns the developing pericytes. This study, carried out on human foetal brains by immunofluorescence and confocal microscopy, demonstrates that NG2 is a useful marker for identification of developing pericytes, whereas α-smooth muscle actin is better expressed by differentiated pericytes; the MMP-2 active form may be considered a very early marker of pericytes involved in the vascular basal lamina dissolution and PDGF receptor , a marker of pericyte recruitment during vessel maturation. The results taken as a whole indicate that pericytes extensively cover the brain developing microvessels and are involved in the angiogenic sprouts and in the formation of the first vessel precursors invading the neuropil.

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Differential expression of lectin binding sites in equine compact and expanded cumuli oocytes

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Equine oocytes can be recovered surrounded by compact (Cp) or expanded (Ex) cumuli. Cp-oocytes have lower meiotic competence, slower rate of maturation, reduced ability to respond to activation stimuli and reduced male pronucleus formation rate after ICSI when compared to Ex-oocytes.[1] Glycoconjugates play a key role in oocyte maturation and fertilization. We here investigated the glycoconjugate expression in equine Cp and Ex cumuli by lectin histochemistry. Cumulus-enclosed oocytes from abbattoir ovaries were fixed in Bouin's solution and embedded in paraffin wax after *in vitro* maturation. Sections (5 μm thick) were stained with 11 lectins (SNA, PNA, DBA, RCA_{120}, SBA, HPA, Con A, GSA I-B_4, GSA II, UEA I, LTA). The ooplasm of Cp-oocytes showed a major reactivity for SNA, SBA, HPA, GSA I-B_4 with respect to Ex ones, whereas both type of oocytes displayed same affinity for DBA, Con A, GSA II and no binding sites for PNA, DBA, RCA_{120}, UEA I, LTA. The zona pellucida of both Cp and Ex oocytes were unreactive for GSA I-B_4, GSA II, UEA I, LTA whereas in Cp oocytes showed no binding sites for SNA and PNA, a lower staining with RCA_{120} and a higher reactivity for HPA than Ex ones. Corona radiata cells from both oocytes types did not react with DBA, showed same reactivity with SNA, RCA_{120}, GSA I-B_4, and GSA II. In Cp-oocytes, it did not react with PNA but showed major affinity for SBA, HPA, Con A, UEA I, and LTA. The different glycoprotein pattern observed between equine Cp and Ex oocytes may be related to their different meiotic and developmental competence.

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Adhesive aspects and apoptotic induction during monocyte-macrophage differentiation

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In this work we have used a cell model represented by some differentiation stages of monocytic THP-1 cell line induced by increasing concentration (6-60 nM) of phorbol ester PMA. The progressive acquisition of macrophage properties was related to changes of adhesive capability and reorganization of microfilament and microtubular networks. The apoptogenic action of Actinomycin D was found mainly in monocytic-like cells and it was evident a decreased activity correlated with the progressive cell adhesion and differentiation into macrophages. On the other hand, the use of the antimicrotubular drug Vinblastine induced apoptosis, mainly in terminally differentiated cells, characterized by a higher adhesion to the substrate. In these cells, immunofluorescence techniques revealed the presence of adhesive contacts (vinculin labelling) and high expression of CD11b/CD18, a member of the β2 integrin family, involved in several functions of monocytes and macrophages related to cell-cell and cell-substrate interactions. In general, more adherent cells are less sensitive to apoptogenic stimuli unaffecting cytoskeleton; this cellular condition could elicit anchorage-dependent survival signals. In this contest, a higher cytoskeletal organization and adhesive structure acquisition could be of critical importance. Apoptogenic stimuli affecting cytoskeleton delete the resistance to cell death in more adherent cells: the

consequent morphostructural alterations, detectable by SEM/TEM approaches, and mainly the forced suspension of cells, are responsible for anoikis. Referring to these biological aspects, morphofunctional features of the cells, related to the differentiation level, could also determine a different sensitivity to the same apoptogenic stimulus.

Detection and immunohistochemical investigation of GFP-stably transfected adult mesenchymal bone marrow stem cells transplanted into the rat heart

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Growing attention is being brought to the use of stem cell transplantation for the treatment of various diseases. The possibility of labelling stem cells with green fluorescent protein (GFP) before their transplantation has opened new and promising perspectives for their use in experimental research since autofluorescence make much easier their detection in the receiving tissues. Adult mesenchymal stem cells (MSCs) obtainable from the bone marrow are one of the most studied stem cell population because of their ability to self-renew and to give rise to various differentiated cell types, and because of the relative ease with which they can be obtained and cultured. In particular, MSCs transplantation has been proposed as a possible treatment strategy for increasing the repair process in the ischemic heart. On the basis of these premises, we have carried out an immunohistochemical characterization of MSCs obtained from transgenic rats overexpressing the enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus enhancer and the chicken b-actin promoter derived from an expression vector, pCAGGS. Autofluorescent MSCs were characterized and analysed both *in vitro* and *in vivo* after their injection in normal rat heart. In vivo characterization of MSCs was carried out after immunolabelling with immunohistochemical markers of both stem cells and myocardiocites in order to investigate MSCs' integration in the receiving organ. The results of this study provide a basis for the experimental investigation of the effectiveness of MSCs transplantation therapy for the repair of the damaged heart.

Immunohistochemical data on the human vitreous cells

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It is known the vitreous occupying about four-fifths of the eye ball and it is colourless and apparently structureless. It is constituted of about 99% of water, collagen fibrils of type II and jaluronic matrix. The literature reports that in the vitreous there is only one tipe of cells: the hyalocytes, rounded cells, in particulary being condensed cortically. Besides notices about their origin are very few. The eye diseases can to interest the vitreous directly or undirectly: in all cases the vitreous suffering from different pathology must be submitted to surgical operation. The purpose of this study is to give a contribution to the morphological knowledge of the cells of the vitreous on the bases of their immunohistochemistry marking. For the present study we utilized the human vitreous from patients suffering from diseases such as emovitreous, retinal detachment, traumatic vitreous, etc.: 30 specimens of vitreous were analyzed for this research: the fragment are submitted to action of cytocentrifuge (1000/min), the slides with the cells immediately fixed in methanol and so with the following substances incubated: GFAP (AB-1) MONOC; CD 68 (Anti-human Macrophage); LYSOZYMA (Muraminidase) and stained with haematossilineeosina and observed by light microscope. The obtained results seem to identify in the vitreous three different cellular types: hyalocytes, cells positive to GFAP, cells positive to Lysozyme. In some case it is also possible to identify pigment epithelial cells from retina. The data obtained in our study show that in the pathologic vitreous are present not only hyalocites, but other cell types.

Extramandibular fat bodies of the striped dolphin (*Stenella coeruleoalba*): a morphological and ^{13}C NMR study

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Odontocetes echolocate by producing clicking sounds and receiving and interpreting the resulting echo, thus obtaining information on animals or objects out of their sighting range. It is widely accepted that the lower jaw acts as a specialized receiver that picks up and conducts the echoes bouncing back from the environments to the auditory bullae. Externally, the posterolateral region of the jaw is covered with a fat deposit which is continuous with the lipids of the melon and of the channel inside the jaw. The molecular and histological structure of the fat bodies covering of lower posterior jaw of the striped dolphin *(Stenella coeruleoalba)* was investigated by means of morphological and liquid state NMR techniques. The analyses of samples belonging to adult and juvenile individuals were performed with the aim of seeking the presence of age-related differences that may reflect different sound reception properties. Samples of extramandibular fat of stranded dolphins were excised within 24 hours from the finding and divided in two lots: the former was formalin buffered-fixed, paraffin embedded and stained for morphological analysis; the latter was used for the NMR analysis. In our study, the NMR analysis showed differences between adults and juvenile in the percentage of isobranched fatty acids. The morphologic analyses revealed that, in both adults and juvenile, this fatty tissue is similar to univacuolar adipose tissue. However, in the juvenile a muscular component was present, while only in adult subjects, enlarged and irregularly shaped cavities, structurally organized as veins, may be seen within the adipose tissue. The veins may regulate blood flow in response to changing water temperature and stabilize thermal gradient within the lipids of the jaw. These findings suggest that the molecular components and the histological organization can be an index of the of the gradual maturation of the organ with age.

