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PROCEEDINGS OF THE 32nd NATIONAL CONGRESS OF THE ITALIAN SOCIETY OF HISTOCHEMISTRY

Messina, May 31 – June 2, 2007

Policlinico Universitario "G. Martino"
Università degli Studi di Messina

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PROCEEDINGS OF THE 32nd NATIONAL CONGRESS OF THE ITALIAN SOCIETY OF HISTOCHEMISTRY

Messina, May 31 – June 2, 2007
President: Giuseppe Anastasi

OPENING LECTURE

Immunohistochemistry of matrix proteins in calcified tissues: functional biochemistry on section

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Université de Montréal, Canada

The organic matrix of calcified tissues comprises collagenous and/or noncollagenous proteins NCPs. *The physicochemical characteristics of these matrix proteins, as well as their temporal expression pattern and spatial distribution are key elements that determine their ability to interact with mineral, cells and other proteins.* Identification and precise mapping of matrix proteins is thus essential for determining their function, formulating coherent hypotheses on their mechanism(s) of action, and developing novel therapeutic approaches based on biologics. Fibrillar collagen can be readily identified by its conspicuous structure, however, NCPs, in general, do not individually exhibit characteristic structural features that permit to identify them and morphologically determine their localization. We have used immunocytochemistry, a form of *biochemistry on section*, to address this situation because it allows to correlate composition with structure. The laboratory has pioneered the application of colloidal gold labelings because this approach has a high spatial resolution and is quantitative. In this presentation, selected studies that highlight the function of NCPs in bone and teeth will be reviewed.

Supported by the Canadian Institutes for Health Research

SYMPOSIUM I
Calcified tissue

Histochemistry and immunohistochemistry of *crystal ghosts*

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Università di Roma "La Sapienza", Italy

The organic-inorganic relationships which occur during the early stages of the calcification process in biological tissues provide information on the organic structures which can be involved in the deposition of the inorganic substance. The Post-Embedding Decalcification and Staining (PEDS) method allows the removal of the inorganic component without altering the organic structures, which are unmasked and can be stained, so becoming visible under the transmission electron microscope.[1] The PEDS method shows that the areas of initial calcification in cartilage, bone, tooth enamel and dentin, mollusc shells, sea urchin embryo spicules, and several other normally or pathologically calcified tissues contain organic structures which have a close resemblance with the original inorganic crystals but lack their electron density and only become visible after staining (for this reason they have been called crystal ghosts).[1,2] Histochemical and immunohistochemical studies have shown that the crystal ghosts of the calcification nodules of epiphyseal cartilage correspond to aggrecan acidic molecules. Acidic, polymeric molecules are characteristically found in all areas of initial calcification in other hard tissues. These findings support the possibility that biological calcifications occur through a biochemical process implying the epitaxial reaction of inorganic ions with anionic groups of polymers, with formation of organic-inorganic nanoparticles which then coalesce into crystal-like structures. During this process, the acidic polymers behave as templates of the definitive structures and are gradually degraded and removed, so leading to true inorganic crystals (reviewed and discussed by Bonucci).[3]

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BAG-75, a biomarker for initial sites of mineral nucleation in primary bone

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Primary bone is the form of bone found in the embryo, in healing fractures, and at sites of adaptive skeletal growth in response to biomechanical strain. A key developmental characteristic of primary bone is its capacity to produce a mineralized matrix directly from a soft mesenchymal precursor. Bone acidic glycoprotein-75 (BAG-75) is an extracellular phosphorylated glycoprotein whose expression is restricted predominantly to primary bone. Interestingly, BAG-75 is localized to areas of new developing bone prior to mineralization and the pattern of its distribution predicts the limits of subsequent calcification (Gorski *et al.*, 2004). These structures appear similar to crystal ghost aggregates, bone nodules, or calcospherulites identified by others. Mineralizing osteoblastic cells in culture nucleate hydroxyapatite crystals within spherical supramolecular structures termed biomineralization foci (BMF) enriched in BAG-75 and bone sialoprotein (BSP) (Midura *et al.*, 2004). Our working hypothesis is that BMF are responsible for the initial nucleation of mineral in primary bone by providing a protected environment enriched in proteins that promote mineralization while excluding inhibitors of mineralization. To investigate the mechanism of nucleation we have undertaken a proteomic analysis of isolated BMF from UMR 106 osteoblastic cultures. Mineralized BMF were purified by laser capture microscopy and subjected to mass spectroscopic peptide mapping and western blotting analyses. As predicted, purified BMF were enriched in BAG-75 and BSP, however, unexpectedly, 50 kDa fragments of both proteins were also localized specifically to mineralized BMF. Treatment of UMR cultures with a series of different protease inhibitors identified only one capable of blocking mineralization and cleavage of BAG-75 and BSP, covalent serine protease inhibitor AEBSF [4-(2-aminoethyl)benzenesulfonylfluoride HCl]. Sensitivity to AEBSF was 10-fold greater immediately before mineralization began than during an earlier proliferation/differentiation phase suggesting its action is late during the process of nucleation. 2-D PAGE analysis of treated and untreated cultures revealed that a potentially activating cleavage of procollagen C-proteinase enhancer protein, which stimulates collagen fibrillogenesis, and 1, 25-vitamin D3 MARRS protein/ERp57, necessary for cellular calcium and phosphorus uptake, was also blocked by AEBSF. In addition, mineralization of cultured primary mouse calvarial cells was inhibited by AEBSF indicating the requirement for serine protease cleavage is a general feature of osteoblastic cells. In an effort to extend these findings *in vivo*, we analyzed the sera of rats undergoing a peak of induced bone formation 21 days after ovariectomy. Importantly, the level of BAG-75 50 kDa fragment in ovariectomized rats was increased an average of 3-fold over sham operated controls and ovariectomized rats treated daily with replacement estrogen. Comparable assays for osteocalcin, another bone non-collagenous protein, increased only 27%. Our results suggest for the first time that nucleation of mineral crystals in developing bone is dependent upon limited fragmentation catalyzed by a yet-to-be-identified serine protease. *In vitro* and *in vivo* studies place the 50 kDa fragment for BAG-75 spatially and temporally at the site of mineral crystal nucleation. We hypothesize that the observed cleavages serve to activate specific functions, e.g., nucleating activity, collagen fibrillogenesis, and calcium or phosphorus ion transport, which will mediate mineral crystal nucleation, and, subsequent propagation and expansion of the mineral phase. Alternatively, limited fragmentation may facilitate assembly of functional nucleation complexes. In summary, our results imply the existence of a multi-step extracellular biochemical pathway responsible for the nucleation of mineral crystals in developing bone.

SYMPOSIUM II
Neuromuscular pathology: immunohistochemistry techniques

Recent immunocytochemical and biomolecular acquisition of knowledge in muscular histopathology of myotonic dystrophies

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Myotonic dystrophies (DMs) are dominantly inherited multisystemic disorders caused by two similar noncoding repeats expansion. Myotonic dystrophy type 1 (DM1) is caused by a CTG expansion in the 3′ untranslated region of the *DMPK* gene,[1] whereas type 2 (DM2) is caused by a CCTG expansion in intron 1 of the *ZNF9* gene.[2] The immunohistochemical staining of fast or slow myosin has shown very small type 2 fibers (diameter ≤20 μm) in DM2 muscle not discernible with ATPase histochemical staining.[3] The molecular pathogenesis of DMs is the nuclear accumulation of mutant mRNA containing CUG/CCUG repeats as ribonuclear inclusions (RIs). In a study on 17 DM2 patients we have demonstrated that a differential diagnosis between DM1 and DM2 can be performed by FISH on muscle sections using the specific (CAGG)5 probe which reveals the presence of ribonuclear inclusions in DM2 but not in DM1 patients.[4] RIs interact with specific RNA-binding proteins, such as muscleblind-like 1 protein (MBNL1), that regulate alternative splicing. An immunofluorescence study from 7 DM1 and 8 DM2 muscle biopsies has revealed the presence of nuclear accumulations of MBNL1 as protein foci which colocalize precisely with RIs.[5] MBNL1 could represent also a good histopathological marker for the DMs.

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Pathogenesis of laminopathies with myopathic or progeric phenotype

N.M. Maraldi
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LMNA gene mutations cause a number of diseases, collectively called laminopathies, included into five classes, by considering their organ system involvement: a) striated muscle; b) peripheral nerves; c) adipose tissue; d) multiple tissue premature senescence; e) overlapping phenotypes.[1] In the progeric laminopathies, *LMNA* mutations result into the accumulation in the nucleus of unprocessed prelamin A.[2] This causes an altered distribution of LBR and destabilization of two heterochromatin-associated proteins, HP1β and H3K9. These defects can be also induced into normal cells by drugs that interfere with lamin A post-translational processing, or by transfection with *LMNA* mutants that lead to accumulation of prelamin A. These results suggest that the accumulation of farnesylated lamin A precursor elicits a toxic effect that results into irregular nuclear profiles with deep envelope invaginations, loss of peripheral heterochromatin, and reduction of whole transcriptional activity.[3] Cell lines obtained from patients affected by the Hutchinson-Gilford progeria syndrome (HGPS), a severe disorder which results in the patient death in the second decade, were treated with the farnesyl transferase inhibitor mevinolin, and the histone deacetylase inhibitor tricostatin A. This treatment greatly reduced the accumulation of mutated prelamin A, leading to rescue of the nuclear shape, heterochromatin organization and transcriptional activity. These results demonstrate that, in the progeric group of laminopathies, nuclear defects are related to accumulation of unprocessed prelamin A and that they can be rectified by a pharmacological treatment.

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NF-κB inhibitors and VEGF gene transfer stimulate muscle regeneration in *mdx* mice

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Nuclear factor-κB (NF-κB) is an ubiquitous transcription factor regulating the expression of a plethora of genes involved in inflammatory, immune and acute stress responses. Several lines of evidence suggest a role of NF-κB in muscle degeneration and regeneration in Duchenne muscular dystrophy (DMD) and *mdx* mice. We investigated the effects of pharmacological blockade of NF-κB on functional, biochemical and morphological parameters in *mdx* mice. NF-κB inhibitors treated *mdx* mice showed: 1) an amelioration in functional parameters with an increased forelimb strength and strength normalized to weight, and decreased fatigue; 2) a reduction of muscle necrosis and an enhancement of regeneration. Moreover IRFI 042 blunted NF-κB DNA-binding activity and TNF-α expression, reduced serum CK levels, decremented muscle conjugated dienes content and augmented muscle reduced glutathione. Our data suggest that oxidative stress/lipid peroxidation represents one of the mechanisms activating NF-κB and the consequent pathogenetic cascade in *mdx* muscles and that the inhibition of this cascade have beneficial effects on muscle function. Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis. Several studies support its role in myogenesis. We tested the effect of VEGF delivered by adeno-associated-virus (AAV) vectors on functional and morphological parameters in *mdx* mice. Treated *mdx* mice showed higher strength and strength normalised to weight. VEGF-treated muscles showed a reduction of necrotic area and an increase of small centrally nucleated fibers area and of cells positive for regeneration markers. We report the novel observation of a beneficial effect of VEGF in *mdx* mice. Further studies are needed to better clarify the NF-κB inhibitors and VEGF mechanisms and their possible therapeutic implications.

Muscular pathologies as indicators of sarcoglycan-integrin complex role

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Sarcoglycans and integrins are proteins of dystrophin-glycoprotein complex (DGC) and vinculin-talin-integrin system, respectively. These proteins, parts of costameres, regulate the interaction between the cytoskeleton and the extracellular matrix in adult skeletal muscle, providing structural support to the sarcolemma and protecting the muscle fibers from any possible damage provoked by continuing cycles of contraction and relaxation. In recent years, the demonstration that mutations

in each locus gene of sarcoglycans cause limb girdle muscular dystrophy (LGMD) in human skeletal muscle, characterized above all by the normal expression of the dystrophin, provides evidence for a key role of the sarcoglycan subcomplex in muscle fiber viability, similar to dystrophin. Although numerous studies have performed on these two type proteins about LGMD, there are insufficient data on morphological expression and patterns of immunostaining of sarcoglycan subcomplex related to integrins. Previously, we analyzed the human skeletal muscle fibers in normal conditions[1,2] showing that exists a bidirectional signalling between sarcoglycans and integrins, that sarcoglycan subcomplex is subdivided in two subunits, α-, and β–γ–δ. Our previous results, made up in skeletal and cardiac muscle, also showed that the costameres can be localized both in the region of the sarcolemma over the I band and in the region of the sarcolemma over the A band. In our opinion this condition is caused by biochemical type of fibers (fast or slow) and then the slow fibers could be characterized by localization of costameric proteins in the region over the I band, while fast fibers by localization of costameres in the region over A band. On this basis we performed an immunofluorescence and molecular study on adult human skeletal muscle affected by any neuromuscular pathology, as LGMD or sensitive-motor polyneuropathy, in order to better define and confirm the possible structural alterations of two systems. The results obtained on LGMD, integrated with filamin2 analysis, demonstrated, for first time in human skeletal muscle,[3] the existence of two distinct subunits in sarcoglycans subcomplex; the presence of a bidirectional signalling between sarcoglycans and integrins and the interaction of filamin2 with both sarcoglycans and integrins. The results obtained on sensitive-motor polyneuropathy, revealed that muscular atrophy and inactivity conditions cause a lack of neural agrin and then a lack muscle agrin, provoking a lower expression of integrins.[4] This condition could determine a quantitative modification of transmembrane receptors and the loss of a7B-integrin could be replaced and reinforced by enhanced expression of the a7A-integrin to restore the muscle fiber viability.

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SYMPOSIUM III
Biotechnologies in pathology

Diagnosis of lymphoid lesions using cytomorphology and flow cytometry

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Dipartimento di Anatomia Patologica e Citopatologia, Università di Napoli "Federico II", Napoli, Italy

Flow cytometry (FC) has proven useful in the evaluation of non Hodgkin's lymphomas (NHL) on samples obtained by fine-needle cytology (FNC).[1-3] Objective of this study is to evaluate the application of FC and FNC to the diagnosis and sub-classification of NHL. FC was used to analyze 650 FNCs of lymphoproliferative processes using a panel of following fluoresceinated antibodies and a three-color Becton Dickinson (San José, CA) FACS scan. When a defined diagnosis of NHL was performed, the combination of cytological features and different expression and co-expression of the antibodies were used to classify, when possible, the specific subtype. Combined FC and FNC provided a diagnosis of benign reactive hyperplasia (BRH) in 286 cases, primary NHL (pNHL) in 148 cases and NHL recurrence (rNHL) in 163 cases, suspicious NHL in 42 cases and inadequate in 11 cases. Clinical control confirmed the diagnoses of rNHL, histological (93 cases) and clinical control (55 cases) confirmed the 148 primary NHL and BRH; 55 cases were lost to the follow-up. In 42 suspicious cases, microscopic features were suggestive of NHL but FC gave unsatisfactory results. Out of 311 cases diagnosed NHL, the evaluation of FC data and the cytological features suggested a specific subtype in 180 cases. All the others, with negative or equivocal phenotype, were diagnosed just NHL not otherwise specifiable. All the FNC/FC diagnoses were confirmed by histology and clinical follow-up except for 55 cases lost to follow-up. FNC offers vital cells to FC suitable for a timely and accurate diagnosis and sub-classifiction of NHL.

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A survey of laser-assisted microdissection systems in pathologic and forensic applications

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The cellular heterogeneity represents a problem in nucleic acid analysis of human tissues performed by extractive procedures. To better correlate the topologic organization of the cells and the molecular analysis, laser microdissection has been applied in pathologic and forensic fields. In particular, laser capture microdissection and laser microbeam microdissection are the two major systems developed. The basic principle of the first system is the capture of cells onto a thermoplastic membrane transiently melted by a low-power narrow-beam infrared laser directed at the cells of interest under microscope control; conversely, laser microbeam microdissection utilizes a pulsed very narrow-beam ultraviolet light laser to cut small tissue fragments as well as single cells. In pathology, laser microdissection allows to detect DNA alterations on morphologically selected modified cells. This is particularly relevant in the analysis of neoplastic tissues, in which together with the main tumour clone, a variable mixture of stromal, endothelial and inflammatory cells and other non-neoplastic components may

be present, masking thus tumour cell-specific DNA alterations. Moreover, RNA and gene expression studies may be improved by this technique. In forensic applications, laser microdissection technique has been proved to be a very powerful tool to isolate specific target cells from smears prepared utilizing material collected after a sexual assault or from mixed biological traces present in the crime scene. Finally, the possibility to perform a genomic analysis of low copy number DNA from few cells harvested by laser microdissection represents a further aid to solve forensic problems.

Histopathological and biomolecular approach to brain tumours

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Primary tumors of CNS are a complex and heterogeneous group of neoplasms with different biological behaviour. Among them, diffuse gliomas and meningiomas are those more frequently occurring in adult patients. Diffuse gliomas (i.e. astrocytomas, oligodendrogliomas and oligoastrocytomas) represent a spectrum with progressive degree of anaplasia. Numerous genetic and epigenetic changes occur during tumor progression and some of them represent today important prognostic factors and possible molecular targets for therapy. Among these loss of heterozigosity for 1p, 19q, 10q, p53 mutations, EGFR amplifications and PTEN loss. Similarly meningiomas may progress to high grade lesions. Various histologic and molecular features characterize such progression. Benign meningiomas are associated with mutations of NF1 gene on chromosome 22. As they increase in anaplasia additional genetic events occur, including loss of 1p, 10q and 14q. Moreover adjunctive parameters such as the loss of progesterone receptors, content of tenascin, and microvessels density seems to correlate with tumor progression in meningiomas.

Apoptotic death of peripheral T-lymphocytes during HIV infection

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An unresolved question in HIV pathogenesis is if and to what extent homeostatic T cell response, in a context of viral induced T cell death, can contribute to the progressive CD4 T cell depletion, As a model of homeostatic T cell turnover we propose the lymphocyte aging studied in peripheral T cells by measuring the progressive accumulation of cell cycle inhibitory proteins (p16, p21 and p53) and structural nucleolar proteins (particularly C23, and nucleolin) whose accumulation is a good indicator of proliferative aging. Cell morphometry, pattern of regulatory and structural protein expression, ultrastructural lesions of peripheral T lymphocytes from HIV infected patients are very divergent from the model of physiological T cell turnover. In particular, lymphocytes from HIV+ patients show a dramatic discrepancy in the expression of inhibitors and regulators of cyclin dependent kinases thus suggesting a general unscheduled expression of cell cycle proteins, in a context of a general mitotic disaster of T cell population.

