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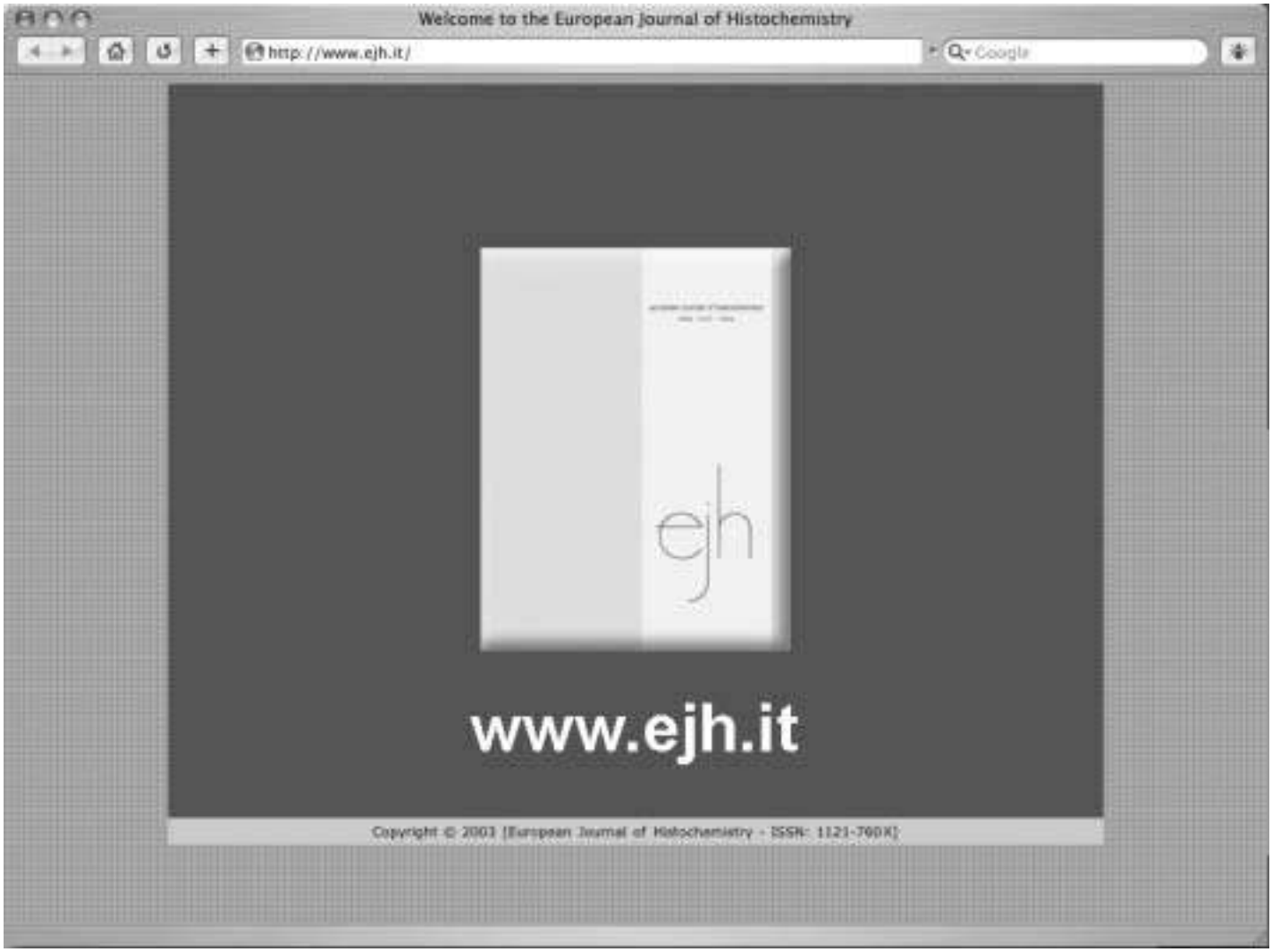
PROCEEDINGS OF THE 30TH NATIONAL CONGRESS OF THE ITALIAN SOCIETY OF HISTOCHEMISTRY

Rapallo (Genova, Italy),
Teatro delle Clarisse,
25-28 Maggio 2003

Editors
Carla Falugi, Grazia Tagliafierro,
Bianca Maria Uva

under the auspices of
the University of Pavia, Italy



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

Beridze T. Satellite DNA. Springer-Verlag, Berlin, 1982.

Mc Conkey DJ, Orrenius S. Cellular signaling in thymocyte apoptosis. In: Tomei LD, Cope FO, eds. Apoptosis: The Molecular Basis of Cell Death. Curr Comm Cell and Mol Biol, vol. 3. Cold Spring Harbor Laboratory Press, New York, 1991, pp. 227-46.

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OPENING LECTURE

A novel function for neuropeptides: the control of neurosteroid biosynthesis

H. Vaudry,* J.L. Do Rego,* D. Beaujean,* J.Y. Seong,° R. Fredriksson,§ V. Luu-The,# H.B. Kwon,° D. Larhammar,§ A. Burlet,+ M.C. Tonon,* G. Pelletier#

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Neuroactive steroids synthesized in the brain, called *neurosteroids*, represent a new class of neuromodulators that regulate various behavioral and neuroendocrine processes. Although the distribution of steroidogenic enzymes and the identification of the biosynthetic pathways leading to neurosteroid formation have now been almost completely elucidated, the neuronal mechanisms that regulate the production of neurosteroids remain almost totally unknown. Therefore, we have decided to investigate the effects of various neurotransmitters and neuropeptides on the biosynthesis of neurosteroids, using as a model the European green frog *Rana esculenta*. This project involves the characterization of the neurotransmitters or neuropeptides contained in nerve fibers that contact steroid-synthesizing neurons, the identification of the neurotransmitter or neuropeptide receptors expressed by steroidogenic neurons, the investigation of the effects of these neurotransmitters and neuropeptides on the biosynthesis of neurosteroids, and the pharmacological characterization of the receptors involved in their mechanism of action. Using this multidisciplinary approach, we have found that GABA, acting through $GABA_A$ receptors, inhibits the biosynthesis of 3-keto-17α-hydroxysteroids. We have also shown that the neuropeptides ODN and TTN (two endozepines) stimulate 3b-hydroxysteroid dehydrogenase-containing neurons through activation of central- and peripheral-type benzodiazepine receptors, respectively. We have recently observed that NPY inhibits the biosynthesis of pregnenolone sulfate (D^5PS) and dehydroepiandrosterone sulfate (DHEAS), and that this effect is mediated via the Y1 receptor subtype, while GnRH stimulates the formation of D5PS and DHEAS, and vasotocin activates the synthesis of D^4-3-ketosteroids and D^5-3β-hydroxysteroids. Since neurosteroids have been implicated in the control of a number of behavioral and metabolic activities such as response to novelty, food consumption, sexual activity, anxiety, depression, body temperature and blood pressure, our data strongly suggest that some of the neurophysiological effects of endozepines, NPY, GnRH and vasotocin could be mediated through modulation of neurosteroid biosynthesis.

Supported by INSERM (U413), France-Québec exchange programs (INSERM-FRSQ), a France-Korea exchange program (INSERM-KOSEF) and the Conseil Régional de Haute-Normandie.

MAFFO VIALLI AWARD LECTURE

Ultrastructural cytochemical analyses of nuclear functional architecture

S. Fakan
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The fine structural organization of the cell nucleus *in situ* has been extensively studied during the last four decades. Methods of ultrastructural cytochemistry, such as enzymatic extraction and preferential or specific contrasting techniques, allowed one to obtain information about the nature of major nuclear structural constituents. Combined with high resolution autoradiography making use of radioactive precursors of nucleic acids and proteins, this approach was essential for analysing the kinetics of these nuclear constituents and of the role of nuclear structural domains in nuclear functions. Further development of techniques of immunoelectron microscopy and molecular *in situ* hybridization, together with the use of nucleic acid metabolic precursors labeled with non-radioactive markers, have opened new ways for investigating functional features of different nuclear domains.

DNA replication. Chromosome domains

The use of non radioactive DNA precursors, such as BrdU, for labeling cells in culture, followed by immunocytochemical visualization on ultrathin sections, allowed one not only to confirm previous data provided by ultrastructural autoradiography (*reviewed in S. Fakan, The Cell Nucleus, vol. 5, p. 3, ed. H. Busch, Academic Press, 1978*), but to investigate at higher resolution the intranuclear distribution of newly synthesized DNA. Localization of replication sites following short labeling periods, or pulse chase experiments making use of different markers (IdU or CldU) showed that DNA synthesis takes place on the periphery of condensed chromatin areas and that the newly synthesized DNA is subsequently internalized into these areas (*F. Jaunin et al., Exp. Cell Res., 260, 313, 2000*).

In order to study individual interphase chromosome domains, cultured cells are labeled with BrdU for a period of S phase duration and subsequently let to grow for several cell cycles. This approach gives rise to nuclei containing only few labeled individual interphase chromosome domains. Although it does not allow one to analyse territories of specific chromosomes, it offers a high resolution tool for investigating individual chromosome domains with regard to their intranuclear neighborhood (*A.E. Visser et al., J. Cell Sci., 113, 2585, 2000*).

Transcription sites and RNA processing

Labeling of cells in culture, making use either of radioactive RNA precursors and ultrastructural autoradiography or of brominated precursors and immunocytochemical visualisation, showed that in the nucleolus, the dense fibrillar component contains newly synthesized pre-rRNA. In the nucleoplasm, the perichromatin region has been demonstrated as the major site of pre-mRNA synthesis and perichromatin RNP fibrils as in situ forms of hnRNA (pre-mRNA) transcripts (*D. Cmarko et al., Mol. Biol. Cell, 10, 211, 1999*). HnRNP core proteins, RNA polymerase II, poly(A) polymerase and a series of transcription and pre-mRNA processing factors have been shown to be associated with these domains, suggesting that they are also sites of most pre-mRNA processing steps. Many pre-mRNA splicing factors have been visualized in clusters of interchromatin granules. Since this nuclear domain is virtually devoid of newly synthesized RNA, hnRNP, various transcription factors and poly(A) polymerase, and it contains many splicing factors, it does not represent a major pre-mRNA processing site, but rather a place for accumulation and storage of splicing factors. Similarly, coiled (Cajal) bodies accumulate some splicing factors, but also factors involved in pre-rRNA processing. Moreover, their occasional association with nucleoli makes them an enigmatic structural domain and their role in nuclear functions still remains unclear.

In conclusion, ultrastructural cytochemical analyses of functional nuclear architecture provide essential information which cannot be obtained by other approaches. In combination with light microscopic studies on living cells, they certainly represent a major tool for future investigations.

NEW MICROSCOPIES AND HISTOCHEMICAL METHODOLOGIES

Flow cytometric detection of Bcl-2 and p53 intracellular antigens

B. Canonico, S. Papa, F. Luchetti, M. Della Felice, C. Felici, C. Masoni, F. Bastianelli, L. Zamai
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Several techniques has been proposed for flow cytometric evaluation of intracellular antigens (*Lanza et al., Cytometry 1997;30:134*). This approach is particularly important for detection at the single cell level of proteins which correlate to tumour progression. Bcl-2 and p53 are two of the most relevant proteins associated in this respect (*William et al., Cell 1991;65:1097-8*). In the present study we have compared five different cell fixation-permeabilisation protocols and nine fluorochrome-conjugated (FITC or PE) monoclonal antibodies (mAb): four mAb directed against Bcl-2 and five against p53. For detection of Bcl-2 we have analysed three Bcl-2 positive cell lines (K562, Daudi and MCF-7), and peripheral blood samples obtained from nine healthy subjects. To this regard, to distinguish internal positive (lymphocytes) and negative control cells (granulocytes), it was necessary to perform simultaneous detection of surface and intracellular antigens. For detection of p53 three cell lines, two p53 positive (*Raji and CEM*) and one p53 negative (HL-60), were analysed. Using these cells we have performed a combined analysis of the efficiency of monoclonal antibodies and sample preparation techniques. In conclusion, clones 124-FITC and Bcl-2/100-PE (Bcl-2), and clones BP53.12-FITC and G59-12-PE (p53) provided the highest specific fluorescence intensity of the respective markers independently on cell preparation protocols. Importantly, our results show that mAb background may depend on the specific fixation/permeabilisation kit and that mAb titration using negative and positive control cells is essential to determine the specificity and the sensitivity of the mAb used (*Zamai et al., J. Biol Regul Homeost Agents 2002;16:289*).

Structural-molecular changes of the neural tissue evaluated by autofluorescence microscopy

A.C. Croce,° S. Fiorani,° M.B. Pisu,* G. Bottiroli,°
G. Bernocchi*
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During postnatal development of rat cerebellum molecular and morphological changes lead to the definitive cerebellum cytoarchitecture. Normal development is altered by treatment with cytotoxic drugs, such as cisplatin. The cytostatic, injected to 10-days old rats, induced 24 h after treatment a high incidence of apoptotic cells in the external germinative matrix and a marked delay of Purkinje cell differentiation in neocerebellar lobules. Clear changes of the molecular features of developmental morphogens were also found (*Pisu et al., Guioli et al., Proceedings of FENS Forum, 2002*). Autofluorescence properties of biological tissues, strictly dependent to the nature, amount and distribution of endogenous fluorophores, in relation with structure (proteins) and metabolic activity (NADH, flavins), can provide a tool for a direct diagnosis of the morpho-functional conditions. Cerebellum autofluorescence was investigated for diagnostic purposes by means of microspectrofluorometry and high-sensitivity imaging, to study the involvement of the target layers at different tissue depth. Small differences in the spectral shape occurred among the layers of cerebellum of untreated rats. No appreciable differences were found among the layers of treated rats, that, in turn, exhibited a broadening of the spectra in comparison with the control. The spectral curve fitting, that allows to evaluate the contribution of each fluorophores to the whole emission, similarly to an in-situ biochemical analysis, indicated a relative increase in the oxidised flavins in treated rats.

The research was supported by MIUR Cofin grant 2002 to G. Bernocchi.

The exciting case of *two-photon*: from microscopy of the cell to single molecules

A. Diaspro
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Although conceived over twenty years ago and developed in its modern form ten years ago, two-photon excitation (TPE) fluorescence microscopy can be considered a young technique in far-field fluorescence optical microscopy, taking advantages over both widefield and confocal laser scanning microscopy (CLSM), for the study of the three-dimensional (3D) and dynamic properties of biological systems. Moreover, a comparatively new form of spectroscopy, related to the use of tiny excitation volumes, has grown together with the development of confocal and two-photon excitation microscopy. This is in tune with the fact that the advent of two-photon excitation microscopes fostered the application of techniques such as Fluorescence Correlation Spectroscopy (FCS), Fluorescence Lifetime (FL) to single biological molecules and to the characterization of tissues, second harmonic generation (SHG) from non centrosymmetric biological structures. There are two popular approaches to realise TPE imaging architectures based on a CLSM, namely: descanned and non-descanned mode. The former uses the very same optical pathway and mechanism employed in CLSM. Pinholes are removed or set to their maximum aperture and the emission signal is captured using the galvanometric scanning mirrors. The latter allows a significant signal-to-noise ratio increase. The signal is collected using dichroic mirrors on the emission path or external detectors without passing through the galvanometric scanning mirrors. More recently two-photon (and multi-photon) excitation with pulsed IR lasers has been exploited to enhance the background rejection and reduce the excitation volumes with respect to the more conventional single-photon confocal techniques allowing single molecule studies. Main features and components of the LAMBS (*Laboratory for Advanced Microscopy, Bioimaging and Spectroscopy*) TPE architecture will be outlined including applications to imaging of different biological specimnes and biomolecules. Emphasis will be also given on the charming possibility of multimodal imaging due to the mapping of lifetime, second harmonic generation and fluorescence polarization anisotropy. A special case of optical switching of a class of GFP will be discussed.

Acknowledgements: the LAMBS TPE architecture is supported by INFM grants.

Studying signalling in single living cells using genetic encoded biosensors with FRET, FLIM, SPIM and FRAP

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By introducing chimeric proteins containing green fluorescent protein (GFP) fused to signaling proteins into living cells and analyzing the properties of the fluorescence light emitted by these molecules with new fluorescence microscopic techniques, it is possible to protein-dynamics, cytoskeleton assembly, and protein-protein or protein-lipid interactions with high spatiotemporal resolution. Using 4D-confocal microscopy, microtubule dynamic instability was monitored and quantified in tobacco Bright Yellow 2 (BY2) cells. Strikingly, during preprophase band formation, the growth rate and catastrophe frequency of plant microtubules were doubled.[1] FRET microscopy (fluorescence spectral imaging microscopy (FSPIM) and fluorescence lifetime imaging microscopy (FLIM)[2] was used for analyzing heterodimerization of CFP- and YFP-tagged transcription factors in nuclei of single living cells,[3] and for studying aggregation of plasma membrane proteins. We recently initiated a research line for monitoring lipid-signaling dynamics using GFP-fusions of lipid-binding protein-domains such as pleckstrin-homology, C1- or C2-domains. Multicolor imaging allowed us to simultaneously monitor dynamics of multiple signaling events.

1. Dhonukshe P, Gadella TWJ Jr. Alteration of microtubule dynamic instability during preprophase band formation revealed by yello w fluorescent protein-CLIP170 microtubule plus-end labeling, Plant Cell 2003;15:597-611.
2. Gadella TWJ Jr, et al. GFP-based FRET microscopy in living plant cells. Trends Plant Sci 1999;4:287-91.
3. Immink RGH, Gadella TWJ Jr, et al. Analysis of MADS box protein-protein interactions in living plant cells, Proc Natl Acad Sci USA 2002;99:2416-21.

SEM analysis of the effect of transient fixatives on the blood cell membranes

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The chemical or physical stabilization (fixation) of biological samples represents a fundamental step in terms of protocol analysis standardization, it allows the setting up of right control samples in any routine activity and consents a variable delay between the taking and analysis of the specimen. Beside the long term stabilisation, it is now possible to apply a short term stabilisation that seems able, in terms of membrane antigenicity, to preserve blood cells at least for 10 days (Transfix®). This reagent was added to a donor peripheral blood in the indicated volume. Treated and untreated samples were maintained at 4°C for 10 days. After 0, 2, 4, 7, 10 days, samples were prepared for the Scanning Electron Microscopy. The Transfix® treatment seems to preserve the red blood cell shape even after 4 days, when the 50% of the untreated red blood cells appear deeply modified, assuming the echinocyte and poichilocyte aspects. After 7 and 10 days, 100% of untreated blood cells are in echinocyte form, whereas the fixative allow to maintain the regular cell shape in 50% of cells. These pure morphological data are fully in agreement with the cytofluorimetric evidences at the same incubation time, about the possible role in the temporary stabilization of the lipid – protein interface in the inner part of the cell membrane (*Bikoue et al., J Immunol Methods. 266:19, 2002*) due to the presence of heavy metals in the Transfix® formulation, without the creation of an antigen – masking lattice as usually happens by means of conventional non penetrating fixatives.

Fluorescence microscopy improved by light emitting diode excitation

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Fluorescence microscopy is largely based on the use of mercury or xenon arc lamps able to provide a wide spectrum of wavelengths from UV to the far red. For some particular applications like confocal microscopy as well as flow cytometry the peculiar characteristics of lasers have been applied with great advantages. Light emitting diodes (LEDs) since many years have successfully replaced conventional low power incandescent lamps in a large variety of applications. The gigantic progress of solid state technology has allowed the development and manufacturing of high power LED able to provide optical radiant flux of more than 0.5 W inside a narrow spectral band over almost the whole visible spectrum. Being almost all the LED emission included in a narrow spectral band (typically 15-25 nm) it makes sense to evaluate the new generation of these light emitters as possible excitation source in fluorescence microscopy. Two different series of "Luxeon" LED (Lumileds Lighting LLC) respectively of 1W and 5W have been tested. As light condenser, a special narrow beam optics has been supplied by Fraen Corporation. A variety of these special optical condenser have been on purpose developed and realized in plastic aimed to collect as much as possible light, from the emitting area, as a parallel beam to be sent to the objective lens. Experiments aimed to test both the optical efficiency and LEDs performances have been carried out on a fluorescence microscope (Leitz Orthoplan) originally equipped with a mercury arc lamp (Osram HBO 100W). Two different excitation conditions have been tested: 1) blue, and 2) green, with the respective filters/mirror settings. 1) BG12, TK500, 2) IF 546 (25nm), TK590 mounted in the epilluminator of the microscope. Instrumental and *visual* comparison between LEDs and lamp excitations have been performed. The first one had been carried out with a power meter located under the objective microscope, while visual comparison had been performed by means of various biological samples labelled with FITC and PI. The results obtained allow to conclude that the excitation performances of the two tested illumination systems are definitely comparable. Many derived advantages are evident: from the simplified, maintenance-free (better than 50.000 operating hours), low-cost excitation device, to the fully portable fluorescence microscope simply operated by rechargeable battery. The possibility of chopped excitation for fluorescence fading reduction as well as for long lifetime fluorescent probe applications is still under investigation.

Dual UV-blue excitation flow cytometry of the dye pair HO33342-PI allow the positioning of damaged cells along the cycle phases

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The discrimination of live/dead cells as well as the detection of apoptosis is a frequent need in many area of experimental biology. Furthermore cell proliferation is linked to apoptosis

since several genes during the cell life can stimulate proliferation while others may modulate the apoptotic pathway. Only few methods (i.e. TUNEL) are now available for studies on close correlation between apoptosis and proliferation. Therefore there is a great interest in methodological approaches able to correlate apoptosis to the cell cycle phases. Nowadays a large variety of methodological approaches have been proposed to detect and enumerate apoptotic cells by flow cytometry. Among others, those based on the cell membrane modifications induced in the early phases of the apoptotic process are the most established and applied. The dye pair Hoechst 33342 (HO) and Propidium Iodide (PI), thanks to their peculiar characteristics to be respectively permeable and impermeable to the intact cell membrane, seems to be very useful. Unfortunately the spectral interaction of these dyes generates a consistent *energy transfer* from HO to PI. Therefore the co-presence of the dyes in a nucleus results in a modification in the intensity of both the emitted fluorescence. In order to correlate the damaged cells (red fluorescence.) to the cell cycle phases (blue fluorescence.) we have tested different staining protocols aimed to reduce as much as possible the interference of the involved dyes. In a DHD/K12TRb colon carcinoma cell culture model we had been able to detect apoptotic cells as well as their location in the cell cycle phases using a very low PI concentration. By means of a flow cytometer Partec PAS, equipped with HBO lamp and argon ion laser, a double UV/blue excitation has been performed, able to discriminate blue (live) cells from the damaged (blue-red) ones even at 0.05 mg/ml PI. The same instrumental setting is going to be used for other multicolor analyses including annexin V-FITC as well as the possibility to make a correlated analysis to a phenotype marker.