Erk-mediated CREB activation in survival response of human K562 erythroleukemia cells exposed to ionising radiation/etoposide combined treatment

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The knowledge of the threshold over which the cells do not resist to ionising radiation /etoposide combined treatment and of some of the molecular mechanisms which drive the response of cancer cells to such agents could help the setting up of therapy protocols. Actually a key role for CREB, cyclic nucleotide response element binding protein (CREB) multigenic family composed by several nuclear transcription factors involved in c-AMP signalling, implicated in numerous cell functions including differentiation, proliferation, apoptosis, survival and adaptive response, has been suggested in hematopoiesis, cell proliferation and acute leukemias.[1,2] Here we report that the survival of K562 to 1.5 Gy ionising radiation ± etoposide is driven by

CREB phosphorylation. In particular, this response, evidenced mainly upon 1.5 Gy, by clonogenic assay and cell cycle analysis, could involve Erk phosphorylation which, regulating p90 RSK activation, determines CREB phosphorylation on Serine-133, as already evidenced in other experimental models.[3] This event is furtherly supported by the parallel evidence of p38 MAP kinase activity down-modulation along with low caspase-3 activity, no modification of Bax and Bcl2 levels. Thus the evidence that endogenous CREB activation could trigger a potent survival signal against cellular stress in K562 erythroleukemia cells exposed to 1.5 Gy ± etoposide, leads us suggest that the use of specific inhibitors against CREB, such as modified phosphoryothionate ODN corresponding to the CREB-1 sequence,[4] could improve the efficacy of anticancer therapy.

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GAD immunoreactivity in the cerebellar cortex under experimental conditions: the contribution of computer-aided techniques

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Three groups of Wistar rats were maintained during prenatal development in the following experimental conditions: 1. exposure to a low concentration of CO; 2. administration of a low dose of WIN, a CB-1 receptor agonist; 3. treatment with low doses of both CO and WIN. To evaluate the effects of these conditions on the development of the cerebellar cortex, highly susceptible to the effects of CO and/or Cannabinoids, we qualitatively and quantitatively analyzed in the cortex of all treated animals and controls the microscopic structure and the distribution of GAD, the GABA synthesis enzyme and functional marker of many cortical neurons. Sections from the cerebellar vermis of all animals were stained with Toluidine Blue (TB) or tested with an anti GAD antiserum. Quantitative analyses were carried out using a computerized image analyzer (VIDAS). In TB-stained sections, the cortex thickness and, in randomly selected fields of 18,000 μm^2, reference area (RA), the numbers of neuronal bodies per layer were calculated. In the GAD-immunostained sections, measurements per RA were made of the numbers of bodies and axons terminals, their relative areas, and the total areas of GAD immunoreactivity (IR). Differences among the different experimental groups and controls were statistically evaluated by the ANOVA test. Qualitative analyses of the cerebellar cortex of all treated animals showed no structural alterations nor differences in the distribution patterns of GAD IR in comparison with the controls. Instead, the computer-aided quantitative analysis of GAD IR, indicated significant changes among the different groups. These changes reflect effects of the experimental conditions on the development of some neurons of the cerebellar cortex, affecting their ability to express GAD and possibly to synthesize GABA.

Histochemistry of fleshy fruits polyphenols, cryostabilization and GMA embedding

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Benefits of a diet rich in fruits and vegetables must be attributed to the synergistic effect of the complex mixture of various phytochemicals present in some kind of foods. Several thousands of these compounds differing in molecular size, polarity, and solubility are here present. Their bioavailability is affected by the distribution in different subcellular organelles, cells, tissues and organs; there are also significant differences in individual phenolics among fruit species, cultivars of the same fruit, region and orchard productions, and also during storage and food processing. For example, apple polyphenols are concentrated in the pericarp, so that process of peeling wastes the bulk of polyphenols. Moreover, in intact plant tissues, cell walls, polyphenols and enzymes are present in distinct compartments, but when cells are ruptured, these three elements come in contact, and polyphenols can react with polysaccharides and cell wall components. Thus the chemical, qualitative and quantitative information on polyphenols has to be supported by information on the precise localization in the fruits tissues and also in their cellular and extracellular compartments. To reveal the precise localization of polyphenols our cryostabilization method - based on Ethylene Glycol (at -20°C) and Glycol Methacrylate embedding, at low temperature (6-10°C) - has been applied to some fruits (apples, wine grapes, grapefruits, apricots, kiwi, cherries, tomatoes). Here we report the preliminary positive results offered by this inexpensive and easy performing method.

RAGE-mediated neuroprotective action of S100B against beta amyloid-induced toxicity

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At the concentrations normally found in the brain extracellular space the glial-derived protein, S100B, protects neurons against neurotoxic agents by interacting with RAGE (receptor for advanced glycation end products).[1] However, at relatively high concentrations the protein is neurotoxic causing neuronal death via excessive stimulation of RAGE.[2] Since S100B is found within senile plaques in Alzheimer's disease,[3] we asked whether it plays a protective role against A, amyloid-induced neurotoxicity. To this aim we treated neuroblastoma cultures with toxic amounts of Aβ25-35 amyloid peptide. Our results show that at nanomolar concentrations S100B protects cells against Aβ-mediated cytotoxicity, as assessed by MTT and TUNEL experiments, by countering the Aβ-mediated decrease in the anti-apoptotic factor Bcl-2. This effect depends on S100B binding to RAGE because S100B is unable to contrast Aβ-mediated neurotoxicity in neurons overexpressing a RAGE mutant lacking the cytosolic and transducing domain.

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Lysozyme-like immunoreactivity in Sus scrofa Leydig cells

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Lysozyme (LSZ) is a glycosyl hydrolase that catalyzes the hydrolysis of peptidoglycan of the cell walls of many bacteria. For this reason lysozyme may also be used as an antimicrobial agent. LSZ has been isolated in a variety of tissues and secretions (lacrimal glands, serous cells of many glands, lactating mammal tissue, tears, saliva, milk, Paneth cells, myeloid cells, histiocytes and macrophages, ...).[1,2] In order to differentiate macrophages from Leydyg cells into the swine testis we used an antibodies-set specific for the former also included the anti-LSZ. Testis samples were collected from four adult pigs, fixed in Bouin, dehydrated, embedded in Paraplast and cut on microtome in 5μm thick sections. Immunostaining was performed with ABC system. Since quantitative ratio between macrophages and Leydyg cells is recognized as 1:4[3] we expected just a limited number of interstitial cells stained for LSZ. Amazingly, the anti-LSZ stained most interstitial cells. In order to explain this result we placed the following questions: there is a cross reactivity between employed antibody and a protein with high LSZ sequence homology? or, is LSZ effectively contained in the stained cells? Moreover, is the LSZ synthesized by these cells? or the enzyme is transported and/or stored into them. The on-line databases research allowed us to find, as expected, that α-lactalbumin (LA) had a significant (42%) sequence identity with the LSZ. LA is a protein that modulates the activity of numerous β-galactosiltransferase allowing, as an example, the synthesis of the lactose from part of the lactose synthetase. In our laboratory further studies are still in progress in order to establish the nature of this antibody immunoreactivity. Possible functions of LSZ, or eventually LA, in the testis are difficult to clarify on the base of morphologic data only.

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Distribution of Fatty Acid Amide Hydrolase (FAAH) in the small intestine of the mouse

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Fatty acid amide hydrolase (FAAH) catalyzes the hydrolysis both of the endocannabinoids (which are known to inhibit intestinal motility, through CB1 receptors) and other bioactive fatty acid amides (palmitoylethanolamide, oleamide and oleoylethanolamide), which might affect intestinal motility.[1] FAAH activity has been detected in the rodent intestine[2] and was found to be increased in the croton oil model of intestinal inflammation.[3] Since the distribution of this enzyme is still unknown, this study, using whole mount preparations, aimed to elucidate the presence of the enzyme FAAH in the small intestine of the mouse. FAAH immunoreactivity (IR) was detected in some myenteric neurons in all regions of the small intestine. IR was moderate and limited to the soma. No FAAH-IR was observed in the submucous plexus. In conclusion, the present study reveals the presence of FAAH-IR in the myenteric neurons in the small intestine. It suggests that 1) FAAH-containing neurons of the myenteric plexus partecipate in the physiological degradation of endocannabinoids acting through CB_1 receptors, as well as of other fatty acide amide molecules, such as palmitoylethanolamide, oleamide and oleoylethanolamide, whose biological effects seem to be independent of CB_1 receptors and whose presence in the gastrointestinal tract[4-5] has been demonstrated, and 2) it contributes to the physiological inhibition of intestinal motility.