SYMPOSIUM IV
Rediscovering the potential of fluorescence microscopy

Fluorescence resonance energy transfer microscopy: a look at cellular molecular arrangement

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Fluorescence resonance energy transfer (FRET) is a photophysical phenomenon for which the excitation energy of a fluorophore (donor) is transferred non-radiatively to another fluorophore (acceptor). FRET efficiency (eFRET) depends on fluorophores distance (within 120Å), reciprocal orientation, and excitation/emission spectral overlapping. The supramolecular spatial arrangements of biological substrates can thus be investigated through FRET analysis, provided that donor and acceptor bind the target biomolecules with high specificity. At microscope, donor-/acceptor-tagged antibodies or lectines, and more recently the genetically encoded autofluorescent proteins -green fluorescent proteins (GFP)- allow an *in situ*, analysis of structures and interactions of plasma membrane and proteins. Chromatin structural arrangements can be investigated by direct labelling of DNA. The spectral properties of Hoechst 33258 and propidium iodide can be exploited for a eFRET analysis by microspectrofluorometry, imaging, flow cytometry, through acceptor sensitized emission measurements. This procedure has been applied to investigate the supermolecular changes of chromatin under different functional activities: cultured human normal fibroblasts in the transition to G_0 to G_1 phases (activation of cell cycle progression specific genes); cultured thymocytes undergoing spontaneous of induced apoptosis (chromatin condensation, DNA cleavage). In the G_0 G_1 transition, an increase in eFRET preceded the changes in the proliferation markers expression (Ki67, statin), indicating the chromatin rearrangement in parallel with activation of cycle-related gene. In thymocytes undergoing apoptosis eFRET increasee preceded the cell morphological alterations.

Fluorescence microscopy enhanced by multi-LED excitation

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Light Emitting Diodes (LEDs) technology greatly improved in these last few years. From small lighting indicators of few mW, now LEDs are also available in the range of some W 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 built up and tested. The results obtained indicates that the excitation performances of 3W 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) LEDs, delivering up to some hundreds mW within narrow spectral bands (15nm). Compared with the standard lamp excitation, FITC labelling observation is consistently improved by blue LED excitation, whereas the optical power emitted by a mercury arc lamp in this spectral region is not very high. Further improvements

have been recently introduced with a new optical design aimed to locate three LEDs in a module still operating in *transmitted excitation* offering great flexibility of excitation performances. The three color excitation bands can be delivered to the sample both simultaneously or sequentially in order to match the variety of fluorescent probes applied to the sample. The simultaneous excitation had been made possible thanks to a proper triple-band barrier filter developed *on purpose* by Fraen and made by Chroma company. Each color band can be easily modulated in power so that the more brilliant as well as the faintly probes can be kept in the same order of emission intensity for a more detailed observation of the sample.

New advanced techniques in fluorescence application (FRAP, FLIM, FLIP)

M. Brich
Not available

Two-Photon microscopy: imaging *in vivo*

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In vivo imaging has a long history, although the range of its applications is relatively limited. For fluorescence imaging in live animals photodamage is the major problem. The development of multiphoton picosecond pulsed laser scanning fluorescence microscopy provides several advantages in this regard. The excitation of visible wavelength fluorophores by the simultaneous absorption of two or three photons allows the use of near-infrared wavelength light, which has relatively low absorption by biological tissues. The requirement for high photon densities ensures that fluorophores are excited only near the focal point, and this confines photobleaching and photodamage to the vicinity of the focal plane. Multiphoton microscopy provides the opportunity to visualize cellular process in their natural environment, including the neurons of mammals. Using two photon scanning microscopy and time-lapse imaging of transgenic mice expressing yellow fluorescent protein, alterations of neuronal behaviour from high plasticity during development to remarkable stability in the adult mice were observed, providing a potential structural basis for long-term information storage. These approaches were also used to investigate the molecular mechanisms underlying experience-dependent plasticity. Multiphoton microscopy has also been used to release caged compounds in subfemtoliter volumes, to measure calcium transients 500 micron deep in mouse brain, and to quantify blood flow. Currently, multiphoton microscopy, with its high resolution and narrow focal plane, is the best high-resolution, noninvasive means of imaging in living animals for direct visualization of tissue morphology, cell metabolism, and disease states. We can expect that continued improvements in many underlying technologies will keep fluorescent imaging at the forefront of morphological studies.

FREE COMMUNICATIONS

Regulation of breast cancer migration to bone: role of RANK/RANKL/OPG

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Bone distruction is aprimaly mediated by osteoclast bone resorption, and cancer cells stimulate the formation and activation of osteoclasts next to metastatic foci.[1] Accumulating evidences indicate that the receptor activator of NF-κB ligand (RANKL) is the ultimate extracellular mediator that stimulates osteoclast differentiation into mature osteoclasts.[1] In contrast, osteoprotegerin (OPG) inihibits osteoclast differentiation into development. In order to elucidate a mechanism for cancer induced- osteoclastogenesis cells from human breast cancer line MCF-7 will be directly cocultured with human osteoblast and monocyte cells. In according to other scientists[3] we postulate that bone distruction by brest cancer could support osteoclastogenesis regulating RANK/RANKL/OPG pathway. Inihibition of RANKL/RANK by bisphosphonates[4] (i.e neridronic acid) offer a promising therapeutic target for interfering with tumor metastasis and progression in bones and giving to patients with cancer much better quality of life.

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Analysis of cell/titanium interactions by confocal laser scanning microscopy

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The analysis of cell/implant interface is a crucial issue in the study of new biomaterials. We evaluated morphology and organization of osteoblasts grown on titanium disks with smooth (SS) or sand blasted (SLA) surface by confocal laser scanning microscopy (CLSM). Experiments were carried out on living or on fixed cells. Confocal system was the Zeiss LSM 510 Meta. A flow chamber, equipped with a microincubator and adapted from a previous one,[1] was designed to visualize living osteoblasts grown on biomaterials. The study of titanium surface topography was carried out by reflection-CLSM; analysis of shape of living cells loaded with Calcein-AM[2] and of integrin β1 expression were performed by fluorescence-CLSM. Titanium surface of SS disks was characterised by shallow and parallel grooves. The simultaneous visualization of the material and living cells showed that cells grown on SS followed the direction of the grooves on the titanium while cells grown on SLA fitted their shape to the underlying topography of the support. Three-dimensional reconstructions visualized that cells grown on SS were flat and scattered while those on SLA were taller and globular. Cells grown on SS showed integrin-β1 dispersed at the cell-titanium interface, while those on SLA had integrin-β1 localized onto surface irregularities, with peaks clearly surrounded. In summary we propose CLSM as a useful tool in the study of cell/biomaterial interactions: it provides vertical scans of the sample, it visualizes planes inside the cells and it shows the surface beneath them. Implementation with the flow chamber proved effective in the analysis of living osteoblasts, thus representing a promising tool for real time long term observation.

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A multiple approach to the study of skeletal muscle apoptosis

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While some types of neuromuscular disorders clearly involve extensive muscle fiber necrosis which can be easily identified at light microscopy, apoptotic death is much less evident in routine histological preparations. However, this type of cell deletion has been widely reported to significantly account for muscle fiber atrophy,[1] even if the sincytial architecture of skeletal muscle suggests the possible existence of discrete cytoplasmic domains, independently controlled by the different myonuclei.[2] The characterization of muscle apoptosis is then important both considering the peculiarity of the model and its incidence in pathology. In this study cell death has been induced in C2C12 skeletal muscle cells by means of UV-B radiations, a universally known apoptotic trigger, and staurosporine, a PKC inhibitor. Undifferentiated myoblasts and differentiated myotubes, cultured as previously described,[3] were processed and their response was monitored by reverted microscope. Cytotoxicity was assayed by MTT test[4] and apoptosis was studied by means of scanning and transmission electron microscopy, as well as by TUNEL reaction. As expected, a minor sensitivity can be evidenced in differentiated cells than in myoblasts, but in both experimental conditions, particularly after UV-B treatment, cells show the majority of apoptotic features. Intriguingly, in myotubes apoptotic, necrotic and normal nuclei appear within the same fiber. Therefore, this model appears relatively sensitive to apoptotic induction, representing an effective tool to highlight muscle apoptosis and its role in pathology.

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Expression of transglutaminase 2 does not differentiate focal myositis from generalized inflammatory myopathies

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Idiopathic inflammatory myopathies (IIM), including dermatomyositis (DM), polymyositis (PM), sporadic inclusion-body myositis (s-IBM) and focal myositis (FM) are a heterogeneous group of autoimmune disorders of skeletal muscle.[1] To date there are not immunological markers which can help to differentiate FM from systemic inflammatory myopathies.[2] An increased transglutaminase 2 (TG2) expression has been found in DM, PM and s-IBM.[3] We re-examined tissue material we have gathered in the course of our previous studies on IIM,[4] investigating muscle expression of TG2 in patients with FM in comparison with DM, PM and s-IBM using immunocytochemistry and real time RT-PCR. Immunocytochemistry revealed an increased TG2 signal in endomysial vessels, in atrophic and regenerating muscle fibres in DM, PM and s-IBM; in s-IBM

vacuoles were immunostained too. In FM, TG2 expression was restricted to endomysial vessels only. Real time RT-PCR study confirmed a significatively increased expression of TG2 in all IIM muscles examined, expecially in PM and DM muscles. Our data suggest that TG2 expression does not represent a distinctive marker to differentiate FM from generalized IIM. TG2 overexpression in inflamed skeletal muscle seems do not contribute to the restriction of the inflammatory process in FM.

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c-Kit positive cells isolated from adult rat myocardium can organize them-selves into a tissue-like cell mass

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In the last few years, different populations with a cardiac potential have been isolated from human, rat and mouse adult and embryonic myocardium. Considering the recently published literature,[1-5] we have isolated, by differential adhesion method, a population of small c-kit highly-proliferating cells. After a first tissue digestion with collagenase type II, we let fibroblast-like cells adhere to the plasticware and, after a few days, we collected non- or low-adherent cells from the medium and maintained them in culture for a long period. The phenotype of these cells was assessed by immunocytochemistry, RT-PCR, cytofluorimetry and cell-based ELISA. We were able to assess the expression of adult stem cell, embryonic stem cell markers and cardiomyocyte cytoskeleton markers. Furthermore the behavior of these cells in 3D cultures demonstrated the heterogeneity of the population, that they are able to organize themselves into a tissue-like cell mass and that they can produce a high concentration of naturally produced extracellular matrix. Finally we were able also to demonstrate that this population can be subdivided into two subpopulations, one Nestin^{POS} and one α-SMC-actin.^{POS}

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Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines

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Muscle regeneration is a coordinate process in which multiple factors are sequentially activated to maintain and preserve muscle structure and function upon injured stimuli. Inflammation is clearly a critical component of muscle physiology and is an important phase in the regenerative process. Inflammatory responses resulting from tissue injury or infection generally resolve in beneficial self-limiting, healing process. To date much evidence reveals that the magnitude of the inflammatory response is crucial to keep the organism homeostasis and deregulation of it can promote disease. Thus, the inflammatory response must be resolved to allow muscle repair. We have previously reported that transgenic expression of a locally acting IGF-1 isoform (mIGF-1) safely enhanced and preserved muscle fibres integrity in senescent and dystrophic muscle, suggesting that mIGF-1 acts as a survival factor by prolonging the regenerative potential of skeletal muscle through increases in satellite cell activity. More recently we characterized the specific effect of mIGF-1 on the modulation of the inflammatory response during muscle injury and in mdx dystrophic muscle. In particular we observed that local expression of mIGF-1 transgene accelerates the regenerative process of injured skeletal muscle and protect dystrophic muscle, modulating the inflammatory response and limiting fibrosis. At the molecular level, mIGF-1 expression significantly down-regulated proinflammatory cytokines, such as TNF-α and IL-1β, and modulated the expression of CC chemokines involved in the recruitment of monocytes/macrophages. Analysis of the underlying molecular mechanisms revealed that mIGF-1 expression modulated key players of inflammatory response, MIF, HMGB1 and NF-κB. The rapid restoration of injured mIGF-1 transgenic muscle was also associated with connective tissue remodelling and a rapid recovery of functional properties. By modulating the inflammatory response and reducing fibrosis, supplemental mIGF-1 creates a qualitatively different environment for sustaining more efficient muscle regeneration and repair.

Histochemical analysis of damage and regeneration in skeletal muscle wasting

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Muscle wasting is associated to variety of neuromuscular pathologies and chronic disease states. Cachexia is a severe form of muscle wasting, triggered by elevated levels of cytokines, which interferes with the management of the primary disease and accounts for the death of a significant percentage of patients. In order to identify pathways able to enhance muscle repair, we analyzed the effects cytokines on skeletal muscle damage and regenerative capacity. We exploited the properties of Evans Blue Dye (delivered by ip injection) to detect sarcolemmal damage in a murine model of cachexia. Fiber damage-associated inflammation was revealed by immuno-labeling of Ig in the endomysium. These events resulted in decreased muscle fiber number in cross-cryosections. Following experimentally-induced damage, intramuscular TNF injection significantly decreased the number and size of the regenerating fibers, an effect abolished by treatment with a general caspase inhibitor. We developed a novel *in situ* assay to localize caspase activity in skeletal muscle. Active caspases were highlighted in interstitial cells expressing stem cell markers in the absence of apoptotic phenomena (the latter investigated by both TUNEL and TEM analysis). TNF-dependent impairment of regeneration was rescued by muscle gene delivery of the dominant negative form of PW1 (a TNF effector) or the V1a receptor (to sensitize muscle to the myogenic factor Vasopressin). We conclude that unbalance between increased damage and decreased regeneration in muscle can contribute to muscle wasting in cachexia and propose that it is possible to counteract cytokine effects on muscle regeneration by pharmacological and gene therapy approaches.

Role of cytoskeleton/stretch-activated channels (SACs) interactions in skeletal myogenesis

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In the present study, we investigated the functional interaction between stress fibres (SF) and stretch-activated channels (SACs) and its possible role in the regulation of myoblast differentiation in the presence or absence of sphingosine 1-phosphate. A clear temporal correlation between SF formation and SAC activation was found in differentiating C2C12 myoblasts. Inhibition of actin polymerization with the specific Rho kinase inhibitor Y-27632, significantly decreased SAC sensitivity in these cells, suggesting a role for Rho-dependent actin remodelling in the regulation of the channel opening. The alteration of cytoskeletal/SAC functional correlation had also deleterious effects on myogenic differentiation of C2C12 cells as judged by combined confocal, biochemical and electrophysiological analyses. Indeed, the treatment with Y-27632, inhibited the expression of the myogenic markers (myogenin and sarcomeric proteins) and myoblast-myotube transition. The treatment with the channel blocker, $GdCl_3$, also affected myogenesis in these cells. It impaired, in fact, myoblast phenotypic maturation (i.e. reduced the expression of a-sarcomeric actin and skeletal myosin and the activity of creatine kinase) but did not modify promoter activity and protein expression levels of myogenin. The results of this study, beside being in agreement with the general idea that cytoskeletal remodeling is essential for muscle differentiation, describe a novel pathway whereby the formation of SF and their contraction, generate a mechanical tension to the plasma membrane, activate SACs and trigger Ca^{2+}-dependent signals, thus influencing the phenotypic maturation of myoblasts.

Hepatic progenitor cells: state of the art and future perspectives

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Background. Liver regeneration is supported by resident hepatic progenitor cells (HPC). HPC are known to proliferate in most liver diseases. Recently, HPC have become the object of many researches since these cells may offer a new therapeutic approach for controlling the evolution of liver diseases. The aim of the present work is to update the evolving concepts about hepatic stem cells biology, their characterization, and their therapeutic potential. *Methods.* a)Immunohistochemistry for cytocheratin (CK)-7, CK-8, CK-18, CK-19, (OV)-6 and chromogranin-A was performed in formalin fixed biopsies of normal liver and of primary biliary cirrhosis (PBC) patients; b) Light and electron microscopy of the canaliculo-ductular junction area. *Results and Conclusions.* A clear positivity of HPC for CK-7 and CK-19 immunostaining was observed in normal and PBC livers; these were located in canaliculo-ductular junctions and were characterized by an oval nucleus and a scant cytoplasm. In PBC HPC differentiate towards cholangiocytes and hepatocytes with formation of cells showing an intermediate phenotype: a) reactive ductules, formed by immature cholangiocytes that strongly expressed CK-7; b) intermediate hepatocytes, characterized by a stronger CK-7 immunoreactivity near the cell membrane and a weak immunoreactivity of the cytoplasm. In conclusion, the immunolocalization of quiescent and proliferating HPC allows to study the dynamic interactions between HPC, surrounding parenchyma and different signals mediating cell proliferation. HPC manipulation as well as modulation of ductal reaction and cholangiocyte proliferation could represent a strategy for controlling the evolution of chronic liver diseases.

Morphological features of hepatic cyst epithelium in autosomal dominant polycystic kidney disease (ADPKD)

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Background. Hepatic cysts are lined by epithelial cells with characteristics of biliary epithelium with enhanced secretory and proliferative activities. Cyst growth and expansion have been recently shown to be driven by increased seric or cystic-fluid levels of a number of growth factors and cytokines. The aim of our study was to investigate the morphological features of hepatic cyst epithelium in ADPKD, with specific focus on the morphology of primary cilia and on the involvement of estrogens and IGF1. *Methods.* a) Immunofluorescence and immunohistochemistry for estrogen (E), IGF1 (insulin like growth factor 1), IGF1 (IGF1-R) and GH (GH-R) receptors, PCNA, pAKT; b) scanning electron microscopy of the apical surface of hepatic cysts were performed in six ADPKD livers; c) Western Blot for evaluation of E and IGF1 was performed in cyst-derived epithelial cells (LCDE). *Results and Conclusions.* The hepatic cyst epithelium of ADPKD patients is characterized by absence of both primary cilia and microvilli in large cysts and by rare or shortened cilia in smaller cysts. The cyst epithelium showed marked immunohistochemical expression of E, IGF1, IGF1-R, GH-R, PCNA and pAKT. E and IGF1 support proliferation of LCDE while antagonists of E and IGF1 inhibit the proliferative effect of serum administration. In conclusion, the hepatic cyst epithelium of ADPKD patients displays structural abnormalities of primary cilia and is sensitive to the proliferative effects of E and IGF1. Our study gives the morphological basis for clinical observations showing how formation and progression of hepatic cysts is highly sensitive to estrogen changes.