Selective silver precipitation and malachite green uptake reveal calcium-binding sites and lipid involvement on calcified aortic valve thin sections

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Previous study on porcine aortic valves implantated in rat subcutis showed that glutaraldehyde-Cuprolinic blue reactions (GA-CB), at low pH, induce tissue unmasking from calcium and subsequent visualization of cellsand matrix-vesicles-like bodies outlined by peculiar, CB-reactive layers; because of anionic nature and differential chemical/enzymatic extra activity, these structures were assumed to be composed by acidic phospholipids (*Ortolani et al. Connect Tiss Res 2002; 43:44-55; Histochem J 34:41-50*). In the present investigation, pre-embedding GA-CB reactions followed by post-embedding von Kossa silver staining (GA-CB-S) showed major metal precipitation just occurring on the pericellular CB-reactive layers, and minor one at three additional sites: (i) nuclear heterocromatin; (ii) juxtacellular, filamentous material; and (iii) collagen fibrils. Moreover, glutaraldehyde-malachite green (GA-MG) pre-embedding reactions, at lowered pH, followed by osmium post-fixation gave rise to the appearance of pericellular osmium-MG-reactive layers, which were comparable to the silver-CB-reactive ones. These data show that a unique process of cell degeneration occurs in this calcification model, in which acidic phospholipids accumulate at cell surface replacing cell membranes and acting as major apatite nucleator. In addition, the overall data are consistent with the concept that common steps would exist for the various pathways in normal and pathological calcification.

GABA-A receptor subunits identified in paramecium by immunoflurescence confocal microscopy

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The presence of opioid, β-adrenergic and cholinergic receptors has been demonstrated in ciliated protozoa, but little is known about GABA receptors. This study is focused on identifying the γ-aminobutyric acid (GABA)A receptor subunits present in *Paramecium*. The ionotropic GABA-A receptors are heteropentameric complexes assembled from at least 21 subunits grouped into several classes: α1-α6, β1-β4, γ1-γ4, ρ1-ρ3, ε, λ, π, and θ subunits. Co-expression of an α, β and γ subunit is required to form a native GABA-A receptor. The type of α, β, γ, λ, ε, or θ subunit grants specific pharmacological or functional properties to the receptor and also determines the cellular domain to which the receptor can be targeted. A detailed mapping of GABA-A receptor subunits (α1, α2, α3, α6, β2/3, γ2, ε, λ, and θ) in *P. primaurelia* was carried out using subunit-specific antibodies. As analyzed by confocal laser microscopy, different subunits displayed the same punctuate labeling along the plasma membrane and throughout the cytoplasm. Double immunofluorescence staining showed that the majority of receptors in *P. primaurelia* contains a single α isoform and that δ and ε subunits can replace γ subunit. Furthermore, a co-localization of α, γ2 and β2/3 subunits allowed us to identify different GABA-A receptor subtypes.

Rose Bengal acetate as a fluorogenic substrate for photosensitization: intracellular location, organelle damage and induction of apoptosis

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Rose Bengal (RB) is a powerful photosensitizer (PS) due to its ability to produce singlet oxygen, under suitable excitation. The addition of an acetate group (RB Acetate, RBAc) improves drug uptake and inactivates both fluorescence and the PS properties. Inside the cell, specific esterases remove the Ac group thus restoring the native, active molecule. Previous studies on C6 cells indicated the endosomes and the endoplasmic reticulum as the first sites where the restored RB molecules are located; this is consistent with the internalization pathway involving membrane trafficking, and with the high esterase activity of these organelles. In this study, HeLa cells were used to investigate the dynamics of subcellular damage and the ability of RBAc to induce cell death. Cells were loaded with RB (10^{-6} to $5x10^{-5}$ M; 30 min to 12h) and irradiated at 540 nm (light doses: 0.2 to 1.6 J/cm^2). The induced cell changes were monitored under vital conditions, using time-lapse phase-contrast microscopy. Different cytochemical and spectrofluorometric techniques were used to detect the intracellular localization of RB and the organelle damage. Apoptosis already occurred under mild treatment conditions while, for longer exposure times, the cytoskeleton too was affected which suggests an intracellular relocation of RB during time.

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Pupil function engineering for confocal microscopy

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The ability to control and tune form and polarization of the point spread function of an objective lens is extremely desirable. It is well known, for example, that the imaging properties of both conventional and confocal optical microscopes may be tuned in a variety of ways by introducing apodising filters into the pupil plane of the objective lens. A particular example of importance is the ability to dramatically extend the depth of focus by the use of an annular pupil plane aperture without compromising lateral resolution. However the use of such a stop is optically very inefficient since most of the incident light is blocked by the annular filter and so cannot contribute the image signal. We will describe a light efficient method of producing annular or ring illumination and show how it may be introduced into two-photon microscopy. This may be of advantage since optical sectioning is unavoidable when two-photon excitation is used. As well as the ability to control the shape of the point spared function it is often useful to be able to control it's state of polarization. We will describe a method whereby we are able to tune the polarization state in the pupil plane in a very flexible manner. Both azimouthally and radially polarized beams will be considered by way of example. The ability to tune the strength of the axial component of the point spared function electric field by annular illumination will also be demonstrated.

CELL DIFFERENTIATION MARKERS: PLANT CELLS

Interactions between plants and mycorrhizal fungi: a dialogue among cells

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Mycorrhizas are the most common symbioses in the world: they involve about 6000 fungal species, distributed through all the fungal phyla and associated with the roots of 90% of plants, including forest and crop plants. Thanks to mycorrhizal symbiosis and nutrient exchanges, regulated by complex molecular signals, the plant improves its vegetative growth, while the fungus accomplishes its life cycle. Cellular analyses demonstrate that during the colonisation process the cellular organisation of the two eukaryotes is completely remodelled. Plant mutants impaired in arbuscular mycorrhizal (AM) symbiosis are a powerful tool to identify genetically defined steps in the development of the symbiotic interaction as well as to decipher the dialogue between partners during the early events of the interactions. Many *Lotus japonicus* lines have been generated which contain mutations in several *SYM* loci: these exhibit blocking of the colonisation process at various stages. The role of the *Lotus japonicus LjSym4* gene during the symbiosis with *Gigaspora margarita*, an AM fungus, has been investigated by using cell and molecular biology approaches on plants harbouring the mutant alleles *LjSym4-1* and *LjSym4-2*. In addition to a major block located at the surface of the epidermal cells, the mutant *LjSym4-1* shows a second block located at the wall of the cortical cells. The attempts of colonisation process of *LjSym4-2* by the fungus eventually result in death of the epidermal cells. Since RO species play a central role in the defence of plants, RO species were hypothesised to be produced during the early contact events. An important peroxide production was demonstrated by cytological analysis mostly inside the hyphae contacting the surface of the mutant *LjSym4-2* as well as the expression of a fungal gene coding for a regulated superoxide dismutase. The results suggest that the dialogue between AM fungi and plant hosts is a multistep process, where many checkpoints have to be overcome to make sure that the fungal partner is the right one to establish the symbiosis.

Ovule development in plants

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In recent years the ovule has emerged as a model system to study the genetic and molecular basis of organogenesis. The knowledge of ovule morphology and the understanding of the patterns of development are the first steps to study the mechanisms involved in ovule maturation and successful fertilization. The emergence of *Arabidopsis thaliana* as model system for a genetic and molecular approach to investigate developmental processes, enabled to identify a number of mutants and related genes involved in proper ovule development. However the basis of ovule specification is best understood in *Petunia hybrida* where two ovule specific MADS-box genes, FBP7 and FBP11 have been identify and characterized in detail. It has been demonstrated that these two transcription factors belong to the MADS-box gene family are necessary and sufficient to induce ovule development in *Petunia*. Simultaneous inhibition of the expression of these two genes by cosuppression led to the homeotic conversion of ovules into stigma-style structures. Furthermore, ectopic expression of FBP7 or FBP11 in *Petunia* induced ovule formation on the sepals and petals. We have study ovule development by making a comprehensive morphological description of ovule development in Petunia wild type and in the mutants in which these important factors have been down regulated or ectopically expressed.

Lipoxygenase localisation in strawberry fruits

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Lipoxygenases (LOX E.C.1.13.11.12) are a family of non-heme-iron-containing fatty acid dioxygenases that are ubiquitous in plants and animals. These enzymes catalyse the oxygenation of polyenoic fatty acids which contain the (1Z,4Z)-pentadiene system, to produce conjugated diene hydroperoxy fatty acids (HPO) (*Varvas et al 1999 J Biol Chem 15; Feussner & Wasternak 2002 Annu Rev Plant Biol, 53*). HPO are then metabolised via several secondary pathways to form a plethora of bioactive compounds collectively called *oxilipins.* In plants, oxilipins are reported to be related either to development or to the stress involved in wound healing, pest resistance or signalling, and they also seem to have antimicrobial and antifungal functions. In strawberry, LOX activity increases during receptacle ripening and highly reactive hydroperoxides are produced as a consequence, these load in part, to volatile aldehydes and alcohols biosynthesis which are putative signalling molecules thought to mediate plant-plant as well as plant-insect interaction (*Leone et al 2002 Hort Acta, in press*). The temporal and spatial expression of LOX during berry ripening seems to be of fundamental importance in providing clues as to the physiological function of various LOXs and LOX-derived products. In this study, histological and intracellular localisation of LOXs was detected by immunofluorescence using anti-LOX antibody and AntiIgG-AlexaFluor688 conjugated. Analyses of strawberry fruit sections, by confocal laser scanning microscope, showed labelling associated to membranes and lipid bodies in receptacle tissue, may be related to the modification and remodelling of the endomembrane system during ripening. LOX accumulation was also detected inside the achenes during strawberry fruit ripening.

Immunoelectron microscopy techniques applied to the study of plant diseases

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Immuno-electron microscopy (IEM) techniques were used in plant pathology both for diagnosis and the study of plant/pathogen interactions. IEM techniques, despite the difficulties involved in producing antisera (especially monoclonal antibodies), are useful evaluating pathogen biodiversity, distinguishing pathogens from host organelles when the former are very small and/or few and the host tissue necrotic, studying infections caused by two or more morphologically very similar pathogens, and localizing inside the plant cells substances

related to pathogen infections. Some different applications of these techniques are reported. Phytoplasmas, prokaryotic plant pathogens, are pleomorphic in shape, due to the lack of the cell wall; they are impossible to identify and classify using the traditional morphological methods. Phytoplasmas were labelled using IEM techniques, localizing the target epitopes, in infected leaf tissues of *Catharanthus roseus L.*, and *Chrysanthemum leuchanthemum L.* Another application of IEM is to probe in situ the product of plant/pathogen interactions, such as virus-coded non structural proteins present in infected cells. A classical example is provided by pinwheels and bundles, cytoplasmic proteic inclusions typically associated with viruses belonging to the Potiviridae group (i.e. Maize Dwarf Mosaic Virus = MDMV). Protein distribution in maize leaf tissues infected with MDMV alone and associated with a virus belonging to a different taxonomic group, Barley Yellow Dwarf Virus (BYDV) was analyzed. The third application was used to study the interaction between apple plants and the agent of Scab disease, *Venturia inaequalis* (Cooke) Aderh. A membrane protein produced by some resistant varieties of apple in response to *V. inaequalis* infection was detected using IEM techniques and a monoclonal antibody produced against a synthetic peptide based on the DNA sequence of the induced gene. In all three experiments, infected leaf samples were fixed in a formaldehyde/glutaraldehyde mixture, dehydrated in acetone and embedded in LR Gold resin. Then a post-embedding technique was used. Monoclonal antibodies against the pathogens (or proteins) and secondary antimouse antibodies coated with colloidal gold were used, and they gave a precise localization and identification of antigens in thin sections from infected leaf tissues.

Imaging the dynamic organization of organelles in living fungi

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A new perspective on the organisation and dynamics of organelles in hyphae is arising from studies involving live-cell imaging at high spatial resolution. We have developed 'lose-dose' imaging techniques using laser scanning confocal microscopy and two-photon microscopy which allow the analysis of living hyphae using a range of vital fluorescent probes without significantly compromising hyphal growth or organisation. Vital fluorescent dyes, or recombinant GFP specifically targeted to organelles or fused to proteins, have been used. Organelles that we are routinely imaging in living hyphae, and the best dyes we have found to stain these organelles, are: the Spitzenkörper (FM4-64), mitochondria (Rhodamine 123, FM1-43 and DASPMI), vacuoles (DFFDA, FM4-64) and the endoplasmic reticulum (ER-tracker). We have also used targeted GFP to image nuclei, mitochondria, ER and Golgi, and GFP fused to proteins to image microtubules and a protein phosphatase. Double labelling has proved very successful with certain probe combinations. Besides showing the organisation and dynamics of a range of different organelles in this presentation, emphasis will be placed on our understanding of how these organelles are integrated into the vesicle trafficking network during hyphal tip growth. We have recently produced an educational CD-ROM entitled *The Biology of Living Fungi*. This CD-ROM contains many of the movies shown in this lecture and can be purchased at minimal cost from the following website: *http://www.fungalcell.org/*.

Morphological and histochemical study on grape berry, fixed or cryostabilized

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Slices from ripen table grapevine berries (healthy or infected by *Botrytis cinerea* Pers.) were briefly fixed in buffered Formaldehyde 1% or alternatively cryostabilized in cold ethylene glycol (*Dore et al. 1999, Eur. J. Histochem. 43, suppl. 2*), thus avoiding any chemical fixation or solvent dehydration. Samples were then embedded in polar resin (Technovit 8100, Kulzer) and sectioned at 2.5 µm. Some histochemical techniques were tested, among them: Toluidine blue, Periodic Acid Schiff, Periodic Acid Silver Methenamine, DAPI for nuclei, Nile red for wax, Fe^{3+} salts for tannic substances. Moreover alkaline phosphatase (EC 3.1.3.1; Palk) and Polygalacturonase (EC 3.2.1.15; PG) activities were revealed using 5-Bromo-4-chloro-3-indolyl phosphate/Nitro BT (Sigma FAST BCIP/NBT) substrate for Palk and a new tetrazolium salt reduction method for PG (*Dore et al. 2001, Eur. J. Histochem 45, suppl. 1*). Enzymes activity was clearly detectable in the tissues of the infected berries, due to the penetration of *B. c. iphae*, always strongly positive. Both histological processing techniques allowed the localization and study of Palk, while PG activity was more evident in the cryostabilized samples. In healthy berries Palk was not detectable and PG was detectable only in the first cellular layers of the pericarp of cryostabilized samples.

Localization and quantitative determination of enzymatic activity in grapevine berries healthy and infected by *Botrytis cinerea* pers.

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The fruit soft and juicy structure and low enzyme concentration make difficult the histochemical revelation of Polygalacturonase (EC 3.2.1.15), one of the enzymes involved in fruit softening. The detection of the enzyme activity become easier in grapevine berries at the first stages of *Botrytis cinerea* Pers. infection, due to the strong activity of fungal enzyme. Grapevine berries healthy and infected by *Botrytis cinerea* were examined using ethylene glycol cryostabilization followed by inclusion in hydrophilic resin (GMA: Technovit 7100, Kulzer) (*Dore et al.,1999, Eur. J. Histochem. 43/suppl 2*). Slices of 2.5 µm thickness were incubated in a 0.5% solution of Polygalacturonic acid at room temperature; enzyme activity was revealed by the reduction of tetrazolium salt (TNBT). PG activity was also quantified in grape extracts measuring the diffusion of the enzyme by staining with ruthenium red in agar plates containing Polygalacturonic acids. In healthy berries, the histochemical assay showed enzymatic activity only in the first cell layers of the pericarp, very near to cell wall. In infected berries, PG activity was more diffused and evident mainly in the areas infected by fungal hyphae. Moreover in this way it was also possible to identify the areas of fungal penetration. The observations by LM were confirmed by quantitative determination: juice extracted from healthy berries showed very low activity, lower than 0.05 mg/mL, while in infected berries the activity was higher than 5.0 mg/mL. The histochemical data obtained using the fluorochrome Nile Red confirmed the lytic activity of *Botrytis cinerea* hyphae on the wax layer of the berry.

The ripening process studied using histochemistry on cryostabilized apple tissues

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We applied cryostabilization and polar resin (Technovit 7100, Kulzer) embedding technique (*Dore et al.,1999, Eur. J. Histochem. 43/suppl 2*) to *Braeburn* and *Red Delicious* apple cultivar, from the turnes dark colour to four months post-harvest. Some of the fruits were stored in relative humidity, O_2 and temperature (+4°C) controlled cellar, others were stored in a low temperature cellar (about +4/8°C). The good morphological preservation of fruit flesh eased the application of an optical micrometer to obtain quantitative evaluation of the thickness of cuticular wax and of the walls of pericarp cells in Toluidine blue stained slices. During the storage we observed an increase of wax layer and a decrease of cell wall thickness in both cultivar, but variations were more rapid in the apples stored in the cellar and the cultivar behaved differently. Our measures strongly support the agronomical observations about the influence of storage conditions on different apple cultivar: the economical storage in cellars can be applied only to *Braeburn* apples. Moreover we confirm the potentiality of hydrophilic resin histology and histochemistry to study the ripening and post-harvest processes in fleshy fruits.

Quantitative study of shade influence on hazelnut production using an hystological method

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A study designed to quantify the effect of shade on production was undertaken in two different training systems of the *Tonda Gentile delle Langhe* hazelnut in 2002. Five trees of each training system (free vase and double hedge) were used for the trial. Flower density for female inflorescences was recorded at bloom on four branches/tree in different compass directions. Fruits on the marked branches were counted in summer to obtain nut set. Stomata were counted in five leaves sampled from the upper and middle canopy of each tree. Three fields per peel were considered and mean stomatal frequency was calculated. A portion of about 1 cm^2 of each leaves was criostabilized using EG solution and embedded in polar resin GMA Technovit 7100 Kulzer. Slices of 2.5 µm thickness were examined using staining procedures such as Toluidine Blu and Nile Red. Leaf lamina highness and palisade tissue were measured using optical micrometer. Numbers of female inflorescence were similar (25.3/m in double hedge, 25.5/m in free vase), but nut set was different (17.7/m in free vase, 14.7/m in double hedge). The comparison between leaves of the two training systems showed some relevant difference in stomata percentage, leaf lamina and palisade tissue. Stomata percentage was higher in free vase than in double hedge (+4.9% in upper canopy and +9.2% in middle canopy). Leaves sampled on upper canopy have a leaf lamina larger in free vase than in double hedge (+16.1%), as well as palisade tissue (+29.1%). In middle canopy leaves measures of free vase were respectively higher of +26.6% and +19.9% than in double hedge. Free vase was the more productive training system, probably because leaves are well illuminated especially in middle canopy, as showed by histological measures.

CELL DIFFERENTIATION MARKERS: NEURAL CELLS

AM404, an inhibitor of cannabinoid transport, decreases pain induced fos-immunoreactivity in the spinal cord of rat

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The pharmacological treatment of chronic neuropathic pain is an important problem for the clinicians. It has been recently shown that cannabinoids, like anandamide, are involved in the nociceptive processes. In this study we evaluated the effects of the administration of AM404, an inhibitor of anandamide reuptake, monitoring the expression of c-fos, a protooncogene widely used as morphological marker of pain activated neurons. Experiments were performed on 40 male Sprague Dawley rats subdivided in two groups. The left sciatic nerve of the first group was tied, while the second group was used as control. All the animals were then subdivided in four groups for the pharmacological study: 1) untreated animals (saline injected); 2) AM404 treated; 3) untreated and stimulated by non-noxious stimulation of the left hindpaw; 4) AM404 treated and stimulated by non-noxious stimulation. Fos expression was monitored 14 day after tying and two hours after not-noxious stimulation. All animals were perfused, their spinal cords transversely cut by cryostat and treated for Fos-immunohistochemistry. Our results showed that non-noxious stimulation increased Fos-positivity in the dorsal superficial laminae of the spinal cord of tied rats but not in control animals thus indicating the presence of hyperalgesia in tied rats. The administration of AM404 significantly reduced the Fos induction. These results suggest that AM404 can be an useful drug to treat the neuropathic pain.

Emx2 in adult neural staminal cells

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Emx2 is a vertebrate homeobox gene involved in the control of central nervous system development. In the formation of the cerebral cortex, Emx2 expression is restricted mainly to the ventricular germinal zone fading away in the first post-mitotic neurons. This expression pattern, the severe alteration of the cortex in mutant mice suggest a role for Emx2 in controlling proliferation and migration of neural precursors. Emx2 persists troughout adult life in the neurogenic areas, hippocampus and subventricular zone. We have found that Emx2 is also expressed at high levels in adult neural stem cells *in vitro* and that its expression disappears when this cells diffrentiate. Overexpressionof Emx2 in neural stem cells *in vitro* has an antiproliferative effect but does not affect differentiation. Our results suggest that Emx2 may act promoting a mode of asymmetric division increasing the size of the transit amplifying population. Experiments aiming to identify genes downstream of Emx2 in neural stem cells inndicate that its overexpression leads to a decreased expression of genes related to stemness and to an increased expression of genes expressed in more differentiated cells.

β-tubulin immunofluorescence as a tool for the study of nervous system and ciliary apparatus morphogenesis during ascidian development

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Ascidian are primitive chordates, whose knowledge is basic for understanding the evolutionary development of higher chordates. Solitary ascidians release large numbers of bilaterally cleaving, determinative eggs. The embryogenesis proceeds rapidly: in *Phallusia mammillata* neurulation starts about eight hours after fertilization and in one day a conventional tadpole-type larva hatches. The nonfeeding larva swims for a few hours and then metamorphoses in a juvenile. A functional nervous system develops in the larva and results to be formed of two parts: the brain vesicle is located in the trunk, dorsally to the gut primordium, and contains two sensory organs: otolith and ocellus; the tubular spinal cord lies dorsal to the notochord and extends the lenght of the tail. We used an anti β-tubulin monoclonal antibody (clone 2-28-33) for immunofluorescence followed by confocal microscopy in order to study the morphogenesis of the nervous structures (primary neurons, ganglia, neural tube) during embryogenesis and larval development. Rhodaminated phalloidin has been used to put in evidence the filamentous actin of muscles and of hemocytes. The same techniques have been used in order to study the morphogenesis of the ciliary apparatus and of the nervous fibers connections during development of the syphons and of the gills in metamorphosing larva and in metamorphosed juveniles.

Serotonin neurotransmitter in *Balanus amphitrite* cyprid and its role in settlement

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Recently it was demonstrated that serotonin and dopamine are involved in the regulation of larval settlement (*Okano et al., 1996*); their presence was identified in the barnacle cyprids through HPLC only (*Yamamoto et al., 1999*). The aim of the present research was to study the presence and distribution of serotonin in the barnacle cyprid by immunohistochemical methods, and its effect on larval settlement by experimental tests. Cyprids were obtained from a laboratory culture of *B. amphitrite* collected in the gulf of Genova (Italy). Dewaxed sections of paraformaldehyde fixed cyprids were used for immunofluorescence and immunoperoxidase reactions using a polyclonal anti-serotonin antiserum. Serotonin-like immunoreactivity was localised in both neuron cell bodies and nerve fibres in the central nervous system. Immunoreactive nerve terminals were also detected at the periphery, at the base of the cement gland. To test its role during settlement, the cyprids were treated with different logarithmic concentrations of serotonin, fluexitine (serotonin re-uptake inhibitor), and dopamine. Settlement tests were performed according to standard Rittschof method: serotonin did not show significative effects (Dunnett test vs. control), while both fluexitine and dopamine treatment affect settlement. The immunohistochemical results confirm the role of neurotransmitter and/or neuromodulator for this bioactive molecule, while the experimental tests strongly suggest its involvement in the settlement process, probably exerting an role on the release of the cement gland secretion.