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Evaluation of NK cytotoxic activity: distinction between cytolysis and apoptosis by flow cytometry

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NK cells constitute potent effectors of the first phase of immune defence.[1] NK cell activity against tumor and virus infected cells is finely tuned by stimulatory and inhibitory receptors[2] and is performed by means of: 1. perforin-mediated Ca^{++}-dependent osmotic lysis; 2. perforin/granzyme-mediated Ca^{++}-dependent apoptotic killing; 3.TNF family ligand (Fas ligand-, TNF-α-, and TRAIL-)-mediated Ca^{++}-independent apoptotic killing.[1,3] Cytotoxic activity of IL-2 stimulated and unstimulated NK cells has been evaluated using a cytofluorimetric test able to distinguish among the different kind of NK cell cytotoxicity. Briefly, target (K562 or Jurkat) cells were stained with DiOC18 green fluorescent probe, and then incubated 2-6 hour at different effector-target ratios in the absence or presence of EGTA (a Ca^{++} chelator able to block Ca^{++}-dependent granule release). During the last 30 min of incubation, PI at 50 ug/mL has been added to reveal: (i) $DiOC18^+/PI^{bright}$ osmotic-lysed target cells; (ii) $DiOC18^+/PI^{dim}$ apoptotic target cells and, (iii) $DiOC18^+/PI^-$ living target cells. Results indicate that perforin/granzyme-mediated secretory/necrotic killing is preferentially induced on K562 cells by unstimulated NK and LAK cells, while all NK cytotoxic mechanisms in concert are utilized against Jurkat cells by LAK cells. In conclusion, the kind of NK cytotoxic activity performed depends on either NK cell activation or target cell sensitivity.

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Phospholipase C-β2 expression correlates with tumoral features of human breast cancer

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The phosphoinositide-dependent phospholipase C (PLC) is involved in tumorigenic features of a wide variety of tissues. Concerning breast cancer, even if a role of PLC in modulating motility and invasiveness of tumor cells has been reported, the fate of each PLC isozyme in tumoral transformation of breast cells has not been explored. To assess whether specific PLC isoforms may have a role in modulating breast cancer characteristics, we have performed immunochemical and immunohistochemical analysis on both human breast cancer-derived cell lines with different phenotype and invasiveness and primary breast tumor tissues with different histo-pathological charac-

teristics. The results demonstrate that, of the different PLC isoforms, β2 is highly expressed in tumors in comparison with nontransformed cells. In particular, PLC-β2 levels correlate with tumor cell progression from the luminal epithelial-like portrait to the mesenchimal-like one, as well as with the proliferation rate and invasiveness of the different tumors. In order to establish if PLC-β2 may have a role in induction and/or progression of breast cancer, we have modulated the protein expression demonstrating that PLC-β2 is not able to induce or modify tumorigenesis in nontrasformed or low invasive cells, but it may further increase the invasiveness of highly tumorigenic breast cancer cell lines. These data indicate that PLC-β2 strongly correlates with some properties of breast tumor cells, like malignant phenotype and proliferation rate, and suggest that it may be involved in regulating their invasiveness and metastatic potential.

Expression of estrogen receptors and IGF-1 in the liver cystic epithelium from patients with polycystic liver disease

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Human polycystic liver disease is an inherited condition characterized by the presence of multiple cysts of biliary origin in liver parenchyma. Mutation either PKD1 or PKD2 genes are associated with polycystic kidney disease, while a rare mutation for protein kinase C substrate 80K-H displays no renal involvement. Hepatic cysts are rarely observed prior to puberty; they arise with the onset of puberty, they are more prevalent in women and increase in number and size with the age. The liver cysts rise as a consequence of genetic mutation of Polycystin 1 and 2 proteins genes. We evaluated the expression of PC-1 and 2, the role of Er-α and β; IGF-1 and IGF-1R in the hepatic cysts. 1.Immunohistochemistry for PC-1, PC-2, Er-α and β, IGF-1 and IGF-1R on liver cists from three patients. 2.Scanning electron microscopy of the cystic epithelium. The cystic epithelium expresses PC-1 and PC-2, Er- β but not α. Cholangiocytes and the cystic epithelium express IGF-1 and IGF-1R. Ultrastructural observations by SEM show the presence of cilia in the epithelial cells of the biliary duct but not in the cystic epithelium. In polycystic liver disease, men and women have equal lifetime risk to develop hepatic cysts but women show greater numbers and larger sizes of hepatic cysts correlated with both pregnancy and use of exogenous steroid hormones. It is well known that the liver cysts arise from uncontrolled proliferation of intrahepatic bile duct epithelial cells. In primary biliary cirrhosis we have recently shown that proliferated cholangiocytes express both estrogen receptors α (Er-α) and β (Er-β) subtypes, insulin growth factor-1 (IGF-1) and its receptor (IGF-1R).Considering the above data and that there are few effective medical and surgical therapies, careful attention should be paid in the hypothesis of theorical approaches with the use of selective estrogen modulators in the proliferating cystic cholangiocytes observed in the polycystic liver disease.

Direct gene transfer strategy, via brain internal capsule, in Tay-Sachs disease: demonstration of the distribution through X-Gal staining

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Therapy for neurodegenerative lysosomal Tay-Sachs (TS) disease would require active Hexosaminidase A (Hex-A)[1] production in the Central Nervous System and a therapeutic approach faster than human disease progression. In fact, in typical clinical form, rapid mental and motor deterioration, starting within the first year of life, leads to death within 2-3 years. Currently, TS treatment is restricted to supportive care.[2] We combined, in a TS animal model,[3] the efficacy of a non-replicating Herpes simplex vector encoding for the Hex A alpha-subunit (HSV-T0alphaHex) and the anatomical features of the brain internal capsule to distribute the missing enzyme optimally. We analyzed the diffusion of the vector in the brain following the viral vector distribution also by monitoring the X-gal staining in coronal, transversal and sagittal brain serial sections. We observed blue cells also in the encephalic trunk and in the spinal cord (T8 level, the farthest). With this strategy, for the first time, we re-established the Hex A activity in both injected and controlateral hemispheres, in the cerebellum and spinal cord of the TS animal model. In our studies, no adverse effects were observed due to the viral vector, injection site or gene expression. Based on these results, we feel confident that the same approach could be applied to TS and other similar diseases involving an enzyme defect.

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Ki-67 expression in rumen tissue of ovine fed on genetically modified maize

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The rumen surface is characterized by numerous papillae, increasing the surface area and the absorption capacity of the organ. The papillae vary in shape and size, from short and pointed to long and foliate, exhibiting a stratified squamous epithelium consisting of four strata: stratum basale, spinosum, granulosum, and corneum. The stratum basale is the layer of cells immediately adjacent to the basal lamina and these cells show an intensive mitotic activity. The Ki-67 is a member of the class of proteins strictly correlated with cell proliferations, expressed during all active phases of cell cycle (G1, S, G2, and mitosis). It has been widely used as a proliferation marker of cells in human and animal tumors. The expression of this protein has been studied in the stratum basale rumen cells of ovine fed a diet containing genetically modified maize and compared to ovine whose diet did not include genetically modified maize by immunohistochemistry. The study has been carried out on animals receiving maize from weaning to 2 years of age. The results revealed that the nuclear protein Ki-67 is more expressed in genetically modified fed sheep, compared to control group. The factors causing these modifications remain still unknown and further investigations are needed to clarify this observations.