Immunoistochemical detection of T and B cells in skin of systemic sclerosis patients

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A relatively spare literature deals with the nature of skin infiltrating cells in patients affected by systemic sclerosis (SSc). For this reason, the aim of our study was to assess by immunohistochemistry the presence and the nature of dermal lymphocyte infiltrates in clinically involved and not involved skin of SSc patients. Tissue biopsies derived from 7 patients with diffuse skin disease (dSSc) and 4 with limited disease (ISSc), were fixed in 10% neutral buffered formalin and embedded in paraffin. All specimens were histologically assessed using standard haematoxylin and eosin sections. In order to investigate the identity and localization of lymphocytes, immunohistochemistry was performed using the monoclonal antibodies anti-CD3 and anti-CD20, for T and B cells respectively. Detection was achieved with Super Picture Polymer kit using DAB as the chromogen. CD3 lymphocytes were found in all the involved and not involved skin biopsies. The mean number of CD3 positive cells was 58.7±17.1 in involved skin and 35.9±15.5 in not involved skin (p=0.018).

Considering dSSc patient subgroup, involved skin specimens showed a higher number of CD3 positive cells (64.0±12.8) with respect to lSSc patients (49.5±10.9; p=0.032). Furthermore, CD20 positive cells were found in 3/11 (27.3%) of involved skin biopsies and in 1/ 11 (9. 1%) of not involved skin specimens. Our data supports the hypothesis that T lymphocyte infiltrate may play a key role in the pathogenesis of SSc. Moreover, it may represent a discriminating element for diagnosis of more aggressive disease.

Altered pattern of vascular connexin expression in atherogenesis

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Initiation and progression of the atherosclerotic plaque involve complex patterns of interaction between the cells of the arterial wall, in which cytokines, chemokines, and growth factors are known to play a critical role. Apart from these paracrine signaling mechanisms, another form of cell-to-cell interaction involves direct intercellular communication via gap junctions.[1] Within vascular tissue, there are four proteins involved in gap junctions and they are called connexins (Cx37, Cx40, Cx43, Cx45).[2] Nowadays, information regarding the role of vascular connexins in atherogenesis is fragmentary. So, we sought to investigate the expression patterns of the 3 vascular connexins (Cx37, Cx40 and Cx43) in aorta of ApoE deficient (ApoE°) mice aged 6, 10, 16 and 20 weeks. Our results show an increase of Cx43 and a decrease of Cx40 and Cx37 expression. These preliminary data demonstrate that vascular connexins are differentially expressed by atheroma associated cells within lesion raising the intriguing possibility that the direct cell-cell communication via gap junctions may also contribute to the still-obscure pathogenesis of atherosclerosis.

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GABAA-ergic forebrain pathways following mechano-gastric receptors stimulation in the rat

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Gastric mechanical functionality is an important aspect of digestive physiology characterised by proper receptor units. Mechano-gastric stimuli are widely integrated into neuronal circuits that interest visceral nuclei of hindbrain, and also several central brain area. In the present work we have detected in the rat forebrain the neuronal pattern responsive to mechanogastric receptors stimulation. In order to perform gastric physiological mechanical stimuli, low pressure gastric distensions were applied in anaesthetized rats. Moreover different protocols of dilatation/deflation were performed to differently stimulate mechanogastric receptors. Inside several activated nuclei, neurons expressing $GABA_A$ receptors, having $\alpha 1$ or $\alpha 3$ subunits in their pentameric structure, have been detected. The influence of vagal and splanchnic afferents was tested by means of bilateral vagal or splanchnic nervotomy. The mapping of activated neurons has been investigated using double colorimetric immunohistochemistry for $GABA_A$-$\alpha 1$ or -$\alpha 3$ subunits and c-Fos. Nuclei activation elicited by mechano-gastric stimulation shows involvement of several midbrain and forebrain nuclei activation. Activation of sovraxial brain nuclei identifies a neuronal pattern playing a role in controlling emotive behaviour associated to feeding. In comparison to intact rats, gastric dilatation in vagotomised rats evidences an almost exclusive dependence of nuclei pattern by vagal afferences, but gastric dilatation in splanchnicotomised rats evidences an important role of splanchnic afferences in determining a correct and balanced expression of activated neuron in the mechanogastric pathway.

Immunohistochemical localisation of cChat and pChat in the nervous system of invertebrates

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Acetylcholine is one of the most anciently known neurotransmitter widely distributed in the animal kingdom. The most reliable method to visualize the cholinergic neurons is the localisation by immunohistochemistry of its synthesizing enzyme choline acetyltransferase (Chat). Two molecular forms of that enzyme, the common type (cChat) and the peripheral type (pChat) have been recently distinguished[1] and two polyclonal antisera specifically recognizing and distinguishing each of them have been produced and characterized.[2,3] Since the cChat is widely present in the central nervous system, the pChat is mostly distributed in the mammalian peripheral nervous system. Since so far in vertebrates lower than mammals the presence of pChat might been hardly demonstrated, the current idea is that the cChat is the form phylogenetically more ancient and the pChat some recent splice variant of it. The present immunohistochemical study, carried out by the use of the two antisera, reopens the debate because it shows that both enzymes could be easily demonstrated in the nervous system of various invertebrates where they are widely and distinctly distributed and often present in different neuronal groups of the same ganglia. Among the invertebrate species we present here some results obtained on two mollusca, the giant garden slug (*Limax maximus*, Linn.) and the octopus (*Octopus vulgaris*, Cuvier) which Chat-positive neurons so far never could be shown by commercial Chat antisera.

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JNK and nestin expression in glioblastoma and periturnor areas: possible prognostic implications

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The c-Jun N-terminal Kinase (JNK) family has been implicated in glioblastoma multiforme (GBM) development. Moreover, it has been hypothesized that GBM may derive from neuroepithelial stem/progenitor cell transformation. This study was performed to determine JNK and nestin localization in GBM and in peritumor tissue and to assess their prognostic implications. Total (t), activated (p) JNK and nestin expression was investigated by immunohistochemistry in 20 cases. Samples were derived from the tumor (1st area) and from the

tissue at a distance <1 cm (2nd area) and between 1 and 3.5 cm (3rd area) from the tumor border. Total JNKs, observed both in the nucleus and in the cytoplasm, were expressed in the majority of the cells of the three areas. Phosphorylated JNKs, mostly located in the nuclei, were found in a variable percentage of cells in both GBM and peritumor tissue. The nestin cytoplasmic immunoreactivity was present in tumor specimens in a variable percentage of cells, while in the surrounding tissue only few cells showed immunoreactivity. Univariate analysis indicated that when the patients were dichotomized by the median value of pJNK/nestin and pJNK/tJNK/nestin ratios, the survival time was longer (19 vs 12 months, p=0.01) for patients whose ratio in the 2nd area was ≥2.619% and ≥0.026%, respectively. The same parameters showed an independent prognostic value in multivariate analysis. The JNK and nestin expression indicate that peritumor tissue, independently of the presence of neoplastic cells, may present signs of transformation. Moreover, pJNK/nestin and pJNK/JNK/nestin ratios in the peritumor tissue seem to have a prognostic implication in GBM patients.

Investigation of markers to estimate numbers of neurons and glial cells in human myenteric ganglia

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An important requirement in pathological diagnostics in the human enteric nervous system (ENS) is the estimation of the total numbers of neurons and glial cells. While myenteric ganglia wholemounts are the best preparations for total cell counting, archivial wax-embedded samples are often the only available material. In order to obtain valid counting of myeteric ganglia cells on paraffin sections, detailed morphological studies are required taking into account some technical notes. To examine the number of myenteric neurons and glial cells in paraffin sections in human colon. Normal colon specimens were obtained from 8 controls undergoing surgery for colon cancer. The myenteric ganglia were evaluated in paraffin serial cross-sections: CB, HuC/D and NFL or S100 and GFAP stainings were used to examine body numbers of neural and glial cells, respectively. The suitability of two neuronal markers, CB and HuC/D, was compared for staining and counting human myenteric neurons: 89.5% of all neurons were HuC/D-reactive (85.6% double-labelled, 3.9% immunostained only with HuC/D) whereas 10.5% were stained only for CB. The expression of NFL was correlated with HuC/D: 50.0% of all neurons were NFL-reactive and only 39.5% were NFL/HuC/D double stained. Thus both markers, CB and HuC/D, were comparably reliable in representing the total myenteric neurons and S100 detection allowed a better evaluation of glial cell bodies than GFAP did. The number of estimated myenteric cells is comparable to that reported in wholemount preparations thus confirming the validity of these evaluations on sections from archivial colon paraffin-wax blocks.

VEGF and VEGF receptors in human medulloblastoma

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Neoangiogenesis seems to be a critical factor for medulloblastoma (MB) growth and progression and a prerequisite for metastases.[1] VEGF is one of the most powerful mitogen for endothelial cells in CNS tumours.[2] Although VEGF receptors were initially found on endothelial cells, recent studies have shown that tumour cells of different origins express VEGF receptors. This suggests that VEGF may act as an autocrine signal.[3] The aim of the study was to investigate the role of VEGF/VEGFR signalling on MB cell growth. 13 paraffin embedded cases of MB were analysed to determine the expression of VEGF,VEGFR-1 and VEGFR-2. Immunohistochemical analysis showed an intense and diffuse cytoplasmic expression of VEGF. VEGFR-1 expression was lower and more heterogeneous than VEGF but with several cells reacting intensely. VEGFR-2 expression was lower than VEGF and VEGFR-1. All targets showed a stronger staining in the intefollicular than in the follicular areas. Positive reaction for VEGF and its receptors was also seen around the endothelia of tumor-suppling vessels. Semiquantitative analysis showed a moderate high expression levels of VEGF and VEGFR. Our findings support an autocrine role of VEGF also *in vivo* given the concomitant expression of VEGF and its cognate receptors in MB cells.

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Synaptic clustering of gephyrin and $GABA_A$ receptors in cerebellar Purkinje cells during development

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The scaffolding protein gephyrin is directly involved in the accumulation of glycine receptors at postsynaptic sites during neuronal maturation. Gephyrin is also localized at GABAergic synapses, however studies on cultured neurons have suggested that gephyrin is not required for the initial clustering of $GABA_A$ receptors. In this study, we investigated the development of gephyrin and $GABA_A$ receptors in rat cerebellar Purkinje cells, from P7 to adult. At early postnatal stages, we observed a diffuse immunoreactivity for the α1 subunit of $GABA_A$ receptors, which was co-extensive with the dendritic arborization of Purkinje cells. In addition, fluorescent clusters were superimposed on the diffuse labelling, suggesting that the α1 subunit was aggregated at synaptic sites. Gephyrin clusters were also present at the earliest stages of synapse formation and their number increased during development. Double-labelling revealed that a1 subunit-positive clusters were almost invariably colocalized with gephyrin, suggesting that the clustering of gephyrin occurs in parallel with that of $GABA_A$ receptors. Thus gephyrin may play a role in the early stages of GABAergic synapse formation. Strikingly we found that, while gephyrin clusters in the molecular layer persisted until adulthood, they disappeared from

the cell body of Purkinje cells at approximately P14. This finding suggests that gephyrin plays a developmentally regulated role at basket cell synapses and reveals striking differences in the molecular organization of GABAergic synapses in the cell body and dendrites of Purkinje cells.

High data output correlative microscopy (HDO-CLEM) and 3D correlative microscopy (3D-CLEM)

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Correlative light-electron microscopy (CLEM) allows the analysis of the same structures by fluorescence light microscopy (FLM) and by electron microscopy (EM), and solves several limitations of fluorescence microscopy. Unfortunately, the current procedures are extremely time consuming, and are not suitable for the acquisition of statistically relevant data. In the view of eventually designing a High throughput method for CLEM, we have first set the conditions for a High Data Output CLEM (HDO-CLEM). Here we present this new HDO-CLEM and also its application in a innovative 3D CLEM. Advantages of this technique are: 1. hundred times more events that can be correlated in each single microscopy session; 2. 3D correlation between FLM and EM can be obtained; 3. sample preparation improvement with a consequent high rate of success.

Propagation, expansion, and multilineage differentiation of human mesenchymal stem cells from dermal progenitors

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Mesenchymal stem cells (MSCs) have become one of the most studied stem cells, especially toward the healing of diseased and damaged tissues and organs. MSCs can be readily isolated from a number of adult tissues by means of minimally invasive approaches. We isolated multipotential MSCs from skin biopsies of healthy adult donors following informed consent. Progeny of these cells can be established as a single dermal MSC line which may be differentiated into adipogenic, osteogenic, and myogenic lineages, consistent with the data reported in other works. In particular we analyzed the differentiation of human dermal MSCs (hDMSCs) into osteogenic progenitors. hDMSCs were maintained in culture until confluence and then induced to differentiate by medium comprising ostogenic supplements (dexamethasone, β-glycerophosphate and L-ascorbic acid). The cells, other than phenotypically, were characterised by an increase of $[Ca^{2+}]_i$ and NO production, both closely involved in the osteogenesis. The evaluation of biochemical and morphological determinations showed that the hDMSCs underwent an *in vitro* osteogenic differentiation, resulting in the appearance of active osteoblast-like cells together with the formation of calcified deposits. In conclusion, human dermal MSCs can be used for the engineering of tissues *in vivo*, providing a valuable resource for tissue repair.

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Histochemical studies on canine globoid cell leukodystrophy

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Globoid cell (Krabbe) leukodystrophy (GLD) is a neurological disease described in humans and dogs. Central nervous system (CNS), liver and kidney from two cases were processed. Haematoxylin and eosin, Luxol-fast blue and lectin histochemistry were performed on paraffin embedded samples. Paraformaldheyde fixed tissues were infiltrated in glycol methacrylate and stained with Periodic Acid Schiff (PAS), Periodic Acid Silver Methenamine (PASM), Cresyl violet, Toluidine blue (TB), Alcian blue (AB) and Nile red (NR). On the same specimens, acid (PAC) and alkaline (PALK) phosphatase activities were tested. Pathological lesions were limited to CNS, with perivascular accumulation of PAS positive, BT methacromatic globoid cells. Histochemical staining for lectins revealed strong positivity using *RCAI*, *RCAII* and *WGA*, mild positivity using *Con A*. Krabbe cells exhibit a strong PAC and no PALK activities, and strong staining also with NR, in particular around the PAS/PASM positive deposits. Abnormal intracellular deposits were clearly visible in kidney glomeruli and distal tubules; resulting positive for BT, but no metachromasy was evident. PAS/PASM positive deposits were unaffected by diastase treatment and non fluorescent after NR; no enzyme activity was detected. The observation of abnormal intracellular material was possible also in the liver parenchyma. Then the stored material in Krabbe cells is mainly composed by glycolipids not containing relevant sulphated groups. The abnormal intracellular deposits in the kidney and liver suggests the accumulation of material released from pathological degeneration of the CNS.

Fluorescence microscopy practical applications to fruit-bearing plants biology

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Fluorescence microscopy is often efficiently applied in the agriculture. In compatibility studies between different varieties to crossbreeding, for genetic improvement, pollen vitality is examined by the fluorochromatic reaction test. This test assess the plasmalemma integrity revealing the esterasic activity on fluorescein diacetate. Compatibility between different varieties were examined by aniline blue, to estimate pollen germination in the stylar tissue by fluorescence microscopy. In fact pollen tubes have strongly fluorescing callose plugs. In somatic embryogenesis, autofluorescence can be used to distinguish between normal and abnormal embryos. Abnormal embryos, in the early stages of their development, present lignified tracheids and suberine deposits on external surface layers. On somatic embryos semi-thin sections, the application of DAPI (4',6-diamidino-2-phenylindole), is used to point out tissues intensity of mitotic activity. Fluorescent microscopy can be applied to fresh tissues or fixed and included ones. In our studies we suc-

cessful used a technique of cryostabilization in ethylene glycol, at low temperatures, and inclusion in glycol methacrylate hydrophilic resin (Technovit 7100, GMA). Also the morphology of leaves can be studied applying fluorescent microscopy to GMA embedded sections. Different leaves structure (palisade and spongy tissue) is associated with different training systems and different sun light exposition. In this way the autofluorescence, due to the presence of chlorophyll a and b and other pigments (such as xanthophylls and carotenoids) can be observed, but subsequently fluorochrome staining, using DAPI or Nile red, may be applied.

Modulation of transcription during hibernation

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Hibernating mammals represent an interesting physiological model to investigate the adaptive morpho-functional modifications of the pre-mRNA transcriptional and processing machinery under extreme metabolic conditions. Previous studies on hibernating rodents revealed that the cell nucleus undergoes important structural reorganisation during the hypometabolic period; however, the nuclear constituents involved in pre-mRNA processing do not show evident structural modifications. To elucidate this point, we investigated, by ultrastructural immunocytochemistry, the subnuclear distribution of some transcriptional, splicing and cleavage factors in liver and brown adipose tissue of euthermic, hibernating and arousing dormice (*Muscardinus avellanarius*). Our results show that, during hibernation, transcriptional activity significantly decreases and pre-mRNA processing factors undergo an intranuclear redistribution; moreover, in hepatocytes pre-mRNA processing preferentially stops at early stages, whereas in brown adipocytes large amounts of mature mRNAs are stored. Upon arousal, maturation of pre-mRNA seems to be restored before transcription and already mature mRNAs are immediately utilised. This suggests a programmed intranuclear redistribution of such molecules aimed to an efficient and rapid restoration of pre-mRNA processing upon arousal, and related to the reactivation of peculiar cellular activities.

Fluoro-edenite fibres induce lung cell apoptosis: an *in vivo* study

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We previously showed that apoptosis in the lungs of sheep exposed to fluoro-edenite fibres is induced via the receptor pathway. The present study was performed to gain further insights into the mechanisms of activation of programmed cell death induced by the fibres. Fluoro-edenite fibres are similar in size and morphology to some amphibolic asbestos fibres. They have been found in benmoreitic lavas, in the local stone quarry, in building materials and in road pavements at Biancavilla, a town in eastern Sicily (Italy), where epidemiological surveys evidenced a cluster of mortality from pleural mesothelioma. Inhalation of asbestos fibres can cause chronic inflammation and carcinogenesis. Since fluoro-edenite has been shown to activate the apoptotic process we set out to characterize the expression of apoptosis-regulating proteins in fluoro-edenite-exposed lung disease and sought to determine if apoptosis results from fluoro-edenite exposure. Lung tissue from healthy sheep habitually grazing near Biancavilla was processed for immunohistochemical localisation of bcl-2 and bax. Results showed epithelial and interstitial bax overexpression, especially in cells directly in contact with the fibres, and negative bcl-2 immunoexpression. TUNEL-positive cells were detected in alveoli and connective tissue. Our results are consistent with the hypothesis that apoptosis is an important mechanism for removing cells with unrepairable fluoro-edenite-induced genetic changes that predispose them to a neoplastic evolution.