G6PD supports the NADPH cytochrome P450 reductase in the cerebellum Purkinje cells

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The glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting step of the hexose monophosphate shunt (HMS), which provides pentose phosphates for nucleic acid synthesis and NADPH for reductive biosynthesis. In previous works, we described the localization of G6PD in the cerebellum: G6PD was present in the granular layer, in the molecular layer, but expecially in the Purkinje cells. It has been demonstrated that Purkinje cells are able to synthesize neurosteroids. Since NADPH cytochrome P450 reductase is involved in the de novo synthesis of neurosteroids, we hypothesized that NADPH, produced during the HMS, supports the NADPH cytochrome P450 reductase activity. In this work, we studied the presence and distribution of G6PD and NADPH cytochrome P450 reductase in the cerebellum, by means of histochemical and immunohistochemical techniques. The highest concentration of both G6PD and NADPH cytochrome P450 reductase was found in the Purkinje cells; the molecular layer showed a similar diffuse staining for both enzyme; in the granular layer the Golgi cells had marked immunocytochemical staining of both proteins, whereas granule cells showed a scarce immunostaining for both enzymes. The intense and matched staining of G6PD and NADPH cytochrome P450 reductase in the Purkinje cells supports the hypothesis that both enzymes have a pivotal role in the synthesis of neurosteroids, which may be involved in the neuronal and glial growth and neuronal synaptic connections.

Distribution and ontogeny of FMRFamide-like peptide in *Balanus amphitrite*

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The tetrapeptide FMRFamide (Phe-Met-Arg-Phe-NH2), was immunohistochemically detected in the nervous system of different crustaceans. Few data are until now available about its presence during development. In the present study we considered by immunohistochemical method the time of appearance and distribution of FMRFamide like peptides in the nauplii, cyprids and adults of the barnacle *Balanus amphitrite*. Barnacles are one of the most primitive and highly specialised crustaceans. They are characterised by sessile adults, six naupliar planctotrophic stages and one lecithotrophic larval stage which is competent for settlement, the cyprid. The nauplii and cyprids were obtained from a laboratory culture of *B. amphitrite* collected in the Gulf of Genova (Italy). Immunopositivity was obtained using the polyclonal anti-FMRFamide antiserum (1:400, Affiniti, UK) on histological sections and whole mount specimens. FMRFamide-like immunopositivity was observed in the nervous system of both larval and adult specimens. Immunoreactive (IR) neuron cells were detected in the brain, suboesophageal and gut ganglia. Besides neuropil area, IR nerve fibres were found in the gut wall, near muscular cells. Furthermore in the larval specimens IR nerve fibres were also located in the terminal horns. hypodermal glands and in the paired compound eyes. These results point out that FMRFamide or FMRFamide-like peptides appear very early during development: in the larval stages it seems to be involved in sensory and gut functions, while in the adults in the regulation of gut functions only.

Immunohistochemical detection of FMRFamide related peptides (FaRPs) in the cnidarian *Eunicella singularis* and *Eunicella cavolini*

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Cnidarians have the simplest nervous system in the animal kingdom constituted by a diffuse peptidergic nerve net. Numerous peptides have been and they seem to play important roles as neurotransmitters and neuromodulators (*Grimmelikhuijzen, 1996*). The present work was performed to investigate by immunohistochemical methods the presence of FMRFamide-like peptides (FaRPs) in two sea-fans *E. singularis* and *E. cavolini* (Coelenterata: Octocorallia). Using SCUBA diving, fans shaped gorgonians were collected randomly from vertical rocky substrata near Punta Bacoli (Peninsula Sorrentina) and within a depth range of 20 to 25 m. The immunoreaction was carried out using an anti FMRFamide polyclonal antiserum (1:200, Affinity, UK). In both species a strong and widely distributed FaRP immunoreactivity was detected. Numerous FaRP immunoreactive (IR) cells were observed among the epithelial cells of the basal intestinal portions. Few FaRP IR cells were also located among the epidermal cells lining tentacles. Beneath these epithelia a rich FaRP IR network of neuron cells and nerve fibres were also seen. From our results we can confirm that these peptides can act as neurotransmitter and/or neuromodulator on muscular elements. Furthermore we can also hypothesize that they can act as local hormonal substances on neighbouring intestinal cells.

Neuropeptides and ontogeny of the nervous system

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Neuropeptides are essential components of the nervous system. In the brain, they are present in a high number and distributed in all the cerebral regions. They form specific nervous circuits and are involved in the regulation of nervous and neuroendocrine functions. Several neuropeptides act as regulators of cellular proliferation, neuronal growth and protein synthesis suggesting their involvement in neurotrophic and neuroprotective actions. This hypothesis is in agreement with their presence in the brain of embryos during the first developmental stages. Recently, the ontogeny of the following neuropeptides has been studied in the brain of frog: somatostatin, neuropeptide Y (NPY), pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP) and R-RFamide. The results indicate that these neuropeptides (i) appear in the brain early during development, (ii) some of them are transiently expressed in specific brain regions, (iii) their distribution gradually increase during the different time of development. The organization of the neuropeptidergic systems suggests that the neuropeptides may act, already during the first stages of development, as regulators of brain activities and may affect the maturation and differentiation of the brain.

Ontogeny of the [Pro2, Met13]somatostatin (SS2) precursor in the brain, pituitary and olfactory organ of the frog *Rana esculenta*

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We have previously demonstrated the occurrence of two somatostatin isoforms in the brain of the frog *R. esculenta* i.e. somatostatin-14 (SS1) and the [Pro2, Met13]somatostatin-14 variant (SS2). In the present study, we have used an antibody directed against the N-terminal flanking region of the SS2 precursor (PSS254-66) to investigate the ontogeny of the SS2 neuronal systems in the frog brain, pituitary and olfactory organ, from post-hatching to juvenile stages. PSS254-66-immunoreactive (ir) perikarya first appeared at stages IV-VII in the ventral hypothalamus and distal lobe of the pituitary. Ir fibers occurred at these stages in the olfactory bulbs, vomeronasal nerve, rostral telencephalon and hypothalamus. Subsequently, new ir cell bodies appeared in the nucleus of the diagonal band of Broca, anterior preoptic area and posterior tuberculum (stages VIII-XII), in the anterior periventricular preoptic nucleus and the intermediate lobe of the pituitary (stages XIII-XV), and in the medial periventricular preoptic n. and in the olfactory organ (stages XIX-XX). The occurrence of PSS254-66 immunoreactivity soon after hatching indicates that the somatostatin variant SS2 may exert neurotrophic activity. Some populations of PSS254-66-containing perikarya were not found in adult animals suggesting that SS2 may be involved in the control of proliferation, differentiation and/or migration of cells during ontogeny. The presence of positive fibers in several regions of the brain, including the olfactory bulb, telencephalon, periventricular preoptic nucleus and thalamus, suggests that SS2 may also act as a neurotransmitter and/or neuromodulator during ontogenesis. Finally, the existence of ir cells in the pituitary soon after hatching supports the view that, from this developmental stage, SS2, in addition to SS1, may play a role in the control of pituitary hormone secretion.

Ontogeny and distribution of ANF-like peptides in the brain of *Raja clavata*

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Atrial natriuretic factor (ANF) is a neuropeptide that occurs in various tissues with regulatory functions on the hydro-saline balance and blood pressure. Recently it has been proposed that ANF can exert also a regulatory role on neural cells proliferation and differentiation. For this reason the time of appearance and distribution of ANF-like peptides during development was investigated. *Raja clavata* eggs, laid in captivity in the tanks of Acquario di Genova, were kept until the desired stages, in the aquaria of DIBISAA (University of Genova) at controlled light and temperature. Coronal and sagittal brain serial sections of embryos, newborns and juveniles were immunostained by the polyclonal ANF antiserum (1:400 Affiniti). Computer aided 3D-representation was performed with the public domain software ImageJ *http://rsb.info.nih.gov/ij/*. Faint immunopositive (ir) neurons appeared in the 17 week embryos. They increased in number and immunopositivity in the tectum of mesencephalon and in the auricolae during development. Few and short nerve fibers appeared in the auriculae, in the mesencephalon and isthmus after hatching. ANF-like ir neurons decreased in the juveniles. ANF-like ir nerve fibers were detected around blood vessels only. The immunoistochemical data confirm that ANF-like peptides act as neurotransmitter or neuromodulator in the brain of *R. clavata*. Furthermore the presence of numerous ANF-like ir neurons during developmental stages suggest an involvement of this peptide in the organization and differentiation of mesencephalon tectum and auriculae cerebelli.

Chordate origin of the vertebrate central nervous system and evolution of regional identity

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Studies on the nervous system of protochordates have provided recent insights into the phylogenetic origin of the vertebrate nervous system. In particular it has been proved that several of the genetic mechanisms for establishing and patterning the vertebrate nervous system already operated in the ancestral chordate. At the same time, evidence from comparative morphology and embryology suggests the nature of the anatomical and mechanistic changes that must have occurred. Moreover the expression patterns of developmental genes allow to outline the main evolutionary pathways of the vertebrate rhombomeric segmentation.

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Rearrangement of the inhibitory and excitatory circuits in the rat developing cerebellum after injury

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Plasticity reorganization of neural tissue after injury is achieved by a cascade of processes that involve molecules and receptors. The aim of this research was the analysis of both morphological and neurochemical patterns of the dendrite development and synaptogenesis of Purkinje cells, after EGL (external granular layer) regeneration that has been recently observed in the late changes of cerebellar histogenesis after cisplatin treatment (*Guioli et al., Proceedings of FENS Forum 2002*). The main object was to analyse the immunoreactivity to markers of inhibitory and excitatory synapses of Purkinje cells, besides to markers of the Purkinje dendrite tree and molecular layer interneurons, at the late stage (20 days) after cisplatin treatment. In the neocerebellar (VI-VIII) lobules, mild granule cell ectopia and rearranged synaptogenesis patterns between Purkinje cells and interneurons were found. The feature of this reorganization, that represents a strategy to promote the recovery of functionality after damages, was obtained by labeling with anti-GAD65 and anti-GluR2/3 antibodies. A peculiar reorientation of Purkinje cell dendrite branches involves signal molecules besides the morfological changes previously shown after cisplatin treatment (*Scherini and Bernocchi, Prog Neurobiol 42, 161-196, 1994*) and ionizing irradiation (*Altman, J Comp Neurol 149, 181-192, 1973*).

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β-amyloid/presenilin/ubiquitin/[Ca^{2+}] system in aging brain

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The decline in brain performances characterizing aging may be associated with alterations of cell function following the activation of a plurality of signal pathways working in cascade. In particular, alterations in beta-amyloid/presenilin/ubiquitin/[Ca^{2+}] system characterize most of neurodegenerative diseases that appear during aging. In this view, we have undertaken a study of the changes in the expression of some proteins of this system considering different brain areas of 28-month-old rats compared with 4-month-old animals. In addition, we have considered 28-month-old rats raised, starting from 12 months of age, under *every-other-day* diet or with a diet additioned with N-acetylcysteine (NAC). After amyloid peptide immunocytochemistry, small inclusions were present in the cytoplasm of neurons of 28-month-old rats. In NAC-treated rats the inclusions increased in number and size. On the contrary, amyloid precursor protein and presenilin immunoreactions did not show any variation, demonstrating that the beta-amyloid cleavage system was preserved. In addition, in 28-month-old and NAC rats, the immunoreactivity for ubiquitin showed small ubiquitin deposits in the cytoplasm of neurons, with a pattern similar to that of beta-amyloid. After immunocytochemistry for the most representative calcium buffering proteins (i.e. calbindin, calretinin and parvalbumin) changes in the number of positive neurons in the neocortex and striatum of old rats in comparison with young animals were revealed. However, the changes were not consistent in all areas and for the different proteins. Intracellular ubiquitin positive inclusions may reflet a segregation of damaged and undegraded proteins. In fact, it is known that beta-amyloid can interfere and inhibit the proteasome activity. In addition, a perturbation of $[Ca^{2+}]_i$, as demonstrated by changes in calcium binding protein expression, may induce ROS production, which in turn are inhibitors of proteasome activity.

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Different expression of c-fos immunoreactivity in two experimental models of inflammatory pain

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Mono-arthritis induced by Complete Freund's adjuvant (CFA) in rat is an animal model which reproduces human inflammatory pain and which is widely used. In the literature two different protocols of injection: intraplantar or intrarticular (tibiotarsical joint) were reported. In this study we evaluated the different effects both of intraplantar and intrarticular CFA injection in the left hindpaw monitoring the Fos expression in spinal cord neurons. Fos is a marker of neuronal activity widely used as neuronal marker of pain activated neurons. Experiments were performed on 40 male Sprague Dawley rats subdivided into four groups. The first and the second group were injected with 150 µL CFA and saline respectively in the left hindpaw.The third and the fourth group were injected with 50 µL CFA and saline respectively in the left tibiotarsical joint. After 1 and 4 days the animals were perfused, their spinal cords removed, transversely cut by cryostat and the sections were treated by Fos-immunohistochemistry. After intraplantar injection we observed a clear increase in Fos-positivity both at one and four days, mainly in laminae I-VII. After intrarticular injection Fos-expression increased only at one day and the increase was high only in the caudal segment of the spinal cord. These results show that the time and distribution of neuronal Fos expression are different in the two models, suggesting a distinct pattern of neuronal activation of inflammatory pain.

Expression and localization of neuromarkers in the human salivary glands

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It is known that neurotransmission involves the release and action of more than one messenger, an event referred to as pluri-chemical transmission. The aim of this study is to examine the presence of putative neurotransmitters and other neuronal markers in the cell bodies and nerve fibers of human salivary glands. Pieces of tissue surgically removed owing to different diseases are immunostained using antibodies against choline acetyltransferase (ChAt), calcitonin gene-related peptide (CGRP), protein gene product (PGP) 9.5, somatostatin (SOM), substance P (SP), protein S-100, vasoactive intestinal peptide (VIP), tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DBH). Both indirect immunofluorescence and immunoperoxidase techniques are used to identify immunoreactive sites. ChAt is present in numerous cell bodies and in single fibres. A moderate presence of VIP immunoreactive fibres are found around acini and duct system. CGRP and SP positivity is observed in some single fibres. SOM positivity are found in very rare single fibres. The TH and DBH positivity is located in few bundles and single fibres adjacent to ducts, acini and blood vessels. Our results demonstrate that in the human salivary glands other neurotransmitters co-exist with conventional neurotransmitters to control the secretory activity and blood flow. The cathecolaminergic fibres (TH and DBH positive) could play an integral role in physiological adaptation and contribute to the homeostasis, particularly under the "stress conditions".

Calretinin-like calcium binding protein in the retina and optic lobe of octopus

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The octopus retina has a common morphological pattern with retina of the rest of cephalopds, but it ownes also proper features similar to those of vertebrates. It appears to be unique consisting of a simple layer of receptor cells which project to the optic lobe directly. Calretinin expression has not been studied in the Octopus visual system formerly. The aim of this study is to describe the distribution of calretinin-like immunoreactive elements in the retina and optic lobe and also to confirm their expression by non-isotopic hybridocytochemistry. Fresh specimen were processed for Western blot, and formalin-fixed, paraffin-embedded sections of retina and optic lobe were processed for immunocytochemistry and *in situ* hybridization. Positive results on Western blots of both retina and optic lobe tissues were obtained with a rabbit calretinin antibody. The molecular weight of the protein matched that reported for vertebrates. Immunoreactivity was found in the optic lobe's deep retina and the plexiform layer as well as in the photoreceptor proximal/supportive cell region, and these observations were confirmed by *in situ* hybridization. Our experiments provided evidence that the distribution of calretinin-like expression shows considerable specificity for retinal cells and optic lobe neurones.

CELL DIFFERENTIATION MARKERS: HAEMATOPOIETIC CELLS

Lectin cytochemistry of the colonial ascidian *Botryllus schlosseri* haemocytes

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Ascidian haemocytes are involved in many biological functions, such as immune defence, non-self recognition, tunic synthesis, catabolite storage, synthesis and secretion of humoral factors, asexual reproduction. Although widely studied, doubts still exist about their classification and functions. In the blood of the colonial ascidian *Botryllus schlosseri*, at least eight different cell-types are present, traditionally grouped in four categories, i.e. limphocyte-like cells, amoebocytes, vacuolated cells (morula cells and macrophage-like cells), storage cells (nephrocytes and pigment cells). Previous investigations on their histoenzymatic characteristics of *Botryllus* blood cells led us to distinguish two class of immunocytes, i.e. morula cells and their precursors, able to mount cytotoxic reactions against non-self cells or particles, and hyaline amoebocytes and macrophage-like cells, involved in phagocytosis of foreign material. To further characterise haemocyte-types and their mutual relationships, we used a series of lectins as cell surface markers. Results indicate that haemocytes share a similar response towards most of the lectins, being labelled or not labelled. Phagocytes have mannose residues, recognised by *Narcissus pseudonarcissus* agglutinin, on the membranes of their vacuoles, strengthening our previous hypothesis of a common differentiation pathway of these cell-types. Morula cells are the only haemocytes recognised by peanut agglutinin, specific for the galactose-N-acetyl-galactosamine residues, whereas granular amoebocytes show the presence of N-acetyl-glucosamine and N-acetyl-lactosamine on their surface. Only pigment cells showed labelling by *Ulex europaeus* agglutinin-I which recognises fucose.

HSP10 selective preference for myeloid and megakaryocytic precursors in normal human bone marrow

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Heat shock proteins (HSPs) are a family of proteins implied in cell homeostasis of many tissues. Indeed, they have many roles during cell life, as to respond to harmful insults, to process immune and inflammatory reactions, to regulate the cell proliferation and differentiation, to regulate the gene expression, and to control the cell death. HSP60 is a mitochondrial chaperonin, highly preserved during evolution, responsible of protein folding. Its function is strictly dependent by HSP10 in both prokaryotic and eukaryotic elements. Few works focus on their putative role in the regulation of cellular proliferation and differentiation of normal and pathological human tissues (1,2). We investigated the presence and the expression of HSP60 and HSP10 in a series of 20 normal human bone marrow (NHBM) by immunohistochemistry. HSP60 resulted negative, probably because of it is under the detectable threshold, using immunohistochemistry, in NHBM. By contrast, HSP10 showed a selective positivity for myeloid and megakaryocytic lineages. The positivity was restricted to precursor cells, while mature elements were constantly negative. These results parallel our previous observations and we postulate that this molecule could play a role in bone marrow cell differentiation other than be a mitochondrial co-chaperonin. Finally, our preliminary data address further investigations on the role of HSP10 during cellular homeostasis.

Expression of granulocyte macrophage colony stimulating factor receptor on human cardiac tissue from normal and heart transplantated subjects

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Several observations show the possibility that cardiomyocytes can proliferate and this proliferative capacity could be suitably controlled. Recently we demonstrated that some haemopoietic growth factors are able to modulate the proliferation and the cellular activity in some cell populations. Here we report the expression of the receptor for granulocyte-macrophage colony stimulating factor (GM-CSFR) on cardiac tissue from normal and heart trasplantated subjects, in order to evaluate an hypothetical role of GM-CSF in the cardiac remodelling. The expression of GM-CSFR was studied by immunofluorescence and by immunoblotting. Quantitative analysis was performed by scanner densytometry. GM-CSFR was variously expressed in cardiac tissue samples we examined. In particular the receptor migrated (by Western Blotting) in a band with apparent molecular weight of 84 Kda; immunopositivity is evident both on some cardiomyocytes and on endothelia. Our purpose is to better characterize such sub-population of GM-CSFR-positive cardiomiocytes in order to relate the expression of the receptor with the morfofunctional characteristic of cells. In particular, we will considerate if the GM-CSFR expression on cardiomiocytes may indicate the possibility to enter in a proliferative pathway or a kind of reaction to injury or if it is simply related with their differentiated state.

Cytochemical characterisation of free amoebocytes in the siphons of an ascidian

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We recently observed, over the tunic covering the inner side of both the siphons of the compound ascidian *Botryllus schlosseri* (Urochordata), the presence of granular amoebocytes, completely exposed to seawater. In order to understand the origin and biological role of these unusual amoebocytes we have investigated them by electron microscope and cytochemistry. They are mononucleate and with numerous round granules, various in content and have long filopodia, which contact the cuticle protrusions of the tunic in the siphon. Cytochemical, cytoenzymatic and immunocytochemical assays were carried out at light microscope on sections and in parallel experiments on fixed cultured haemocytes. Results showed that the phagocytic blood cells, i.e. hyaline amoebocytes, macrophage-like cells and signet-ring cells, and the free amoebocytes of the siphons, showed affinity to the a-mannose specific agglutinin of *Narcissus pseudonarcissus* (NPA), and exhibited the hydrolytic activities of acid phosphatase and non-specific esterases inside lysosomal vesicles and large vacuoles. No phenoloxidase activity, typical of the cytotoxic blood cell line (morula cells) of

this species, was detected. Moreover, anti-CD39 immunocytochemical assay for mammalian macrophages labelled the lysosomes of both phagocytes and free amoebocytes, whereas the positivity to anti-CD57 antibody for mammalian NK occurred in the morula cells but not in the amoebocytes. We consider these cells represent *sentinel-cells* belonging to the phagocytic line of the immune system since they share with the blood phagocytes the hydrolytic enzyme pattern, and the labelling by both NPA and anti-CD39 antibody, and can recognise and phagocytise target particles experimentally administered.

Stem cell activation and autofluorescence of the liver of MMTV-neu (erbB-2) mice

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Re-expression of fetal phenotypes was reported in the liver of tumor hosts. We studied de-differentiation markers and the autofluorescence of liver of transgenic MMTVneu mice (model of breast carcinoma). In control liver a normal differentiation phenotype was seen. The percentage of Vit A emission (~480 nm) was higher in pericentral (PC) than in periportal (PP) areas. The ratio of free to bound NAD(P)H peak intensities (oxygenation index) was higher in PP than in PC. Red autofluorescence (~670 nm) in Kupffer cells, ascribed to hemocateresis product(s), was in keeping with Perl's reaction. In transgenic mouse liver the oxygenation index was unaltered in PP and decreased in PC; the Vit A contribution to autofluorescence decreased and Perl's reaction was negative. Most hepatocytes were AFP^+. AFP is a growth-factor and biological response modifier for undifferentiated cells. PP and peri-Glisson regions promoted the arrest of $CD34^+$ cells and intense hemopoiesis, with erythroblasts displaying the red (~630 nm) autofluorescence of heme precursors. Hepatocytes in PP were $CK19^+$ and γ-GT^+. Portal tracts contained AFP^+, $CK19^+$, γ-GT^+, $CD34^+$ (oval cell markers) cells, and bile ducts showed intense proliferation. PC contained large dysplastic hepatocytes. Our data point towards tumor induced-stem cell activation, retrodifferentiation in PP, terminal differentiation in PC and presence of pre-carcinogenic features.