Chromatin remodeling during final follicular growth phase: influence on gap-junctional coupling between oocyte and cumulus cell and developmental competence

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During folliculogenesis, chromatin in germinal vesicle (GV) of mouse oocytes undergoes dynamic changes and its progressive condensation has been related to the achievement of developmental potential. The final phase of oocyte growth is also characterized by modifications of the nucleolus, which is transformed into an inactive remnant. During this phase, the presence of cumulus cells is essential for the process of chromatin condensation and the acquisition of meiotic competence. Aim of this study was to investigate on the possible relationship between the chromatin remodeling process, the acquisition of developmental competence and the coupling status between oocyte and cumulus cells in bovine species. Following fluorescence nuclear staining, we identified four discrete stages of GV (0-3), characterized by a progressive increase of chromatin condensation. Most of the oocytes collected from early antral follicles were GV0, while GV1 GV2 and GV3 were similarly distributed in middle antral follicles. Ultra structural analysis revealed changes in the nucleolar structure passing from GV0 to GV3 stage. By dye coupling assay, most of GV0 oocytes showed fully open communications while the number of GV3 oocytes with closed communications was significantly higher than GV1 and GV2. However, GV0 oocytes were unable to progress through metaphase II while GV2 and GV3 showed the highest embryonic developmental capability. We suggest that also in bovine, the progressive chromatin condensation is related to oocyte developmental competence acquisition and that communications between oocyte and cumulus cells could be implicated in chromatin remodeling process.

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Involvement of PI-3K/AKT and NF-kB/IkBα pathways in the response of Jurkat T cells to TRAIL or etoposide single treatment

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TNF-related apoptosis-inducing ligand (TRAIL) is a cytokine capable of inducing apoptosis in many types of neoplastic cells[1] and Etoposide is an antineoplastic agent wich targets the DNA unwinding enzyme Topoisomerase II.[2] The aim of this work was to evaluate the signalling pathways activated by the teatment with TRAIL or Etoposide of Jurkat T cells, already known to be sensitive to both chemotherapeutic agents.[3,4] Both Etoposide and TRAIL induced an early (4-24 h) reduction in cell viability but, while upon Etoposide administration there was a progressive decrease in viability up to 96 h, after TRAIL treatment an increasing rate of suviving cells was detected from 48 h onwards. Consistently, western blot analysis of TRAIL-treated samples showed both Akt-1 and IκBα phosphorylation. In turn, Etoposide treatment downregulated Akt-1 phosphorylation inducing a parallel Bax increase, IAP decrease and caspase-3 activation that determined the persistence of the apoptotic state along with the occurrence of cell death by necrosis. Thus, the existence of a balance between death and survival responses in the cell fraction resistant to the cytotoxic action of TRAIL suggests the possibility of exploiting new pharmacological strategies to push the balance in favour of cell death.

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Immunodetection of X ray-damage markers in red blood cells of fishes

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A sensitive method for detection of mutagenic effect following X-rays in humans has been proposed by Gong *et al.* (Health Ph.77,1999). They demonstrated that X-rays, lead to an increase in expression of transferrin receptor (CD71) and a decrease in expression of glycophorin A (GPA) on the membrane of mammalian erythrocytes, correlated with the received x-ray dose. Aim of this research is to reveal the occurrence of CD71 and GPA on torpedoes erythrocytes, after X-irradiation, to obtain a sensitive method for assessing X-ray water pollution. Benthonic Elasmobranchs such as torpedoes, subjected to different single doses of total-body X-ray (10-120 Gy) displayed severe anaemia and leucopoenia and in haemopoietic tissues, the injured cells showed vesiculation, disruption and dysplastic nuclear changes, such as nuclear fragmentation. Red blood cell recovery following autologous haemotransplantation was completed in 28 days, while white blood cell recovery was slower (Pica *et al.*, Comp. Haematol. Int.10, 2000). Erythrocytes of Elasmobranchs, like in all non mammalian vertebrates, are complete cells that maintain both the nucleus and the other organelles in mature stages, and their mitochondria exhibited canonical functional respiratory chain (Pica *et al.*, CBP 128, 2001). Methods: FACS evaluation have been performed on blood of 2 *Torpedo marmorata* and 3 *T. ocellata* before and after 90 Gy X irradiation (sublethal dose), after incubation with anti CD71 and anti GPA antibodies. Preliminary results displayed an increase of Fluorescence medium intensity (MFI) of both the markers and an increase in percentage of immunoreactive cells 7, 14 and 21 days after X-irradiation. Fluorescence microscope observations on smears of the same cells measured at FACS, confirmed an increase in number of CD71-immunoreactive RBCs. Observations on blood samples from specimens exposed to decreasing doses of X rays are still in progress.

Adrenomedullin immunoreactivity in the human carotid body

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Adrenomedullin (AM) is a multifunctional regulatory peptide, widely distributed in tissues and organs, including central and peripheral nervous systems. AM has been recently found in the rat carotid body, and we studied by immunocytochemistry the expression of AM in human carotid bodies, sampled at autopsy from 16 adult subjects (mean age ± SD: 44.3±3.4 years) and from 6 fetuses (mean gestational age ± SD: 167 ± 11 days). No AM immunoreactivity was visible in the type II cells of both series. The percentage of immunoreactive type I cells was higher in the adult subjects (32.3±7.7%) with respect to the fetuses (11.8±2.7%, $p<0.001$). Dark cells

showed a higher percentage of positive immunoreaction with respect to light cells, both in adult subjects (61.7±13.4% vs 19.2±5.2%) and in fetuses (25.3±4.4% vs 6.2±2.0%). AM may play a role in the regulation of chemoreceptor discharge through paracrine releasing action and/or vasodilator effect. The low expression of AM in fetuses may be ascribed to the absence of pulmonary respiration with lack of regulatory role of the carotid body during the prenatal period.

Localized expression of E-cadherin, β-catenin and IQGAP1 in cultured human oral epithelial cells: an *in vitro* study on the development of intercellular adhesion

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The first steps of the intercellular junctions formation in cultured human epithelial cells have been analyzed in detail. It is thought that following microvilli formation, intercellular junctions develop through adhesion between interdigitating microvilli of epithelial cells, followed by membranous localization of the components of the cadherin/catenin complex. Once E-cadherin localizes onto the plasma membrane, in presence of Ca^{++}, its extracytoplasmic moiety interacts with the extracytoplasmic moiety of similar molecules on surrounding cells. On the cytoplasmic side of the plasma membrane, the "adapter" molecules α-, β-, and γ-catenin catenin bind the cytoplasmic tail of E-cadherin and are necessary to link cytoskeletal filaments with the cytoplasmic adhesion plaque. Additional membrane-associated molecules, including p120 and IQGAP1, may play a regulatory role at the level of intercellular adhesion sites. In the present paper, we observed by transmission (TEM) and scanning (SEM) electron microscopy the development of intercellular adhesion in monolayer cultures of keratinocytes isolated from human gingival biopsies. In a second phase, we applied confocal laser scanning microscopy (CLSM) to determine the membranous expression of E-cadherin, β-catenin, and IQGAP1 in the same cell types. By TEM and SEM, development of intercellular junctions began with contact of interdigitating microvilli. By CLSM, the formation of intercellular adhesion was accompanied by membranous localization of the three adhesion proteins analyzed, including E-cadherin, β-catenin, and IQGAP1. We may conclude that, in the conditions tested in this study, intercellular adhesion in human oral epithelial cells proceeds through membranous expression of the three adhesion molecules described.

Invading islands in oral squamous cell carcinomas positive for SMAC/DIABLO and cytochrome c: an immunohistochemical study on the possible activation of apoptotic pathways

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In oral squamous cell carcinomas (OSCC), malignant cells are characterized by continuous proliferation and invasion of the underlying tissue. Consistently, these cells are unable to differentiate and do not express markers of terminal epithelial differentiation. Some invading epithelial cells, however, may reactivate their genetic differentiation program. These cells will express newly synthesized markers of terminal differentiation, aggregate in compact colonies, and develop layers, similarly to stratified epithelia. It is not known whether these cell colonies may represent areas of increased apoptosis within the cancer. The purpose of this work was to determine whether evidence of apoptotic signal exists in these islands. To that purpose, we analyzed the expression of two mitochondrial proteins, which are released into the cytoplasm in the first phases of apoptosis. Formalin-fixed, paraffin embedded biopsies of OSCC were incubated with primary antibodies against second mitochondria-derived activator of caspases/ direct-inhibitor-of-apoptosis protein-binding protein with low pI (SMAC/DIABLO, Imgenex, San Diego, CA) and cytochrome c (Dako, Glostrup, DK). After incubation with secondary antibodies, immunoreactions were visualized with peroxidase substrate (DAB, Sigma Fast) and counterstained with ematossilin. Internal positive control was obtained by the positive staining of the striated muscle fibers. Lymphocytes, endothelial wall of veins and lymphatic vessels showed no staining. Moderate positive staining was present in the upper layers of normal and dysplastic epithelial tissue. In the body of the cancer, undifferentiated invading cells showed no staining. Compact colonies showed positive immunoreaction to both antibodies, i.e., SMAC/DIABLO and cytochrome c. Additional studies are necessary to determine whether expression of the two mitochondrial proteins is caused by increased differentiation or apoptotic pathway activation.

