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PROCEEDINGS OF THE 33rd NATIONAL CONGRESS OF THE ITALIAN SOCIETY OF HISTOCHEMISTRY

Rome, June 8 – June 10, 2009

Dipartimento di Anatomia Umana

Sapienza Università di Roma

Honorary President

Tindaro G. Renda

under the auspices of
the University of Pavia, Italy

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Celebration of Prof. V. Erspamer's Centennial

Maffo Vialli Award Lecture

OF FROGS AND MEN: FOLLOWING VITTORIO ERSPAMER'S AND TINDARO RENDA'S FOOTSTEPS

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The concentration of many neuropeptides in the brain of amphibians is several orders of magnitude higher than in the brains of mammals. We have taken advantage of this singular situation to isolate many regulatory peptides from the brain of the European green frog Rana esculenta, following in that the footsteps of Vittorio Erspamer and Tindaro Renda. Through this program, we have characterized a series of biologically active peptides that are orthologous to mammalian neuroendocrine peptides including α-MSH, γ-MSH, two GnRH variants, CRH, NPY, PACAP, two tachykinins, CGRP, CNP, GRP and ODN. This project has also led to the discovery of several novel neuroendocrine peptides that were first isolated from brain tissue and have subsequently been identified in mammals. Notably, we have characterized (1) the somatostatin-14 (S14) isoform [Pro^2,Met^{13}]S14 together with authentic S14, thereby providing the first evidence for the occurrence of two somatostatin variants in the brain of a single species; (2) the first tetrapod urotensin II, thus demonstrating that this peptide was not the appanage of the fish caudal neurosecretory organ; (3) secretoneurin, a peptide derived from the post-translational processing of secretogranin II; and (4) 26RFa, a novel member of the Arg-Phe-NH$_2$ family of regulatory peptides. Orthologs of all these frog neuropeptides have now been identified in human and have been shown to exert important regulatory effects in mammals.

Supported by grants from INSERM (U413), the International Associated Laboratory Samuel de Champlain, the European Institute for Peptide Research (IFRMP 23), the platform for Cell Imaging of Haute-Normandie (PRIMACEN) and the Région Haute-Normandie.

VITTORIO ERSPAMER'S PATH FROM COMPARATIVE HISTOCHEMISTRY TO NEUROPHARMACOLOGY

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During his long scientific career, Vittorio Erspamer isolated and identified dozens of peptides of biological and pharmacological interest. Erspamer's contributions to the characterization of almost each different molecule have been discussed and recognized. However, most of those who commented his investigations paid little attention to the theoretical and methodological tools that Ersparmer used to discover what Viktor Mutt has called *a continent to explore*. In fact, from what has been written on his histochemical and pharmacological research, Erspamer emerges as a skilful experimenter, guided by an inductive logic of discovery, where, for example, the origin of his interest in biologically active substances in the amphibian remains unaccounted. Actually, the reading of Erspamer's early studies and of some comments he made about his researches, it appears that a singular curiosity for the phylogenetic distribution and the biological significance of chemical substances extractable from tissues of different amphibian species inspired his investigation. Moreover, he used a systematic descriptive-comparative approach in analyzing the chemical and biological properties of the substances identified. Erspamer acquired his interests and methodology during the training in Pavia, and it is of some historical and epistemological interest to question his awareness that during his studies on the nature and function of enteramin the approach he was using hindered his understanding of the relationship between serotonin and enteramin. Afterwards, Erspamer was able rethink his biological assumptions and to redirect his methodological approach for effectively achieving also pharmacological objectives.