Neuroreceptor regulation of megakaryocyte differentiation from haematopoietic stem cells

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Megakaryocyte differentiation from haematopoietic stem cells requires strict regulation to ensure correct circulating platelet numbers. Numerous haematopoietic growth factors and cytokines are involved in regulating megakaryocyte growth and differentiation, however the precise signalling pathways are not clearly understood. Thrombopoietin (TPO) has been identified as the primary regulator of megakaryocyte development and mice lacking the TPO receptor (c-Mpl) display a significant reduction in megakaryocyte number. However, c-Mpl knockout mice do not exhibit major bleeding abnormalities and the megakaryocytes and platelets that remain are functionally normal, suggesting that other signalling pathways are involved in megakaryocytopoiesis and platelet release. One newly identified signalling molecule implicated in megakaryocyte differentiation is the neurotransmitter glutamate. Glutamate is the major excitatory amino acid in the central nervous system (CNS), which interacts with a range of ionotropic and metabotropic receptors to mediate key CNS function including synaptogenesis, learning and memory formation. We have recently demonstrated that primary human megakaryocytes express ionotropic NMDA-type glutamate receptor subunits NR1, NR2A, NR2D and NR3A, which form functional channels *in vitro* and *in vivo*. Megakaryocytic NMDA receptor signalling appears to be TPO-independent and operates through calcium calmodulin-dependent protein kinase II and Erk1/2 intracellular signalling pathways. Furthermore, $CD34^+$ hematopoietic progenitor cells, extracted from umbilical cord blood by magnetic immuno-isolation and cultured in the presence of TPO, fail to differentiate into megakaryocytes following NMDA receptor blockade. These findings suggest that neuronal-like signalling mechanisms are involved in haematopoietic stem cell specialisation and megakaryocytic differentiation, which may impact on current and future therapies for haematological disorders.

Mitochondrial ferritin expression in erythroid cells of congenital and acquired sideroblastic anemias

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We have recently reported an unusual intronless gene on chromosome 5q23.1 encoding a 242-AA precursor of a ferritin H-like protein. This 30-kDa protein is targeted to mitochondria and processed to a 22-kDa subunit that assembles into typical ferritin shells and has ferroxidase activity. This new mitochondrial ferritin (MtF) may play an important role in regulating mitochondrial iron homeostasis and heme synthesis. We studied MtF expression in patients with sideroblastic anemias (SA), disorders characterized by ineffective erythropoiesis with iron accumulation in the mitochondria of erythroblasts. Bone marrow erythroblasts of 11 normal controls and 23 patients, 7 with refractory anemia without ring sideroblasts, 13 with acquired idiopathic SA and 3 with hereditary SA, were analysed for the distribution of H ferritin (HF) and of MtF using immunocytochemical methods. About one fourth of normal erythroblasts showed diffuse cytoplasmic positivity for HF, but very few were positive for MtF. Similar patterns were found in anemic patients without ring sideroblasts. By contrast, in SA many erythroblasts were positive for MtF, which appeared as granules ringing the nucleus. Double immunocytochemical staining confirmed the different cellular distribution of HF and MtF. There was a highly significant relationship between the percentage of MtF positive erythroblasts and that of ring sideroblasts. RT-PCR studies demonstrated the presence of MtF mRNA in circulating reticulocytes of 2 patients with hereditary SA but not in controls. These findings suggest that most of the iron deposited in perinuclear mitochondria of ring sideroblasts is present in the form of MtF and that MtF might be a specific marker of SA.

Cyclooxygenase-2 expression during human megakaryopoiesis

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Cyclooxygenase (COX)-1 or -2 and prostaglandin (PG) synthases catalyze the formation of various PGs and thromboxane (TX) A2. We have investigated the expression and activity of COX-1 and -2 during human megakaryocytopoiesis.We analyzed megakaryocytes from bone marrow biopsies and derived from thrombopoietin-treated $CD34^+$ hemopoietic progenitor cells in culture. Platelets were obtained from healthy donors and patients with high platelet regeneration because of immune thrombocytopenia or peripheral blood stem cell transplantation. By immunocytochemistry, COX-1 was observed in $CD34^+$ cells and in megakaryocytes at each stage of maturation, whereas COX-2 was induced after 6 days of culture, and remained detectable in mature megakaryocytes. $CD34^+$ cells synthesized more PGE2 than TXB2, whereas the reverse was true in mature megakaryocytes. By immunostaining, COX-2 was observed in <10% of circulating platelets from healthy controls, whereas up to 60% of COX-2-positive platelets were found in patients. A selective COX-2 inhibitor reduced platelet production of both PGE2 and TXB2 to a significantly greater extent in patients than in healthy subjects. Finally, we found that COX-2 and the inducible PGE-synthase were coexpressed in mature megakaryocytes and in platelets. We conclude that both COX-isoforms contribute to prostanoid formation during human megakaryocytopoiesis and that COX-2- derived PGE2 and TXA2 may play an unrecognized role in inflammatory and hemostatic responses in clinical syndromes associated with high platelet turnover.

Influence of magnetic field exposure on the U937 cells differentiation

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The human pro-monocytic U937 cells can be induced to differentiation by a large number of substances: like TPA, DMSO, Zn and low glutamine concentration. During differentiation U937 cells shift from suspension to adhesion growth and as a consequence from a round shape with short microvilli, to a macrophage-like cells morphology. In addition differentiated U937 cells have an intense phagocytic activity. Taking into mind the increasing environmental electromagnetic pollution, aim of the present work has been the study of the U937 cells differentiation under 6mT static magnetic fields (SMFs) exposure, already reported to interfere with a typical process of differentiation, i.e. apoptosis, by modulating calcium ions influx. U937 cells differentiation under static magnetic fields was followed by Nitro blue tethrazolium (NBT) test, marker of macrophage differentiation, by conventional and confocal light microscopy and by electron microscopy observations. The higher degree of differentiation was achieved at the second day of incubation with TPA (DMSO, Zn and glutamine deprivation were less effective) in presence or absence of SMFs exposure. Fully differentiated cells attached to the culture dishes and formed long cytoplasmic protrusions. The cells differentiated under SMFs were more prone to attach to the substratum and showed a more elongated or stellated shape than those differentiated in absence of SMFs. As a consequence the cytoskeleton modified accordingly to the shape changes. Differentiation as well as exposure to SMFs affect distribution and quantity of sugars surface. In addition differentiated U937 cells can phagocyte latex particles as well as apoptotic lymphocytes, but the phagocytic activity was low in those cells differentiated under SMFs exposure. In conclusion the static SMFs exposure favos the adhesion but not the differentiation of U937 cells, as the modifications of the cell surface and the cytoskeleton indicate.

NK cell interactions during innate immune responses

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Recently it has been demonstrated that various NK receptors are involved in controlling the interactions of NK cells with other actors of innate immunity. in particular evidence has been provided for a potent cross-talk between NK and dendritic cells (DC) that results in a bidirectional circuit of activation/differentiation. this mutual control is dependent upon direct cell to cell contact involving different surface receptors and soluble factors. thus, while normal cells are usually resistant to the nk-mediated attack, a remarkable exception is represented by dendritic cells (DC). in their immature form (iDC), they are susceptible to nk-mediated lysis because of the expression of low levels of surface mhc-class i molecules. since the process of dc maturation (mDC) is characterized by the surface expression of high levels of MHC-class I molecules, mDC become resistant to NK cells. exposure to live bacteria induces rapid DC maturation and, thus, resistance to NK cells. the cross-talk between DC and NK cells is more complex and involves also a DC-dependent NK cell activation and proliferation. thus, two important players of the innate immunity may be involved in a coordinated regulation of critical events occurring at the interface between innate and adaptive immunity. regarding the possibility of exploiting NK cells in therapy, an important field of interest is bone marrow transplantation. a major role for alloreactive NK cells (i.e. donor's NK cells that are not inhibited by the HLA-class I alleles of the recipient) was shown in acute myeloid leukemia patients undergoing allogenic bone marrow grafting. in these patients, donor's alloreactive NK cells not only mediated graft-versus-leukemia (thus preventing leukemic relapses) but also abolished graft-versus-host responses by killing DC of the patient. these findings highlight important new perspectives on bone marrow transplantation.

Different expression of apoptosis during monocyte/macrophage differentiation induced by PMA

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Phorbol esters (e.g. PMA) are able to induce monocyte/macrophage-like differentiation in leukemic cell lines: in the monocytic THP-1 culture, here used, one of the aspects of differentiation process consists of cell adhesion to the growth substrate. In the present work we have analyzed (by cytofluorometric, immunofluorescence, SEM and TEM techniques) some cytokinetic, cytoskeletal and cell surface features during the progressive transition from cell suspension to adherent cell condition, obtained by treatment with increasing PMA concen-

trations (6-60 nM). These conditions of stimulation promoted a gradual appearance of cell adhesive contacts, microfilament redistribution, lysosome and endocytosis expression, and inhibition of cell proliferation. In this experimental model, cell death, induced by Actinomycin-D and Vinblastine, was analyzed by comparing some apoptotic expressions during the progression of cell differentiation (monocytes against macrophages with different adhesion ability). The apoptogenic action of Actinomycin-D (mainly evident in the sub-2c region) was shown to decrease with the progressive cell adhesion and differentiation: this resistance towards the cell death appeared to be related to the microfilament and microtubule network reorganization, particularly evident in the cultures with the greatest adhesive capability. On the other hand, the use of Vinblastine, an antimicrotubular substance, induced apoptosis with a higher frequency (mainly evident in the sub-4c region) in the terminally differentiated cells. These results suggest that apoptosis induction/expression can be strictly related to both the functional conditions of the cells (not adherent-undifferentiated phagocytes and adherent-differentiated phagocytes) and the different subcellular target of the drug.

CELL HISTOCHEMICAL MARKERS: NEOPLASTIC CELLS

Molecules belonging to the cholinergic neurotransmitter system in pleural malignant mesotheliomas.

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In recent years, neurotransmitter molecules were often found in non-nervous cells and tissues, undergoing differentiation. In particular, neoplastic cell growth is often accompanied by enhanced acetyl and pseudocholinesterase activity and gene amplification. Pleural mesothelioma (MPM) is a malignant tumour spreading in the chest cavity and sometimes in the lung. Asbestos exposure is the most common risk factor, but other promoting factors are known. Generally, the combination of smoking and asbestos exposure greatly increases the risk of this kind of cancer, due to the effect of nicotine on cell proliferation, mediated by both nicotinic and muscarinic acetylcholine receptors. Molecules belonging to the cholinergic system were identified by histochemical, immunohistochemical and molecular biology methods on selected surgical samples and cultured human cell lines. As a first result, cholinesterase activity, mainly due to the presence of the enzymes acetylcholinesterase (AChE) and propionyl-cholinesterase (PrChE) was found in *normal* pleurae used as a control, but neither in the MPM surgical samples (in both homogenates and sections), nor in MSTO 211-H cells. On the contrary, muscarinic acetylcholine receptors (mAChRs), and in particular those belonging to the m2 form were found in malignant mesothelioma cells and tissues, but not in normal pleura. The findings, together with the findings available in the literature, might support the hypothesis that the scarce AChE activity causes an overflow of acetylcholine (ACh) at the receptors, that are amplified in the tumour cells, thus causing intracellular responses playing a possible role in cell proliferation.

Fine structural analysis of prostatic cancer cells PC3 under opioid treatment

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Previous studies demonstrated that some opioid agonists are able to inhibit the growth of human prostatic cancer cell lines in a dose-dependent manner, suggesting a role of such molecules in the reduction of prostatic cancer cell proliferation. In order to investigate the intracellular effects of opioid treatments, we incubated the PC3 epithelial cell line (derived from a bone marrow metastasis of a prostate adenocarcinoma) with 10^{-3}M and 10^{-5}M [D-Ala2,D-Leu5]enkephalin (DADLE) for 24 hs and 48 hs. The cells were counted and then processed for electron microscopy. Our observations demonstrated several ultrastructural modifications in all treated cells in comparison to controls. In detail, in the cytoplasm the Golgi complexes became smaller, numerous residual bodies appeared, and the mitochondria displayed less evident cristae and many matrix granules; in the nucleus, perichromatin fibrils and granules underwent quantitative changes, nucleoli became more compact and heterochromatin areas increased. These structural modifications, already detectable after 24 hs, became quite evident after 48 hs, and indicate a significant reduction in metabolic activities. This is in accordance with the decreased cell proliferation observed in all treated samples. Anti-Leu5-enkephalin immunolabelling revealed the presence of DADLE molecules in both cytoplasm and nuclei of PC3 cells after 24 hs and 48 hs, suggesting that DADLE may be internalised, probably interacting with k opioid receptors present in this prostatic cell line. However, the mechanisms by which DADLE influences the cellular metabolism remain unknown.

Immunohistochemical characterization of a new atypic antibody in a paraneoplastic neurological syndromes

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Paraneoplastic neurological syndromes (PNS) are inflammatory disorders of the central and peripheral nervous system that occur in association with cancer but are not a direct result of neoplasia or of metastasis to the nervous system. PNS are found most often in association with small cell lung cancer and sometimes with tumour of breast, uterus and ovary. Patients with PNS develop autoantibodies in their serum and cerebrospinal fluid that react with neuronal antigens that in some cases are shared with the tumour. Serum immunohistochemical screening on nervous tissue reveals the presence of different types of antibodies which, at least in some cases, identify characteristic neurological syndromes and specific types of cancer. On rat cerebellar frozen sections and with an anti-human IgG and avidin-biotin immunoperoxidase technique, we examined the serum of a patient with cerebellar degeneration and Merkel cells carcinoma. Rat cerebellum is widely used to identify antineuronal antibodies produced in humans. Immunohistochemical analysis showed a fibrillary reactivity localized in molecular and granular layers and around the Purkinje cells. This pattern of the reaction may be considered as atypic, because after the immunoblot analysis with recombinant neuronal proteins, the antibody did not recognize know antigens. Then we biotinylated the atypic antibody and studied the reactivity on frozen and paraffin-embedded sections of Merkel cells carcinoma with a direct avidin-biotin immunoperoxidase method. The positivity on Merkel cells revealed that the same antigen was expressed both on the tumour and the nervous tissue. We found a new atypic antibody that represents a causal link between a tumour and a neurological disease, whose onset preceded the discovery of the neoplasia.

The human skin keratoacanthoma as a model for the relationship between cell proliferation and cell death-differentiation

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Some features of the programmed cell death (DNA cleavage, nuclear piknosis/karyorrexis, caspase-3 and Bcl-2/Bax expression) were examined during the keratinocyte differentiation in relation to the cell proliferation (PCNA and Ki-67 expression) in six cases of human skin keratoacanthoma. The study was performed both in light (conventional/fluorescence) and electron (transmission) microscopy by means of morphological and cytochemical approaches. In the normal skin areas the terminal differentiation of the epidermis was a consequence of the nuclear degeneration (e.g. highest TUNEL L.I.) in the granular cell layer, with a complete absence of nuclei in the cells of the horny layer (corneocytes). In the neoplastic areas a different keratinocyte differentiation (partial or complete), also in the identical lesion, was found. During the mature stage of the neoplastic lesion, a correlation between the degree of the epidermis growth (e.g. high PCNA L.I. of both the basal and suprabasal spinous layers) and the incomplete maturation (e.g. absence of the granular layer and shedding of a thick horny layer mainly composed by apototic cells) appeared generally expressed. During the regressive stage all the typical epidermis layers reappeared and the superficial keratinocytes, completly differentiated, resulted sometimes covered by a residual layer of apoptotic cells belonging to the previous generation of the incompletely differentiated neoplastic cells. Our observations indicate the fundamental role of some mechanisms of the apoptotic process as a prerequisite for the keratoacanthoma spontaneous regression throughout the re-epithelialization with cells showing a normal morpho-phenotype.

The phosphoinositide 3-kinase/Akt pathway regulates cell cycle progression of HL60 human leukemia cells through cytoplasmic relocalization of the cyclin-dependent kinase inhibitor p27kip1 and control of cyclin D1 expression

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The serine/threonine protein kinase Akt plays a pivotal role in tumorigenesis because it affects the growth and survival of cancer cells. Akt is a downstream effector of phosphoinositide 3-kinase (PI3K), a family of enzymes which is recruited upon growth factor receptor activation and produces 3' phosphoinositide lipids. Several laboratories have demonstrated that Akt inhibits transcriptional activation of a number of related forkhead transcription factors now referred to as Fox01, Fox03, and Fox04. Akt-regulated forkhead transcription factors are involved in the control of the expression of both the cdk inhibitor $p27^{Kip1}$ and Bim, a pro-apoptotic member of the Bcl-2 family. Very little information is available concerning the importance PI3K/Akt pathway in HL60 human leukemia cells. Here, we present our findings showing that the PI3K/Akt axis regulates cell cycle progression of HL60 cells through multiple mechanisms also involving the control of forkhead transcription factors Fox01 and Fox03. To this end, we took advantage of a HL60 cell clone (HL60AR cells) with a constitutively activated PI3K/Akt axis. When compared with parental (PT) HL60 cells, HL60AR cells displayed higher levels of phosphorylated Fox01 and Fox03, as well as cyclin D1, whereas the amount of total Fox01 and Fox03 was unchanged. In AR cells forkhead factors localized predominantly in the cytoplasm, whereas in PT cells they were mostly nuclear. AR cells proliferated faster than PT cells and showed a lower amount of the cdk inhibitor $p27^{Kip1}$ which was mainly found in the cytoplasm.

HSP60 and HSP10 expression in the *dysplasia-carcinoma sequence* of uterine cervix

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Pre-cancerous (dysplastic) lesions always precede squamous cervical cancer. In the new classification of dysplastic lesions of uterine cervix (Bethesda system) there are present only two entities: L-SIL (squamous intraepithelial lesion, low-grade) and H-SIL (high grade). Heat shock protein (Hsp) family includes a class of chaperons and their over-expression can be induced by a variety of cellular stresses. In particular, Hsp60 and Hsp10 are mitochondrial proteins mediating a wide range of intracellular activities, not all well understood. Their putative role in carcinogenesis is now growing. The aim of the present study was to examine the presence and the expression of Hsp60 and Hsp10 during the cervical carcinogenesis. We performed both immunostaining and Western blot analysis for Hsp60 and Hsp10. Positivity for Hsps was growing from normal cervical epithelium through SILs to carcinoma, as showed by both immunohistochemistry and Western blotting, being the maximum positivity present in carcinomas. By contrast, interposed stromal cells resulted commonly negative. These data could have diagnostic and prognostic significance. Moreover, we can postulate that Hsp60 and 10 have a role during cervical carcinogenesis different than the requirement to regenerate mitochondria during cell proliferation or after high mitochondrial activity, as in normal cells, but the knowledge of this role needs of further investigations.

Histochemical diagnosis of pulmonary metastatic biphasic synovial sarcoma

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Synovial sarcoma is a malign soft tissue tumor that commonly metastasises in various sites. We studied a rare case of metastatic pulmonary biphasic synovial sarcoma in which a wide panel of histochemical and immunocytochemical analyses were performed to exclude other neoplasms, among which malignant mesothelioma. The tumor was PAS and alcian blue positive, digestion resistant. Reticulin highlighted the biphasic pattern. The immunophenotype was consistent with synovial sarcoma but not discriminative. In particular, CK8, CK18 and CK19 were strongly positive in both epithelial and spindle components, with the exception of CK7 that was moderately present in the epithelial cells and absent in the spindled. A strong positivity of Ber-Ep4 was present in the epithelial component, while calretinin showed a moderate positivity in epithelial pattern and was scarcely found in spindle cells. Moreover, a

moderate Bcl-2 positivity was reported mostly in the spindled component. Finally, CEA and CD34 were absent. In the presented case, histochemical data resulted more helpful than immunohistochemistry to address diagnosis. Nevertheless, histological pattern, histochemical and immunohistochemical features needed to be assessed in combination to perform diagnosis. Finally, we compared histochemical and immunocytochemical results obtained in the metastatic tumor with those found in the primary lesion and we registered a discrepancy in Ber-EP4 and calretinin expression; indeed we found a moderate-strong positivity of these antibody only in metastasis, while in primary tumor they were absent. These data are probably due to the biologic variability of this sarcoma and we address other molecular studies to better understand this discrepancy.

Pattern of expression of cyclin d1 in different mouse tissues

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Cyclin D1 is a key regulatory protein of the cell cycle required for cell progression through G1 phase to S phase. Cyclin D1 acts in cooperation with its major catalytic partners, cyclin-dependent kinase, cdk4 and cdk6 and facilitates progression through the G1 phase in part phosphorylating the retinoblastoma protein. Four mammalian G1 cyclins have been enumerated to date: cyclins D1, D2, and D3 (D-type cyclins) and cyclin E. Among them, cyclin D1 is unique, since it was originally identified as an oncogene, PRAD1. These G1 cyclins and their cdks were also shown play some important roles in cell differentiation and transformation. In dividing cells, the expression of cyclin genes and the subsequent activation of cyclin-dependent kinases are closely associated with progression through and exit from the cell cycle. However, in cells such as neurons, which have permanently exited the cell cycle, very little is known concerning the expression of these genes or the functions of their proteins. We have shown that cyclin D1 has a wide distribution in mouse tissues by western blot analysis and immunohistochemistry although its expression level and localization may vary among different tissues. Moreover, we have shown different sub-cellular localization of this protein in several tissues by immunoelectron microscopy. These findings suggest that cyclin D1 may play multiple roles within specific tissues probably interacting with several substrates.

Identification of genetic markers involved in glioma tumor progression

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Gliomas are heterogeneous intracranial glial neoplasms ranging from the highly aggressive malignant glioblastoma multiforme to the less invasive low grade astrocytoma. Generally, malignant transformation of neuroepithelial cells is a multistep process driven by the sequential acquisition of genetic alterations. Among these, differential gene expression in glial tumors often involves extracellular matrix/basement membrane components. We have therefore investigated the behaviour of two outer membrane glycoproteins mainly investigated and differently involved in neurodegenerative associated diseases, namely the human prion (PrP) and prion-doppel (Dpl). Their expression profiles and their immunohistochemistry were evaluated in different histopathological grades of glioma tumors as well as in glioblastoma-derived cell lines. In conclusion, we reported that Dpl expression profiles in glial tumors parallel with grade of malignancy, also highlighting an unexpected Dpl localization within the tumor cells. These data could suggest for Dpl a potential role in brain neoplasia.

Immunohistochemical expression of HLA antigens and components of the antigen processing machinery in astrocytic tumors

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The selection of MHC class I-deficient variants is a frequently observed mechanism used by tumor cells to escape immunosurveillance in experimental and spontaneous tumours. In this paper we investigated, by immunohistochemistry, the expression of HLA antigens in a copious panel (88) of malignant astrocytic tumours, histopathologically classified according to the WHO classification of brain tumours. We used a panel of 5 experimental monoclonal antibodies: anti-HLA class I, anti-β2-microglobulin, anti-HLA-A, anti-HLA-A2, A28, anti-HLA-B7. Our results indicated that HLA class I downregulation is frequent in astrocytic tumours and, in particular, there is correlation between tumour malignancy and HLA class I antigen downregulation. In order to determine the mechanisms of the impaired HLA class I surface expression in high grade astrocytoma, 44 biopsies of astrocytic tumours were analysed by immunohistochemistry for their expression of peptide transporter associated with antigen processing (TAP1 and TAP2), LMP2, LMP7 and LMP10 proteasoma subunits and the chaperones tapasin, calnexina and calreticulin. The reduced expression levels of tapasin found in 20% of GBL samples bring to the conclusion that this molecule deficits can be considered as an integral part of the immune escape mechanisms of human high grade astrocytic tumours.