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Apoptosis in spermatozoa of infertile men, clinical correlations

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The methods for evaluation of male infertility include not only routine investigations, standardized by the WHO,[1] but also complementary techniques, developed over the last years, in order to improve the predictive value of seminal analysis for natural conception and assisted reproduction.[2-5] With reference to these new methods, studies suggest that sperm with certain levels of DNA fragmentation serve as a strong predictor of reduced male fertility.[6-7] We studied subjects who underwent seminal fluid evaluation, because of an infertility condition, at the Department of Biomedical Sciences of the University of Sassari. The samples collected by masturbation were evaluated according to the World Health Organisation (1999). The samples was washed twice in PBS and cytocentrifuged for 10 min at 1800 rpm on polylysine-coated slides that were fixed in methanol at room temperature. The apoptosis was evaluated using the TUNEL (*In Situ* Cell Death Detection Kit, Fluorescein, Roche, Cat.No. 1 684 795). At fluorescent miscroscopy are counted at least 300 cells. Quantitative evaluation of apoptosis by the TUNEL method confirmed that apoptosis did not seem to be correlated with sperm concentration or morphology; however, we found a higher apoptotic rate in semen from patients affected by andrologic diseases, such as varicocele, than from those with alteration of semen characteristics. Apoptosis analysis might be used in infertile patients in order to understand the etiology of unexplained infertility and to improve therapeutic effectiveness.

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Morphological features in organoptypic cultures of normal human breast skin after gamma rays: cell adhesion and apoptosis

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Skin is a target organ for crucial side-effects of routine radiotherapy. The pathophysiology of the cutaneous radiation reaction is still unknown. Organotypic cultures of normal human breast skin can help in defining the immediate radiation response otherwise difficult to study in human volunteers.[1] Using this experimental model, we reported a significant inhibition of epidermal proliferation 24 hrs after the exposure to a single dose of ionising rays.[2] In the present study we evaluated the early radiation effect on intercellular adhesion and apoptosis. Bioptic fragments (n=8) were obtained from cosmetic surgery of young healthy women, cultured epidermal side up in Transwell, and harvested 24 or 48 hours after exposure to a single 2 Gy dose of γ-rays. Samples were processed for transmission electron microscopy and immunofluorescence to investigate desmoglein 1 (Dsg1) and p53 expression. Throughout the epithelial compartment, Dsg1 distribution pattern was similar in all non irradiated and irradiated samples. Preliminary observations indicated that no desmosomal ultrastructural modifications occurred, suggesting that intercellular adhesion is not yet affected. At 24 hrs, in both irradiated and non irradiated samples p53 immunofluorescence was similarly detected in scattered positive keratinocytes in the spinous layer. At 48 hrs, p53 was expressed starting from basal layer, with a slight p53 staining increase in irradiated samples. Our results can help in improving the knowledge of regulatory processes as a major prerequisite for radiobiological rationale effective interventions.

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Are hyalocytes deriving from monocytic origin cells? A morphological and immunocytochemical study

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Proliferative vitreoretinopathy (PVR) is the most common cause of failure of surgery for rhegmatogenous retinal detachment. This non neoplastic intraocular proliferation can be defined as the growth and contraction of cellular membranes within the vitreous cavity and on both retinal surfaces. As PVR occurs in 5% to 10% of all rhegmatogenous retinal detachments, it represents one of the most important causes of blindness in developed countries Several experimental studies have confirmed the hypothesis that PVR is a dysregulated wound healing process induced by a retinal break in which several different cell types are overstimulated by growth factors and cytokines. The cellular basis of PVR has been the subject of numerous studies over the last two decades: acording to the efforts produced in such works, we consider that only through a full understanding of the cytology of the epiretinal membranes we can conceive a prevention of this disorder. Ten fragments from patients suffering from proliferative vitreoretinopathy have been utilized. The specimen submitted to the process for resin inclusion and for immunocytochemical study have been observed by light and electron microscope. Together with fibroblasts, retinal pigment epithelium, macrophages, lymphocytes, and inflammatory cells, retinal glial cells (Muller and astrocytes) represent a major cellular constituent of fibroproliferative tissue associated with PVR. This study suggests neither ultrastructural studies, biological cultures, not the presence of numerous isolated factors are sufficient to determine the cell type of origin of cells in PVR. It should be pointed out that among the first cells to be exposed to these growth factors and other stimuli for cell migration and proliferation are hyalocytes, the cells of the vitreous body which are located in the posterior vitreous cortex. As cells of monocytic origin, these resident elements may play an important role as mediators of the inflammatory response, recruiting the various involved cells that ultimately result in PVR membranes. The immunocytochemical study show these cells positive marked to CD68, marker of monocytic cell and this confirms the monocytic origin of the hyalocytes.

Nuclear ribonucleoproteins (RNPs) and nucleolus-associated proteins undergo dynamic segregation and release during apoptosis

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The most important RNP-containing structures in the nucleus are the nucleolus, perichromatin fibrils (PF), perichromatin granules (PG), and interchromatin granules (IG). PF, IG and PG are the sites where RNA transcription, splicing and storage take place. PF and PG locate at the periphery of condensed chromatin, and IG in the interchromatin space; their intranuclear position is necessary for nuclear RNAs to be properly processed. During apoptosis, the collaps of chromatin is responsible for the disruption of the whole nuclear architecture, including the RNP domains. We found that, in early apoptosis, PF, IG and PG segregate in the interchromatin space, where they form characteristic fibro-granular clusters in which different RNP protein components co-localize ectopically (we have called these aggregates HERDS, for Heterogeneous Ectopic RNP-Derived Structures). Some nuclear RNP proteins (e.g., hnRNPs, U1-70-kDa snRNP, several ribosomal proteins) are cleaved during apoptosis, whereas other (e.g., the antigen Sm, Ro and La) are not degraded; the same occurs for different non-RNP nucleolar proteins (nucleolin, the antigen UBF, and PARP-1 are cleaved, whereas fibrillarin, B23, C23, topoisomerase I, and phosphorylated c-Myc are not). Immunolabeling experiments at light and electron microscopy showed that during apoptosis several among these nuclear proteins move into the cytoplasm with a different kinetics, and can be finally found in apoptotic blebs; their heterogeneous content essentially depends on the dynamic sequence by which the molecular aggregates of nuclear origin migrate toward the cell surface.

Oxidative stress and apoptosis markers in pterygium: immunohistochemical study

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Ultraviolet radiation is known to cause oxidative DNA damage and is thought to be a major factor implicated in the pathogenesis of pterygium, a benign invasive lesion of the bulbar conjunctiva. Among all the photo-oxidative DNA products, the 8-hydroxydeoxyguanosine (8-OHdG) is regarded a sensitive and stable biomarker for evaluating the degree of DNA damage. The protein p53 is a major cell stress regulator that acts to integrate signals from a wide range of cellular stresses. UV radiation can cause mutations in the p53 tumor suppressor gene, that, when inactivated through mutation and loss of heterozygosity, can lead to cell proliferation and genomic instability. In many types of UV-radiation damaged cells, p53 is overexpressed and immunohistochemically detectable. Our recent data demonstrated the concomitant presence of increased levels of p53 in 8-OHdG immunoreactive cells, providing evidence that pterygium is a tumor-like growth disorder, related to faulty apoptosis. Apoptosis is preceded by increased generation of reactive oxygen species, is associated with p53 activation, and decreased levels of survivin. Survivin is a member of the inhibitor of apoptosis protein family, overexpressed in most human malignancies and implicated in the cellular stress response. It is well-known that the functional loss of wild-type p53 is associated with up-regulation of survivin expression in several types of tumors, however there is no evidence of interaction between mechanisms regulating their expression.

The purpose of this study was to investigate, by immunohistochemistry, a possible correlation between 8-OHdG, p53 and survivin in a group of 31 Ecuadorian primary pterygia. The results will be discussed.

MBP and IL 5 as markers of eosinophilic granulocytes in circulating blood of the sea turtle *Caretta caretta*

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Eosinophils play a key role in defense against parasites and serve as an important mediator cell in hypersensitivity diseases. Eosinophils of the sea turtles are, generally, rare in the differential count (about 4%) and contain both eosinophilic and colourless granules in their cytoplasm. They were studied by immunocytochemical detection of major basic protein (MBP) and interleukin-5 (IL5) at L.M and TEM in leukocytes of 6 specimens of *Caretta caretta*. It is well known in Mammals that MBP is a potent enzyme against helminths and is toxic towards bacteria. It activates neutrophils and macrophages, and is implicated in asthma. It forms the electron-dense crystalline core of the specific granules of mature peripheral blood eosinophils.[1] IL5 is a growth and differentiation factor,[2] activator and chemoattractant for eosinophils and, as a consequence, is considered a pivotal cytokine in allergen-mediated eosinophilic responses. Both blood smears and ultrathin sections of the buffy coat were treated with anti-MBP and anti IL5 polyclonal antibodies revealed by ABC method on the smears and by immunogold method for TEM. At LM both MBP and IL5 immunoreactivities were localized in the cytoplasmic granules. IL5 was detected in the cytoplasm of the lymphocytes too. These results were confirmed by TEM observations by showing an intense gold labelling of MBP in the granules, even if most of them are lacking of crystalloid core. IL5 detection displayed less intense immuno- reactivity in the eosinophilic granules and in the lymphocytes.

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Molecular signalling driving the effect of the combination of TRAIL and Tyrphostin (AG490) in leukemic cells

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The ability of TNF-related apoptosis-inducing ligand (TRAIL) to selectively kill a variety of cancer cells has been recently described, but one of the major concerns in the successful treatment is the occurrence of resistance and possible side toxic effects evoked by the treatment.[1,2] Here we report that in human primary T leukemia cells, as well as in a T cell line, the combinatorial treatment with TRAIL and Tyrphostin (AG490)[3,4] increased the toxic effect of TRAIL on leukemic cells, allowing treatments with TRAIL several folds lower than the doses commonly used. Of note, the combinatorial treatment did not exert toxic effects on normal T cells, while the treatment with AG490 alone, did not produce significant modification of the tumour cell apoptosis. Here we report the molecular signalling related to the above described biological effects.

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Interaction of megakaryocytes and fibroblasts triggers megakaryocyte para-apoptosis in GATA-1^{low} mice

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In the GATA-1^{low} mice the pathological correlation of myelofibrosis develops with age.The MK die because of pathological emperipolesis of neutrophils and release of TGF-β in the microenvironment. On this basis, GATA-1^{low} mice could presumably develop fibrosis due to TGF-β-mediated fibroblast activation. This hypothesis is further analyzed here by determining the amount of TGF-β present in the microenvironment and by characterizing the state of fibroblast activation in spleens from GATA-1^{low} mice during disease progression.such a presense of TGF-β in the microenvironment is demonstrated by the progressive increase with age of TGF-β gold particle in spleen of these animals(from~20 in 6 mth.old mice to ~230 in 13 mth.old mice). In the extracellular space,TGF-β gold particles were specifically associated with the non-overlapping regions of the collegen polymers. With disease progression,an increase is noted in TGFβ-gold particle not only in all cells, including fibroblasts, but also in collagen fibers. In addition, the fibroblasts develop long protrusions by meens of wich they astablish a close contact with megakaryocytes by either short or long range cell peripolesis. In both cases, the protrusions

penetrated within the MK releasing fibronectin and collagen in its cytoplasm. The megakaryocytes therefore die from para-apoptosis caused by the accumulation of collagen in their cytoplasm. The emperipolesis of neutrophils and the peripolesis of fibroblasts are two independent processes which are, hovever, not mutually exclusive. These data indicate that the primary defect induced by the GATA-1^{low} mutation at the MK level triggers two pathological cell-MK interaction: the first one, neutrophil emperipolesis, and the second one, fibroblast peripolesis. We suggest that both the altered growth factor milieu of the microenvironment, and pathological fibroblast-MK peripolesis contribute to the pathobiology of myelofibrosis in GATA-1^{low} mice.

Molecular characterization and gene expression of serine-protease HtrA1 in the lizard *Podarcis sicula*

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The human HtrA family of serine proteases appears to be involved in important functions such as cell growth, apoptosis, and in cell fate control via regulated protein metabolism.[1] HtrA1 is a secreted protein involved in the degradation of extracellular matrix proteins important in tumor progression and invasion. The differential expression of HtrA1 in human and mouse tissues[2,3] and the observation that the transcription of this gene is highly regulated between fetal liver and postnatal liver[4] strongly suggest that HtrA1 exerts its control function on cell growth not only in neoplastic cells but also under physiological conditions. We have first determined the nucleotide sequence by RT-PCR. Secondly, we have studied the expression and distribution profile in several tissues by RT-PCR, western blotting and immunohistochemistry showing that HtrA1 had a widespread pattern of expression although a different tissue distribution and/or level of expression in several organs was observed. Our data concerning HtrA1 expression and distribution in lizard tissues demonstrate that the expression of this protease is modulated in tissues with different physiological activities such as stomach and brain and probably this protease could be involved in differentiation processes and cell growth.

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Apoptotic and anti-apoptotic proteins in cutaneous malignant melanoma

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Survivin has been implicated in multiple essential functions, including cell division, apoptosis, cellular stress response and checkpoint mechanisms of genomic integrity. Its expression is regulated in a cell cycle-dependent manner and it is most abundant in cells at G2-M phase of the cell cycle. Survivin seems to protect normal or transformed cells from apoptosis although the mechanism through which survivin antagonizes cell death is controversial. Apoptosis is induced by p53, protein involved in cell cycle checkpoint mechanisms, in response to DNA damage, preventing cell cycle progression. p53 up-regulates the expression of various genes contributing to cell cycle arrest, DNA repair, or apoptosis. Although survivin and p53 are both modulators of the opposing cellular processes of proliferation and apoptosis, there is currently no evidence of interaction between mechanisms regulating their expression. Wild-type p53 seems to repress transcription of the survivin gene and the functional loss of wild-type p53 results associated with up-regulation of survivin expression in human cancers. A cell cycle suppressor, as p16, seems to interact with survivin. In human hepatoma cell lines, it has been demonstrated that Survivin interacts with Cdk4 during the cell cycle, showing a competitive interaction of Survivin with Cdk4/p16INK4a complex to progress the cell cycle. This study was designed to examine, immunohistochemically, the relationship between the survivin expression and p53 or p16 in 58 primary cutaneous melanomas. The results will be discussed.

Immunohistochemical expression of iNOS and metalloproteinases MMP2 and MMP9 in abdominal inflammatory aortic aneurysms

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Abdominal aortic aneurysms (AAA) is a degenerative vascular disease characterized by localized dilatation of the aortic wall as a result of altered matrix composition (elastin and collagen degradation). Howewer the pathogenesis of the changes is elusive and unclear. Some experimental evidences suggest that iNOS (who synthesize a large amount of NO in inflammatory processes) and the metalloproteinases (MMP) are implicated in the pathogenesis of AA but the relationship between NO and MMP to aneurismal disease is currently unknown. The aim of this study is to investigate the immunohistochemical expression of iNOS and MMP2 and MMP9 in human probable inflammatory aneurysmal tissues. Specimens of 10 AAA were obtained during surgical procedure. Furthermore during aorto-coronaric bypass were obtained 10 punches of normal aorta in the side of joint of bypass. All the specimens were fixed in Bouin's mixture and embedded in paraffin; obtained sections were processed with anti MMP9 (Chemicon International), monoclonal anti MMP2 (Chemicon International), anti iNOS (Transduction laboratories) by EnVision+System HRP (AEC) (Dako Cytomation). Our results underline that in tunica media of aortic aneurysm in comparison with control tissues the MMP2, MMP9 and iNOS immunoreactivity are significantly higher. These studies provides a morphoistochemical basis to further support the role of NO and metalloproteinases in the pathogenesis of AAA and suggest the hypotesis that iNOS, produced in high levels by inflammatory cytokines, can stimulate the matrix metalloproteinases during aneurysm formation.

Immunohistochemical expression of somatostatin receptors sst2A in human meningiomas: is there any relevance with clinico-pathological parameters?

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Somatostatin receptors consist of a family of at least five different subtypes (*sst*); in particular, two isoforms of *sst2*, *sst2a* and *sst2b*, have been isolated. The availability of their corresponding *sst2* antibodies allows the differential diagnosis

between *sst2*-expressing *versus sst2*-negative tumours, as well as the evaluation of the potential therapeutic efficacy of octreotide and other somatostatin analogues. Herein we have determined the pattern of *sst* expression in 26 formalin-fixed paraffin-embedded surgical samples of human meningiomas (11 male, 15 female; age range 31-84 yrs), classified according to WHO 2000. Data on the tumour site, histological grade and microvessels density (MVD), on growth fraction determined by KI-67 LI and on Simpson's grade of surgical resection were also available. Four μm thick parallel sections, previously microwaved in 10 mM citric acid (pH 6.0; 3 cycles for 15 min), were incubated overnight at 4°C with polyclonal antibodies anti-somatostatin receptors *sst1*, *sst2a*, *sst2b*, *sst3*, *sst4*, *sst5* (BIOTREND, Germany; w.d. 1:3500). The staining was categorized as strong, moderate, weak or negative when it was easily or not easily seen under a low-power objective along the neoplastic cells plasma membrane. A diffuse strong to moderate intense immunoreactivity was found in 18/26 meningiomas by *sst2*. Only a focal staining was observed with *sst3* or *sst4* antisera, while no staining for any *sst* was noted in normal meninges. Statistical analysis revealed that a higher MVD, revealed by CD34 and CD105, as well as a Ki-67 LI greater than 4% were significantly associated to *sst2a* positive meningiomas in comparison to *sst2a* negative tumours.

Immunolocalization of lactoferrin in human bone tumours: an investigation by monoclonal antiserum

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Lactoferrin (Lf), a major iron-binding protein, has been immunohistochemically revealed in many human tissues and neoplasms, with unexplained biological meaning. Moreover, *in vitro*, Lf has been considered as one of the most potent regulators of bone mass, stimulating the proliferation of osteblasts and cartilage cells. We have investigated the Lf immunopattern in 34 formalin-fixed paraffin-embedded consecutive unselected bone neoplastic samples, obtained from an equal number of patients (16 male, 18 female; age range 9-69 years; mean age 24.79 yrs); the corresponding staging as well as macroscopic morphology of tumours were available. Histologically, 13 were condromas, 7 giant cells tumours, 6 fibromas with or without ossification, 8 miscellaneous ones (4 osteoid osteoma, 1 myeloma, 1 osteosarcoma, 1 condrosarcoma, 1 adamantinoma). On 4 μm thick sections, Lf immunostaining was revealed by a monoclonal mouse anti-human Lf (Biodesign International, USA; w.d. 1:75) for 60 min. at room temperature. Quantification of immunoreactions was performed by using the intensity-distribution (ID) score based on both the percentage and the staining intensity of positive neoplastic cells. An evident cytoplasmic immunoreactivity for Lf was constantly encountered in giant cells tumours with a variable ID score, while Lf was occasionally found in myeloma and adamantinoma cases. No evidence of Lf immunostaining was noted in other bone tumours. No relationships between immunohistochemical data or sex, age of patients as well as the neoplastic site were recorded. In the light of our results, Lf immunoexpression wouldn't play only an anabolic effect on bone formation since its ability to inhibit osteoclastogenesis seems questionable.