Dehydration induces oxidative stress in *Brassica* seeds

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Orthodox seeds are dehydrated before storage in gene banks. Their longevity depends on temperature and seed moisture content (m.c.) being sufficiently low to enable survival without deterioration. Successful seed drying depends on achieving a balance between deterioration caused by storage at high moisture and damage due to drying itself.[1] This damage may not be reflected by changes in the germination percentage. *Brassica* seeds dehydrated to 3% m.c. germinate in the same percentage as seeds with 6 or 15% m.c., however their deterioration is indicated by a loss of salt stress tolerance when imbibed in 0.2 M NaCl at 30°C for 6-12 h.[2]

Seed deterioration is associated with an increase in reactive oxygen species (ROS) and a decrease in the activity of antioxidant enzymes (superoxide dismutase, catalase and peroxidase)[3,4] involved in protective mechanisms.

In this study we found that *Brassica* seeds dehydrated to 3% m.c. prevalently released ROS in the integument, unlike control seeds with 6 and 15% m.c. Antioxidant enzymes, localized histochemically in the integument and embryo of control seeds, were slightly represented in seeds with 3% m.c. These findings, supported by proteome analysis, show that dehydration determined ROS production and a decline in antioxidant enzyme activity.

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Tyrosine hydroxylase immunoreactivity changes induced by 6OH-dopamine treatment on human neuroblastoma SH-SY5Y cells

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The human dopaminergic neuroblastoma cells, SH-SY5Y subline cells were obtained from University of Heidelberg, HD, and were maintained in a humidified atmosphere containing 5% CO_2. Cells were seeded in 10 mm dishes in DMEM media supplemented with 10% heat inactivated fetal bovine serum, 50 units/mL penicillin, 50 mg streptomycin, and 2 mM glutamine. After 48h of seeding the cells were exposed to freshly prepared 6OH-DA an ascorbic acid solution (10^{-4} M, 0.01% w/v) in cold media containing 2% serum) for 4-24h. Cells used as control were exposed to freshly prepared ascorbic acid solution at the same concentration used for the test compound. Immunocytochemistry was performed according to the method described in brief. The cells were washed and then fixed with methanol for 2 min. at -20°C. The cell were then treated to block non-specific binding. After several washing cells were incubated (15h at 4°C) with anti-Tyrosine Hydroxylase (TH) (MAB5280 mouse anti-TH) monoclonal antibodies diluted 1:100 in 0.1% Triton X100 PBS, (Chemicon©) The next day, after washing with PBS, cells were incubated with a secondary antibody (DAKO E0433 Goat anti Mouse) diluted 1:1000, for 1h at room. temperature. Cells were washed with PBS and then incubated with Alexa Fluor-Extravidine (Sigma) 1:1500 in PBS for 1h. Images of immunoreactive cells were digitalized by a CCD digital camera) connected to a Nikon E800 microscope (obj. 40x) and then analyzed by a Macintosh PC with the software NIH image. Preliminary results pointed out that after 24 h of 6OH-DA treatment SH-SY5Y cells showed an increase of TH immunoreactivity in comparison with controls, suggesting a possible pathogenetic role of this enzyme in early Parkinson's disease.

Immunohistochemical profile of neurotransmitters and neurotrophin in human palatine tonsils

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Lymphoid tissue is implicated in the host response to a wide range of stressors and it is supposed to interact with the nervous system. Several lymphatic organs, like palatine tonsils are supplied by many peptidergic nerve endings, associated with different kinds of cells and macrophages. Although it is well established that different neuropeptides exert significant effects on vascular and other lymphoid districts, the role played by some neuromediators on the differentiation and release of locally active substances in the human tonsil tissue is still controversial. We studied the innervation of human tonsils in 10 patients, by immunohistochemical techniques. The ChAT, nNOS, VIP, SP, CGRP and neurotrophins (NTs) immunoreactivity was studied by immunohistochemistry and immuno-TEM. A wide expression of the analysed neurotransmitters is detected in macrophages, lymphocytes, blood vessels and nerve fibers. Moreover our data describe the expression of different neurotransmitters in human tonsil lymphoid aggregates in which both lymphocytes and macrophages express NT receptors and synthesize NTs. Our results show the presence of an extensive network of innervation in the human tonsil tissue, confirming a possible link between the neural modulation of the mucosae-associated lymphoid tissues and the related physiological/pathophysiological mechanisms in human tonsil. NTs could exert a role in regulating immunological and functional activity in palatine tonsils, acting as a paracrine-autocrine mediators of cell to cell communication and/or as a regulators of lymphocytes-macrophages interactions.

Moreover the interpretation of possible existence of relationships among different neurotransmitters and lymphocytes, macrophages, epithelial cells and nervous fibers tested by the expression of some neurotransmitters and neurotrophins (NTs) with their own receptors may be useful in order to provide interesting models in experimental and clinical research.

Invasive growth: a genetic program for stem cells and cancer

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Invasive growth is a physiological program, which leads to morphogenetic movements and three-dimensional organization of tissues during development and organ regeneration. Aberrant execution of this program in time and space is otherwise responsible for anarchic cell spread through tissues, the hallmark of cancer malignancy.[1] The invasive growth program is controlled by Scatter Factors, the ligands for tyrosine kinase receptors of the *MET* oncogene family.[1] A critical feature of *MET* expression is that, in adult tissues, it might be present, or, in some cases even confined, to stem cells. In the hemopoietic system, we specifically found *MET* expression in the early progenitor/stem cell population. In the skeletal muscle, *MET* is expressed by myoblasts, to be downregulated during differentiation into striated fibers; *MET* is however expressed in human rhabdomysarcoma, suggesting that this tumor directly derives from transformation of myogenic precursors1. In the mouse, we found that *MET* is specifically expressed in putative stem cells of the small intestine, and that its expression is increased in intestinal adenomatous polyps, consistently with the expansion of a stem-like cell population. Overexpression is the most common form of *MET* oncogene activation.[1] We found that hypoxia is a key mechanism inducing *MET* transcription and the ensuing invasive growth program, either in physiological or pathological conditions.[2] We also elucidated the effector machinery of invasive growth. This includes the unexpected activation of hemostasis genes, leading to fibrin deposition, that forms a primitive matrix supporting cell growth, spread, and vascularization.[3]

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Ultrastructural analysis during wt and GATA-1^{low} mice megakaryocytes maturation

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Megakaryocytes are specialized cells of the blood responsible for platelet production. They originate from committed progenitor cells, usually localized in the marrow,through a complex maturation process, during which MK precursors progressively increase in size,while synchronous morphologic changes

in the cytoplasm and nucleus. At the ultrastructural level, the major cytoplasmic modifications are represented by massive compartimentalization into discrete regions, delimited by intrusions of the plasma membranes,bound to give rise to the demarcation membrane system (DMS). The DMS will, in turn, internalise platelet-specific α granules, giving rise to proplatelets through a process defined thrombocytopoiesis. On the basis of distinct ultrastructural characteristics, murine and human MK precursors are divided into 4 classes: the promegakaryoblast, a small monuclear cell expressing already platelet-specific proteins (vWF); the megakaryoblast (stage I), with a large oval or kidney-shaped nucleus and several nucleoli, whose cytoplasm presents abundant ribosomes and a well-developed RER; the promegakaryocyte (stage II), with an irregularly shaped nucleus and a more abundant cytoplasm, containing a rudimental DMS; and mature megakaryocytes (stage III) that contain a multilobed nucleus surroundedby abundant cytoplasm. The complex process of MK maturation is controlled by growth factors and transcription factors (GATA-1). In particular, an essential role for GATA-1 in megakaryocytopoiesis has been established by the observation that mice laking regulatory regions of the GATA-1 gene are thrombocytopenic. The defects induced by the GATA-1^{low} mutation in MK precursors include *in vivo* and *in vitro* hyperproliferation, markedly reduced expression of lineage-specific genes and ultrastructural abnormalities,suggesting retarded cytoplasm maturation. Ultrastructural studies, performed on spleen sections,show morphological differences between wt and GATA-1^{low} megakaryocytes at different maturative stages.