GnRHreceptor expression in human prostate cancer cells: effect of hormones and growth factors

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In this study we investigated the presence of receptors for Gonadotropin-Releasing Hormone (GnRH-R) in both androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) prostate cancer cells by immunocytochemistry. We also explored the effect of Leuprorelin Acetate (LA), a GnRH agonist, and other agents on receptor content. LNCaP cells were treated with LA (10^{-11} or 10^{-6}M), 10^{-9}M Dihydrotestosterone (DHT) or their combination. PC-3 cells were exposed to LA (10^{-11} or 10^{-6}M) and Epidermal Growth Factor (EGF) (1 or 10 ng/mL), alone or in association. After six days of treatment the cells were processed with immunocytochemical methods and the GnRH-R monoclonal antibody (clone A9E4, Novocastra Laboratories Ltd, UK). Indirect immunostaining was achieved by ABC technique. Both LNCaP and PC-3 cells expressed GnRH-R. Untreated LNCaP cells showed a high percentage of cells with moderate staining intensity and a low percentage

with either weak or strong intensity. Treatment with LA alone did not modify GnRH-R expression compared with controls. The percentage of cells with strong intensity increased in LNCaP cells following addition of DHT. This effect was not counteracted by LA. Untreated PC-3 cells showed a higher percentage of cells with strong staining intensity than did LNCaP cells. EGF and LA did not affect GnRH-R expression either alone or in combination. The persistence of GnRH-R in both LNCaP and PC-3 cells after LA treatment suggests that these receptors might be used as targets for further treatment with GnRH analogs.

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A salycilate compound, aurintricarboxylic acid, cooperatively elicits survival signals in lymphoma cells treated with TNF-α

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Previous results demonstrated that the occurrence of death in lymphoma cells by tumor necrosis factor-α (TNF-α) is paralleled by the activation of the cytoplasmic Janus tyrosine kinases Jak1 and Tyk2 along with the recruitment of a specific set of latent cytosolic transcription factors Stat3 and Stat5b. Here we demonstrate that the balance of survival signals in the presence of TNF-α was altered by the interaction of TNF-α with a salicylate compound, aurintricarboxylic acid (ATA). Apoptosis effected by TNF-α alone was suppressed in the presence of ATA and this event was paralleled by phosphorylation of Janus kinase 2 (Jak2), associated with a distinct switch in the pattern of signal transducers and activators of transcription phosphorylations (Stat1, Stat2 and Stat4). Coincidentally, phosphorylation and nuclear translocation of the nuclear factor kappa B (NF-kB) occurred. Of note, ATA alone did not produce substantial modifications with respect to untreated cells. Since ATA forms membrane-impermeable oligomers in solution, and was not effector of Jak/Stat activation in the absence of TNF-α, the novel evidence of signal shifts implicates a cooperative influence of ATA and TNF-α on the dynamics of surface receptors and interactions with upstream signal transducers.

Immunohistochemical detection of p53 and p16 in malignant melanoma from different geographical regions

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The frequency of malignant melanoma has steadily increased; UV solar irradiation, linked to latitude, has been found to be correlated with the incidence of melanoma. Epidemiological studies have related the rate of melanoma to ethnographic and geographical factors. This suggests that genetic factors, interacting with environmental agents, such as the exposure to UV rays, appear to be the most relevant to the incidence of the melanoma. To better understand the influence exerted by various geographical and ethnographic factors we compared two regions, Azuay province in Ecuador and Sardinia in Italy, with high risk for the disease, primarily because of their geographic position. We carried out the immunohistochemical detection of p53 and p16, encoded by the most commonly mutated genes in human skin cancer, which function in separate pathways of growth control. The p53 is a central element in cell-cycle control, apoptosis, DNA replication and repair. The p16 is considered to be a potent tumor suppressor and its inactivation can occur in a wide range of human cancers. Microtome sections of formalin fixed and paraffin embedded biopsy specimens of primary melanoma were treated for the immunohistochemical demonstration of $p16^{INK4a}$ and p53 using the alkaline phosphatase streptavidin method. In immunohistochemical p16 detection, cytoplasmic staining, although generally low, was present in most of melanomas, but it was considered in the evaluation only if accompanied by distinct nuclear staining. Immunoreaction for p53 tends to give stronger staining of the nuclei only, but in most of the positive samples the cell number, both in tumor and surrounding epithelial cells, was poor. Our data suggest that type specific geographical differences exist among the two populations.

Immunohistochemical markers in solid tumors: role in diagnosis, prognosis and as predictors of response to therapy

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Immunohistochemical markers of human solid tumors may have both a diagnostic and a prognostic role and may be used as markers of response to specific therapy. Differentiation markers are applied in the routine differential diagnosis of tumors that originate from different cells but show similar morphological features (e.g spindle cell carcinoma versus sarcoma). The preoperatory diagnosis of the epithelial or mesenchymal origin of a tumor will pave the way to different therapeutic approach. In these cases, the pathologist can rely on simple markers such as the cytoskeletal proteins (keratin, vimentin). In addition, the same markers are useful for the diagnosis of lesions that originate from the same cell, but show different evolution (hyperplasia versus neoplasia). Other differentiation markers suggest the functional activity of tumor cells, in other words the ability of a cell to produce proteins that can be used both as diagnostic markers and as follow-up markers of the disease (endocrine or apocrine differentiation of tumors). Other markers, such as steroid hormone receptors, have multiple roles. Estrogen and progesterone receptor expression in metastatic cells indicate their origin from a breast carcinoma. In primary breast carcinoma, the same receptors are used as prognostic markers being correlated with well differentiated tumors and as markers of therapeutic response to hormonal treatment. Finally an important role as prognostic markers is shown by different onco-proteins. A specific diagnostic role is recognized to CD117 or c-kit, the receptor tyrosine kinase that is the protein product of the c-kit proto-oncogene. c-kit is specifically expressed by gastro-intestinal stromal tumors (GIST), which originate from Cajal cells. In addition, the immunohistochemical expression of c-kit is requested by oncologist for a specific treatment of malignant GIST with a signal transduction inhibitor. Another typical example is the over-expression of c-erbB2 receptor protein by aggressive carcinoma of different organs. In breast carcinoma c-erbB2 over-expression and HER2/neu gene amplification are important parameters in order to select patients with advanced breast cancer that can be treated with a humanized monoclonal antibody (Herceptin), which inhibits tumor growth through specific binding to the extracellular receptor domain.

Clathrin coated pits in membrane rafts: breaking a prejudice

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How receptor tyrosine kinase (RTK) signalling is connected to their internalisation is largely unknown. Apparently, the two events are topologically distinct. Membrane microdomains, the so-called lipid rafts, function as platforms to concentrate active receptors and assemble the signal transduction machinery and are also considered to be the starting site of non-clathrin dependent endocytosis. Receptor endocytosis, in most cases, is instead carried out by different specialised structures, the clathrin-coated pits. RTKs are thought to exit lipid rafts to enter coated pits, in order to be removed from the cell surface. This largely accepted *exit into pits* model, however, does not account for the molecular mechanisms responsible for the translocation of the receptor from one compartment to the other. Here we show that the epidermal growth factor receptor (EGFR) is internalised through clathrin-coated pits that form within rafts. Thus, specialised membrane microdomains have the ability to assemble both the molecular machineries necessary for intracellular propagation of effector signals and for receptor internalisation via clathrin coated pits.

Hepatocyte in gastric and bowel carcinomas: an immunohistochemical study

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The murine monoclonal antibody, hepatocyte has been demonstrated as a sensitive, diagnostic tool for hepatocellular carcinoma (HCC).[1] It reacts with a not yet fully described epitope in mitochondrial fraction of normal and neoplastic hepatocytes. Most of the metastatic liver tumors arise from the gastrointestinal tract and their diagnosis is often difficult only on the basis of the histological features. The aim of our study was to evaluate by immunohistochemistry the specificity of hepatocyte testing 39 gastric carcinomas, including five cases with hepatoid differentiation and 18 colorectal adenocarcinomas. Our results showed a positive immunostaining in 26/39 (66.6%) and in 9/18 (50%) of the colorectal carcinomas. All of the hepatoid gastric carcinomas stained positively. Therefore we think that hepatocyte is a highly sensitive marker of hepatocellular differentiation, but the reaction with neoplastic cells of gastrointestinal carcinomas reveals a low grade of specificity, thus limiting its use as marker of differential diagnosis between hcc and metastatic lesions arising from the gastrointestinal tract.

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Analysis immunoistochemical of the HLA class I molecules in human glioma

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The *Major Histocompatibility Complex* (MHC) antigens are found in some human malignant cells and it has been known that malignant transformation of human cells is frequently associated with abnormalities in the expression of these antigens. This human MHC class I downregulation reflects tumour escape mechanism from T-cell immune responses. The expression of HLA class I antigens by a tumour is central to the efficacy of any cell-mediated immunotherapy. We have analysed by immunoistochemistry technique the expression of HLA class I molecules in 45 specimens of fresh cell imprints of malignant brain tumours, differently classified according to the WHO classification: 10 astrocytoma (WHO grade II), 6 anaplastic astrocytoma (WHO grade III), 23 glioblastoma (WHO grade IV), 3 oligodendroglioma (WHO grade II) and 3 anaplastic oligodendroglioma. As control we analysed brain tissues obtained from patients who underwent surgery for an aneurysm. We were used, instead, as probe anti-HLA class I (TP25.99), anti-β2microglobulina (L368), anti-HLA-A (LGIII-147.4.1), anti-HLA-A2, A-28 (KS1) and anti-HLA-B7 (KS4). The results indicated that HLA class I antigen downregulation is frequent in malignant brain tumour and in particularly HLA class I molecules frequency is grading-dependent: we observed a strong downregulation of these antigens in high gliomas (WHO grade IV) in contrast to a minor downregulation in low grade astrocytoma (WHO grade II). In addition, we analysed the expression of components of the class I antigen processing machinery in 22 specimens classified as follow: 6 astrocytoma (WHO grade II), 6 anaplastic astrocytoma (WHO grade III), 8 glioblastoma (WHO grade IV), 1 oligodendroglioma (WHO grade II) and 1 anaplastic oligodendroglioma (WHO grade III). The anti-LMP2 (SY-1), anti-LMP7 (SY-3), anti-TAP1 (TO-1), anti-TAP2 (SY-2) were used as probe. Yet, the results don't show significant abnormalities in components involved in antigen processing.

CELL MARKERS OF STRESS AND POLLUTION

The response to oxidative stress by human bronchial epithelial cells

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Oxidative stress plays an important role in the development of many human diseases. The bronchial epithelium is directly exposed to many oxidants that may damage its integrity. Abnormal responses during the repair processes may lead to airway persistent inflammation, as during asthma or cancer. The aim of our work was to study *in vitro* the response to oxidative stress by normal and tumoral bronchial epithelial cells (16HBE and H292, respectively). In particular, we focused the attention on the expression of inducible nitrogen oxide synthase (iNOS). 16HBE and H292 cells were treated with 200 and 300 µM of H_2O_2 for 24 and 48 hrs. They were fixed with paraformaldehyde and stained with methylene blue. The cell number was measured to wavelength of 630nm. Moreover, we performed Western blotting for iNOS on cell lysates. At morphologic analysis, 16HBE resulted more resistant to oxidative stress than H292 although their survival was dependent by addition of growth factors after 48 hours. By contrast, the cell number of untreated H292 increased after 48 hours. The cell number decreased in a time and dose-dependent manner in both 16HBE and H292. The apoptotic index was elevated in both treated cell lines. The number of apoptotic cells increased in a time- and dose-dependent manner. The expression of iNOS increased in both cell lines treated with H_2O_2 compared with controls after 24 and 48 hour of incubation. Moreover, the expression of iNOS did not show any difference in both 16HBE and H292 treated with 200 and 300 µM of H_2O_2 for 24 and 48 hours of treatment. The expression of iNOS is upregulated by oxidative stress in normal and tumoral cells. We could explain the different response of 16HBE and H292 to the oxidative stress with the presence of antioxidant mechanisms, but we need of further experiments to confirm this hypothesis.

Ischemia/reperfusion effects on pig liver metabolism and autofluorescence

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Liver transplantation involves ischemic cold preservation followed by reperfusion, which affect the organ metabolism and may result in irreversible tissue damage. *In vivo* autofluorescence detection is a non-invasive method to monitor organ metabolism. We related the autofluorescence properties during pig liver transplantation to markers of metabolism and oxidative stress, as demonstrated in biopsies. As compared to the pre-transplantation stage (T0; glycogen-free), in the ischemic phase (T1) the autofluorescence amplitude increased due to the relative contribution of NAD(P)H and decreased to the basal level during reperfusion (T2). T1 caused portal edema, sinusoid dilatation, drop of SDH activity, activation of NOS activity in a few Ito cells, ROS production by a few sinusoidal cells, decrease of GGT activity in bile ducts and in portal hepatocytes. In T2, the parenchyma architecture was almost restored, a few portal hepatocytes had glycogen, SDH and GGT activity increased in portal hepatocytes with respect to T1, GGT was released into bile and the number of ROS^+ and NOS^+ sinusoidal and stromal cells further increased. The histochemical data support the information given by autofluorescence analysis, and suggest NO-mediated vasodilatation in response to hypoxia, and oxidative stress and damage to bile ducts during reperfusion. *MIUR-COFIN2001; CNR Project Biotechnology.*

Effects of SLS sublethal concentration on gill morphology and Na^+,K^+ - ATPase activity in *Coris julis* (*Labridae*)

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The noxious effects induced by anionic detergents on marine organisms have been the subject of different studies. Information relating to aquatic vertebrates is still rather sparse, and studies carried out on Teleostei appear limited to freshwater species. For this study, we analyzed, from a morphological and ultrastructural viewpoint, the gill apparatus of a labride, *Coris julis*, under normal conditions and after exposure to a non lethal concentration of SLS. We calculated for this species an LC_{50} of 5 mg/L. In order to identify the probable biochemical mechanisms target of toxicity we have evaluated and compared enzymatic activity of the Na^+/K^+-ATPase in standard and experimental conditions. The immunocytochemical study aimed at localising Na^+/K^+-ATPase revealed the activity of this enzyme in the CC, which appear to be placed along the entire principal filament. Ultrastructural analysis after 96 hours of exposure to SLS has revealed conspicuous alterations on the epithelium. SEM observations show the loss of the structural asset of the surface cells. MO and TEM observations allowed us to see the reduction in the number of CC on the principal filament, the appearance of ample intercellular lacunae and cellular degeneration phenomena. The observations also show the appearance of CC on the secondary lamellae, attested by Na^+/K^+-ATPase localization. Furthermore, occasional fusion process between adjacent lamellae appears. Damages are also present on the vascular component.

Do thymic epithelial cells exert a local control of oxidative stress?

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The biological mechanism, on which is based the generetion of MHC-restricted and tolerant thymic repertoire, requires a 90-95% deletion of harmful lymphoid cells. This causes indeed a relatively high lipid peroxidation, which in turn generates the need of oxidative stress control. The presence of the anti-oxidant enzyme, CuZn-superoxide dismutase, in the thymus has been studied by means of immunochemical methods, and primary cultures of thymic epithelial and fibroblast cells have been assessed to show the enzyme release. Double immunocytochemical methods confirmed that epithelial cells, as well as many thymic stromal cells, contain CuZn-superoxide dismutase, while ELISA assay allowed a measurement of the enzyme release under normal and stress condition. We think that the control of oxidative stress within the thymic microenvironment may be locally exerted by CuZn-superoxide dismutase with a major contribution of epithelial cells.

Effects of static magnetic fields exposure on HepG2 cells

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The data of literature, concerning the electromagnetic-related alteration of cellular functions, although are quite heterogenous in terms of fields intensity (from 10^{-7} to 10T), type of field (static or oscillatory) and subjects exposed to EMFs (isolated cells, humans), nonetheless suggest a link between EMFs exposition and tumorigenicity. In this work the biological effects of 6mT static magnetic fields (SMFs) were studied in HepG2 cells with respect to morphological modifications of cell shape, sugar residues, cytoskeleton alterations and induction of apoptosis. Application for up to 24hrs of SMF modified the cell shape: cells growth under SMFs rounded up (focal adhesions were altered) and incresed the number of pseudopia. The number of cells showing these modifications increased with the time of exposure to SMF. Two other cell components were modified by the SMF exposure: cell surface antigens and cytoskeleton. After 4 hrs of continuos esposure to SMF actin filament network, labelled with falloidin-FITC conjugates, showed modifications indicating depolymerization of the filaments. Cell surfaces sugar residues (δ-galactose, δ-mannose) are extensively rearranged as consequence of the SMFs exposure. The intensity of the lectin-FITC conjugates increased progressively with the time of SMFs exposure. HepG2 cells can be induced to a high rate of apoptosis, a type of cell death characterized by morphological features (chromatin condensation, cell blebbing, apoptotic bodies, etc) by a number of drugs. When the cells are induced to apoptosis simultaneously with SMFs exposure, a reduced rate of cell death was measured. 24 hrs of SMFs exposure before incubation with apoptotic inducers abolish apoptosis of about 70%.

Cell shape, cell surface and cytoskeleton as indicators of magnetic pollution

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The growing interest in the influence of magnetic fields (MFs) on life process is deriving from the concern about their possible harmful effects on human health. More and more evidence has been gathered during the last years about hazardous consequences of the so-called electromagnetic pollution. Here we report data supporting biological effects of 6mT static MFs on different cell types. Indeed, some of them (cell shape, cell surface, cytoskeleton, but also apoptotic rate, calcium ions, gene products expression) could be considered good indicators of magnetic pollution. Cells exposed to SMFs (in the presence or absence of apoptosis-inducing drugs) were analyzed by transmission (TEM) and scanning (SEM) electron microscopy. Lectin cytochemistry and falloidin- FITC conjugates were used to analyze structural modifications in the plasma membrane and in the cytoskeleton. Static MFs exposure induces rearrangement of the cell shape. For example, abundant lamellar shaped microvilli were observed upon 24 hrs of enduring exposure to static MFs in contrast to the normally rough surface of U937 cells with many as well as short microvilli. Conversely, lymphocytes lost their round shape and became irregularly elongate. Lamellar microvilli were clearly observed before the distortion of cell shape, that was found at longer times of exposition. In our experiments, static MFs reduced the smoothness of the cell surface and partially impeded modifications of distribution of cell surface glycan occurring during apoptosis. Cell shape as well as modifications of plasma membrane structure were time dependent on static MFs exposition. MFs exposure promoted de-arrangement of F-actin filaments that could be, in turn, responsible of cell surface modifications. Modifications induced by the exposure to static MFs were irrespective of the presence or absence of apoptotic drugs and of the cell type. However, the involvement of these modifications in the onset of diseases can further be elucidated.

Histochemistry and enzymes in mitochondria-rich cells of *Rana esculenta* skin, exposed to osmotic stress

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Frog skin has an important osmoregulatory function because it is involved in the water and ions transport processes. Mitochondria-rich cells (MR cells) are rich in carbonic anhydrase and are specialized for proton and bicarbonate secretion. The aim of this study is to obtain more informations about morphological features and density variations of MR cells, as consequence of experimental adaptations in water or dry medium. Samples are fixed in paraformaldehyde 1% in 0.1 M cacodylate buffer, pH 7.3 and then embedded in hydrophilic resin (Technovit 7100-Kulzer). On the sections we have applied several reactions: Acid Fuchsin and Toluidine Blue stain, Fast green and Naphtol yellow stain, Kernechtrot stain, Alkaline phosphatase (PALK, EC 3.1.3.1) and Carbonic anhydrase (CA, EC 4.2.1.1) detection. In water experimental condition, MR cells present an elongated shape and have an high density. Maintaining the animals in a dry medium the number of wider shape MR cells decreases. In both conditions MR cells are negative for PALK. A strong CA reaction is observed in most of MR cells in water condition, while CA activity is less evident in skin dry acclimation. The histochemical approach supports the important role of MR cells in different stressing environmental conditions and evidences a functional regulatory response.

Determinations of biological markers of stress in mugilidae from brackish environments

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The aim of the present research was to define those bio-physiological parameters that can be related to pathologies caused by heavy metal exposure. Specimens of the mullets *Liza aurata* e *Chelon labrosus* from two different polluted brackish environments (lagoon of Faro e Ganzirri, Messina) and one non polluted control area (Lake of Fogliano, Latina) were considered. In addition to the field study, experimental Hg, Cd e Pb exposure of selected mullets from low impact reference areas were also carry out. The histopathological, biochemical and immunohistochemical analysis performed in the differently sampled specimens, put in evidence the presence of those biological active substances that can be involved in the osmoregulatory, respiratory, digestive, reproductive and nervous functions The alteration of their presence and/or distributive pattern, compared to those observed in mullets from non- pollut-

ed control area, was considered as precocious marker of the heavy metal exposure effects. The immunohistochemical dectection of serotonin, VIP, galanin and of the NOS anbd AchE biosynthetic enzymes seem to be varied, as well as the metallothionein, glutathion and porfirin content Furthermore the biochemical data put in evidence the linvolvement of the liver, gills, kidney and gut in the accumulation and/or detossification process of heavy metals. The correlation and integration of the different data obtained using different investigative methods allow us to put into evidence the most useful biomarkers for the detection of toxic agent disease and to fournish a detailed analysis of the pollution status in the choosen brackish environments and of possible restorative interventions.

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Hepatotoxic effects of some contaminants in the liver of *Rana esculenta* and detoxificating involvement of Kupffer cells

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The toxic effects on animals following their exposure to contaminants depends on certain factors, i.e. the kind of contaminant, its concentration, concomitant toxic agents, duration of exposure, organism sensitivity. In this study we aimed to assess, both in the animals' natural environment and experimentally, the effects of some contaminants on the liver of the frog *Rana esculenta*. We assessed contaminant concentration in adult frogs sampled from two rice-fields: one flooded with polluted waters, the other flooded with unpolluted spring water. The two groups differed in their concentration of Chromium (Cr VI) and of heptachlor epoxide (HE). On this basis, we exposed tadpoles to different concentrations of CrVI (0.5 ppm, 5 ppm, 50 ppm) and of HE (4 ppb, 40 ppb, 400 ppb) in experimental ponds, in order to study their toxicity and effects. Samples of liver were processed for both morphological and histochemical testing and two parenchymal cell components, i.e. hepatocytes and Kupffer cells (KCs), were examined. We paid particular attention to KCs, evaluating their melanin content and some enzyme activity (G6PDH and catalase), involved in the detoxifying action. The most significant modifications observed regarded an increase of melanin content/distribution and an increase of catalase activity, in large and small KCs, respectively. In hepatocytes, no significant alterations were noted, except for experimental groups treated with the highest doses of contaminants. The data obtained point to an important detoxifying role of KCs through melanosynthesis and enzyme action.

Galanin and serotonin immunoreactivity in the gut of experimentally treated *Liza aurata* from low and high polluted area

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The exposition to heavy metals is often considered a possible cause of degenerative pathologies in nervous system, especially in mammalians. The effects of these exposure in the gut of fishes are not completely known. In particular no data are available about the influence of the previous polluted condition in the fish adaptative response to experimental heavy metal treatment. Specimens of *Liza aurata* collected from the brackish natural environment of Marinello (low human impact) and from Ganzirri (high human impact) were exposed in aquaria to a non-lethal concentration of Hg, Cd and Pb. Controls were also considered. The presence of galanin and serotonin, was investigated by immunohistochemical techniques. In heavy metals exposed specimens, from both the two sites considered, numerous galanin immunoreactive (ir) nerve fibres were detected. They were located along the gut wall both in lamina propriety, sub mucosa and muscular layer. Fewer ire nerve fibres were detected in the control aquaria. In control and heavy metal exposed specimens from low polluted environment numerous serotonin ire nerve elements and endocrine cells were found while they were less frequently seen in control and heavy metal exposed specimens from high polluted environment. The results obtained suggest that the alteration of galanin immunoreactivity can be considered as a marker of short-term exposure to heavy metals, while variations in serotonin immunoreactivity can be detected after long-term or chronic exposure.