Microvessels density evaluation in human meningiomas: an immunohistochemical investigation by the specific marker for neoangiogenesis CD105 (endoglin)

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Microvessels density (MVD) reflects the entity of the neoangiogenetic process and it has been investigated in many malignancies by endothelial markers such as FVIII, CD31 and CD34; nevertheless, these markers react also with pre-existing normal host vessels, resulting not precisely adequate to define tumour MVD. Since mortality and morbidity of human meningiomas depend upon the tumour vascularity as well as on the extent of peri-tumoral vasogenic oedema, we have evaluated MVD by the specific marker for neo-angiogenesis endoglin (CD105) in 54 formalin-fixed paraffin-embedded surgical samples of human meningiomas (22 male, 32 female; age range 21-84 years), classified according to WHO 2000. For each case, the site, histological grade, Simpson's grade of surgical resection and growth fraction determined by KI-67 LI were available. On 4mm thick sections, pre-treated with proteinase K for 15 min at room temperature, CD105 monoclonal mouse antibody (DAKO Corporation, Denmark; 1:50) was applied overnight at 4°C. CD105 stained vessels were counted (400X) in the three most vascularized areas by two independent observers; the mean value of three counts was then recorded as the MVD of the section. CD105 positive vessels were identified in 70% meningiomas with different MVD values. Significant differences in CD105-MVD counts were achieved between grade I and II tumours, these latter ones displaying higher MVD values; moreover, significantly higher MVD counts were recorded in cases of meningiomas showing Ki-67 LI greater than 4%. CD105 might be proposed as a target for selective immuno-therapies blocking tumour blood supply in meningiomas.

Actinic keratosis associated with squamous and basal cell carcinomas: an evaluation of neoplastic progression by a standardized AgNOR analysis

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The AgNOR technique allows to visualize at the light microscopic level a set of argyrophilic non-histone proteins localized in the nucleolar organizer region (AgNOR) and their quantity has been demonstrated to be strictly related to the rapidity of cell proliferation. Furthermore, the prognostic value of AgNOR quantity as an independent variable, able to predict the recurrences and/or the overall survival, has been documented in skin tumors. Actinic keratosis (AK) is a common skin lesion considered the earliest stage in the development of skin cancer with a 10% estimated rate of malignant transformation into squamous cell carcinoma (SCC). To investigate the neoplastic progression in different stages of AK, a standardized AgNOR analysis has been performed on 94 cases of AK, 35 of which were associated with SCC or basal cell carcinoma (BCC); moreover, 31 cases of SCC and 22 cases of BCC were also analyzed. The mean area of AgNORs per cell (NORA) was evaluated in each case; using as a cut-off the mean NORA value in AK (3.996 μm^2), the cases were divided in low- and high-AgNOR-expressor (AgNOR status). In AK samples, a progressive increase of the mean NORA value from Stage I to Stage IV was encountered. A highly significant difference was found in the comparison of AK cases with or without SCC, while no

differences between AK cases with or without BCC were noted. A highly significant association between AgNOR status and coexistence of SCC was encountered in AK; no association was appreciable between AgNOR status and BCC. Our data document the standardised AgNOR analysis represents a strong negative predictor for the association between SCC and AK.

Modifications of the peptides ANP and oxytocin in the rat hypothalamic paraventricular nucleus during the physical exercises

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Recent studies showed that plasmatic ANP levels change during the physical exercises; in regard to brain no literature data are reported, we previously evidenced that in the rat hypothalamic supraoptic nucleus, ANP and oxytocin increase during the exercises; in this paper we examine the ANP and oxytocin in the rat hypothalamic paraventricular nucleus, PVN. Wistar rats were trained by a physical exercise type *power* exercise using a rung ladder varying the load-weight fastened to the tail of the individual; the exercises lasts 20 minuts everyday, for 15, 30, and 45 days. The animals were sacrificed after each time, some animals were sacrificed 15 days after the end of exercises. Brains are removed fixed in the Bouin's fluid and processed for the immunostaning with the ANP and oxytocin antibodies. In the ANP-immunostained sections in the PVN it is observed that ANP decreases at 30° of the exercises, and increases from 30° to 45° day. Further, ANP increase is noted at 15° days after the end of the exercises. In oxytocin-immunostained sections it is observed an increase at 30° day of exercises and no decrease is noted, differently than ANP. It known ANP regulating the body fluids by vasodilatation and diuresis. We hypothesize that in PVN, at 30° day, the ANP peptide decreases, so the vasodilatation and the diuresis diminish and consequently retention of fluids occurs. Therefore, ANP might involved by the vasodilatation in diminishing the body temperature, that increases during continued exercises. The oxytocin, at 30° of exercises, in the PVN increases, differently than ANP, according to relationship between the two peptides.

Immunoreactivity to Syntaxin, SNAP-25 and NSF in the rat cerebellar cortex

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In the mammalian cerebellar cortex, excitatory (glutamatergic) and inhibitory (GABAergic) synapses exist. It is unclear whether there are differences in the distribution of proteins involved, according to SNARE (SolubleN-ethylmaleimide-factorAttachmentREceptor) hypothesis, in vesicle docking, activation and release, between excitatory and inhibitory synapses. This study aimed to analyze immunohistochemically the distribution in the rat cerebellar cortex of Syntaxin (SYN) and SNAP-25 (SyNaptosomalAssociatedProtein-25), SNARE proteins of presynaptic membranes, and of NSF (N-ethylmaleimideSolubleFactor), a cytoplasmic ATPase protein involved in vesicle activation. Distributions of the immunoreactivity (IR) to SYN and SNAP-25 were superimposable. In all layers, the IRs were observed homogeneously distributed in the neuropil, resulting in a 'background of positivity', interposed among immunonegative neuronal bodies and processes. NSF IR was observed throughout the cortex in perikarya and processes of various neuron types and in puncta attributable to axon terminals. Double immunolabelling for SNAP-25 and GAD revealed a co-localization within a low number of puncta. These results supply data on the distribution of some SNARE proteins in the rat cerebellar cortex. IRs to SYN/SNAP-25 are diffuse in the neuropil, but only partly overlap the IR to GAD, labelling glutamatergic rather than GABAergic synapses. IR to NSF is diffuse in the neuronal cytoplasm, indicating that it is not strictly active at synapses. Due to their different distributions, SYN, SNAP-25 and NSF likely play different roles at cerebellar cortex synapses.

S100 and GFAP immunoreactivity of astroglia in the *Diplodus sargus* brain

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S100_ protein and glial fibrillary acidic protein (GFAP) are widely distributed in astrocytes[1] but also in ependymal and radial glia.[1,2] These proteins are then used as reliable markers of all astroglial cells. In teleost fishes, the studies on the astroglia are still relatively scarce.[1,2,3] In this paper we report the study on the astroglial architecture in the brain of a teleost fish, *Diplodus sargus*, by the immunohistochemical staining of S100_ protein and GFAP. The immunodetection of GFAP and S100 showed the presence of an astroglial architecture various in the sargus brain. It was represented mainly by radial glia and fibres, which were more numerous and large in the mesencephalon and rhombencephalon, but very scarce in the telencephalon. In fact in this region we revealed few and thin fibres immunoreactive only to anti-GFAP. In the other regions of the brain indeed we observed structures both GFAP(+) and S100(+). The immunodetection of these protein revealed generally a major number of GFAP(+) structures occasionally also S100(+). However, the immunolabeling of GFAP was more strong than the S100 immunolabeling. Radial glia was especially evident in the optic tectum and in the white matter of the cerebellum. The occurrence of bundles of GFAP(+) fibres, but also S100(+), was observed in the periventricular region, in the inferior lobes of the hypothalamus, in the dorsal thalamus and in the optic tectum. The infudibular region and the neurohypophysis showed also intensely GFAP(+) fibres, processes of the pituicytic cells, which appeared also S100(+).

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Cadmium effects on PRL cells in the pituitary gland of *Podarcis sicula*

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In spite of their important role as bioindicators there is few information about the cytotoxic effects of cadmium at the pituitary level in reptilian species. In order to fill this gap, we have studied the possible morphological changes induced from $CdCl_2$ on the prolactin cells in the pituitary gland of *Podarcis sicula*. Two types of experiments were carried out: a group of lizards were exposed to an acute treatment by an intraperitoneal injection of a single and massive dose (2 mg/Kg-BW) of $CdCl_2$; another one was indeed treated for four months with $CdCl_2$ at dose of 1 mg/kg-BW in the drinking water (chronic treatment). Serial sections of 6 μm of the pituitary gland were processed for routine histological and immunohistochemical staining by

ABC technique. The cytotoxic effects of cadmium were more evident on the PRL cells of *P.sicula* of the chronic treatment. In fact, in the lizards at 2, 7 and 16 days of the acute treatment, few differences was revealed in the shape, number and distribution of these cells respect at the same cells of control animals. In the PRL cells of the chronic treatment indeed we observed significant difference: during the treatment a meaningful increase in number of PRL cells was appreciate just at 30 days; the cells appeared in the rostral and medial pars distalis, like in the control specimens, but also in the caudal pars distalis with an increase in immunostaining intensity. In lizards, a protracted exposition to cadmium involves evidently an inhibitory effect on the PRL secretion as in the rat[1] through an interaction with the prolactin molecule, that is sensitive to divalent metals as was shown *in vitro*.[2]

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Occurrence and localization of S100 protein in the central nervous system of adult zebrafish *(Danio rerio)*

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The S100 proteins are a family of low molecular mass (10–14 KDa) EF-hand-containing calcium binding proteins that constitutes of at least 20 proteins with calcium binding ability. The S100 plays an important role in the regulation of several cell processes such as motility, growth, differentiation, progression of the cell cycle, transcription, and secretion.[1] Recent data have demonstrated the involvement of S-100 in acute neurological disorders such as global hypoxia, ischaemic or haemorrhagic stroke and traumatic brain injury.[2] The expression of S100 in the mammalian central nervous system has been demonstrated,[3] while the data about the expression of this protein in the fish brain were scarce. Here we investigate, using western-blot and immunohistochemistry, the occurrence and cellular localization of S-100 protein throughout the CNS of adult zebrafish. In the zebrafish CNS, S100 protein has been observed in glial cells, particularly radial glial cells lining the walls of cerebral ventricles and the canalis centralis of the spinal cord. Also, the Purkinje's cells of the cerebellar cortex, as well as the neurons in the deep cerebellar nuclei were S100 protein positive. All together these results claim for an evolutionary preservation of S100 protein. In any case, and interestingly, S100 protein was demonstrated to be an excellent and specific marker for cells lining the CNS cavities, and therefore it might serve for future studies in hydrocephalus mutations of zebrafish.

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Aggrecan labels perineuronal nets associated with pyramidal neurons and interneurons of human cerebral cortex

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The extracellular matrix (ECM) of the central nervous system is formed by a complex mixture of several types of proteoglycans including the keratan/chondroitin sulfate proteoglycan aggrecan. An antibody recognizing the whole human aggrecan molecule, denoted 5D3, has been utilized to analyse by immunohistochemistry and confocal microscopy localization and distribution of aggrecan in the human cerebral cortex. 5D3 aggrecan is present in low concentration in the diffuse ECM, whereas it concentrates, as component of the net-like structures called perineuronal nets (PNNs), around the body of medium- and large-sized pyramidal neurons of cortex layers III and V as well as of small and large interneurons of the layers III, IV, and VI. Different types of aggrecan-containing PNNs are recognizable, from an incomplete patchy pattern to a complete coat, which extensively enwraps the cell body, dendrites, and axons proximal tracts, and appears deposited at synapses and glial contacts. Among the aggrecan PNN neurons, subsets of parvalbumin and calbindin interneurons are identified in cortical layers III, V and VI, whereas the aggrecan coat is absent on calretinin-type neurons. The results demonstrate that aggrecan PNNs are present throughout the cerebral cortex layers according to a topographical and neuron-specific distribution.

The akt/mammalian target of rapamycin (mTOR) signal transduction pathway is activated in high risk myelodysplastic syndromes and influences cell survival and proliferation

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The Akt/mTOR signaling pathway is important for both cell growth and survival. In fact, it has been strongly implicated in mechanisms related to neoplastic transformation, through enhancement of cell proliferation and survival. Myelodysplastic syndromes (MDS) are a group of heterogeneous hematopoietic stem cell disorders characterized by ineffective hematopoiesis and by a high risk of evolution into acute myeloid leukemia (AML). It is still unclear what is the pathogenesis of the MDS evolution into AML, although some recent studies indicate that aberrant activation of survival signaling pathways could be involved. Our investigation, performed by means of immunofluorescent staining, reports an activation of the Akt/mTOR pathway in high risk MDS patients. Interestingly, not only mTOR was activated, but also its downstream targets, 4E-BP1 and p70S6K. Treatment with the selective mTOR inhibitor, rapamycin, significantly increased apoptotic cell death of CD33+ cells from high risk MDS patients. Rapamycin was ineffective in cells from healthy donors or low risk MDS. Moreover, incubation of high risk MDS patient CD34+ cells with rapamycin, decreased the *in vitro* clonogenic capability of these cells. In contrast, the PI-3-K inhibitor LY294002 did not significantly affect the clonogenic activity of high risk MDS cells. Our results indicate that the Akt/mTOR pathway is critical for cell survival and proliferation in high risk MDS patients. Therefore, this signaling network could become an interesting therapeutic target for treating more advanced MDS cases.

Hippocampal neuropathology in murine model of bacterial meningitis

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We evalued regional neuropathologic changes in hippocampal regions (HI) of adult and aged wild type and K.O. male mice inoculated intracerebrally with *Streptococcus pneumoniae*. Brains of infected mice were excised, and sectioned along an horizontal plane. The cryosections were assessed by Hematoxilyn and Eosin, acethylcholinesterase (Ache) histochemistry and TUNEL staining for apoptosis. Our data demonstrate that pneumococcal meningitis induces cell death having characteristic features of apoptosis in (HI). In our study we observed that in infected mice neuronal cells were depleted from *Dentatus gyrus* (DG) and *Stratum oriens* (SO) of HI and TUNEL-positive neurons were detected in the same brain areas. Moreover the AchE staining suggests that pneumococcal meningitis has a direct action on cholinergic neurons.

Our study may be useful to correlate brain injury mainly affecting the cortex and hippocampus with the host response as a consequence of bacterial meningitis.

Neurotensin receptor type 1 immunoreactivity in peripheral ganglia and carotid body

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Neurotensin (NT) is a neuropeptide which is widely distributed in the central nervous system. Three different NT receptor types, termed NTR1, NTR2 and NTR3, have been identified to date; NTR1 being the high-affinity receptor. There are no immunohistochemical data regarding NTR1 presence in peripheral ganglia and carotid body. Thus, the aim of the present study was to investigate through immunohistochemistry the presence and location of NTR1 in peripheral ganglia and carotid body of both human and rat, with particular reference to the different cell types and internalization processes. Materials consisted of dorsal root ganglia, Gasser's ganglia, superior cervical ganglia, pelvic parasympathetic ganglia, enteric ganglia, and carotid bodies obtained at autopsy from 16 adult subjects (10 males, 6 females; mean age 44.3 years, SD±3.4) and 5 adult rats. Nuclear NTR1 immunostaining was found in ganglionic cells of all types of peripheral ganglia, indicating a modulatory role of NT in these ganglia. Nuclear or nucleo-cytoplasmic positivity was detected in glomic type I cells, while type II cells were negative. Nuclear immunostaining is consistent with NTR1 internalization in both peripheral ganglia and carotid body. Our findings suggest that NT produced by type I cells act in an autocrine or paracrine way on the same cell type, playing a modulatory role on chemoception.

The extracellular signal-regulated kinase signalling pathway in the human carotid body and peripheral ganglia

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Extracellular signal-regulated kinase (ERK) is activated by phosphorylation and links various extracellular signals to nuclear events. We studied ERK expression and activation, by means of anti-ERK and –pERK immunohistochemistry, in carotid bodies and peripheral ganglia (sympathetic, Gasser's and dorsal root ganglia), sampled at autopsy from 8 adults (mean age 52.5 years) and 4 foetuses (mean gestational age 177 days). Mean percentages (± Standard Deviation) of ERK- and pERK-positive glomic type I cells were higher in adults (59.4±8.5% and 15.9±5.1%, respectively) than in foetuses (19.5±8.6% and 5.3±2.8%). It may be hypothesised that the ERK signalling pathway in the carotid body is activated by neuromodulator/neurotrophic factors and plays a role in producing long-term cellular modifications. The immaturity of the ERK signalling pathway in foetal glomic cells may be ascribed to the lack of pulmonary respiration and of the regulatory role of the carotid body. Higher ERK immunoreactivity was also found in adult sympathetic and somatic ganglia (70.6±10.6% and 51.1±13.6%) than in foetuses (59.0±11.0% and 22.3±8.8%). The pERK immunoreaction was detected only in adult sympathetic and somatic ganglia (9.6±3.7% and 9.9±4.9%), demonstrating that this pathway is not yet fully operative at a foetal stage.

A proteomic–based investigation identifies lamin A/C Ser404 as a bona fide nuclear substrate of Akt/Protein Kinase B

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Akt is a central activator of multiple signalling pathways that phosphorylates substrates coupled to a large number of stimuli. A proteomic–based search for nuclear substrates of Akt was undertaken, exploiting 2D-electrophoresis/MS in combination with an anti-Akt phosphoSubstrate antibody. This analysis identified pre-lamin A Ser404 as a new nuclear substrate of Akt. Mutations at several sites in A-type lamins cause autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD-2). We show here that Akt failed to phosphorylate pre-lamin A in primary cells from an EDMD-2 patient with lamin mutated in the Akt consensus motif. Remarkably, expression of S404A pre-lamin A in primary cells from healthy tissue was sufficient to cause the same nuclear abnormalities that are an hallmark of the EDMD-2 primary cells carrying the above mutation. Our data demonstrate that pre-lamin A is a novel nuclear substrate of Akt, and implicate Akt phosphorylation of pre-lamin A in the correct assembly of the nuclear lamina.