GFAP immunoreactive structures in the hypothalamo-hypophyseal axis of the teleost *Diplodus sargus*

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The glial fibrillary acidic protein (GFAP) is the characteristic cytoskeletal protein of astroglia. It is a reliable molecular marker for all astroglial cells and shows also considerable stability in its antigenic characteristics across vertebrate phylogeny. The study of astroglial cells in the hypothalamo-hypophyseal axis of the teleost fishes is particularly significant because in these vertebrates there is no distinct portal system. The adenohypophysis, unlike the other vertebrates, is directly innervated by neurosecretory fibres and the pituicytes, typical astroglial cells of the neurohypophysis, may have an active role in the release of neurohormones. We report the occurrence and distribution of GFAP immunoreactive structures in the hypothalamo-hypophyseal axis of the teleost *Diplodus sargus*. For this study was used a polyclonal anti-cow GFAP antiserum (DAKO): deparaffined, dehydrated sections were immunostained using the ABC technique and the Immunogold technique was applied for the ultrastructural immunodetection. GFAP-immunoreactive fibres appeared particularly organized in bundles close to the ventricles and the hypophyseal stalk. These fibres were generally thin but they appeared more thick and numerous in the hypophyseal stalk where we observed as they were projected from hypothalamus towards neurohypophysis. Thin and isolated GFAP-immunoreactive fibres, corrisponding to the pituicytic processes, were found in the all neurohypophysis where was possible also to observe the pituicytic cellular bodies immunostained. This evidence was also confirmed by ultrastructural immunodetection: the gold particles appeared on the cellular body and on the long processes of the pituicytes of Dark type.

A new morphometric method to evaluate the process of exocytosis by high resolution scanning electron microscopy

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To investigate morphologically the dynamics of salivary secretion we have set up an *in vitro* incubation method of human salivary glands samples. By using an inorganic incubation medium we succeeded not only in detecting the morphological changes induced by various secretagogues, but also in identifying the salivary peptides secreted into the medium during stimulation. Furthermore, thanks to our osmium maceration technique which, following removal of all cytoplasmic organelles, exposes the entire inner surfaces of the luminal membrane, we visualized, by HRSEM, the exocytosis- related changes of the portions of the luminal plasmalema involved into secretion. Thus, since from our previous ultrastructural observations it resulted that, following secretory stimulation (30 min), pits increased, whereas microvilli were greatly reduced, we quantified morphometrically the secretory response induced by secretagogues. We calculated, on HR-SEM images, the mean value of n° of the holes corresponding to microvilli and that of the pits/microbuds (considered as the morphological aspect of membrane recycling of the exocytosed granules), for μm^2 of luminal membrane inner surface, by a non-parametric test (Mann-Whitney U test). The significance level was $p<0.05$. Our results allowed both the quantitative evaluation, at the cellular level, of the effect of a given drug on secretion, and the comparison of the relevant effects of different drugs on the same gland.

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Effects of magnetic fields on osteoblasts grown onto polyurethan porous scaffold

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The histogenesis of bone tissue is strongly influenced by physical stress, including torsion, compression and magnetic fields. Recently, advances in tissue engineering have permitted the formation *in vitro* of bone-like artificial equivalents. Our aim was to investigate the effects of magnetic field on cell proliferation and differentiation in a model of bone-like tissue. SAOS-2 cells, a human osteosarcoma cell line, were seeded onto a three-dimensional polyuretane foam. Some coltures were stimulated by pulsed magnetic-field. Samples were fixed, wax embedded and sectioned. The antigens investigated included bone specific markers, such as collagen I, decorin, osteopontin, and alkaline phosphatase. Moreover, Western blot analyses were performed for the same antigens. In unstimulated cultures, osteoblast cells were adherent to pore surfaces and organized in monolayer; conversely, in stimulated coltures, cells displayed a multilayer organization and almost no pore surfaces were free of cells. The number of cells was significantly higher in stimulated cultures compared to controls. In both stimulated and control bone-like constructs, cells were immunoreactive for osteoblast markers. The immunoreaction was both intracellular and extracellular. Our data suggest that the application of magnetic field may be used to stimulate osteoblast growth in bone-like constructs *in vitro*, while maintaining cell differentiation. Further investigations will be addressed to clarify the modification of the extracellular matrix produced by osteoblast cells in this bone-like equivalents.

Histochemical characterization and histomorphometric distribution of mucins in the rat gastric mucosa

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Mucus protects gastric mucosa from the potentially hostile acid and peptic secretions, or irritants in ingested materials. Indeed, the anatomical and functional integrity of gastric mucosa depend on the balance between aggressive and defensive mechanisms. The present study characterized histochemically gastric mucins and investigated histomorphometrically their distribution in the rat stomach. Female Sprague-Dawley rats (180-200 g bw) were used. Each stomach was opened along the greater curvature, pinned upon a cork plate, fixed in formalin and cut in 2-mm parallel strips which were sequentially superimposed on a glass slide, covered with melted 3% agar and processed for histology. Six μm-thick sections were then cut and stained with periodic acid-Schiff (PAS), or Alcian blue solutions at pH 2.5 or pH 1.0 for the histochemical detection of neutral mucins, sialomucins or sulphated mucins, respectively. Three representative areas were observed: greater and lesser curvature; mucosa lying between the two curvatures. PAS positivity was revealed in cells of the superficial epithelium as well as gastric pits, but appeared very weak in the vicinity of the lesser curvature. Alcian blue staining both at pH 1.0 and pH 2.5 mainly marked gastric pit cells, with the exception of the mucosa of the lesser curvature where Alcian blue stained an amorphous material into the lumen of the fundus of gastric glands. The present results demonstrate that in the rat gastric mucosa mucus barrier shows different composition and distribution and might explain the lesion pattern during mucosal injury.

Focal adhesion molecules expression, actin organization and fibrillin deposition by lymphatic endothelial cells cultured on hyaluronan microstructured surfaces

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Lymphatic endothelial cells (LEC) seeded on microstructured surfaces with alternating stripes of hyaluronan (Hyal) and aminosilanized glass (glass-NH2) adhere, align and proliferate on glass-NH2 tracks and avoid Hyal ones.[1] The aim of this study was to evaluate whether alignment on microstructured surfaces affects the expression and distribution of some molecules implied in cell adaptation to the substrate. LEC were isolated from bovine thoracic duct[2] and seeded onto Hyal-glass-NH_2 microstructured surfaces obtained by spin-coating and photoimmobilization of Hyal onto glass-NH_2 in the presence of a chromium photomask with stripes of defined dimensions (25μm). Double labelling for αv-integrins and FAK or β-actin was performed on confluent cells fixed with acetone and permeabilized with Triton-X-100. Fibrillin deposition was evaluated in fixed but not permeabilized cultures 6 days after confluence since its *in vitro* deposition has been shown to increase with time.[3] LEC consistently adhered only to glass-NH_2 tracks. Integrins and FAK co-localized along cell borders and at the leading edge of migrating cells suggesting that cell adhesion to the substrate was integrin-mediated and required focal adhesion formation. Actin fibres aligned parallel to the stripes. Fibrillin microfibrils were also deposited in bundles running parallel to the stripes. We conclude that LEC alignment on Hyal/glass-NH_2 microstrucured surfaces is accompanied by focal adhesion molecules expression and actin reorganization and conditions the pattern of fibrillin microfibrils assembly in the underlying extracellular matrix.

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Synergism between amphetamine derivatives and proteasome inhibitors *in vivo* and *in vitro*

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In recent studies we found that amphetamine derivatives produce *in vivo* neuronal inclusions which share structural and immunological features with those obtained using proteasome inhibitors. Release of dopamine (DA) represents a major biochemical step in the mechanism of action of methamphetamine (MA) and it plays a key role in producing neuronal inclusions both in nigral and striatal cells. Similarly, the presence of endogenous DA is critical for cell death and neuronal inclusions induced by proteasome inhibitors. In the present study we investigated to which extent amphetamine derivatives and proteasome inhibitors share common biochemical features which might lead to the enhancement of their effects. We found that in mice proteasome inhibitors similarly to amphetamine derivatives produce DA release both *in vivo* and *in vitro*, leading to a transient increase of extracellular DA in the microinfused striatum which lasts a few hours after infusion, similarly to what occur following amphetamines. Conversely, amphetamines produce a decrease in proteasome activity. This synergism extends to the neurotoxic effects producing a robust striatal denervation in the case of administration of non toxic doses of MA in combination with proteasome inhibitors. Similarly, at ultrastructural level we found an enhancement of neuronal inclusions which, for the first time, were analyzed by matching scanning and transmission electron microscopy. In conclusion our data provide a direct evidence of multi step synergism between amphetamine derivatives and proteasome inhibitors which involves the enhancement of DA release and the first observation of the 3D ultrastructure of neuronal inclusions.