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Apoptosis and proliferation in the gill epithelium of heavy metal exposed *Liza aurata*

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Heavy metal pollution is a well known important causative factor of branchial damage. Gill epithelium often become thicker and detach from the basal lamina. We investigate the effects of heavy metal exposure on the apoptosis and proliferation of gill epithelial cells in the mugil *Liza aurata*. Mugils were sacrificed after twenty days of high and low Cd, Hg and Pb exposure in aquaria with controlled temperature and salinity. The apoptosis was assessed by terminal deoxynucleotidiltransferase-mediated dUTP nick end labelling and cell proliferation by anti-PCNA immunostaining. Comparing the experimental and control results, the low heavy metal exposed mugils showed the most frequent TUNEL positive nuclei and PCNA immunopositive epithelial cells. The first one were located at the apex , while the latter at the base of lamellae. A throughout distribution was seen in the high heavy metal exposed gills. Our results suggest that the reparative process for the maintenance of gill mucosal continuity is only present in the controls and in the low heavy metal exposed mugils.

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Warm hypoxia effect on metabolism and oxidative stress in rat liver: histochemical evaluation and biochemical correlates

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The damage induced by *warm* ischemia to the liver during surgery or transplantatation is usually supposed to be negligible with respect to that caused by reperfusion. We studied isolated rat liver perfused with N_2- or O_2-saturated medium at

37°C for up to 2 hours. The perfusate was studied for LDH activity released from dead cells and for thiobarbituric acid reactive species (TBARS) derived from oxidative stress. Bile flow was taken as index of liver metabolism and Trypan blue uptake as indicator of hepatocyte necrosis. Metabolism was evaluated in terms of the in situ demonstration of LDH, SDH, AlkPh, PNP, XOR and NOS activity, and oxidative stress in terms of reactive oxygen species (ROS) production. Normoxic perfusion had negligible effects. By contrast, warm hypoxia caused lack of bile flow, hepatocyte death in the midzone, alteration of the activity of all enzymes, increased ROS production by hepatocytes and sinusoidal cells (in keeping with high TBARS concentration in the perfusate) and release of LDH to the perfusate (in keeping with LDH loss from mid-zonal hepatocytes). NOS activity in Ito cells appeared to be correlated to sinusoid dilatation and PNP activity to be a sensitive marker of damage to sinusoidal and bile duct cells. Our results suggest lobular zone and cell type-dependent vulnerability to warm hypoxia and a more serious damage to the parenchyma than that expected from biochemical data.

Neurogenesis in the frog. Effects of environmental cytotoxic substances on proliferation and survival of neural cells

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It is largely accepted that neurogenesis occurs not only during development (*Fritz et al., Int J Devl Neuroscience, 14: 931, 1996*), but also in adult life (*Zupanc, Brain Behav. Evol, 58, 246, 2001; Garcia-Verdugo et al, Brain Res Bull, 57, 765, 2002*). In the brain, ventricle zones are actively proliferating mainly in non mammals. In particular, amphibians maintain an exceptional capacity to regenerate some CNS areas (*Zhang et al., Neuroscience 114, 837, 2002*) and DNA synthesis was also demonstrated in some brain nuclei of adult frogs (*Bernocchi et al, Anat Rec, 228, 461, 1990*). In this research, sections of paraplast embedded brain from adults frogs, that were collected in resurgence water (CL frogs) and contaminated water (CT frogs), were immunocytochemically labelled with PCNA (Proliferative Cell Nuclear Antigen), BAX and BCL2 antibodies to evaluate the influence of environment toxic substances on the proliferative activity in the ventricular zones and the survival of neural cells. A larval stage (25) was also considered. Immunolabelling was found in all the encephalic vesicles of CL adult and larval frogs, but mainly the patterns of proliferation were changed in CT animals. Therefore amphibian nervous tissue is vulnerable to environment citotoxic agents and amphibia can be considered good indicators of the biomonitoring of acquatic environments.

Localization of apoptotic cells and immunolocalization of metallothionein in zinc-treated LEC rat liver and kidneys

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It is known that LEC rats are an animal model for the study of Wilson disease. They accumulate Cu, and also Fe, especially in liver. These metal ions are involved in free radical production, which plays an important role in the regulation and induction of the apoptotic process. Metallothionein (MTs) have a protective role as scavenger of free radicals and are induced by heavy metals, such as Zn and Cu. MT is an intracellular protein that is localized either in the cytoplasm or in the nuclei or both, depending on its abundance in the cell. We have studied the effect of oral Zn treatment (by gavage) after 60 days in the liver and kidneys of 30 male Long-Evans Cinnamon (LEC) rats (5 weeks old) in relation to MT immunolocalization (*Cherian and Banerjee 1991, Methods Enzymol 205: 88*) and presence in apoptotic cells (*Frankfurt and Krishan 2001, J Histochem Cytochem 49: 369*). Fluorescent staining with Mab against ssDNA demonstrated that cells had chromatin condensation and nuclear fragmentation typical of apoptosis. Untreated group sections showed confocal fluorescent signal that highlighted the irregular nuclei and small apoptotic bodies. In contrast, the intensity and quantity of fluorescence decreased in the treated group. Intense immunostaining for MT appeared in the liver of the treated rats, both close to nuclei and cytoplasm of numerous hepatoparenchymal cells whereas the immunoreactivity for MT decreased in the untreated rats. In the cortex of treated rat kidneys, MT was localized in the cytoplasm and nucleus of the proximal tubular cells whereas in untreated rats MT was also present in the distal convoluted tubules near the glomeruli. These observations confirm the important role of Zn, by MT induction, in cellular protection against apoptotic processes as a cellular response to DNA damage by free radicals generated by excess Cu and Fe in LEC rat tissues.

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Effects of genetically modified food on mouse pancreatic acinar cells

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No direct evidence that genetically modified (GM) food may represent a possible danger for health has been reported so far, however, the scientific literature in this field is quite poor and heterogeneous. In this study, we investigated the possible effects of a diet containing GM soybean on mouse exocrine pancreas by combining ultrastructural morphometry, immunoelectron microscopy and biochemistry. Twelve female Swiss mice were fed on a standard laboratory chow containing 14% GM soybean, while 12 control mice were fed on the same diet with wild soybean. Animals were sacrificed at 1, 2, 5 or 8 months of age and samples of pancreas were processed both for electron microscopy and for biochemical determination of enzyme activity. Our observations demonstrated that, although no structural modification occurs in pancreatic acinar cells of mice fed on GM soybean, quantitative changes of some cytoplasmic and nuclear constituents take place in comparison to control animals. Moreover, quantitative analysis of anti-α-amylase labelling showed lower signal densities in GM soybean-fed mice in comparison to control animals; accordingly, the biochemical evaluation of α-amylase in pancreatic tissue confirmed a lower enzyme content in GM soybean-fed mice. On the other hand, serum α-amylase levels were similar in all mice. In conclusion, a diet containing significant amount of GM food seems to influence the pancreatic activity, especially zymogen synthesis and processing, maybe through not well understood regulatory mechanisms involving dietary substrates and intestinal microflora.

Zymography and ortogonal profile of matrix metalloproteinases in human breast secretions

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases involved in the degradation of the extracellular matrix, implicated in physiologic processes and in tumor growth; reports of their bio-molecular expression in breast cancer (BC) specimens prompted us to look for MMPs in nipple aspirate fluid (NAF), an excellent noninvasive and reliable tool to identify biomarkers of BC risk. NAF was collected from nonlactating, nonpregnant women with a disposable breast pump and immediately processed. MMP-2 and MMP-9 levels were quantified with ELISA test in 85 NAFs from women aged 30-55 years. MMP profiles were characterized using monodimensional and ortogonal zymography (SDS-PAGE copolymerized with gelatin) and identified by western blot. On the basis of biochemical markers, two subgroups of NAFs were classified: Type I (23 controls and 45 benign breast disease patients); Type II (12 BC patients, and 5 women with benign breast disease at the time of NAF collection who subsequently developed BC). ELISA and zimographic analyses allowed us to reveal in Type I NAFs MMP concentrations and 4 *zymogen* isoforms similar to that found in healthy plasma (median 27 mg/L, MMP-9 constitutive isoform); whereas in Type II NAFs we revealed *activated* MMP isoforms with significantly higher levels respect to that found in Type I NAFs (median 198 mg/L, $p<0.01$; MMP-2 and -9 constitutive isoforms). Interestingly, in all Type II NAFs we revealed unknown MMP *isoforms* of unexplored origin and function, electrophoretically co-migrating in monodimension but reliably separated through ortogonal zymography. We are studying whether the MMP isoforms found in NAFs may be additional biomarkers to currently screening methods for early detection of BC.

Phthalates as global contaminants: immunodetection, analytical and cytotoxicological aspects

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There is considerable emphasis currently on development of more sensitive frameworks for testing and assessment of endocrine-disrupting substances; compounds such as phthalates are likely early candidates for additional testing since phthalates are now global contaminants and specific phthalates, considered to be hormone disruptors, include di-ethylhexyl phthalate, butyl benzyl phthalate (BBP), di-n-butyl phthalate (DBP), and diethyl phthalate. Immunohistochemical monitoring, by using an antibody that selectively recognizes o-phthalate esters, was centered on organ tissues and cell cultures and it was demonstrated that: i) cells are able to absorb the endocrine disrupting chemicals into the cytoplasm but not into the nucleus; ii) phthalates persist differently in specialized cells of the alimentary canal of fish, amphibian and mammals. Differential calorimetry analysis indicated a first endothermic process with a maximum at 9 min, overlapped by a second more enhanced endothermic process that reach a steady state after 50 min when phthalates are concentrated near the nuclear envelope of Py1a rat osteoblasts. Confocal studies revealed that BBP and DBP exert their effects on FGF-2 promoting a nuclear accumulation, although in a transient manner, and produce some peculiar modifications of actin cytoskeleton changing rat osteoblasts from a spindle shape to a rounded form. Northern blot showed that phthalates did not decrease mRNA β-actin expression, but actin assembly; conversely, an increase of β-actin expression occurred when stimulated cells were serum-free medium maintained for two hours and treated again with phthalates. Appreciable effects were also showed in the type I collagen polymerization.

Gravitational vector changes affect glial cells in culture

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Astrocytes represent the major type of cells in the nervous system, they are required for synaptic formation and maintenance and, for synaptic efficiency. Gravitational force alterations causes severe damages to the cytoskeleton and induces apoptosis in 70% of the cultured glial cells after 1h in simulated microgravity (*Uva et al, Brain Res 2002, 132-9; Uva et al, Eur J Histochem 2002, 46: 209-14*). Aim of the present research was to further investigate on the alterations caused at subcellular level, in cultured glial cells, by simulated microgravity. We used C6 glioma cell line in monolayer cultures kept in a Fokker 3D Clinostat under continuous rotation (60 rpm) for 1h, 20h and 32h (simulated microgravity 0g). At the end of each experiment the cells fixed with 4% paraformaldehyde or 2.5% glutaraldehyde were submitted to immunohistochemistry or electron microscope procedures. Immunohistochemistry was performed using antisera to α-tubulin, to proteins of the inner mitochondrial membrane (AMA), to proteins in transmembrane ion transport (Na^+/K^+ATPase (α-subunit), to the carrier protein $Na^+/K^+/Cl^-$ cotransporter T4 NKCC1 and to the water channel Aquaporin 4) and to subcellular organelles like Golgi apparatus and synaptic vesicles. The results were visualised, with a conventional epifluorescence microscope (Olympus) and a laser scanning confocal microscopy system. Damages at subcellular level were visualised with a transmission electron microscope. Analyses of the cellular shape and surface were carried out with a scanning electron microscope. After 1h at 0 g severe damages were observed in mitochondria and other cell organelles. Immunofluorescence for the ion transport proteins was highly diminished if compared with the controls. After 20h percent altered cells decreased and several normal cells were present, some of which underwent mitosis. We conclude that gravity vector changes may induce only transient alterations in the glia, so that cultured glial cells might adapt to low gravity and restart a normal cycling processes.

Stress biomarkers in mugils living in brackish water environment

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Metal-binding proteins in marine organisms have been proposed as potential biomarkers of environmental exposure to heavy metal contamination. Before applying the use of MTs in a monitoring program, more information is needed on endo- and exogenous factors influencing the protein level. In the present work we want to monitor the Mugilidae biologic conditions, through the histological and immunohistochemical localization of those markers (neurotransmitters, biogenic amines etc) that can allow us to evaluate the environmental stress adaptative response of the fishes. Gill and intestinal epithelia of *Liza aurata* living in the polluted brackish water of Faro and Ganzirri (ME) were considered. As non polluted controls, specimens of *L. aurata* were collected in the lakes of Marinello (ME) and Fogliano (LT). The gills and gut of specimens from brackish polluted area showed significant variations of serotonin and nitrix oxide immunopositivity and numerous TUNEL positive apoptotic centers. Thicker epithelia and more numerous mucous cells than in the controls, were always seen. Even neuroendocrine cells and nervous elements seem to be morphologically modified. The MT levels in liver and gills of the mugilidae Liza aurata from the control and the high polluted areas were also biochemically studied. The highest values of MT levels were determined in fish caught in Ganzirri, the lowest in Fogliano. Furthermore the hepatic levels of MTs are always about 10 times greater than MTs in gills. The lowest values of MT levels were determined in fish caught in Fogliano during the spawning period.

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Pollution alter content of phenolic compounds and chemical elements in vascular plants leaves and pollens

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Air pollution often causes visibile symptoms of leaf and pollen injury. The injury is sometimes associated with an increase in the accessibility of trace elements to vascular plants. We investigated the distribution of elements in plants growing at unpolluted control sites and at sites polluted in urban and rural area of Palermo (Sicily). Samples were taken in two year period (summer and winter). The level of pollution on different sites and seasons was compared and evaluated the cell structure and histochemical cell characteristics. The content of phenols and enzymes histochemically revealed, is positively correlated with air trace elements concentration. A significant decrease in their detection was observed in every place between winter and summer. Biomonitoring and bioindication may be defined as the use of bio-organisms to obtain information on certain characteristics of the biosphere. The relevant information in biomonitoring is commonly deduced from changes of monitor organism and the plants are the best biomonitors. Biomonitoring is a method of quantitative analysis which makes it possible to correlate the amount of the harm done to the plant organism to the concentration of pollutant,while bioindication is a method of qualitative analysis to identify the presence of air pollutants and of their effects. Acid phosphatases or peroxidases activity have for long been regarded as a biochemical markers of stress and in particular of pollution stress; in numerous cases their increment precedes the appearance of macroscopically visible damage and for this reason them represent an important study histochemical parameter within the framework of bioindication researches.

NO production in tomato plants under biotic stress

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Root-knot nematodes *Meloidogyne incognita* cause severe damage in tomato crop. Besides chemical control, plant resistance to nematodes is often the most effective and environmental safe method to control this pest. In some cases, however, tomato crop resistance can be overcome by virulent nematode pathotypes, which are able to reproduce on plant carrying a resistance gene. Such investigations may lead to an understanding of the mechanisms by which the nematode is able to overcome the resistance barriers activated by the plant. A key difference between resistant and susceptible plants is the timely recognition of invading nematodes and effective activation of host defences. There is evidence that nitric oxide (NO), an important signalling and defence molecule in mammals, plays a key role in activating disease resistance in plants by triggering hypersensitive response through NO/H_2O_2 cooperation (*Delledonne et al 2002, Plant Physiol Biochem 40, 605*). The use of NO-reactive fluorescent indicator DAF-2DA, in conjunction with confocal laser microscopy, made possible the real time bio-imaging analysis of intracellular NO in resistant tomato roots infected with a virulent and avirulent line of M. incognita, respectively. Infected roots produced NO with a pattern similar to H2O2 accumulation [detected as a reddish-brown polymer by using the *DAB-uptake* method (*Thordal-Christensen et al 1997, Plant J 11, 1187*)], with a rapid stimulation by both avirulent and virulent lines followed by further strong production only in roots infected with avirulent nematode population. For the first time, these results indicate that NO can be detected in whole roots as another important component of plant defence system that might provide targets for improving plant resistance to nematodes.

Integration of somatic effects and population genetic analyses in mosquitofish exposed to pollutants of Sarno river

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Exposure to environmental contaminants can have various effects on natural populations. Acute or chronic toxic exposure can lead to genetic alterations at either the somatic or the population genetic level. Somatic-level (genotoxic) effects are due to the direct interaction of contaminants with DNA. Chromosomal aberrations are often the result of such interaction and can be revealed by examination of cell preparations for micronuclei test (MN test). Another cell indicator of genotoxic effects is well represented by single cell gel electrophoresis (COMET assay), testing DNA migration into an electrophoretic field. A second type of genetic effect may occur on the genetic composition of the population, in terms of genetic variability or distribution of allele frequencies. The objectives of this study were to assess the biological damage caused by

the exposure of mosquitofish (*Gambusia holbrooki: Cyprinodontiformes, Poecilidae*) to mutagenic substances present in the polluted waters of Sarno river at the somatic (MN and COMET analyses) and population genetic levels (RAPD-PCR analysis), to compare patterns of population differences at these two levels and to use population genetics as a support in cell level effect analysis. We used 22 individuals coming from the Sarno river and 18 specimens from the Astroni natural reserve as negative controls. The fishes from the Sarno showed a statistically significant increase in frequency of micronuclei, as well as in damaged cells evidenced by COMET test, respect to Astroni individuals. The results obtained with RAPD technique revealed that the frequency of two RAPD markers was greater in the contaminated than the reference sites. This observation is consistent with the hypothesis that these DNA markers may originate from genetic elements that provide a selective advantage in contaminated habitats.

Micronuclei test and COMET assay for the evaluation of zebrafish genomic damage induced by pharmaceuticals

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The presence of pharmaceutical products in the internal waters has been reported to be the major sources of these biologically active compounds in the aquatic environment. Drugs are a diverse group of chemicals that have received little attention as potential environmental pollutants and their genotoxicity has been poorly investigated *in vivo.* Fishes constitute the largest group of primarily water-living vertebrates. They have been shown to be particularly sensitive to pollutants interacting with DNA and leading to the formation of DNA adducts, DNA strand breaks, loss and/or chemical modification of DNA bases, because they may concentrate and conserve the substances scattered in the water. Genotoxicity of eritromycin and lincomycin, two antibiotics present in almost all internal waters, was evaluated on specimens of zebrafish (*Danio rerio: Cyprinidae, Teleostei*). The cytogenetic alterations were measured by two tests, the micronuclei test and the COMET assay, that evaluate morphologic damages at different development stages of cell cycle. Cells used in this test were taken from gills, the first organ exposed to contaminants during the breathing, and from liver, in which, as it is well known, several toxic substances accumulate. The tests were performed at different concentrations of pharmaceuticals and after different times of exposure. Finally, the results were analyzed and statistically processed by Scion Image Comet 1.3. The data showed a significant increase in frequency of micronuclei, as well as in damaged cells evidenced by COMET test, after 22 days of treatment and at low level of concentrations. In conclusion, this two combined cytogenetic methods represent a rapid and highly sensitive tool to monitor the genotoxicity, since they are able to detect low-level exposure effects.

NOS-dependent NADPH diaphorase activity in the brain of *Blattella germanica* (Blattaria, Blattellidae): effects of PCB xenobiotics

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NADPH diaphorase (NADPHd) is a histochemical marker for Nitric Oxide Synthase (NOS) and is widely used to identify nitric oxide (NO) producing elements in the brain of all vertebrate classes (*Steinbusch et al., 2000. Functional Neuroanatomy of Nitric Oxide System. Handbook of Chemical Neuroanatomy,. Elsevier, Amsterdam*). The NADPHd staining method in invertebrate has been validated by purification of the locust NOS and demonstration of co-localization for both NOS and NADPHd activities (*Elphick et al, 1994. Nitric Oxide Signalling in the insect nervous system. In: Borkovec, A.B. and Loeb, M.J., eds; Insect Neurochemistry and Neurophysiology, CRC Press, Boca Raton, pp 129-132*). This research was done to determine whether PCBs (Poly-Chlorinated Biphenyls) can interact with NO pathway system in the brain of male nymphs of the cockroach *Blattella germanica*, that is an ubiquitary insect, deeply interacting with human population. The reaction for NADPHd activity was performed on brain cryostatic sections from insects injected with a single dose of 5 µL of triolein containing 1.3 µg DMCA PCBs. Comparing with controls, increased reactivity was found in the neuropilar regions of mushroom bodies and antennal lobes; higher number of glial cells was also observed. In particular, among the glomeruli of the antennal lobes there were very intensively reactive and tortuous fibers that appeared degenerating. These results support a cytotoxic role of NO, whose synthesis increased after PCBs treatment.

Cadmium-induced alterations in teleosts gills and kidney

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Histopathological alterations of specific organs express exogenous impacts on the organisms from environmental pollution. Histological changes occur earlier than other effects and provide a better evaluation of organisms health than biochemical parameters. Exposure to heavy metals involves pathological degeneration in many organs. Cadmium (Cd) is a tossic non-essential metal whose concentration in the environment is continuously increasing. In the aquatic environment it causes severe damages in fishes' gills and kidneys. In the present study impact of Cd on gills aspect, pavement and chloride cells morphology, kidneys morphology, presence of ion transport proteins, water channels and apoptosis were examined in adult *Scorpaenea porcus* after 4 and 10 days of continuous Cd exposure. The investigation was carried out by immunohistochemistry, using antibodies to proteins of the inner mitochondrial membrane (AMA), to proteins of transmembrane ion transport (Na^+/K^+ATPase-α-subunit, the carrier protein $Na^+/K^+/Cl^-$ cotransporter T4 NKCC1), to the water channel Aquaporin 3 and caspase-7, an enzyme of the family of cysteine proteases, whose activation causes death by apoptosis. DNA fragmentation *in situ* was identified by the TUNEL method. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were used to study alterations at gill's and kidney's level. After 4 days of Cd exposure, the gills showed chloride cells sank into the branchial epithelium, partially covered by the pavement cells. Necrotic and apoptotic cells were present in gills and kidneys. Cd accumulations were seen in the cytoplasm and the cell membrane was disrupted. Cell shrinkage and chromatin condensation in the nucleus, positivity to caspase-7 and TUNEL indicated programmed cell death, macrophages were numerous. The presence of Na^+/K^+-ATPase and the carrier protein $Na^+/K^+/Cl^-$ cotransporter were more evident in the non-exposed fishes if compared with the fishes exposed to Cd. After 10 days of Cd exposure apoptotic and necrotic cells were still numerous, however a slight recover of the gills architecture might be seen, on the contrary no recover was detected in the renal tissue.

Studies on lizard thyroid gland after exposure of methylthiofanate: morphological and physiological evaluations

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In recent years, many new chemicals have been introduced in the environment, specifically endocrine-disrupting contaminantes (EDCs) and a number of them are being evaluated for their tossic effect on vertebrates. Although some of the most profound effects of EDCs are elicited in the reproductive glands of the endocrine system, thyroid gland disruption was one of the first observed effects of these chemicals and has since been well documented in the mammals. The purpose of this study is to determine the effects of low and high dose of the fungicide, methylthiophanate (TM) on the thyroid activity in the lizard *Podarcis sicula*. A significant decrease was observed in serum T3 and T4 levels of the lizards which were exposed to TM doses, compared to the serum thyroid hormones levels of the control group. Besides, this fungicide caused histological damages in thyroid gland of treated lizards. There were no differences between the control and TM-treated group of the lizards regarding hepatic T3 and T4 contents and 5'-T4 ORDII-monodeiodinase activity. No changes were observed in serum TSH levels of treated lizards. These results suggest that a high quantity of chronic TM exposure affects thyroid gland.