Calcific patterns in atherosclerotic aortic walls versus stenotic aortic valves. Modified histochemical reactions and immunohistochemical localizations.

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A relevant issue in the study of ectopic calcification affecting different vascular tissues is to elucidate whether and how much common pathogenetic mechanisms, calcific factors and degenerative events are shared mineralization hallmarks.[1] Previously, in calcifying subdermally implanted aortic valves, modified Cuprolinic Blue reactions revealed a distinct cell degenerative pattern culminating in clustering of acidic lipids at cell surfaces with subsequent blebbing of matrix-vesicle-like bodies, acting as major apatite nucleators.[2] Additional co-localization of calcium-binding sites, as ultrastructurally revealed by von Kossa reactions,[3] and of immunogold labelled Annexin-V was also found. In the present work, the above procedures, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method, and immunohistochemical reactions for calcium-binding proteins annexin-V, osteopontin, osteonectin and tenascin-C were used to study calcifying aortic wall and stenotic valve samples. Peculiar CB-and von-Kossa-reactive lipidic layers comparable with those in the experimental model actually resulted as degenerative forms also characterizing these two calcification types. In addition, the whole calcification patterns showed some similarities but also distinct dissimilarities to exist by comparing experimental model, vascular wall atherosclerosis and aortic valve disease.

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Simultaneous fluorescence immunophenotyping and interphase cytogenetics (fiction) in multiple myeloma.

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Multiple myeloma (MM) is a disease characterized by a proliferation of malignant plasma cells (PC) in the bone marrow; survival rate varies greatly but it is generally poor and related to abnormalities of specific chromosomes, as translocations involving the Ig heavy chain gene at 14q32 or partial/total deletion of chromosome 13. Conventional cytogenetics of MM is difficult owing to the low proliferation rate of malignant PC; it has to be considered poorly informative if not associated to separation or immunophenotyping techniques. We present the results of FICTION analysis of patients at the beginning of the disease. This technique combines immunofluorescence and FISH analysis on interphase cells. Immunophenotyping was performed by a primary antibody against CD138 (also called syndecan 1), a heparan sulfate rich membrane glycoprotein expressed on normal and malignant plasma cells only, followed by a secondary antibody AMCA-labeled. Interphase FISH experiments were performed on the same slide using a probe (Texas red-labeled) specific for the RB (13q14.2) locus, associated with Tel (13q) DNA probe (fluoresceine-labeled). *Results:* PC were detected by the intense signal of AMCA, while chromosome 13 probes were analyzed with Texas Red- and FITC-filters. The results obtained with FICTION procedure were compared with those obtained with the FISH analysis on interphase cells and chromosome samples using the same probes.

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Gliosis alters expression and uptake of spinal glial amino acid transporters in neuropathic pain

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Gliosis has been strongly implicated in the development and maintenance of persistent pain states. Astrocytic activation following chronic constriction sciatic nerve injury (CCI) results in behavioural hypersensitivity in mice, a neuropathic pain syndrome, including hyperalgesia, allodynia, and spontaneous pain. Here we demonstrate that in the dorsal horn of the spinal cord, gliosis was accompanied by clear changes of glial aminoacid transporters examined on postoperative days (pd) 3, 7, and 14 by Western blot, Immunohistochemistry and RT PCR. Spinal lumbar protein and mRNA levels of CGRP, CD11b, Glial fibrillary acidic protein (GFAP), Glycine transporter 1 (GlyT1), Glutamate transporter 1 (GLT-1), and Calpain-I, a Ca-dependent protease implicated in the cytoskeletal rearrangement and receptors degradation, were assayed. Both the expression and mRNA level of CD11b raises up to pd 3 to decrease to nearly basal levels on pd 7. In contrast, spinal GFAP mRNA and protein significantly increase on pd 7 lasting up to at least pd 14. Simultaneously, the expression of glial aminoacids transporters (GlyT1 and GLT-1) was reduced on pd 7 and 14 following CCI as revealed by Immunohistochemistry and Western blot. The up regulation of Calpain-I, well correlates with a high turnover condition. Interestingly, HPLC analysis on lumbar spinal cord tissue revealed the increase of glutamate and glycine concentration on pd 7 and 14, this result paralleled the alteration of expression and uptake activity of Glyt1 and GLT-1 transporters. This study clearly demonstrates that peripheral nerve injury induces an early spinal microglial activation that precedes macroglial activation. The reactive gliosis is represented by a delayed but sustained GFAP expression determining a massive cytoskeletal rearrangement. These changes correlates with a marked decrease of the glycine and glutamate glial membrane transporters, followed by a net increase of both neurotransmitters involved in glutamate NMDA receptor activation. It's possible that this cascade of phenomena could affect the glutamatergic system producing such synaptic plasticity changes responsible to determine and maintain a persistent pain state.

Study *in vitro* of platelet-derived growth factor (PDGF) receptor responsiveness to exogenous PDGF stimulation in human astrocytoma cells

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The large family of tyrosine kinase proteins, which behave as components of signal trasduction pathways, plays a central role in several biological processes, such as cell growth, metabolism, differentiation and apoptosis. In particular, platelet-derived

growth factor receptors (PDGFR) regulate several processes in normal cells including differentiation, proliferation and migration and are widely expressed in a variety of neoplasms. In astrocytoma, an increased expression of PDGF and its receptor has been described and a deregulation of PDGFR activity has been linked to development, proliferation and maintenance of these neoplasms. Despite many studies described an increased expression of PDGF and its receptor in different glioma cell lines, only a few have evaluated PDGFR functional capacity by measuring the proliferative response induced by exogenous PDGF. In the present study, PDGFRα expression was evaluated in human astrocytoma cell lines and tissue specimens by immunocytochemistry. The receptor responsiveness to exogenous PDGF was determined in astrocytoma cells with MTT assay. We observed that astrocytoma cells express PDGFRα and respond to PDGF action in a grade-dependent manner. The receptor resulted to be functional since it induces cell proliferation at different ligand concentrations. We can thus conclude that proliferative response of human astrocytoma cells is related to their malignancy and receptor status before PDGF stimulation, suggesting a role for PDGFRα inhibitors as blockers of malignant cell proliferation.

Immunohistochemical localisation of α-synuclein in the rat spinal cord

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α-synuclein is a 140-amino acid protein, richly expressed in the central and peripheral nervous system. It is closely related with Parkinson disease and various other neurodegenerative diseases. Recent studies indicated the presence of α-synuclein deposits within the spinal cord of elderly people.[1,2] Since no data are available in current literature we aimed to investigate the presence of that protein and its distribution patterns in the spinal cord of normal rats. Immunohistochemical protocols were applied using two new monoclonal antibodies (2E3 and 3D5, by Yu[3]), able to localize α-synuclein in different portions of neurons. Whereas 2E3 antibody mainly labelled the axons, the 3D5 antibody labelled the cell nuclei. Furthermore both the antibodies labelled the synaptic terminals. This may be due to a different capability of the antibodies to penetrate the cellular compartments or with a different conformation of the protein within the cells. α-synuclein immunoreactivity was found well distributed in the dorsal horn and especially in the lamina X of all neuromeres. Numerous α-synuclein positive neurons were also shown within the intermedio-lateral nucleus of thoracic tract. Even if the connections of lamina X are still poor understood, our results indicate that this area may be involved in some way with the beginning of neurodegenerative phenomena, according to the Brockmann theory of ascending pathogenesis of Parkinson disease.

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Molecular characterization of PI-PLCβ1 in myelodysplastic syndromes

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The involvement of the PI-PLCβ1 gene in erythroid differentiation lead us to investigate this gene in patients affected by high-risk MDS. It is still unclear what is the pathogenesis of the evolution of MDS into AML, even if an impaired regulation of the PI3K/Akt axis has been recently implicated in mechanisms related to the neoplastic transformation. By using FISH analysis, we have previously evidenced that, in MDS patients with normal GTG banding and a fatal outcome, the PI-PLCβ1 gene undergoes a monoallelic and interstitial deletion. In the present study, we performed a sequence analysis on MDS patients to investigate the PI-PLCβ1 alleles in the MDS patients. We have performed a relative quantification real-time PCR analysis on all of the MDS patients tested for FISH analysis at the time of diagnosis and during the treatment with 5'-Azacitidine. Furthermore, we have evaluated the expression of the PI-PLCβ1 gene on healthy donors and the HL60 cell line, which is useful for testing the accuracy of the technology because of its low expression of PI-PLCβ1. We have seen that all of the MDS patients have higher levels of the PI-PLCβ1 mRNA compared to the HL60 cell line as expected, but lower levels compared to the healthy donors. Furthermore, MDS blasts always express higher levels of PI-PLCβ1b mRNA compared to PI-PLCβ1a mRNA. Our data support the contention that in the MDS patients the PI-PLCβ1 gene expression levels could be responsible for an altered expression of the enzyme, hinting at a possible imbalance of the nuclear versus cytoplasmatic PI-PLC signalling which, in turn, could affect the cell cycle progression of MDS blasts.

Ancient bone remains sectioning in anthropological studies: some technical innovations.

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The proposed sectioning technique may be applied to ancient bone remains even if heavily damaged, so extremely brittles and difficult to manipulate, to obtain sections for optical microscopy. In effect, in forensic, paleopathological, historical and ecological studies is necessary to resolve lamellar or osteonic bone organization. The results of the common cold resin embedding protocol are scant when applied to remains heavily contaminated by biological agents, altered by combustion or water submersion. The histological technique protocol is: a) Dehydratation of the bone in 70% ethanol; b) Inclusion, inside a cylindrical block, by transparent poliestheric three components resin Implex (Remet, Ceretolo di Casalecchio-Bologna), to avoid the disruption of the structures during sectioning; c) Sectioning by hard tissues diamond circular blade microtome (Leitz 1600, Wetzlar). The proposed modifications are: a) Milder infiltration or polymerization conditions, to obtain a better penetration of the resin, thus a more homogeneous specimen to section; b) Fixation of the embedded specimen to a coverslip, before the sectioning, to facilitate the manipulation, thus obtaining 20 micron sections even from very brittle material. The section obtained can be examined by bright-field, polarization-interferential or auto-fluorescence microscopy and eventually stained. The lamellar organization

of the bone may be assessed also using a quantitative evaluation program (NIHS-Image J, freely distributed at URL http://rsb.info.nih.gov/ij/).

Extracellular matrix remodelling in human bronchial epithelial cells exposed to various crystalline silica polyformes

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Exposure to silica particulates causes pulmonary fibrosis associated with excessive extracellular matrix (ECM) production. Human bronchial epithelial cells were exposed to two samples of silica with a different crystal micro-morphology, Silica Powder (Silica P) and no powder Silica (Silica F), in order to compare cellular biological responses in terms of ECM production. To determine whether the silica-related fibrogenic effects were mediated by a fibrogenic factor, expression of basic fibroblast growth factor (FGF2) and its receptor was also evaluated. Scanning microscopy demonstrated Silica P samples had a very fine lamellar crystalline structure with an average size of 0.007-0.014 μm, while Silica F was characterized by larger rounded crystals, between 1-10 μm. Each silica sample promoted a different profibrotic microenvironment. Silica P stimulated collagen production and down-regulated metalloproteases, while Silica F did not affect metalloproteases and mainly stimulated collagen synthesis. Different effects on laminin and collagen V expression suggest Silica P and F may also confer different properties to meshworks of basement membranes, with an impact upon visco-elastic behaviour and dynamic lung parenchymal mechanisms. Since only Silica F induced an increase in FGF2 receptor number, the two silica samples had different effects on FGF2 signalling pathways. The data suggest that various micromorphology of Silica particles affects remodelling of ECM and molecular mechanisms of dust pathogenicity.

Sarcoglycan and dystroglycan expression in epithelial cells. An immmunohistochemical study.

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The sarcoglycans (α-, β-, γ-, and δ-) are four proteins that are parts of dystrophin-glycoprotein complex (DGC) and that cluster together to form a complex which is localized in the cell membrane of skeletal, cardiac and smooth muscle[1] conferring stability to muscle fiber membrane; in particular α- and γ-sarcoglycan are expressed exclusively in skeletal and cardiac muscle and then are specific-muscle, whereas β- e δ-sarcoglycan are more widely distributed in various tissues, both foetal and adult. A fifht sarcogycan, ε-sarcoglycan, homologous to a-sarcoglycan is widely expressed in most tissues and so is not included in the DGC complex.[2] Dystroglycans are a group of two proteins, α- and β-dystroglycan, parts of DGC, that are receptors for the basement membrane components and that link the extracellular matrix protein laminin-2 to the intracellular cytoskeleton. Previous studies demonstrated that dystroglycans are involved in linking basement membranes to epithelial and muscle cells and that play a key role for the maintenance of tissue integrity.[3] On this basis, studying long since these proteins on human skeletal and smooth muscle, we performed, for first time, an immunofluorescence study testing all sarcoglycan and DGC proteins on respiratory, digestive and urinary epithelium. Our results show a constant presence of all tested proteins in all types of epithelium. Considering that all these types of epithelium are submitted at continue mechanical forces and then are continuously subject to stresses, in our opinion, we can hypothesized, for the first time, that the presence of DGC proteins can play a important role in the mediation of force cohesion between the epithelial cells and between epithelial cell and basal lamina. This study open a new line of research, since it is intriguing to analyze the same proteins on other epithelium types which are innervated, as skin, in order to better define the role of innervation on proteins of DGC and the relationship between DGC and agrin, a crucial nerve-derived organizer of postsynaptic differentiation.

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Genetic effect of nicotine on *in vitro* non-syndromic cleft lip with or without cleft palate fibroblasts evaluated by DNA microarray studies

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Among craniofacial malformations, non-syndromic cleft lip with or without cleft palate (NSCLP) is one of the most common oral-facial clefts (OFC). Little is known about its etiology and both genetic and environmental factors are involved in the pathogenesis. Numerous studies showed a positive association between maternal cigarette smoking and OFCs but there is a lack of comparative gene expression analyses in NSCLP and nicotine treated (NT) human normal fibroblasts. By using DNA microarrays containing 19,200 genes, we identified 1923 genes in NSCLP and 554 genes in NT which were differentially regulated when compared to untreated normal fibroblasts. Then we aimed to identify possible convergences and/or divergences between NT and NSCLP expressions. In particular, we found a group of 50 genes that are modulated concordantly (5 up- and 45 down-regulated) in NT and NSCLP fibroblasts and a group of 29 genes that are discordantly modulated (15 up-regulated in NT and down-regulated in NSCLP;14 down-regulated in NT and up-regulated in NSCLP). The data reported are, to our knowledge, the first genetic portrait of human fibroblasts cultured *in vitro* with nicotine and, taken together, our results from NT and NSCLP fibroblasts can contribute to a better understanding of molecular mechanisms underlying abnormal CLP phenotype.

Immunohistochemical expression of some neuropeptides and CB1 cannabinoid receptor in rat lung

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The present study refers to our previous immunohistochemical research about the distribution of leptin, orphanin FQ, orexin A and CB1 cannabinoid receptor in some peripheral tissues where these messengers could carry on other physiological functions different from role played in the Central Nervous System (CNS) in wich as everybody knows involved in the hypothalamic circuits implicated in the regulation of food intake and energetic homeostasis. This study investigates the immunohistochemical expression of these substances in rat lung. We have examined proximal and terminal bronchioles, respiratory bronchioles and alveoli of normal rats. Specimens of lung were fixed in Bouin's mixture and embedded in paraffin; obtained sections were processed with anti leptin (Santa Cruz Biotechnology), anti orexin-A (Chemicon International), anti orphanin FQ (Chemicon International) and anti CB1 (Biosource Europe S.A.) by En-Vision + System HRP (AEC). Our results indicate that leptin, orphanin FQ, orexin A and CB1 receptor are expressed in all pulmonary structures examined, but with different intensity. In addition each substance is immunohistochemically expressed with different intensity in the various cytotypes of bronchiolar epithelium (Clara cells, neuroendocrine cells and ciliated epithelial cells). In the alveoli a discrete immunoreactivity appears in pneumocytes type 2 even though with different intensity for every tested substances. The results obtained provide a morphoistochemical basis to understand the new additional roles played by these substances in the lung with probable autocrine, paracrine and endocrine mechanism.

Lung histopathology in murine model of *Cryptococcus neoformans* infection.

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We evalued histopathologic changes in lungs of adult and aged wild type and r-IFN 1 female mice k.O. inoculated intrathracheally with *Cryptococcus neoformans*. Animals were sacrificed after 35 days postinfection and prepared for histological evaluation by using histochemistry and himmunochemistry techniques. In all infected animals we observed several morphologic changes dependent on the severity of the disease directly correlated with severity of immunocompromission. In moderately infected mice a dense and inflammatory cell infiltrate in alveolar epithelium was present, with criptococci, lymphocytes, plasma cells, histiocytes, and eosinophils. In immunocompromised mice an elevated number of large and melanized criptococci, ECF-1 positive macrophage, eosinophils at various stages of degradation, and Charcot-Leyden crystals were observed. Our data indicate that these characteristics are useful in order to estimate the severity of criptococcal disease in human infection of immunocompromised patients.

Sarcoglycan subcomplex in human smooth muscle of ureter with ipomobility. An immunohistochemical study

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Sarcoglycans (α-, β-, γ-, and δ-) are transmembrane components of the dystrophin-glycoprotein complex (DGC) which links the cytoskeleton to the extracellular matrix in adult muscle fibers. The mutations in each locus gene of sarcoglycans cause limb girdle muscular dystrophy (LGMD), or dystrophies dystrophin-positive, in human skeletal muscle; this condition provides evidence that they seem to be functionally and pathologically important as dystrophin.[1] A widely expressed fifth sarcoglycan with significant homology to α-sarcoglycan, ε-sarcoglycan, has been identified.[2] This sarcoglycan is expressed in both muscle and non-muscle cells, and in embryos as well as adults. Recently, ζ-sarcoglycan, a novel sarcoglycan highly related to γ- and δ-sarcoglycan, has been identified. Previously, it is hypothesized that ε-sarcoglycan might replace a-sarcoglycan in smooth muscle, forming a novel sarcoglycan subcomplex consisting of ε-, β-, γ-, and δ-sarcoglycan. Although numerous studies have been carried out on sarcoglycan subcomplex in smooth muscle, there are discordant hypothesis about the composition of this complex. The only common assumption seems to be the tetrameric arrangement of the sarcoglycan subcomplex. On this basis, in previous our studies,[3,4] we demonstrated, by immunofluorescence and rt-PCR techniques, that all sarcoglycans are expressed, with larger of lower expression, in all districts of human body, hypothesizing a pentameric or exameric arrangement of subcomplex. The larger or lower expression of single sarcoglycan could characterize the distinct districts, according to function and motility of smooth muscle fibers. In order to confirm our previous hypothesis, here we studied, by immunofluorescence and rt-PCR, the smooth muscle fiber from human ureter with ganglia pathology that presents a ipomotility. Our results showed a constant presence of all sarcoglycan and a lower expression of e-sarcoglycan than a-sarcoglycan and other sarcoglycans. This condition seems to confirm our previous hypothesis demonstrating that the larger or lower expression of sarcoglycans depends by motility of smooth muscle and that each sarcoglycan, in exameric structure can be more expressed in order to replace a loss of other sarcoglycans.