VITTORIO ERSPAMER AND PAVIA HISTOCHEMISTRY

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It is well known that Vittorio Erspamer discovered and characterized chemically, functionally and pharmacologically many neuroendocrine factors, from enteramine/serotonin/5-hydroxytryptamine to several monoamine derivatives to a number of 0active peptides from amphibia and other lower species. The cellular origin of such factors was originally investigated in Pavia, first in the *Istituto di Anatomia e Fisiologia Comparata* directed by Maffo Vialli, Erspamer's first head and one of the founders of Histochemistry, and later on at the *Istituto di Anatomia Patologica* by myself with several collaborators. Among results obtained in these studies were: 1) A conclusive proof of the indole nature of enterochromaffin (EC) cells secretory product,[1] an essential step for its bona fide identification with 5hydroxytryptamine, 2) The immunodetection of mammalian endocrine peptides homologous to Erspamer's peptides, such as cholecystokinin (Erspamer's caerulein),[2] substance P (eledoisin) and gastrin releasing peptide or GRP (bombesin),[3,4] in specific endocrine cells of the human gastrointestinal mucosa and lung, as well as in corresponding endocrine tumours. These investigations allowed to characterize ultrastructurally and cytochemically up to 14 morphofunctional types of cells in the gastroenteropancreatic endocrine system,[(5)] a more recent development of Erspamer and Vialli's *enterochromaffin cell system* and to recognize and classify related tumour growths.[(6)] Vittorio Erspamer achievements as well as personal suggestions and encouragements have been seminal for such developments.

1. *Solcia E and Sampietro R Nature 1967, 214:196-7.*
2. *Buffa R et al. Gastroenterology 1976, 70:528-32.*
3. *Wharton J et al. Nature 1978, 273:769-70.*
4. *Buffa R et al. Histochemistry 1982, 76:457-467.*
5. *Solcia E et al. Regul Pept 2000, 93:31-5.*
6. *Solcia E et al. Histological typing of endocrine tumors, Berlin Heidelberg: Springer-Verlag, 2000, pp 56-149.*

SEROTONIN: FROM DISCOVERY TO TREATMENT. A STORY OF TRANSLATIONAL BIOMEDICAL RESEARCH

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Vittorio Erspamer was a biological scientist of many achievements that have lead to greater understanding of the chemical regulation of interactions between cells. One of Vittorio Erspamer's great discoveries was the extraction of an unknown secretory product from enterochromaffin cells which he named enteramine.[1] Later, with the involvement of Irvine Page and Maurice Rapport, it was established that enteramine was 5-hydroxytriptamine (serotonin).[2] The discovery of serotonin opened new avenues of research on the cardiovascular, gastrointestinal and nervous systems involving both animals (invertebrate and vertebrate) and humans. After identification mapping, involving the development of sensitive analytical and elegant histo- and immuno-chemical methods, revealed a limited number of midbrain and brainstem neurones produced widespread innervation of limbic brain areas and spinal cord so providing important possible clues to function. The next stage, coinciding with the expansion of molecular biology, was the surprise identification of at least 14 distinct receptor subtypes of which all, except one, were metabotropic in nature. Subsequent research has concentrated on identifying the function of individual receptors and investigating the functional role of serotonergic systems in an integrated manner. This has lead to new treatments for a range of psychiatric (depression, anxiety, schizophrenia), gastric (IBS) and cardiovascular (migraine) disorders and current research offers the potential of more new exciting drugs just as Vittorio Erspamer predicted in his landmark review published in 1954.[3]

1. *Erspamer V and Vialli M. Boll Soc Med-chir Pavia 1937, 51:357-63.*
2. *Erspamer V and Asero B. Nature 1952, 169:800-1.*
3. *Erspamer V. Pharmacol Revs 1954, 6:425-87.*

VITTORIO ERSPAMER, NAVIGATORE, ESPLORATORE E FREECLIMBER DELLA SCIENZA

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Vittorio Erspamer, come Cristoforo Colombo, ha scoperto un continente da esplorare. Infatti egli ha condotto personalmente le spedizioni scientifiche che, in varie parti del mondo, gli hanno permesso di raccogliere anfibi e molluschi da cui estrarre sostanze ad attività farmacologica. Le centinaia di reperti, raccolti e catalogati scrupolosamente da Erspamer, sono oggi conservati in un Archivio donato alla Sezione di Storia della Medicina della Sapienza, dalla Dott.ssa Giuliana Falconieri, moglie, ricercatrice e collaboratrice di Erspamer.