EXTRACELLULAR MATRIX

Specific immunolocalization supports presence of heterotypic collagen fibrils in dermal connective of *Sepia officinalis* (Cephalopoda, Mollusca)

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We obtained a suspension of isolated collagen fibrils from the integument of *Sepia officinalis* by tissue fragmentation, enzyme treatment, washings and centrifugation. The fibrils were prepared for transmission electron microscope (TEM) examination (a) in suspension and (b) by cutting ultrathin sections of resin-embedded fibril suspensions. Some fibril preparations were treated with dissociating agents (glycerol, acetic acid or urea). After preparation the fibrils were immunostained with colloidal gold for TEM observation. Reactions to antibodies against the following collagens were tested: human, teleost, and cephalopod type I-like collagen; human type III collagen; and human and rat type V collagen. The fibrils showed weak positivity for type I-like collagen of cephalopod and teleost, and for mammalian type V collagen. When treated with antibodies against type I-like and type V collagen simultaneously, the fibrils were positive for both. Examination of resin-embedded material showed that positivity for type V collagen was most often located within the fibrils, whereas positivity for type I collagen was mainly located peripherally. The density of gold particles was much increased on the swollen dissociated fibrils produced after treatment with urea. These findings are the first to indicate that collagen fibrils from an invertebrate are heterotypic.

Ultrastructural features of human dental pulp

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We have studied the ultrastructure of human dental pulp domains in young vs. aged subjects and during inflammatory pathologies.[1] Two different withdrawal techniques were tested: 1) dental pulps from teeth undergoing endodontic therapy, collected using a k-type file #15 and immediately fixed in glutaraldehyde; 2) dental pulps from extracted teeth after a quick fixation of the whole tooth, followed by sectioning along its longitudinal axis and gentle pulp mechanical extraction. All specimens were then processed for tem observation. In group 1 the odontoblastic layer got lost, while it appeared completely preserved in group 2. Numerous myelinic nerves, closely correlated to Schwann cells, appeared in the connective tissue, particularly in the apical region.[2] We found calcified structures,[3] surrounding, or even masking, collagen fibrils.[4] A lot of bacteria were present in pulp connective, presumably belonging to different species.[5] Finally, the odontoblastic nuclei often showed *coiled bodies*. A careful tem examination of human pulp revealed different morphological features related to the method of tissue preparation, age of donors, and current status of inflammation. in particular, flogistic condition seems to affect, in a certain manner, calcium metabolism in dental pulp. moreover *coiled bodies,* consistently and exclusively present in odontoblast nuclei, could represent a possible interesting cellular marker, requiring further specific studies.

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Effect of chronic sympatectomy in a model of rat cardiac hypertrophy: role of the extracellular matrix

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Increased sympathetic nervous system (SNS) activity helps to support cardiovascular function to mantain cardiac output and blood pressure in pressure over-load hypertrophy, thereby preserving flow to vital tissue. However, chronically increased SNS activity, may exert deleterious effects on cardiovascular structure and function by stimulating pathologic myocardial remodeling. Pharmacological blockade of the β-adrenergic receptors has beneficial effect on the progression of the heart failure, reduction mortality, and improving both the hemodynamic and the quality of life. Conversely, it is unclear whether the abolition of sympathetic nerve activity, which may be achieved by chemicl sympathectomy, may induced changes in myocardial structure, particularly related to an imbalance in collagen deposition by fibroblasts and collagen degradation by matrix metalloproteases (MMPs) activity. Aim of the present study was to assess the effect of abrogating sympathetic activity on myocardial interstitial remodeling during experimental pressure over-load hypertrophy. Sprague-Dawley rats were subjected to aortic banding (B) or sham operation (S), to subsequently undergo chronic sympatectomy (Sx, 6-hydroxydopamine 150 mg/kgi.p. twice weekly) or vehicle treatment (Vh). Collagen abundance (fraction %) was measured by Syrius red computer-aided analysis. To assess the extent of collagen degradation, matrix metalloprotease MMP-2 activity (ng/ml/mg prot.) and its specific tissue inhibitor (TIMP-2) concentration (ng/mL/mg prot.) were measured by gel zymography and ELISA techniques, respectively. Then to localize MMP-2 in the left ventricular tissue, paraffine sections were analysed by immunoistochemistry. In animal with chronic sympatectomy we observed an enhanced activity of MMP-2 and reduced activity of TIMP-2 accompained by reduced deposition of collagen. Chronic sympatectomy appears to exert benefical effect on development of fibrotic process during experimental cardiac hypertrophy.

MEK1 is required for invasive growth in MDCK and mIMCD3 epithelial cells in a 3D collagen matrix

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Branching morphogenesis is an essential and controlled invasive growth process during the development of organs such as in the kidney, lung, and pancreas. In kidneys, this process controls epithelialization of the metanephric mesenchyme during the induction of branching structures and in this way determines the number of nephrons in a mature kidney. Various growth factors, such as hepatocyte growth factor (HGF), have been implicated in branching morphogenesis in renal cells, including mIMCD3 (murine inner medullary collecting duct) and MDCK cells. Growth factors which induce branching morphogenesis in renal cells also activate the MEK1/ERK and the PI 3-kinase/Protein Kinase B pathways. We analyzed the potential roles that these two pathways might play in branching morphogenesis and in cystogenesis. We show that PD98059, a specific inhibitor of MEK1, inhibits branching of mIMCD3 cells and HGF-induced branching in MDCK cells. Treatment of MDCK cells cultured in collagen with HGF upregulates phospho-ERK in branching cells. Adenoviral-driven expression of the activated form of MEK1, Ad-MEK1-DD, in mIMCD3 cells, results in the induction of branching structures, whereas non-infected IMCD3 cells exhibit little or no branching. Expression of MEK1-DD, or BD 110, the catalytic subunit of PI-3 Kinase, decreases cystogenesis of MDCK cells. Our data show that, while activation of the MEK1/ERK or PI 3-kinase/PKB pathway inhibits the process of cystogenesis, only activation of the MEK1/ERK pathway is necessary for the induction of branching in renal cells, and therefore may play a more direct role in invasive growth.

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Extra cellular matrix role and ombilical vessels functional alterations in intra uterine growth retard

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During a non-invasive study of foetal condition in intra-uterine growth retard (IUGR), our purpose was to relate eco-color-Doppler flussimetric variations with some changes in placental ECM. Some years ago we demonstrated an increase in collagen type I in these pathologies, with subsequent increased blood flow resistence. In some case, this led to a complete reversion of the blood circulation. As many recent studies proved that morphogenetic proteins such as fibronectin, laminin and tenascin play a complex role in inter-cellular interacions, we decided to exhamine these non-collagenic components of the ECM and relate their distribution to foetal circulation conditions. In particular, our attention was appointed on tenascin, which plays his regulative role on cellular migration both as adhesive and anti-adhesive molecule. In addition, it seems to be involved in immunoprotection of the embryo during the implantation. In normal pregnancies, the presence of tenascin goes towards a natural decrease with placental ageing. It was surprising to observe that tenascin is markedly expressed in many IUGR we studied, in spite of the terminal age of pregnancy. In addition, in all these cases were present marked flussimetric alterations in umbilical artery. This lead us to hypothesize a biomolecular placental immaturity in IUGR foetuses, which could explain the characteristic decrease in the development of these babies.

Interstitial fibrosis is blunted by chronic sympathectomy but not by β-blockade in experimental pressure-overload hypertrophy

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Pressure-overload hypertrophy (POH) is accompanied by an imbalance between left ventricular (LV) collagen deposition by fibroblasts and its degradation by matrix metalloproteases (MMPs) activity, with net collagen accumulation. Although it has been shown that cardiac deterioration is accelerated by chronic sympathetic overactivity via induction of myocyte toxicity and death, little is known about the possible effects of excessive catecholamine exposure on LV extracellular matrix composition and turnover. To assess the effects of abrogating sympathetic activity on myocardial interstitial remodeling during experimental POH, Sprague-Dawley rats were subjected to abdominal aortic banding (B) or sham operation (S) to subsequently undergo chronic beta-blockade (Bb, oral propranolol, 60 mg/kg), chemical sympathectomy (Sx, 6-hydroxydopamine, 150 mg/kg i.p. twice a week) or vehicle treatment (Vh). Ten weeks later, carotid systolic blood pressure (SBP, mmHg), LV echo-derived end diastolic diameter (EDD, mm), and excised lung (LUNGi) and LV (LVi) weight indices (g/100g body weight) were measured. Collagen abundance (fraction, %) was assessed by Syrius red computer-aided analysis. To assess the extent of collagen degradation, MMP-2 activity (ng/ml/mg protein, gel zymography) and its specific tissue inhibitor concentration (TIMP2, ng/ml/mg protein, ELISA) were measured. In the course of experimental POH, chronic sympathectomy (but not beta blockade) profoundly affects the cardiac interstitium, with complete abolition of LV fibrosis accompanied by enhanced MMP-2 activity and markedly reduced expression of its specific inhibitor, TIMP-2. These findings indicate that during the development of experimental hypertensive heart disease sympathetic (over)activity plays a major pro-fibrotic role, which is not mediated by beta-adrenergic effects.

Morphological evaluation of cellular infiltrates induced by subcutis implants of dental materials in the rat

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This *in vivo* experiment consists of the implantation of dental materials in the subcutaneous tissue of rats in order to study the biological reaction induced by the biomaterials and to evaluate the inflammation response. 30 male Sprague Dawley rats were used and divided in two groups: group I was used as control (sham operated); group II was implanted into subcutaneous pockets, along the back of the animals, with the dental alloys. Seven, 14, 21 and 28 days after the implant, the macroscopic presence of any possible erythema, edema, necrosis at the implant site were measured. The animals were killed and the implants, their surrounding subcutaneous tissues and

lymph-nodes were excised and histochemically treated for the cell identification. We used different implant materials: cobalt-chromium alloys for ceramic crown and for removable prostheses. After 7 days the skin and lymph-nodes of animals treated with alloy for ceramic crown, showed a very high number of inflammatory cells. They were present near lymphatic hilus, where blood vessels enter or leave the organ. In the skin they were present mainly in the dermis. After 14 days the pattern changed and the number of these cells was similar to sham operated rats. On the contrary, the number of inflammatory cells was higher after 14, 21 and 28 days both in skin and lymph-nodes of rats treated with alloy for removable prostheses. We found that alloy for ceramic crown produced a rapid response but for short time whereas the alloy for removable prostheses induced a rapid response that persisted also after 28 days. These results suggest that the implant of dental materials determines biological reactions which involve cells inducing immediate hypersensitivity. The duration of activity of inflammatory cells depends on the type of dental materials.

Effects of the chronic digoxin administration on extracellular matrix turnover in a model of cardiac hypertrophy in Guinea pigs

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Digoxin has different positive inotropic actions that justify its therapeutic use in the treatment of heart failure (HF) and atrial fibrillation. The turnover of extracellular matrix (EM) plays a crucial role in the development and in the progression of pressure-overload left ventricular hypertrophy (LVH) to HF. In this regards there is not evidence on the relationship between Digoxin treatment and Matrix Metalloproteinases (MMPs) that plays an important role in EM degradation, are involved in the remodelling of interstitium and are tightly regulated by tissue specific inhibitors (TIMPs). The purpose of this work was to study the effects of Digoxin treatment on LV function and MMPs activity in experimental LV hypertrophy induced by aortic costriction in Guinea Pigs. Guinea pigs subjected to thoracic descending aortic banding were treated with Digoxin (0.5 mg/kg/die oral, DIG=13) or vehicle (Veh=9) for 12 weeks. Gel zymography was used to measure gelatinolytic activity at 72 KDa (MMP-2) and at 92 KDa (MMP-9) in LV samples. The zymograms were scanned by densitometer to analyze the proteolytic regions for each sample. The same samples used for zymography were also used for immunoblotting analyses. The specific inhibitors TIMP-1 and TIMP-2 were assayed by ELISA kits. Then to localize MMPs and TIMPs in the LV, criostatic sections were analysed by immunoistochemistry. In animals treated with Digoxin we observed a reduction of MMP-2 activity and a rise of TIMP-2 inhibitory activity. Digoxin appears to exert beneficial effects on matrix remodelling during experimental LVH.

Cytochemical and immunocytochemical characterization of microvasculature in guinea pig brain maintained *in vitro*

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In this investigation, we have analysed the molecular organization of the microvessel wall of the guinea-pig brain isolated and maintained *in vitro* by arterial perfusion for at least five hours. In particular, using enzyme cytochemistry, lectin histochemistry at microscopical and ultrastructural level and immunohistochemistry, we aimed at: 1) characterizing the endothelial phenotype of arterioles, venules and capillaries; 2) verifying the integrity of the endothelial glycocalyx; 3) studying the distribution of junctional molecules (occludin, ZO-1, vinculin, PECAM-1) and of components of the vascular basal lamina (type IV collagen, laminin, heparan sulfate proteoglycan). Our observations showed that in guinea-pig brain, as in other mammalian species, venule endothelium lacks the alkaline phosphatasic activity that characterizes arteries and capillaries. Moreover, the distribution of all the molecules considered resulted fully comparable in guinea-pig brains maintained *in vitro* and fixed *in vivo*, thus indicating that the transient hypoxia produced by the isolation procedure does not alter the molecular organization of microvessel wall. These findings extend previous physiological and ultrastructural studies confirming that the isolated *in vitro* guinea-pig brain is a powerful tool to address a variety of questions in physiological and pharmacological research on cerebral microvasculature.

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CELL AND TISSUE HISTOCHEMISTRY

Bioadhesion of drug delivery polymers to mucosal structures

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Increasing attention is focused to the use of mucoadhesive drug delivery systems owing to their possibility to promote dosage form residence time as well as to improve an intensified contact with the biological substrate (*Ahuja et al., Drug Dev Ind Pharm 23: 489-515, 1997*). In this context, the present study was designed to explain the interactions between mucous patterns and bioadhesive polymers as Carbopol and Pharmacoat with the mucosa types, the mucus layer composition and the chemical characteristics of polymers. For this purpose, Tensile test, lectin histochemistry combined with sialidase digestion and immunohistochemical techniques were carried out in sublingual, oesophageal and duodenal mucosas. Statistically significant variations (Turkey's test, $P<0.05$) of the Carbopol behaviour evaluated, by detachment force, in all samples and the highest mucoadhesion of Pharmacoat in the duodenal mucosa were correlated with the thick desquamation layer immunodetected in the oesophageal mucosa and the different sialic acid amounts present in the mucosas.

Observations on the morphogenesis of otoliths during larval development in brook lamprey, Lampetra planeri (Linnaeus 1758) (*Ciclostomata Petromyzonidae*)

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Ciclostomata are the most primitive vertebrate and the study of their functional morphology should be relevant also for etic and evolutionary studies. In this contribution we present the first data on ontogenesis and morphogenesis of the otoliths of *L. planeri*, a threatened European species of brook lamprey.

It is well know that the inner ear of the lampreys has two semicircular canals and a single elongate epithelium, the macula communis, instead of the two or three distinct otolithic epithelia found in all other vertebrate groups. The macula communis is covered by a mass of calcareous otoliths. These are crystalline spherites held together by a fibrous ground substance. We have studied the otoliths' morphogenesis of *Lampetra planeri* beginning from 2nd year to the 4th year of ammocoetes larval stages (several of them in incipient metamorphosis) by SEM and by histochemistry stains. We have found both proteins and glicoproteins in the ciliated chamber, in cell secreting of the sacculus, localised also into and around the otoliths. The presence of proteins and glicoproteins is greater in the 2nd year ammocoetes larvae than in the later stages. These results together with SEM and microanalysis data suggest that in origin the otoliths arise near the apex of the cells as small organic masses. Then, they enlarge by deposition of many concentric layers of a dense crystalline substance. Moreover confocal microscopy analyses were performed by using anti-calmodulin and anti-calbindin D28k. CLSM data showed the precise localization of these proteins in a fibrous ground substance that surround and held together the otoliths' mass.

High c-*myc* amplification level modulates the multiple effects of paclitaxel

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Paclitaxel (PTX) leads to cell accumulation in the G2/M phase, polyploidization and apoptosis due to its effects on microtubule stability through the binding to α-tubulin. Since c-myc is involved in the regulation of cell proliferation and apoptosis, we aimed at investigating whether a high c-*myc* amplification level could modulate the multiple effects of PTX. Experiments were performed on SW613-12A1 and -B3 human colon carcinoma cell lines (with either a high or low c-*myc* endogenous amplification level, respectively), and on the B3mycC5 cell line, with an enforced exogenous expression of c-*myc*. Immunocytochemical, cytometric and biochemical methods were used to assess the occurrence of apoptosis and the effect on the cell cycle. We found that a high c-*myc* amplification level potentiates PTX cytotoxicity, confers a multinucleated phenotype and promotes apoptosis at a higher extent, thus suggesting that c-*myc* expression level is relevant in modulating the cell response to PTX. We have recently shown, in HeLa cells, that the phosphorylated form of c-Myc accumulates in the nucleus, as distinct nucleolar and extra-nucleolar spots (*Soldani et al., Eur J Histochem 2002;46:377-80*); here, we demonstrate that in PTX-treated cells, phosphorylated c-Myc undergoes redistribution, becoming diffuse in the nucleoplasm.

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Expression of granulocyte macrophage colony stimulating factor receptor on human cardiac tissue from normal and heart transplantated subjects

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Several observations show the possibility that cardiomyocytes can proliferate and this proliferative capacity could be suitably controlled. Recently we demonstrated that some haematopoietic growth factors are able to modulate the proliferation and the cellular activity in some cell populations. Here we report the expression of the receptor for granulocyte-macrophage colony-stimulating factor (GM-CSFR) on cardiac tissue from normal and heart trasplantated subjects, in order to evaluate an hypothetical role of GM-CSF in the cardiac remodelling. The expression of GM-CSFR was studied by immunofluorescence and by immunoblotting. Quantitative analysis was performed by Scanner Densytometry. GM-CSFR was variously expressed in cardiac tissue samples we examined. In particular the receptor migrated (by Western Blotting) in a band with apparent molecular weight of 84 Kda; immunoposi-

tivity is evident both on some cardiomyocytes and on endothelia. Our purpose is to better characterize such sub-population of GM-CSFR-positive cardiomiocytes in order to relate the expression of the receptor with the morfofunctional characteristic of cells. In particular, we will considerate if the GM-CSFR expression on cardiomiocytes may indicate the possibility to enter in a proliferative pathway or a kind of reaction to injury or if it is simply related with their differentiated state.

PKC a mediated response of rat heart to intermittent hypoxia

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Alterations in oxygen tension determine different responses at tissue and cell level dependent not only on its intensity and duration, but also on cell type and on rate of its tolerance to O_2 level changes. Hypoxia, in fact, gives rise to multiple cellular and systemic physiological responses such as angiogenesis, erythropoiesis and glycolysis (*Guillemin, Cell, 89, 9, 1997*) and is a pathophysiologic component of many disorders including heart attack, stroke and cancer (*Harris, Cancer 2,38, 2002*). Since among the organs which mainly undergo the effects of hypoxic injury heart responds disclosing a relationship between oxygen supply and demand, i.e.myocardial blood flow and oxygen carrying capacity of blood and functional state of cardiac muscle, we try to elucidate, by immunohistochemical and western blotting analyses, the molecular mechanisms leading to morphological responses during development and ageing of rat heart subjected to intermittent hypoxic challenge (10% O_2/90% N_2 followed by 12 hr of reoxygenation) for 12 days. Thus here we report activation of a signal transduction pathway driven by PKC a which can phosphorylate IKB through a IKKS paralleled by an increased expression of HIF and VEGF. These events, involved in the occurrence of continuous state of dynamic adaptation of vasculature to counteract oxygen deficiency in rat neonatal normoxic and hypoxic hearts, suggest a rescue strategy against hypoxia along with an hypertrophic response. Moreover, in hypoxic young heart PKC a activation is paralleled by a substantial Bax homodimerization along with reduced p-IKB a and IAP expression thus suggesting that the young does not carry out arescue strategy, as also old hearts do disclosing the same response in the two experimental conditions.

Immunocytochemical characterization of cytoplasmic aggregates in the spinal cord of transgenic SOD1 mice

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Transgenic mice expressing human superoxide dismutase 1 (SOD1) with a glycine to alanine mutation in position 93 (Tg SOD1 G93A) are a valuable model for the study of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease of unknown etiology that primarily affects spinal motor neurons. As one of the proposed causes of ALS is the intracellular aggregation of misfolded proteins, we examined by light and electron microscopy the ventral spinal cord of transgenic mice at different times of progression of the pathology. In the ventral horn of symptomatic transgenic mice we found hyaline inclusions consisting histologically of an eosinophilic core and a pale halo, and ultrastructurally of accumulations of a) irregularly oriented filaments and b) electrondense material of variable size and irregular profile. The inclusions are absent in mice transgenic for wild type human SOD1 and in non-transgenic control littermates. Immunogold electron microscopy performed to characterize the composition of the aggregates showed that both types of aggregates are labeled by antisera against ubiquitin, a marker of protein degradation normally absent in neuronal cytoplasm, but only the filamentous ones are also labeled by anti-human SOD1 and by antisera against neurofilaments. The results indicate that the mutated human SOD1 selectively accumulates in aggregates containing filaments, where it is ubiquitinated in order to be subsequently degraded by the ubiquitin-proteasome pathway.

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Expression and distribution of serine protease, HtrA1, in human tissues

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The human HtrA family of proteases consists of three members: HtrA1, HtrA2, and HtrA3. In bacteria, HtrA chief role is to recognize and degrade misfolded proteins in the periplasm, combining a dual activity of chaperone and protease. In humans the three HtrA homologues seem to be involved in diverse functions such as cell growth, apoptosis, allergic reactions, fertilization, control of blood pressure, as well as blood clotting. In previous studies using RNA blot hybridisation, it was shown that the expression of HtrA1 was ubiquitous in normal human tissues. In this report we show by immunohistochemistry and in situ hybridization that HtrA1 was expressed widely, although a different tissue distribution and/or level of expression was detected in the different tissues examined. In particular high to medium HtrA1 expression level was detected in mature layers of epidermis, in the secretory breast epithelium, in the liver, in the kidney tubules of cortex and in the epithelium of proliferative endometrium, in contrast to epithelium of secretive endometrium, completely negative for this protein. Finally, we found that HtrA1 expression in the placenta greatly increased from first to third trimester of pregnancy and that the HtrA1 staining shifted from the cytotrophoblast to the syncytiotrophoblast, thus suggesting a role for HtrA1 in the complex mechanism of formation and function of the placenta during pregnancy.