Transient inhibition of noradrenaline synthesis promotes noradrenergic damage by dopaminergic neurotoxins

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Locus coeruleus noradrenergic (NA) depletion plays a crucial role in sustaining the progression of dopaminergic (DA) neurodegeneration. In particular, it has been established that DA damage induced by methamphetamine (METH) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium (MPTP) is enhanced by NA loss, without affecting noradrenergic terminals, supporting the hypothesis of a protective role of NA for DA neurons and the natural resistance of NA axons to dopaminergic neurotoxins. To understand the mechanism which lies this protective effect of NA for the DA system and the resistance of NA neurons, we produced a transient depletion of NA by inhibiting NA synthesis, and examined the extension and selectivity of the neurotoxic damage produced by METH or

MPTP *in vivo*. Mice C57Bl, 9 week-old, were administered with the DA-β-hydroxylase (DBH) inhibitor fusaric acid (40 mg/kg x2, 2 h apart, i.p.) and then were treated with increasing doses of METH (5 mg/kg x2; x3, 2 h apart, i.p.) or MPTP (15 mg/kg x1; x2, 2 h apart, i.p.). Mice were sacrificed 7 days after the treatment and examined at biochemical and morphological level. We found that inhibition of DBH activity in combination with DA neurotoxins produced a massive reduction of NA content in brain areas typically innervated by locus coeruleus nerve fibers, thereby causing an extensive depletion of NA nerve terminals, as assayed by morphological analysis and immunocytochemical investigations. These findings demonstrate that conversion of NA- into DA-containing nerve terminals, obtained by transient inhibition of DBH activity, is able to enhance neurotoxin-induced DA toxicity, and to modify the pattern of METH toxicity involving NA neurons.

Glycosaminoglycans in corneagenous cells of Stomatopoda (Crustacea) eyes

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The eyes of Crustacea are complex structures formed by hundreds or thousands of "ommatidia", each showing a complicated optical and sensorial organization by many cells. Stomatopod eyes are specialized for 3-D vision of each eye, most visual fields overlap. The pattern of superposition subserves localization, recognition and striking of a prey at ultrahigh speed.[1] In Stomatopoda, predators living in holes in the mud bottom or in coral rubble but also moving in the water columns, eyes show adaptation to light variations involving the elongation or shortening of optical (cones) and sensorial cells (retinal structure). Two specialised cells are interposed (corneagenous cells, CGC) between the hard structure of the corneal lens and the tip of each cone; cone and CGC are both refracting structures of the ommatidium.[2,3] In this preliminary study, using some histochemical techniques on slices from glycol methacrylate embedded specimens of *Squilla mantis and Lysiosquillina maculata* eyes, we demonstrate the considerable presence of substances tentatively identified as non sulphated glycosaminoglycans (GAG) only in the CGC. The presence of GAGs detectable in these cells may be related to: -optical properties of the eye; -"hydraulic cushion" function, interposed between the tip of the cones, actively modified in dark/light adaptation of the eyes, and the structure of the cornea; -precursors of the chitinous structure of the cornea, periodically produced during the exoskeleton shedding and organized by N-acetyl glucosamine polymers.[4]

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Correlation between cellular inclusions and cell death induced by proteasome inhibition in PC12 cells

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The impairment of the Ubiquitin-proteasome (UP) system has been often related to the occurrence of neuronal inclusions, which are a constant morphological feature of neurodegenerative disorders. Recently we have described that inhibitors of the UP-system induce selective nigrostriatal degeneration and the formation of multilamellar bodies within nigral neurons. The presence of inclusion bodies after treatment with amphetamine derivatives (MA, MDMA), suggests that the neurotoxicity induced by these drugs of abuse involves the UP-system. To analyze this hypothesis, we selected PC12 cells, which represent an *in vitro* model for dopamine neurons, which degenerate in Parkinson's disease. We treated this cell line with the following neurotoxins: selective inhibitors of the UP-system (lactacystin 0.1-1 μM, epoxomicyn 0.001-1 μM), 1-methylphenylpyridinium (MPP+), amphetamine derivatives, and measured the UP-system activity by a fluorimetric assay. We compared the dose-response curve of the proteasome inhibition with that for the formation of intracellular inclusions and for cell death. We found that the dopaminergic neurotoxins we used, produced both neuronal inclusions and cell death in a dose-dependent manner, but with a different dose-response curve, and that these effects correlate with the inhibition of the UP-system. However, the dose necessary to induce cell death was constantly higher compared with that required to induce inclusion formation. The present data suggest that in dopaminergic neurons, the onset of inclusion bodies and cell death represent different degrees of a similar mechanism involving the impairment of the UP-system.

Mitochondrial DNA extraction from human odontoblasts

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Teeth are the hardest tissue in the human body because of the dental enamel, which makes them resistant to degrading adverse conditions but at the same time it makes difficult to extract organic components from the mineral matrix. Odontoblasts are tall columnar cells located at the periphery of the dental pulp and cell process arise from the cell body and penetrates into the mineralized dentine. Up to now, it is not completely clear if mitochondria are aligned along the odontoblast process or if they are scattered around the odontoblast cell body. It is well known that mtDNA is present in many copies inside the organelle and for this reason is easiest to identify small quantity of it compare to a specific sequence in the nuclear genome. Furthermore, mtDNA has not an efficient repair system and this makes it an important tool in evaluating genotoxic damage due to odontoiatric materials. In order to study if mitochondria could be retrieved from odontoblast processes, we isolated mitochondrial DNA (mtDNA) from human dentine with only odontoblast process or complete with the odontoblast body. Dentine disks were cut out from wisdom teeth extracted for orthodontic reasons from patients ranged between 25-55 years. Starting from enamel we obtained thin dentin disks in which only odontoblast process were supposed to be, and thicker disks of dentin that included the odontoblast body. The dental pulp was used as control. mtDNA was extracted and HVI and HVII ipervariable regions were amplified using specific primers. mtDNA was successfully extracted from all dentin disks which included the odontoblast cell body, while dentin thin samples were not always positive. Pulp samples were always positive. We conclude that mtDNA extraction and amplification from dentin can achieved due to the presence of mitochondria scattered throughout the odontoblastic cell body while the presence of mitochondria in the cytoplasm process could be related to the age of the teeth.

An immunohistochemical investigation of decorin in human dentin matrix: a FEISEM study

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Proteoglycans (PG) are large and complex macromolecules distributed within matrices of mineralized and not mineralized tissues. In human predentin and dentin matrix the major presence of PG is represented by chondroitin 4/6 sulphate, decorin, biglycan and keratan sulphate distributed heterogeneously to stabilize and link major collagen fibrils. The aim of this study was to identify decorin in human dentin matrix by means of an immunolabeling approach with an high resolution field emission scanning electron microscope (FEISEM). Ten non carious teeth were stored in 0.5% chloramines in water at 4°C and used within 1 month after extraction. Crowns were removed by means of a low speed diamond saw (Micromet, Remet, Bologna, Italy) under water irrigation. Specimens were then etching with 10% citric acid for 15 seconds for removing smear layer and expose dentin matrix. *Immunolabeling protocol*: Specimens were rinsed in demonized water, in TBS 0.05 M (pH 7.6). Specimens were pre-incubated with NGS (1:20 in TBS 0.05M pH 7.6). Incubation was performed with an anti-decorin primary antibody (1:100 in TBS 0.05M pH 7.6). Specimens were rinsed with TBS 0.05 M (pH 7.6) and TBS 0.02M (pH 8.2). Gold conjugated secondary antibody was used to visualize sites of antigen-antibody cross reaction. Specimens were fixed in 2,5% buffered glutaraldehyde (pH 7,4), alcohol/HMDS dried, mounted on specimen holder, glued with graphite, carbon sputtered and inspected by FEISEM with boot secondary and backscattered signals. FEISEM micrographs revealed an intricate network of undemineralized collagen fibrils. No crystallites or other debris were visible. The immunolabeling procedure stained decorin approximately at the junction between each periods. Decorin branched to minor fibrils interconnecting collagen structures. The immuno-gold labeling protocol followed in this study allowed to visualized presence and distribution of collagen and associated decorin in dentin matrix. The study also revealed that proteoglycans maintain their antigen integrity. The very high resolution of the FEISEM used, allowed the observation of the three-dimensional appearance of both mineral and organic phase of this tissue.