Vittorio Erspamer nel 1935, scopre l'enteramina, poi rinominata serotonina. Negli anni successivi, egli ritrova l'enteramina e le amine a struttura feniletilaminica anche nella pelle di anfibi sudamericani, animali da cui Erspamer stesso ed i suoi collaboratori estraggono, testano e chiariscono la struttura di numerosi peptidi, che presto si rivelano agonisti di altrettanti nuovi recettori presenti nel cervello dei mammiferi e dell'uomo. Erspamer ha dimostrato che la pelle di anfibio è una vera e propria *farmacia*, infatti le sue indagini hanno contribuito in modo sostanziale al chiarimento della struttura e allo studio farmacologico di peptidi di mammifero già noti, ma non ancora sequenziati e hanno preceduto e pilotato la scoperta di peptidi nuovi nei tessuti di mammifero. La scoperta della bombesina (1970) ha precorso di otto anni l'individuazione dei peptidi bombesino-simili di mammifero; quella della kassinina ha preceduto gli analoghi neurochinina A e neurochinina B; quella della sauvagina ha anticipato di due anni l'identificazione della corticorelina (CRH). Erspamer, professore di Farmacologia alla Sapienza, dal 1967 fino alla sua morte 10 anni fa, ha condotto le sue ricerche peptide dopo peptide, di scoperta in scoperta, nel silenzio e nella tensione dell'esplorare l'ignoto, ed è così che lo vogliamo ricordare, chino sui campioni, nel testare un peptide appena estratto dalla pelle di qualche esotico anfibio.

FROM DELTORPHIN TO TACHYKININS: MY LABORATORY EXPERIENCE WITH PROF. VITTORIO ERSPAMER

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It is really a privilege for me to contribute to the memory of Prof. Vittorio Erspamer. I had the great opportunity to work with him during the last 15 years of his career, until his death. It is not my task to point out and celebrate his extraordinary scientific contribution to Pharmacological sciences, but I just would like to remember the wonderful experience I had working in his laboratory. Since I met him the first time, when I got my degree in 1985, I was particularly impressed by this man

so simple, frank and modest and so shine, even if conscious of the excellence of his research, universally acknowledged by a lot of prizes and awards. At first glance, he appeared brusque but day bay day he then revealed his way to be: a lovable teacher able to speak about science as a storyteller. After few months, his laboratory started to be my second home and him and his lovely wife Giuliana my second family. Those years were incredible on the science side and even formidable on that of life. I cannot forget his enthusiasm when he discovered deltorphins, the first biologically active peptides with a D-aminoacid, in addition to dermorphins, or the incredible effort in studying Tachykinins, and he was already over 80's. It was a great privilege for me to be his last pupil and to contribute to his last, laborious work about the tachykinins family. I will never forget his last days, by that time at home, when with his usual affability, he said to me: don't worry, this work will be published! The review *The Tachykinin peptide family* was at least published, without him. He was an exceptional person whom I miss very much.

VITTORIO ERSPAMER: A MASTER, A FRIEND

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Tindaro and I met Professor V. Erspamer for the first time in the late 1970s. That day, he enlightened us with a straightforward and simple description of his studies on Neuropeptides - after years in which his main interest had been Enteramin - and his view of the frog skin as an almost inexhaustible source of biologically active molecules. He asked us to help him in his research: our first collaboration led to the immunolocalisation of the Sauvagin - the equivalent of the hypothalamic CRH in mammals - in the hypophysis of teleostei fishes. From that day onwards, a passionate scientific collaboration between us blossomed. We shared a strong belief: that phylogenetic and ontogenetic studies should be applied to neurosciences. And slowly, gradually, his apparently unemotional behavior translated into a close friendship and into a scientific endeavour that lasted, with no pause, until 26 October 1999.

Professor Erspamer was a man deeply in love with his work: he was literally tireless and volcanic in his ability to locate and plan new fields and domains to explore. In human relationships, he was not the most expansive man: maybe because, after all, he was shy; he used to jealously preserve and protect his innermost feelings. I will never forget the many evenings we spent in the living room of his beautiful and inspiring Roman apartment, discussing research topics but also, as time went by, more confidential issues. He was also a very quiet and happy man. He used to say *I've been very lucky because I love research, and research never betrayed me*.

Great Masters leave an eternal mark in the minds of their pupils and collaborators. Great friends leave a deep trace in the heart of those who profit from their friendship.