Involvement of CDK9/cyclin T2a in myogenic differentiation

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Mammalian cell cycle progression is regulated by sequential activation and inactivation of cyclin-dependent kinases (cdks). However not all of them are involved in cell cycle regulation. In fact some of these were shown to be involved in the regulation of transcriptional elongation, in regulation of cell differentiation, apoptosis etc. We are currently studying the function of the cdk9/cyclinT2 complex. By fluorescent in situ hybridization (FISH), the gene codifying for cyclin T2a has been mapped on human chromosome 2q21, locus that has been linked to different forms of myopathy. The observation that immunohistochemical expression of cyclin T2a was high in skeletal muscle cells, while was undetectable in two cases of centronuclear myopathy, together with its chromosomal location, might suggest an involvement of the cdk9/cyclin T2a complex in this disease. To further prove the function of cdk9/cyclinT2 complex on myogenic differentiation, we showed the overexpression of cdk9 and its associated cyclin in C2C12 muscle cells increase the MyoD-dependent transcription and stimulates myogenic differentiation. Indeed inhibition of cdk9 activity by a dominant negative form represses the myogenic program.

Preliminary observations of atrial natriuretic factor on the female non-pregnant rabbit breast

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The breast is known to be a gland, whose function is entirely dependent on the steroid hormones estrogen, progesterone, prolactin and oxytocin. In the last years other hormonal factors have been found in the breast and CNP in the endothelial cells of the blood vessels. It is recognized that atrial natriuretic factor (ANF) is released by myocardiocytes on the stimulus of oxytocin. Our research carried out to show a relationship between oxytocin and ANF in the esocrine gland evidenced a relationship between oxytocin and ANF in the normal and hyperplastic prostate. We want to extend the study to breast to verify whether ANF is present, the cytotypes involved in its synthesis and, also, to pint out a probable relationships between oxytocin and ANF. Fragments of female non pregnant rabbit breast are fixed in Bouin's fluid and embedded in paraffin. Some sections (6 micra) were stained by E.E., some by monoclonal ANF antibody in TRIS buffer, and other by policlonal oxytocin antibody. Both immunohistochemical stainings are revelated by AEC. At low magnification the immunostained sections show an immunopositivity for ANF and oxytocin in the epithelium of interlobular ducts; the muscle fibers round the ducts are oxytocinergic. At high magnification a strong ANF immunopositivity in perinuclear area of epithelial cells; in the some cells it is possible to observe positivity also in apical area. The oxytocin immunopositivity is in basal of epithelial cells and in the muscle fibers. These results show, firstly, ANF presence in the ductal epithelium of the breast. Our precedent researches for ANF in the inter-intralobular ducts of rabbit parotid gland evidenced that the cells interlobular produced ANF, which plays a role on the production and ionic composition of the saliv. These preliminary investigations on the breast cannot clarify the action of ANF on the glandular activity, but we may hypothize that ANF of the interlobular ducts would act on the glandular secretion and oxytocin, similarly to miocardiocytes, woulde induced release of ANF.

VIP stimulation of *Podarcis sicula* adrenal gland: physiological or pathological condition?

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The adrenal gland of the lizard *Podarcis sicula* is formed by a dorsal ribbon of chromaffin cells, generally defined as medullary tissue, arranged along a central part of steroidogenic cells considered as cortical tissue. These two tissues produce catecholamines and steroids as part of the hypothalamo-hypophyseal-adrenal gland axis. Recent studies have demonstrated that *Podarcis sicula* adrenal gland is not only under hypothalamo-hypophyseal axis control but that several peptides may influence the physiological activity of the gland; among these, vasoactive intestinal peptide (VIP) is able to enhance strongly both catecholamine and steroid hormone production. We have shown by immunohistochemistry that VIP fibers were localized exclusively around clusters of chromaffin cells in the dorsal ribbon of the lizard adrenal gland. Moreover, a strong positivity for this peptide was observed within ganglial cells and within most chromaffin cells of the gland. We have also shown that VIP administration induced not only a functional enhancement of adrenaline release from adrenergic cells, but also a shift of noradrenaline cells to adrenaline ones. Moreover, we have verified whether strong stimulation by VIP could become deleterious. For this reason, we monitored the pattern of expression of two members of the Bcl-2 family, Bcl-2 and Bax, in control and VIP treated specimens. Furthermore, we also tested if peptide treatment induces apoptosis by TUNEL assay.

Gonadotropic cells in the pituitary gland of *Leuciscus cephalus*

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Two types of gonadotropins, GTH I and GTH II structurally homologous to tetrapod FSH and LH, exist in many teleost species. In salmonid species immunocytochemical studies have demonstrated that these hormones are produced in two distinct cellular types; however in other species it is not clear about the duality of GTH cells. The aim of this work was to analyse the occurrence of the gonadotropic cells in the pituitary gland of the freshwater fish Leuciscus cephalus at light microscopy. The study was performed using the antisera specific against chum salmon α-GTH I, chum salmon α-GTH II (kindly provided by Dr. H. Kawauchi), human FSH and human LH and the technique of ABC. The immmunocytochemical characterization, by the anti-human LH and anti-human FSH antisera, reveals two distinct gonadotropic cells, but also a single cellular type pro-

ducing both the hormones. FSH cells are mainly present in the RPD and in the ventral region of the PPD, instead LH cells are distributed along the PPD. LH cells appear always immunoreactive to the anti-chum salmon α-GTH II, in according to the confirmed homology between these two hormones. No cells show immunoreactivity to the anti chum salmon α-GTH I antiserum. This negative result isn't enough to prove the presence in *L. cephalus* of a single type of gonadotropic cells. The absence of α-GTH I cells could be in fact ascribed to the high degree of difference between amino acid sequences of α-GHT I of the chum salmon, used as antigen, and that of *Leuciscus cephalus.*

Preliminary immunohistochemical study on eNOS in rabbit pleura

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Endothelial eNOS (NOS III) isoform, which oxidize L-arginine to L-citrulline produces nitric oxide (NO). eNOS expressed in endothelial cells is a predominant isoform in the vessel wall. NO is thought an efficient neurotrasmettitor NANC located in specific sites of the bronchial tracts and blood vessels. Because NOS is present in the respiratory system aim of this work is to investigate whether eNOS is present also in the pleurae. Rabbit pleural fragments were fixed in Bouin's fluid and dehydrated and embedded in paraffin. The sections were processed by immunohistochemical methods using a monoclonal eNOS antibody; detector system was used avidin-biotine system. eNOS immunoreactivity is present in the mesothelium of the parietal and visceral pleura. In the submesothelial layer eNOS immunostained cells are closed to blood and linphatic vessels. It is evidenced that is localized in different pulmonary sites (respiratory epithelium, peribronchial muscle fibers, alveolar epithelium) with important roles of a histophysiologic mediation. A discriminant topology related to the appearance and to distribution of NO its isoform is morphofunctional interest.

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Glucose transporter (Glut-1) distribution in brain vessels of the lizard *Podarcis sicula*

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The brain vessels represent the anatomic site of the blood-brain barrier (BBB) of vertebrates. In the lizard *Podarcis sicula* these vessels are paired appearing as hairpin-shaped vascular loops. Scanty information is available on BBB markers in reptiles, especially in relation to paired vessel pattern. We studied the histochemical and subcellular distribution of glucose transporter (Glut-1) in brain vessels of adult lizards. Coronal sections from paraffin embedded brains were incubated with polyclonal anti-Glut-1 according to the immunoperoxidase technique. Ultrathin sections from brain tissue embedded in the hydrophilic resin Bioacryl were exposed to anti-Glut-1 antiserum followed by gold-labelled secondary antibody. Independently of size, all cerebral vessels were organized as vascular pairs and were immunopositive. In each vascular pair, one vessel was heavily immunopositive, whereas the adjoining vessel showed a faint immunoreaction. Pial vessels, which also possess BBB-characteristics, were Glut-1 immunopositive. In the brain, astrocytes and neurons were not stained. This pattern was found in all the regions of the lizard brain. As regards Glut-1 cytochemical localization, immunogold technique showed that gold particles were not uniformly distributed on endothelial cells. The abluminal surface displayed more particles than the luminal one and also the endothelial cytoplasm showed numerous gold particles. Like mammalian cerebral vessels, lizard brain endothelia show a clear polarity in Glut-1 distribution. Nevertheless in each vascular pair the two adjacent vessels appear differently engaged in transferring glucose from blood to the brain interstitial fluid.

Immunolocalisation of caveolin-1 in the oesophageal peptic glands of the red-legged frog *Rana aurora aurora*

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Caveolin-1 is an integral membrane protein with both COOH- and NH- terminal in the cytoplasm. This protein can reside in caveolae, cytoplasm, mitochondria and elements of the secretory pathway (*Li et al., J Cell Sci 114:1397-1408, 2001*). According to its locations, caveolin-1 can either be a membrane or a soluble protein. This latter is probably embedded in a lipoproteic particle. In mammals caveolin-1 has been localised in secretory granules of various serous exocrine glands, such as pancreas, salivary and mammary glands (*Liu et al., Nat Cell Biol 1:369-75, 1999*). We have localised immunohistochemically the caveolin-1 in the gastro-oesophageal tract of the red-legged frog *Rana aurora aurora*. In this frog, as in other Ranidae, there are voluminous oesophageal glands which elaborate both pepsinogen and mucus. Oxyntic cells clustered in the gastric glands produce hydrochloric acid of gastric juice. Immunostaining showed that caveolin-1 is mainly concentrated in the secretory granules of the peptic oesophageal cells. Weak labelling was observed in some goblet cells of the oesophageal epithelium and in gastric oxyntic cells. Our results support the hypothesis that the caveolin-1 is involved in both intracellular and extracellular lipid transports. Caveolin-rich lipoproteins co-secreted with pepsinogen from oesophageal glands may be transported in the gut where specific receptors may exist on the surface of cells that mediate their internalisation.

Cytoskeletal organisation and meiotic competence of immature bovine oocytes cryopreserved whithout cumulus cells

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Cryopreservation of oocytes is an essential tool in the improvement of reproduction technology and biotechnology. Freezing of immature oocytes seems to be more successful than freezing of mature oocytes. However at this stage the presence of cumulus cells limits the efficiency of cryopreservation. Aim of this study was to verify if the removal of cumulus cells before vitrification influences the capability of immature bovine oocytes to resume meiosis and reach metaphase II-stage after thawing. Cumulus cells were removed before freezing using a complete medium (M199+Calf serum) and PBS/PVA as control. After thawing, all the oocytes were matured for 24 hours in the presence of FSH and co-cultured with intact cumulus oocyte complexes. Since microfilaments and microtubules, that provide the framework for chromosomal reorganisation and cell division,

are sensitive to temperature changes, tubulin and actin distribution were analysed using a laser scanning confocal microscopy along *in vitro* maturation after thawing. At the same time nuclear maturation was assessed. Our results indicated that removal of cumulus cells in PBS/PVA negatively affects the reorganisation of cytoskeleton of vitrified oocytes and, consequently, their meiotic competence. On the contrary the use of a complete medium significantly improves the resumption of meiosis in denuded bovine oocytes.

Karyotype characterization of two polypteriformes by banding techniques and fluorescent *in situ* hybridization (FISH)

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While the classification of Osteichthyes into either the ray-finned or the lobe-finned fish is actually defined, the phylogenetic placement of Polypteriformes into one of these two categories remains somewhat uncertain. Polypteriformes have been placed into a number of widely differing taxonomic groups during the last century. Furthermore, few data are actually known about the inner relationships and the karyological and molecular evolution inside the different species belonging to Polypteridae. We examined the karyotype and the DNA from eight specimens of *Erpetoichthys calabaricus* (2n=36, FN=72) and *Polypterus palmas* (2n=36, FN=72). Polypterids lack of microcromosomes and have 36 metacentric and submetacentric chromosomes, that are smaller, but structurally more similar to the lungfishes and to the coelacanth than to the actinopterygians, also if the DNA/cell value (8.9–11.7 pg) is closer to the Latimeria (3.8 pg) than to Dipnoans (80.2–140 pg). We also localized the constitutive heterochromatin by C-banding, followed by CMA3 and DAPI staining and evidenced the NORs positions on two pairs of chromosomes. We detected the presence of telomeric sequences on these metaphase plates by FISH. In the whole group the karyotype resulted strongly conserved, the main differences regarding a probable centromeric fission in the couple 8 of *P. weeksii*, and the last two couples of subtelocentric chromosomes in the *Erpetoichthys genus*. *P. weeksii* would be subjected to more recent speciation events in the group. These data are confirmed by an our previous analysis on mitochondrial genome that supported the taxonomic separations of Polypteriformes from the Chondrosteans and Holosteans, suggesting their placement close to the lobe-finned fishes. Moreover, *P.senegalus* and *P.ornatipinnis* resulted more closely related than *P.palmas*, while Erpetoichthys genus is distinct from the whole group.

Developmental expression of eye regulatory genes in amphioxus

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To advance on controversial issue of eye homology, molecular studies on diverse eyes types have been initiated in Bilateria, other than Insects and Vertebrates. In Deuterostomia Invertebrates, the single pigment cup eyes in amphioxus and ascidians have been studied. We discuss on two eye specification genes conserved in evolution, *Dach* and *Six3*, with respect to development of *Branchiostoma floridae* embryos and larvae. In *Drosophila, dachshund* (*dac*) is required for eye and leg development. In mouse and chick *Dachshund* genes are expressed in the developing eye, central nervous system, somites and limb buds. During amphioxus development, *AmphiDach* is expressed in several tissue or organs that in Vertebrates also express members of the *Dachchund* genes family: developing somites, photoreceptive neurons of the frontal eye complex as well as Hesse organs and cells scattered along nerve cord. Eye development is a multistep process controlled by another family of genes highly conserved throughout evolution, the *Six* family. In amphioxus neurula, *AmphiSix3* transcripts are detected in the anterior epidermis, in the most anterior tip of notochord and in ventral cells of the anterior tip of the nerve cord: these cells are precursor of the photoreceptive neurons of the frontal eye complex, that will also labelled in late larva. Expression is also found in Hatchek's left diverticulum from which it forms the preoral ciliated pit, part of which gives rise to the homologue of Vertebrate anterior pituitary. These data suggest that the genetic network leading to eye formation has been conserved during evolution, in fact amphioxus shares control genes such as *Pax6, Dach,* and *Six3*, and probably also other members of the regulatory cascade required for eye morphogenesis.

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Histochemical approach for the identification of two cellular types in *Unio pictorum mancus* mantle

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Unio pictorum mancus is a filter-feeder freshwater bivalve, actually proposed as a sentinel organism for assessing the effects of pollutants. The mantle is a thin membrane-like tissue that lines the inner surface of the shell and surrounds the body soft parts; it is responsible for the secretion of an organic matrix that mineralizes to form the shell. The purpose of this study is to provide the histochemistry of two cellular types in the connective tissue: filtering cells (FC) and granules forming cells (GFC) to correlate them with some functional aspects of the mantle. The samples are fixed in buffered formaldehyde 1% or alternatively cryostabilized in ethylen glycole, then embedded in hydrophilic resin. On the sections several histochemical and histoenzymatic reactions are performed. The filtering cells can be easily observed only in cryostabilized samples, using reactions such as Toluidine blue and Periodic Acid Silver Methenamine; they are not reactive for tested enzyme activities. Closely around FC there is a reactivity for a new metal chelation technique (*Dore et al. Eur J Histochem 2001;45: 48*), probably associated to the cellular processes of the GFC, which are phagocytic-like cells. The proposed body of the GFC presents alkaline phosphatase and malic dehydrogenase activity, but only in fixed samples; it is also Nile red positive in cryostabilized ones. To confirm our observations the use of electron microscopy appears necessary.

Cytogenetic mapping of globin genes in FISH: a contribution of fluorescence in situ hybridisation to comparative genomics

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Progress in fluorescence *in situ* hybridization techniques makes significant advances in our understanding of genomic structure and evolutionary changes. A striking example is provided by recent cytogenetic mapping of globin genes in teleostean species (*Pisano et al., 2003*). The internal organisation of the α- and β- globin genes, typically consisting of three exons and two introns, is quite well conserved in vertebrates but important differences exist between taxa, at the level of chromosomal organisation. In FISH, as in amphibians, a strict α-β globin gene linkage has been found and the genic clusters are located on a chromosome pair. In birds and mammals the α-globin and the β-globin genes are separated on two different chromosome pairs. Although incomplete, because of the lacking of information on reptiles, the available data on molecular organisation and chromosomal arrangement of globin genes clearly indicate that, as vertebrates evolved, a major mutational event (or events) eliminated the tight linkage of α- and β-globin genes found in fish and amphibians. This important genomic rearrangement is likely to have occurred in the common ancestor of the amniotes.

The synthesis of vitellogenin within the ovarian follicle in *Torpedo marmorata*

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The pattern of vitellogenesis is similar in all non-mammalian vertebrate: liver, under oestrogenic stimulus, synthetizes vitellogenin that, via the maternal circulation, is delivered to the oocyte and here internalized by receptor-mediated endocytosis (*Wallace, 1985, in: "Developmental Biology" vol.1, Oogenesis, L. Browder, ed, pp.127-177; La Fleur, 1999, in: "Encyclopedia of Reproduction", vol.4, pp.985-992, Academic Press*). Contribution to vitellogenesis of different components of ovarian follicle has been also reported. In this regard, it has been suggested in some vertebrates, as well as amphibians (*Wallace, 1985*) and squamate reptiles (*Ghiara and Limatola, 1980, Acta Embryol Morphol Exper 1:5-6; Andreuccetti, 1992, J Morphol 212:1-11*) that ovarian follicle could be involved in yolk synthesis. Recently, in Torpedo marmorata typical morphological pictures of yolk organization and new-formed yolk globules have been found within granulosa cells (*Prisco et al., 2001, in: Perspective in Comparative Endocrinology: unity and diversity, pp.1197-1201, Monduzzi Ed.; Prisco et al., 2002, Gen.Comp.Endocrinol. 128:171-179*). An investigation, performed with immunohistochemical and in situ ibridization techniques during different phases of follicular growth in *Torpedo marmorata,* showed that granulosa cells in both previtellogenic and vitellogenic phase actively synthetize vitellogenin, the first evidence that synthesis of such a protein also occurs in the ovarian follicle.

Overview of FGF-2 and its receptor 2 pathways under prostaglandin stimulation in rat Py1a cell line

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It is known an involvement of some prostaglandins on the expression of FGF-2 and FGFR2 in bone. Indeed, our previous studies indicated that PGF_{2a} and the synthetic analog fluprostenol (Flup) increase FGF-2 mRNA and protein synthesis; moreover, they are involved in the regulation of the expression of FGF-2 mRNA. This regulation follows a protein kinase C (PKC) mediated mechanism in Py1a rat osteoblasts. In fact, data regarding pretreatment with PMA or PKC inhibitor H-7 confirmed the findings. Under prostaglandins stimulation, FGF-2 and its receptor 2 accumulated at nuclear envelope level, then colocalized inside the nucleus. Under PMA and H-7 pretreatment conditions, the protein pathways changed. Incubation with PD-98052 and PGF_{2a} stimulation demonstrated an involvement of MAPK kinase in this process. All these findings were supported by confocal sequential and/or simultaneous double labeling analysis and by molecular data. Parallel immunogold electron microscopy studies confirmed the results obtained at confocal laser scanning microscopy level in addition to a close relation between FGF-2/FGFR2 complex and importin-α. Ultrastructural analyses showed that an increment of coated-pits and coated-vesicles occurs under FGF-2 stimulation but double-sided immunogold labeling revealed that cytoplasmic internalization of FGF-2 and FGFR2 does not involve these structures.

Cellular distribution of RhoA in human embryonic and adult renal tissues and renal cell lines: morphofunctional implications

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Rho signaling proteins control many cellular processes by acting in the transduction pathways of various receptors. Contrary to other members of the Ras superfamily, they display a peculiar property by cycling in various subcellular compartments depending on their metabolic form: the inactive form is in the cytosol and the active form is mainly localized along cell membranes and is involved in the control of many cellular functions (actin reorganization, epithelial cell polarity, differentiation and proliferation). This study aims to assess the cellular distribution of RhoA in human embryonic, fetal and adult renal tissues, in pathological renal samples (IgA nephropathy biopsies, renal carcinoma) and in renal cell lines derived from normal (MDCK), embryonic (SK-NEP-1), and cancerous (ACHN) kidneys. The localization of RhoA was performed by using polyclonal anti-RhoA immunoglobulins with an indirect streptavidin-peroxidase method. Rho activation assay was used for cell culture studies. Anti-RhoA cytoplasmic staining was found in the cytoplasm of mesonephric duct/vesicle and metanephric ampullar-derived cells of embryonic kidneys as well as of tubule and collecting duct cells of fetal and adult kidneys. A membrane-linked RhoA positivity was observed in either ampullar-derived or metanephric blastema cells of fetal kidney, in tubular cells from IgA nephropathy and renal carcinoma. Membrane-linked RhoA as well as the activated GTP-bound

RhoA form was observed only in embryonic-cancerous SK-NEP-1 cells indicating that RhoA is constitutively activated in this cell line. The presence of membrane-linked RhoA in fetal and pathological renal samples suggests the importance of RhoA activation in the control of cellular processes during kidney development and deseases.

Characterization of the alveolar content in several species of Antarctic Notothenioids

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Alveoli in teleosts are large vesicles that fill much of the cytoplasm of previtellogenic oocytes. They are considered homologous of the cortical alveoli of amphibians and their content, rich in glycoconjugates would be involved in chorion hardening. In the present paper we have investigated the nature of the alveolar content in several antarctic Notothenioids. In particular, we have studied four species of Nototheniids (*T. bernacchii, T. lepidorhinus, T. pennellii and T. newnesi*), two species of Bathydraconids (*Gymnodraco acuticeps* and *Cygnodraco mawsoni*) and two species of Channichthyids (*Chionodraco hamatus* and *Cryodraco antarcticus*). By using different staining procedures (silver salts, Sudan black B, PAS) we have been able to demonstrate that the alveolar contents in these three families significantly differ. In particular, we have demonstrated that alveoli in Nototheniids contain a proteinaceos material, non acidic, non lipo-conjugated, that acquires a PAS positivity during oocyte growth. Gel electrophoresis confirms the presence of these glycoconjugates and indicates that they significantly vary in the different species examined. In Bathydraconids and Channichthyids the analyses carried out have demonstrated that the alveolar content is not responsive to any of the stains assayed and, therefore, its nature remains uncertain.

Modulation of transcription and export in an *in vitro* model of hibernation

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DADLE (D-Ala2-D-Leu5) is an enkephalin analogue capable of mimicking the Hibernation Induction Trigger, a protein which drives mammals into hibernation. This peptide binds to opiod receptors and it is capable of increasing the survival time of esplanted organs such lung, liver and heart twice longer than organs kept in saline. Consequently, we have hypothesized that DADLE could have some effects both on cell proliferation and transcription. Moreover, recent data (*Malatesta et al., 1999, 2001; Biggiogera and Pellicciari, 2000*) have shown that, in different vertebrate tissues, RNP-containing structures undergo significant changes during hibernation. We have already observed that DADLE induces a decrease in transcription when Hep-2 cells are treated for 48 hs (*Vecchio et al., submitted*) and in a cell line derived from woodchuck, a hibernating animal. In order to verify a possible modulation of transcription/export we have studied its effects in this cell line. Cells were labeled for 30 min with BrU and then treated with DADLE for 1, 3 or 6 hrs and then observed in fluorescence and at EM. In control cells, newly synthesized RNA is present in the nucleus and in the cytoplasm, as expected, and the nuclear amount decreases progressively. In DADLE treated cells, however, a higher amount of RNA seem to persist within the nucleus. These preliminary data seem to indicate that this hibernation-mimicking peptide is capable not only of decreasing transcription but also to influence RNA export to the cytoplasm.

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