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Immunohistochemical localization of cystic fibrosis transmembrane regulator in taste receptor cells of rat circumvallate papillae

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Taste receptors cells (TRCs) are organized into taste buds embedding in the epithelium of palate, tongue, larynx and pharynx. Several studies have demonstrated that TRCs involved in sweet as well as bitter responses expressed α-gustducin, an α subunit of a G-protein complex. However, this typical taste protein has been identified in other cell types including the chemosensory cells of the gastric and pancreatic mucosa and of the respiratory apparatus. Recently, we have shown that α-gustducin was localized in epithelial cells of the airways where it was also coexpressed with cystic fibrosis transmembrane regulator (CFTR), a Cl- channel which allows transepithelial salt absorption as well as secretion.[1] This finding raises the possibility of immunohistochemical similiarities between taste and airway cells suggesting that TRCs might also express secretory markers. To verify this hypothesis, we performed immunohistochemistry using anti-CFTR antibody on rat circumvallate papillae. The results showed that CFTR was expressed in taste cells with a characteristic bipolar or pear-shaped morphology. The immunoreactivity was predominately in the perinuclear cytoplasm or in the apical process. By laser scanning confocal microscopy, CFTR expressing cells were also α-gustducin positive. These observations could indicate a possible α-gustducin role in regulating CFTR activity. The coexpression of α-gustducin with CFTR in taste cells confirms our previous results in the airways, lending further credence to the notion that chemoreception and secretion may be correlated process.

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PKCα mediated CREB activation in aged rat hearts exposed to hypoxia

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Both aging and hypoxia affect the morphology and the function of rat myocardial tissue. Moreover the heart tries to counteract the impaired function by activating specific signalling cascades. Recently the transcriptional regulation mediated by CREB, 43-46 kDa nuclear factor recognizing the highly conserved sequence known as cAMP responsive element (CRE), has been considered an important mechanism of gene regulation during aging.[1] It's activation, not only dependent on the cell type but also on the stimulus administered,[2,3] results from phosphorylation on serine-133 by several factors, such as PKC, Erk/MAPK and ATM.[4,5] In this study we investigated the possible role of CREB in the *in vivo* response of rat myocardial tissue to hypoxic and to age-related oxidative stress. CREB is activated in parallel to HIF-1α nuclear translocation in the young after hypoxia exposure, while this kind of response is not so dramatic in the old, neither in terms of CREB activation, neither in terms of HIF-1α expression and translocation, suggesting in the old the existence of impaired oxygen-sensing mechanism or adaptation to hypoxia. Moreover in the young a PKCα/Erk pathway seems to be involved in the activation of HIF-1α along with CREB, suggesting an attempt of the young to counteract the damage evoked by hypoxia, while in the old a PKCα/p38 MAPK/CREB pathway could determine the occurrence of aging and aged cell hypoxia response.

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Immunohistochemical evidence of caveolins-1 expression in the human fetal and neonatal striated muscle, but its absence in the adult striated muscle

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Caveolin-1 (Cav-1) is a 22 KDa protein, which exerts essential roles in the regulation of cell proliferation and in transmembrane transport processes. It is mainly expressed in adipocytes, smooth muscle, fibroblasts and endothelial cells. Its expression in striated muscle fibers is controversial. Indeed, most Authors have attributed Cav-1 detection in striated muscle to endothelial cells, adipocytes and fibroblasts secretion. Nonetheless, recent *in vitro* studies have shown that Cav-1 is expressed in L6 myoblasts and maintained during the differentiation process. In view of this, and, since only one study has heretofore explored Cav-1 expression in human striated muscle, the aim of the present study was to evaluate and to compare Cav-1 immunohistochemical expression in the human striated muscles of fetus, newborn and adult. Samples of skeletal muscles of different sites and of myocardium were taken at autopsy from 12 fetuses and 4 newborns and submitted to the immunohistochemical analysis for Cav-1 together with 10 samples of adult skeletal muscle. Myocardial fibers displayed a weak immunoreaction in all samples, from both the newborns and the fetuses, independently of the week of gestation. Conversely, skeletal muscle fibers were only labelled in specimens from fetuses at late gestation and from the newborns, whereas no immunoreaction was evidenced in muscles taken from fetuses at mid- gestation and in the adult samples. This novel and unexpected pattern of Cav-1 expression in human skeletal muscle suggests a role for Cav-1 in terminal differentiation processes, which need to be clarified by further studies.

Extracellular matrix and cardiac remodelling in post-ischemic end-stage human hearts

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The interest in the remodelling of cardiac extra cellular matrix (ECM) is due to the fact that heart failure is the only cardiovascular disease with increasing incidence and prevalence. Recent observations hypothesized that damaged cardiac tissue can remodel as well as regenerate. We investigated the expression of some ECM proteins in 10 healthy and 20 end-stage human hearts to assess their possible role in the regeneration of cardiac tissue. We observed increased expression of fibronectin, collagen I, collagen III and collagen IV, as expected, by immunohistochemistry and biomolecular methods. mRNA expression for the same proteins was investigated by RT-PCR which demonstrated significant variations in the mRNA expression for Tenascin-X as well as Laminin α2-chain. Tenascin-X, highly expressed in heart ECM where positively regulates blood vessels development, is increased in pathologic

hearts confirming the activation of vasculogenesis in heart failure. Laminin α2-chain is a component of muscle cells basal lamina and is decreased in damaged hearts, where cardiomyocytes are the major cell population involved in ischemia. Our findings support the hypothesis that failing hearts react to the ischemic damage through some important modifications of ECM proteins types and distribution. Nevertheless, in our pathological specimens this reaction was not able to completely restore the myocardium structure as regards ECM composition. Further investigations are needed to clarify why the involvement of ECM in cardiac repair cannot restore the physiological conditions.

Smooth muscle cell apoptosis in ascending aortic dilation with aortic valve stenosis: the role of cell-matrix interactions

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Smooth muscle cells (SMCs) apoptosis is a characteristic finding in aortic dilations. We examined the relationships between cellular and extra-cellular changes in the media of dilated aortas with valve stenosis. Our interest was appointed on the changes in the spatial pattern of expression of extracellular matrix (ECM) remodelling proteins and SMC changes. Aortic wall specimens were retrieved from the convexity and the concavity of 8 dilated and 10 aneurismal ascending aortas and from 5 heart donors for control. Morphometry, immunohistochemistry for ECM proteins involved in vascular remodelling, TUNEL detection of apoptotic cells were employed. Bmf (Bcl2-modifying factor), a pro-apoptotic protein responding to alterations of cell-matrix interaction (anoikis) was studied by western blot. The antero-lateral vessel wall showed more pronounced ECM alterations and apoptotic indexes both in bicuspidal and in tricuspidal aorta dilations, with greater increment in Bmf-Bcl2 binding in bicuspidal aortas. The complex balance between survival/proliferative stimuli (fibronectin and tenascin increase) and apoptotic/degradative signals (Bmf) has an important role in the progressive phases of aortic enlargement. The differences in apoptosis and remodelling between greater and lesser curvature suggest that mechanical factors (i.e. expected wall stress overload at the convexity with aortic stenosis) may concur to affect this balance. SMC apoptosis could be initiated by Bmf, a factor involved in cell death due to loss of cell-matrix contacts as in anoikis.

Immunohistochemical and molecular study of sarcoglycan subcomplex in normal human smooth muscle

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The evidence that sarcoglycans seem to be functionally and pathologically important as dystrophin, it has permitted to study these transmembrane proteins more attentively, and in last years numerous studies have been carried out on sarcoglycan subcomplex in smooth muscle. This subcomplex consists of four transmembrane proteins, α-, β-, γ-, and δ-sarcoglycan. The synthesis of all four of the sarcoglycans is required to ensure the proper localization of the complex to the cell surface membrane;[1] then the complex formation and the localization of sarcoglycan subcomplex require all four subunits since that the direct interaction beteewen the sarcoglycans has been demonstrated biochemically by co-immunoprecipitation.[2] A fifth sarcoglycan homologous to α-sarcoglycan, ε-sarcoglycan, is more broadly expressed, showing a wider tissue distribution, also in non-muscle tissues.[3] Previous observations demonstrated that in lung, this glycoprotein was associated with both alveoli and bronchioles and that the sections of urogenital and digestive tracts were ε-sarcoglycan positive.[3] Recently, a novel mammalian sarcoglycan, ζ-sarcoglycan, has been identified and it is a protein highly related to γ- and δ-sarcoglycan mediating membrane stability together other sarcoglycans. Numerous studies have demonstrated that all sarcoglycans can be organized in various subcomplexes but only common assumption of them is a tetramerix arrangement of sarcoglycan sub complex. On this starting point, in our recent immunohistochemical investivgation, carried out on surgical biopsies of human adult visceral smooth muscle, we showed that all sarcoglycans coexist in the same fiber, hypothesizing the presence of pentameric structure.[4] Addressing this issue, in the present work we extend our previous results, with immunofluorescence and molecular techniques, testing all districts of human body (gastroenteric, respiratory, vascular and urogenital tracts) in order to better verify the real arrangement of sarcoglycan subcomplex. Our results, that show the constant presence of all sarcoglycan in all districts, confirm a real pentameric, or considering ζ-sarcoglycan detected with rt-PCR, hexameric model of sarcoglycan subcomplex. This hypothetic new complex, formed by all sarcoglycans, could present a higher or lower expression of single sarcoglycan in conformity with muscle type, skeletal, cardiac, or smooth, or also in conformity with the origin of smooth muscle, gastrointestinal, urogenital, or respiratory tract.

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Relationship between the presence of high-risk HPV DNA and proliferation rate in cervical intraepithelial neoplasia

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The human papilloma virus (HPV) plays a crucial role in carcinogenesis of human uterine cervix; in fact, the infection with high-risk (HR) HPV genotype is closely associated with cervical carcinomas. HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins that alter some cell cycle regulatory genes. In cervical intraepithelial neoplasia (CIN) a correlation between HPV infection and cellular growth fraction determined by Ki-67 immunostaining has been documented, while no data are available regarding the relationship between the presence of HR-HPV and proliferation rate assessed by standardized AgNOR analysis. We have applied this latter histochemical method on 40 formalin-fixed and paraffin-embedded cervical biopsies including 20 low-grade (10 condylomata, 10 CIN 1) and 20

high-grade (10 CIN 2, 10 CIN 3) intraepithelial lesions, evaluating the mean area of AgNORs per nucleus (NORA). Moreover, in each case DNA was extracted (QIAamp DNA mini kit, Qiagen) and PCR-based HPV DNA assays (HPV-HS Bio, AB Analytica) and reverse dot blot genotyping (HPV-Strip, AB Analytica) were performed. HPV DNA was encountered in 16/20 (80%) low grade and in all high-grade intraepithelial lesions. A progressive increase of mean NORA value from low to high grade lesions was documented, and significant differences were noted when CIN 1, CIN 2 and CIN 3 were compared among them. Moreover, a significant difference in NORA value was found when low risk - and HR-HPV DNA cases were analyzed. We conclude that proliferation rate is strongly related to the presence of oncogenic HPV DNA.

The effects of encapsulation on the insulin secretion of NIT-1 cells

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Transplantation of pancreatic islet cells has been shown to be a potential modality of treating insulin-dependent diabetes mellitus. However transplantated cells are rapidly destroyed by immune rejection if not adequately immunoprotected. Over the last three decades, the encapsulation of insulin secreting cells within semipermeable membrane has emerged as a promising approach for type I diabetes treatment. Since its ability to forme a three dimensional network in the presence of millimolar concentration of divalent cations, alginate is the biomaterial commonly used in the fabrication of this immunoisolating membrane. In this investigation we characterized the NIT-1 mouse insulinoma cells in normal cell culture condition using morphological stains, flow citometry, clonogenic assays, growth curve and measuring the insulin secretion. Subsequently we investigated the effects of alginate encapsulation on the cell growth and on the metabolic activity entrapped NIT-1 cells. In particular, growth curves have been used to study the cellular growth while ELISA test has been employed to analyze the insulin secretion. Our data demonstrate that the rodent insulinoma cell retain the functional attributes of normal islets with the advantage that they are easy to isolate and to proliferate in culture. In addition our investigations show that, althoug with some important differences compared to free, the NIT-1 entrapped cells continue to proliferate and maintain their secretory function.

Primary cell cultures of colorectal cancer: histochemical characterization

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Colorectal cancer is a significant cause of morbidity and mortality in Western populations. Epithelial cells of colon play a key role in the patophysiology of a wide variety of large-bowel disorders, including colon cancer.[1] By combining a mechanical method with an enzymatic digestion[2] of surgical resections derived from our Clinical Centre, we obtained tumoral colon epithelium cell cultures. The cells proliferated under the chosen culture conditions and were maintained for several weeks, including subcultivation steps. We characterised the cell morphology by light and phase contrast microscopes and by immunoistochemistry analysis.[3] The tumoral origin of the isolated cells was proved by the detection of common tumor markers, such as p53 and CDX2.[4,5] We also demonstrated the preservation of the secretory function of the cultured cells over the time.[6,7] This validated model of primary epithelial cells from colon cancer will be used to understand the biological and pathological features of human tumoral colonic cells. This will be done by studying the expression of specific proteins in the tumor and by analysing mutations of specific genes in each patient to relate each genetic signature to a precise pharmacological response.

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Autofluorescence characterization of livers with accumulation of lipids

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In the liver transplantation practice, the I/R damage can be influenced by the tissue lipids accumulation degree, in relation with nutritional status or a steatotic condition. An accurate selection of donor livers is thus required within the short times required by transplantation. A powerful approach for a real time investigation of tissue morpho-functional conditions can be provided by autofluorescence analysis. Preliminary studies on human livers showed the dependence of autofluorescence parameters (signal amplitude and spectral shape response to irradiation) on the tissue steatotic degree. Aim of the work is to define the endogenous fluorophores responsible for this behaviour and the potential parameters exploitable for diagnostic purposes. Histochemical and photophysical characterization were performed on cryostatic tissue section of livers from normally fed and starved (24h) rats, as experimental model. Nile Red staining for lipids, and Gold Chloride reaction for vitamin A evidenced a greater amount of both lipids and vitamin A in starved rats. Microspectrofluorimetric analysis (366nm exc.) of unfixed, unstained tissue sections evidenced differences in both spectral shape and response to continuous irradiation between starved and fed rats. Spectral fitting analysis indicated vitamin A as the fluorophore mainly responsible for the signal decay much faster in starved than in control rats, in agreement with its photophysical and chemico-physical properties. The combined analysis of autofluorescence spectral shape and photofading kinetics are promising parameters of the lipids accumulation degree in the liver tissue.

Ageing affects transcription and splicing in rat hepatocytes

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Ageing implies a progressive deterioration of the concerted functions of molecular components, checkpoints and events needed for cell viability and proliferation. It has been demonstrated that ageing involves alterations in the pathways of gene expression, which are not necessarily associated with mutations but can imply impairments in pre-mRNA transcription and/or splicing. In eukaryotic cells, the events leading to the formation of mature mRNA are chronologically and spatially ordered and occur mostly on perichromatin fibrils (PFs), which represent the *in situ* form of nascent transcripts as well as of their splicing and 3' end processing. In previous studies we described an unusual accumulation of PFs in hepatocyte nuclei of old rats, suggesting altered pre-mRNA pathways. In this study we investigated, by means of immunoelectron microscopy, the presence of different pre-mRNA processing factors as well as the incorporation of bromouridine (BrU) in the hepatocytes of adult and old rats, in order to elucidate the nature of the RNP structural constituents which are stored in cell nuclei during ageing and the mechanisms responsible for their accumulation. Our results demonstrate both a decrease in pre-mRNA transcription and a slowing down of PF processing and transport in hepatocyte nuclei during ageing. On the other hand, in the nucleolus no modification in pre-rRNA transcription and early splicing seems to occur.

Modulation of wolframin expression among normal and diabetic human placentae

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The WFS1 gene, encoding a transmembrane glycoprotein of endoplasmic reticulum (ER) called wolframin, is mutated in the Wolfram syndrome, defined by the association of diabete mellitus, optic atrophy, and further organ abnormalities.[1] It has been demonstrated that disruption of the WFS1 gene in mice causes progressive β-cell loss in the pancreas and impaired stimulus-secretion coupling in insulin secretion.[2] However the physiological function of this protein remains totally unknown. We investigated immunohistochemical expression of wolframin in human placenta throughout pregnancy in normal and diabetic pregnant women. In normal placenta there was a modulation of wolframin throughout pregnancy with a strong level of expression during the first trimester and a moderate level in the third trimester. In diabetic women, the wolframin expression was strongly reduced in the third trimester of gestation. The pattern of expression of wolframin in normal placenta suggests that this protein may be required to sustain normal rates of cytotrophoblast cell proliferation during the first trimester of gestation. The decrease of wolframin expression in diabetic placentae may hypothesize that this protein is directly regulated by insulin concentration also in the placenta, suggesting that this protein physiologically maintain the glucose homeostasis in this organ.

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Distribution of wolframin in human foetus tissues

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Wolfram syndrome, also called DIDMOAD is a autosomal recessive disorder characterized by Diabetes Insipisus and Mellitus, Optic Atrophy, Deafness.[1] Genetic analysis has demonstrated that mutations in the WFS1 gene are associated with the DIDMOAD syndrome.[2] The WFS1 gene consists of eight exons coding for a membrane protein of 890 amino acids.[3] Recent studies localized wolframin to endoplasmic reticulum (ER)[4] suggesting a possible role in protein biosynthesis, folding, trafficking and/or regulation of Ca^{2+} homeostasis.[5] Wolframin protein could play a role in modulation of apoptosis that arise from impairment of ER.[6] We used immunohistochemistry to determine the localization of wolframin in a panel of different human foetal tissues (14-35 weeks). Wolframin was ubiquitously expressed in many organs although with different tissue distribution and expression levels. In almost all systems, wolframin expression was faint at the 14-16th week and progressively increased when development proceeded. In conclusion, our data suggest that this protein may have important roles in the maintenance of cellular homeostasis.