Prolactin and growth hormone cells in the reptile *Chalcides chalcides*

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Prolactin (PRL) and growth hormone (GH) cells are specific pituitary cells secerning PRL and GH respectively. These two hormones are members of a family of polypeptide which evolved from a common ancestral gene. Their molecular characteristics are indeed quite similar. Little is still known about PRL and GH cells in the reptiles. In order to develop our previous studies on the pituitary gland of the viviparous reptile *Chalcides chalcides*, in this paper we report the ultrastructural study of PRL and GH cells in this skink. Ultrathin sections of pituitary gland of C. chalcides females during the reproductive period were used. The immunodetection was performed by using the polyclonal anti-PRL and anti-GH antisera and the immunogold technique. PRL cells, present in the rostral and in the medial pars distalis, appeared pyriform or ovoidal, small in size with a mean diameter of 8,5(±1,2)x6,2(±0,9)μm. They showed a round nucleus and a large nucleolus. PRL cells were full of 250-350nm granules, in which was evident homogeneous material with a little area of higher density. GH cells were larger then PRL cells with a mean diameter of 10(±0,95)μm and were only observed in the caudal pars distalis. Their shape was generally globular with a peripheral and large nucleus. Unlike PRL cells, GH cells showed less numerous and smaller granules of 180-220nm. The immunogold labelling was limited in the secretory granules for both cellular types. It's possible to conclude that, in the viviparous reptile, *C. chalcides,* the PRL and GH hormones, although structurally related, are secreted by two cellular types with ultrastructural characteristics quite different.

Immunolocalization of aromatase in human Leydig cell tumor

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Cytochrome P450 aromatase is the enzyme catalyzing the conversion of androgen to estrogens. Leydig cells are the main site of aromatase expression in normal human testis, suggesting a paracrine action of local estrogen biosynthesis in the control of steroidogenesis. However estrogens are involved in cell growth and apoptosis regulation therefore the enhancement of their production could induce tumorogenesis process. Aim of this work has been the investigation of aromatase expression in human Leydig cell tumor, a rare cancer arising from gonadal stroma, found in adults and children. The tumor-bearing testes were obtained from 2 patients with Leydig cell tumors by therapeutic orchidectomy. Paraffin embedded tissues were processed for immunohistochemistry using a rabbit polyclonal antibody generated against human placental cytochrome P450 arom, as primary antibody, and a biotinylated goat-anti-rabbit IgG, as secondary antibody. An intense immunoreactivity was detected in polyedrical packed cells of Leydig cell tumors while neighbouring connective cells were unstained. Previous studies have indicated hormone involvement in human testicular tumorogenesis, but the link between estrogen byosinthesis and testicular neoplasms has scarcely known. The present study has demonstrated, for the first time, aromatase immunolocalization in neoplastic cells of Leydig cell tumor. These results confirmed previous experiments in animal models showing that aromatase overexpression leads to development of testicular Leydig cell tumors. Our findings suggest a possible relation between an increased estrogen production from Leydig cells and testis tumorogenesis promotion. Therefore, the interaction between estrogens and neoplastic process can be also hypothesised in testis as demonstrated in some human breast and ovarian malignancies.

Role of the histochemistry in the diagnosis of myopathies

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Histochemistry represents the first approach to muscle biopsy: fibers cross-sectioning reveals not only the morphological features but also the majority of abnormalities. Histological stain (haematoxylin-eosin, modified Gomori trichrome, PAS, Oil red O) and enzymatic reaction [coenzyme-linked dehydrogenase (NADH), non-coenzyme-linked dehydrogenase (SDH),

cytochrome oxidase (COX), adenosine triphosphatase (ATPase), acid phosphatase] are usually perfomed. Each stain reveals different features and from the analysis of all them the diagnosis can be obtained. At H&E, size variation or grouped atrophic fibers may be seen, such as nuclei number, location, clumped features. Inflammatory infiltrate may also be shown, better visualized at Gomori's stain, as degenerating fibers. Vacuoles (H&E) may be identified with acid phosphatase as derivated from activated lysosomes, as filled with glycogen (PAS) or fat (ORO). Using Gomori's trichrome, cytoplasmic inclusion can be identified as rods, cytoplasmic bodies and ragged red fiber (mitochondrial myopathies). Endomysial fibrosis (H&E, Gomori's) reflects severe damage, progressively transforming the muscle to an endstage connective-tissue scar with fiber remnants or piknotic nuclear clumps (dystrophic changes). Enzymatic reactions permit nomenclature of fiber types, evaluation of fiber dysproportion, grouping and show typical neurogenic/myopathic changes.

By immunohistochemestry proteins located at the transsarcolemmal, nuclear and sarcoplasmic levels may be demonstrated, although their reduction may be more difficult to document (emerin, dysferline). The appereance of proteins not usually seen in mature intact fibers (immature myosin, utrophin) can be revealed, such as protein aggregate (inclusion body myopathies). It is conceivable that histochemical preparations will be more and more integrated by immunohistochemical ones, although the role of the latter in the diagnostic approach still remains not replaceible.

Histochemical techniques in clinical diagnosis of metabolic myopathies

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The metabolic myopathies represent a large group of clinically heterogeneous disorders characterized by a common pathogenic tract, the failure to adequately match skeletal muscle metabolic requirements by energetic fuels.[1] As the main functional and energetic properties in skeletal muscle are finalized to generate contractile activity, it ensues that when a metabolic muscle disorder causes ATP insufficiency it determines a clinical condition characterized by exercise intolerance and symptoms such as muscle contractures, myalgias and rhabdomyolisis. Increased levels of serum creatinkinase and other muscle enzymes indicate skeletal myofibers damage.

The possible causes of a metabolic myopathy can be different, acquired or inherited, but all of them are able to induce a selective defect of one or more steps along a metabolic pathway. In order to make a correct diagnosis several investigations are indicated.[2] Some of them are merely suggestive, others are more specific in indicating the possible metabolic cause. In this context exercise testings in different functional conditions, i.e. during aerobic or anaerobic contractions, are useful when matched to simultaneous determination of blood metabolites, such as lactate or ammonium. However, in order to precisely define the origin of a muscle metabolic disorder further examinations are necessary and, between them, skeletal muscle biopsy plays a fundamental role in this regard. In any case biopsy of skeletal muscle and other eventually involved tissues (skin, cardiac muscle or liver) is indicated only after the clinical evaluation has been performed. Conversely, other investigations, such as biochemical, molecular or genetic analyses will be planned depending on the results of muscle biopsy.

Histochemical assessment of skeletal muscle provides general informations about fiber type proportion, atrophy and hypertrophy factors, presence of degeneration, necrosis, or regeneration. In addition, of particular importance in the diagnosis of metabolic myopathies are staining methods able to reveal presence of storage material, lipid (Oil red O or Sudan Black stainings) or glycogen (Periodic Acid Shiff), or ragged-red fibers (RRF) with the modified Gomori trichrome stain which indicates abnormal accumulations of mitochondria.[3] The absence of either storage material or accumulations of mitochondria does not exclude either a glycogenosis or a lipid myopathy, nor does a mitochondrial disorder; however, when these indicators are present, further biochemical and molecular analyses that may lead to a more definitive diagnosis are advisable. In this context, histo-enzymatic and immuno-histochemical methods appear more useful in defining the precise metabolic step implicated in the disease. These techniques are now indispensable, having showed in the last years a considerable wide field of application thanks to the recent acquisitions in the knowledge of molecular mechanisms underlying metabolic myopathies.

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Artico M, Cavallotti C, Iannetti G, Cavallotti D. Effect of interleukin 1ß on rat thymus microenvironment. Eur J Histochem 2001; 45:357-66.

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