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Human granulosa cells morphology: effects of GnRH agonist and antagonist treatment

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GnRH analogs have been widely used to inhibit gonadotropin pituitary release. Besides the effects on the pituitary-gonadal axis, studies have shown that GnRH has extrapituitary effects, particularly on rat and human ovaries. The aim of this study was to assess morphological changes induced by GnRH agonist (Triptorelin) or antagonist (Cetrorelix) *in vivo* treatment on human granulosa cells (GCs) derived from 20 women undergoing an *in vitro* fertilization (IVF) programme. Two main GCs pools were detected with light- and electron-microscopy examination: large/pale cells with uncondensed chromatin, smooth endoplasmic reticulum and small lipid droplets in the cyto-

plasm, and small/dark cells with more condensed chromatin, abundant rough endoplasmic reticulum and big lipid droplets in the cytoplasm. Morphometric analyses demonstrated in the large/pale cells secretory granules with a mean area of 2.8 mm2, covering the 8.1% of the cell area (184.7 μm^2), whereas in the dark cells secretory granules had a mean area of 4.8 μm^2 and covered the 12.4% of the cell area (171.3 μm^2). Upon Triptorelin treatment large/pale cells were more numerous (83.2±21.2%) than small/dark cells (16.8±4.9%), whereas upon Cetrorelix treatment small/dark cells (54.5±4.7%) prevailed on large/pale cells (45.5±4.6%). In light of the antagonist-dependent significant drop in oestradiol and progesterone soerum levels and the concomitant rise in LH concentration, these results let us hypothesize that antagonist treatment can delay and/or prevent GCs differentiation towards a mature steroidogenic phenotype.

Nuclear-cytoplasmic cooperation in bovine oocytes can be investigated by germinal vesicle transplantation

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The biological processes sustaining cooperation between cytoplasmic and nuclear compartments and leading oocytes to achieve maturational and developmental competencies are still poorly understood. Our objectives were to evaluate the use of reconstructed bovine oocytes by means of germinal vesicle transplantation (GVT) as a cell model to study the interplay between intracellular compartments. Denuded oocytes were treated with IBMX and cytocalasin B, the GV was removed with a small amount of cytoplasm (karyoplast) and transferred in previously enucleated immature oocytes (cytoplast). Karyoplast and cytoplast membranes were electrofused and artificial oocytes were *in vitro* matured. Nuclear and cytoplasmic competencies were analyzed through assessment of meiotic progression and the analysis of cytoskeleton re-organization. The capability of reconstructed oocytes to generate an embryo was evaluated after parthenogenetic activation. Morphological evidences indicated that the correct distribution of microtubules and microfilaments was associated with a regular progression through MII demonstrating a proper nuclear-cytoplasmic cooperation. This was confirmed also by the embryonic developmental capability since 18% of reconstructed oocytes were able to reach the blastocyst stage. In conclusion our results demonstrated that artificial oocytes can represent a suitable tool to explore the biological cooperation between nucleus and cytoplasm in bovine female gametes. *Work supported by FIRST2006 and PRIN2005*

Confocal analysis of mouse-mater protein distribution in human developing oocytes

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MATER (Maternal Antigen That Embryos Require) is a mouse oocyte-specific protein, dependent on the maternal genome, required for early embryonic development beyond the two-cell stage, and at first identified as an antigen associated with ovarian autoimmunity in mice (OP1). The gene products expressed in oocytes play important roles in folliculogenesis, fertilization and pre-implantation development. However, later on during oogenesis, oocytes become transcriptionally inactive, maternal RNA is degraded and the activation of early embryonic development requires pre-existing maternal products from the oocyte. The Mater gene is a single-copy gene transcribed in growing oocytes and, although its transcripts are degraded during meiotic maturation, MATER protein persists into the blastocyst. Tong et al. (Nat. Genet. 26, 267-268. 2000) showed that Mater- null female mice are sterile. They exhibit normal oogenesis, ovarian development, oocyte maturation, ovulation and fertilization, whereas the embryo development is arrested at a two-cell stage. The null males are fertile. The aim of our study is to identify the presence of MATER protein using a specific antibody in human ovarian tissue and isolated oocytes, which were obtained after ovarian stimulation for *in vitro* fertilization procedures. The confocal analysis demonstrates that the oocytes of primordial, primary and multilayered follicles were strongly labelled by the anti-mater antibody (Ab) in human ovarian cryosections. The cytoplasm of the oocyte was strongly stained by the anti-mater Ab mainly in the cortical region. The nucleus was weakly stained and it showed a specific stain of the nucleolar domain in multilayered follicles. Follicular cells were unstained in the primordial and primary follicles whereas they were strongly stained in the multilayered follicles, especially at the nuclear level. The same staining pattern was found in the cumulus oophorus cells. At germinal vesicle stage the nucleus presented a similar appearance with a labelling of chromatin surrounding the nucleolus. Cytoplasm was also strongly stained. In ovulated oocytes anti mater Ab stained only the cytoplasm. We have also performed an immunogold electron microscopic analysis in human ovaries and isolated oocytes to identify the subcellular localization of MATER. In isolated oocytes gold particles, indicating the presence of MATER protein, were found consistently within the cytoplasm and microvilli. In the perichromatin domain a moderate labelling was observed. Moreover a mitochondrial labelling was evident. The presence of gold particles was also detected in corona radiata cells. The analysis of ovarian cortex revealed that MATER was mostly located in oocyte cytoplasm of primordial and primary follicles. A moderate mitochondrial and a low nuclear expression were found. These data agree with the results previously obtained in mouse ovary (Tong et al. Endocrinology, 145, 1427-1434. 2004) and suggest a role for MATER in cytoplasmic metabolism and signalling. MATER nuclear localization in the late phase of folliculogenesis suggests a regulative role of the protein in chromatin rearrangement, which occurs during this process.

The role of serine protease HtrA1 in placental pathologies: hydatidiform mole, choriocarcinoma and preeclampsia

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Several evidences confirm that the transcription of Serine Protease HtrA1 gene is highly regulated in human during development and in adult tissues,[1] suggesting that HtrA1 may exert its control on cell growth not only in neoplastic cells as tumor suppressor,[2] but also under physiological conditions. Intriguingly, it has recently been demonstrated that HtrA1 is upregulated during the progress of human pregnancy, suggesting an important role of this protein in this physiological state. In particular, its subcellular distribution has led to postulate that HtrA1 acts on different targets, such as intracellular growth factors or extracellular matrix proteins, to favour the

correct mechanism of trophoblast maturation during placentation.[3] For this reason, we have hypothesized the possible role of HtrA1 in Choriocarcinoma, Mole and Preeclampsia, diseases due to an incorrect formation/function of the placenta. By immunohistochemistry, we have studied the different expression of this protease in the mentioned pathologies. In Partial Mole, we have observed a modest level of HtrA1 staining in both layers, syncytiotrophoblast and cytotrophoblast, while in the first trimester of control placenta the staining is mainly revealed in the cytotrophoblast. The islands of Partial Mole are all positive, whereas in the first trimester the positivity is less equally distributed. Moreover, in the Partial Mole, circumferential collections of trophoblast are totally positive. In the Complete Mole, as in the Partial Mole, the positivity can be observed in both layers. But, as more pathological the villus is, as more the positivity decreases. From our preliminary data, Choriocarcinoma shows a decrease of HtrA1 staining. Moreover, in regard to Preeclampsia we have detected a stronger HtrA1 signal in the most severe cases, but these results need further investigations.

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Immunohistochemical expression of orphanin FQ, atrial natriuretic peptide and oxytocin in normal human seminal vesicles

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Oxytocin (OXT), atrial natriuretic peptide (ANP) and orphanin FQ (OFQ) are involved in homeostasis of several tissues in human as well as in other mammals. Eight normal human SV were obtained from our histopathologic files. In particular, they were obtained from radical prostatectomies for prostate neoplasm. In each case, histopathologic examination exclude tumour involvement of SV, as well as the occurrence of other pathologies. We performed an immunohistochemical study by streptavidin-biotin method, using primary polyclonal antibodies for OFQ (Chemicon, Cat. No. AB5515, dilution 1:500), ANP (Cymbus Biotechnology LTD, Cat. No. CBL66, dilution 1:600) and OXT (Chemicon, Cat. No. AB911, dilution 1:800). Immunoexpression was assessed from three different observers (MLU, MB and AG) in the different tissutal component of SV (epithelium; corion; muscular layer) by a -/+++ scale where (-) was absence of immunopositivity and (+/+++) was the intensity of immunoexpression. OFQ was expressed by epithelium (+++) and muscular layer (+). OXT was present into the muscular layer (+) and venulae of muscular layer (++). ANP was found in epithelium (+++) and muscular layer (+). This preliminary results may indicate that these peptides could be involved in homeostasis of SV, as already showed in other organs of genitourinary system; further studies may clarify also their involvement in SV pathophysiology and senescence.

Endoglin (CD105) expression in human fetal tissues

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Endoglin, a component of transforming growth factor (TGF) receptor complex, is a cell surface transmembrane glycoprotein which is present on vascular endothelium, syncytiotrophoblast and tissues macrophages. Endoglin is highly expressed in the neovasculature of regenerating and inflamed tissues or tumors in association with an hypoxic condition. During the intrauterine life, normal development of foetal tissues takes place in a relative hypoxic environment which is beneficial for organogenesis and vascular development. Therefore, in the present study we investigated the expression of endoglin in the first trimester human fetal tissues, when primitive intraparenchymal vascular system develops. Ten human fetuses, ranging from 12-14 weeks of gestation, were obtained from elective terminations of pregnancies. All tissues samples, fixed in 4% neutral-buffered formalin, were processed for immunohistochemistry using the avidin-biotin (ABC) immunoperoxidase method. After pre-treatment with proteinase K (S3020, Dako), sections were incubated overnight at 4C° with CD105 primary monoclonal antibody (clone SN6h, w.d. 1:50, Dako). Sections of human term placenta and renal cell carcinoma were also tested as positive controls. A marked endoglin immunopositivity was documented in the microvessels endothelial cells of the spleen, pancreas, lungs, heart, gastrointestinal apparatus and testis. Moreover, some mesenchymal stem cells also stained positive for endoglin in the lung and heart tissues. The role of endoglin on mesenchymal stem cells may be in mediating TGF-β signalling during differentiation in microenvironment of tissues. No immunoreactivity was observed in the liver, meninges and umbilical cord.

HEMA-DOWN regulates procollagen α 1 type I protein in human gingival fibroblasts

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Previous investigations have shown that free monomers such as 2-hydroxyethyl methacrylate (HEMA) are released by resin composites into the oral cavity, inducing cell damage. Although there are many studies about the cytotoxic effects of resin monomers the majority of them are based on cell proliferation assays while few investigations analysed the effects of resin based materials on the synthesis and expression of the main proteins produced by fibroblasts. The aim of this work was to evaluate the effects of a sub-lethal concentration of HEMA in the synthesis and expression of procollagen α1 type I (pro α1) protein produced by cells. Primary culture of human gingival fibroblasts were exposed to 3mM of HEMA for 24 h, 72 h and 96h. RT-PCR and western blotting analysis were carried out to evaluate the variability in the expression and synthesis of pro α1 protein. A real time PCR was performed to quantify the differences in the expression of pro α1 mRNA (COL1A1). Western blotting data demonstrate a strong reduction of the protein after 24 h of HEMA treatment, and any signal was detectable after 72 h and 96 h. RT-PCR shows an high level of COL1A1 mRNA after 24 h and a weak signal after 72 h and 96 h. Real time PCR demonstrated a reduction of 96% between controls and cells treated with HEMA starting from 24 h, while the reduction is of 99% after 96 h of exposition. These data demonstrate that sub lethal concentrations of HEMA can affect cell activity without inducing cell death. Real

time PCR is able to visualize differences in protein expression even after short times of resin exposition suggesting that biocompatibility should be evaluated by more sensitive tests.

Photosensitization with the fluorogenic substrate, hypocrellin B acetate induces multiple organelle photodamage in HeLa cells

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Hypocrellin B (HypB) is a powerful photosensitizer (PS) which induces cell photodamage and death following light irradiation at the proper wavelength. HypB has been chemically modified by adding acetate groups, in order to improve its intracellular accumulation. Once inside the cells, HypB-Ac acts as a fluorogenic substrate, the added chemical groups being removed by cellular esterases with restoration of the native photoactive HypB. HeLa cells were here used to investigate the photophysical and photodynamic properties, and the cytotoxic action of HypB-Ac. The addition of the acetate group facilitates the compound intracellular accumulation and the restored photoactive molecules redistribute in the cytoplasm in the form of both diffuse fluorescence and brightly fluoresceing spots which likely correspond to lysosomes (as suggested by the distribution pattern of Lysotracker). For a wide range of concentrations (10^{-8} to 10^{-5}M) no direct phototoxic effect of HypB-Ac on cells was observed, in the absence of irradiation. On the contrary, following incubation with 10^{-6} M HypB-Ac and irradiation with a light emitting diode (LED; Fraen, Milan, Italy) at 480 nm, dramatic changes take place in the cytoplasm: the lysosomes, endoplasmic reticulum, Golgi apparatus, as well as the cytoskeleton are especially involved and massive cell death was observed from 24 to 96 hr post irradiation. Organelle-specific PSs are known to be powerful cytotoxic agents. In the case of HypB-Ac, the ability to induce cell death apparently depends on multiple organelle photodamage as much as we have observed to occur with another fluorogenic substrate, rose Bengal acetate.

Correlative TEM/FEI-SEM analysis of type I and type III collagen and chondroitin sulphate in human predentin

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The aim of the study was to identify type I collagen, type III collagen and chondroitin sulphate within human predentin by means of a correlative analysis under high resolution SEM and TEM. Human dentin disks were submitted to either a pre-embedding or a post-embedding immunolabelling procedure using monoclonal primary antibodies anti-type I collagen, anti-type III collagen and anti-chondroitin 4/6 sulphate. Gold conjugated secondary antibody anti-mouse IgG and IgM were used to visualize sites of antigen-antibody reaction. Correlative labelling patterns were obtained for type I and III collagen and chondroitin sulphate under FEI-SEM and TEM. The FEI-SEM analysis revealed an intricate three-dimensional network of type I collagen and the presence of chondroitin sulphate clarifying the intimate relationship between the two main components of the predentine organic matrix. The TEM analysis revealed odontoblasts exhibiting intracellular labelling for chondroitin sulphate, which became more intense and diffuse over the predentine organic matrix. The same diffuse immunoreaction was revealed for type I collagen, whereas a weak immunolocalization of type III collagen was found scattered throughout the predentine layer and over the collagen fibrils. Pre-embedding and post-embedding immunohistochemical approaches have led to the visualization of collagen fibrils and chondroitin sulphate labelling distribution within the predentine layer. Further investigations are needed to elucidate collagen-proteoglycans interaction in the organic matrix of human dentin.

Adult human dental pulp stem cells (DPSC): preliminary observations for selecting and conditioning DPSC according osteogenic aims

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In order to approach an important problem concerning regenerative medicine, i.e. bone harvest for autologous grafting, we have recently planned a series of investigations involving the selection of mesenchymal stem cells, derived from adult human dental pulp, to commit towards osteogenic differentiating patterns, with the aim to obtain a potential source of autologous bone produced *in vitro*. Dental pulp was extracted from human permanent teeth (second or third molars) of adult patients (aged 18 to 25 years). After a gentle digestion with type-1 collagenase and dispase, the cells were cultured for 7 to 20 days and identified with three markers of stem cells: CD34, STRO-1 and c-kit, combined with fluorocromes and observed with confocal microscope. The choice to identify and to select these type of stem cells lays in the fact, already reported in literature, that CD34, STRO-1 and c-kit positive stem cells are capable to differentiate towards several mesenchymal/stromal-derived cell types, among which osteoblasts, myoblasts and adipocytes. The successive steps of our procedure will imply the separation of CD34+, STRO-1+ and c-kit+ cells by means of the *magnetic-beats method* and their *in vitro*-culture using a medium enriched with factors conditioning for osteogenic differentiation. Recently, some authors have obtained *in vitro* newly-formed bone tissue with a similar procedure; the novelty of our proposal is to applied a protocol of mechanical loading to the medium, in order to mime the shearing stress occurring *in vivo* in bone fluid compartment during the transduction of mechanical loads into biological signals. A computerized model of such protocol of culture showed the real possibility of obtaining *in vitro* bone formation to apply in regenerative medicine.

The performance of human periodontal ligament mesenchymal stem cells on xenogenic biomaterials

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Recent studies have shown that mesenchymal stem cells obtained from periodontal ligament (PDL-MSCs) are multipotent cells that have similar features of the bone marrow and dental pulp MSCs and are capable of proliferating and producing different types of tissue such as bone and tooth associated-tissues.[1,2] Human PDL-MSCs expanded *ex vivo* were induced to osteogenesis, seeded in three-dimensional biocompatible scaffolds (fibrin sponge, bovine and porcine-derived substitutes) and examined using light, scanning and transmission electron microscopy. Morphological observations showed extensive growth of cellular biomass partially covering the scaffolds after 4 weeks of incubation in mineralization medium.

These findings indicated that periodontal ligament can be an easily and efficient autologous source of stem cells with a high expansion capacity and ability to differentiate in osteogenic cells that can colonize and grow connected to bio-compatible scaffold.[3,4] It can be suggested that the use of PDL-MSCs for generating graft biomaterials is advantageous for bone tissue engineering in regenerative dentistry.

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Cleft lip with or without cleft palate: implication of the heavy chain of non-muscle myosin IIA

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Non-syndromic cleft lip with or without palate (CL/P) is one of the most common malformations among live births but most of the genetic components and environmental factors involved remain to be identified. Among the different causes, we considered MYH9, the gene encoding for the heavy chain of non-muscle myosin IIA, as a potential candidate because we found it abundantly and specifically expressed in epithelial cells of palatal shelves before fusion. After fusion we showed that, its expression level decreases and becomes limited to epithelial triangles before disappearing as fusion is completed. In order to understand whether MYH9 plays a role in CL/P etiology, a family-based association analysis was performed in 218 case/parent triads using SNP markers. Pairwise and multilocus haplotype analyses identified linkage disequilibrium between polymorphism alleles at the MYH9 locus and the disease. The strongest deviation from a null hypothesis of random sharing was obtained with two adjacent SNPs, rs3752462 and rs2009930, (global P value = .00001), indicating that MYH9 might be a predisposing factor for CL/P, though its pathogenetic role needs to be more accurately investigated.

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