For us, Professor Erspamer was both an unforgettable Master, and an unforgettable friend.

IN MEMORIAM
PROF. TINDARO GIUSEPPE RENDA

On April 25th 2008 Professor Tindaro Giuseppe Renda suddenly and prematurely passed away in Rome. He was born in Cefalù (Palermo, Italy) on September 7th 1940. He obtained his Medical Doctor degree with *summa cum laude* at the University of Messina on July 29th 1965. For his remarkable attitudes, even before the degree, his Master Professor Mario Franceschini early involved him in research and teaching activities.

He was Assistant Professor of Human Anatomy at the Medical Faculty of Messina during the period 1965-1971. On December 1971 he moved to the Medical Faculty of Sapienza University in Rome wherein he gave rise to the laboratory of immunohistochemistry, nowadays one of the sections of the Department of Human Anatomy. He was Associate Professor of Anatomy between 1971-1979 and then Full Professor since 1980, besides coordinating activities of a medical course of Human Anatomy. He was Director of the Institute of Human Anatomy (1985-1988), then becoming Director of the Department of Human Anatomy (2002-2008) at the I Faculty of Medicine, Sapienza University of Rome. His scientific curriculum much reflects a curious and rigorous personality. Meeting Professor Vittorio Erspamer as well as the colleagues Professors Toshihiro Maeda and Hiroshi Kimura (Shiga University of Medical Science, Otsu, Japan) represented for him really crucial events. Professor Tindaro Giuseppe Renda is author of numerous scientific papers on topics regarding phylogenesis, ontogenesis, and neurosciences. He was member of the Editorial Board of the European Journal of Histochemistry, official journal of the Italian Society of Histochemistry. Together with other Italian colleagues he also published a textbook on Human Anatomy, which is now the most diffuse text of Anatomy among Italian medical faculties.

Besides devoting to teaching Human Anatomy in pre- and post-degree courses, he got actively involved in an experimental *parallel* course, in which he put the same dedication, and the same rigour and enthusiasm diffused in his researches in that both were considered by him as being inseparable academic achievements. These outstanding personal qualities, together with a notorious service spirit as well as a big sense of humanities, make students, pupils, and collaborators, from both the academic and scientific community, became well aware and vividly experience his sudden pass away.

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Symposium I: Recent Advances in Peptides and Neurotransmitters

BOMBESIN-LIKE PEPTIDES IN ACUTE AND CHRONIC LUNG INJURY

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Pulmonary neuroendocrine cells (PNECs) are epithelial cells that secrete bioactive peptides including gastrin-releasing peptide (GRP), the major pulmonary bombesin-like peptide. GRP is expressed at high levels in fetal lung, where it promotes fetal lung development. After birth, GRP levels usually drop. Pathological PNEC hyperplasia can result from exposure to hypoxia, free radicals, or infection. We showed that GRP mediates lung injury in baboon models of bronchopulmonary dysplasia (BPD), chronic lung disease of newborns, associated with PNEC hyperplasia: GRP blockade abrogates acute and chronic lung injury in BPD. The observation that GRP mediates lung injury in BPD is highly relevant to asthma because half of BPD patients develop pediatric asthma. We hypothesize that GRP mediates asthma, which is a major medical condition of intermittent airways obstruction. Asthma could be initiated and/or promoted by GRP, triggered by inhaled pollutants or allergens. Bombesin and GRP are potent, immediate bronchoconstrictors *in vitro*. PNEC hyperplasia occurs in guinea pigs given systemic antigen, then PNEC degranulation follows aerosol challenge. We now test whether GRP mediates asthmatic responses *in vivo* (airway hyperreactivity, AHR, and inflammation using two mouse models: 1. Ozone exposure, like air pollution, and 2. Ovalbumin (OVA) immunization, like allergic asthma. GRP blockade before ozone or OVA challenge abrogates AHR and bronchoalveolar lavage (BAL) macrophages, neutrophils and eosinophils. GRP blockade also reduces bronchiolar inflammation and decreases levels of 18 BAL cytokines from Th1, Th2, Th17, macrophages, neutrophils, and epithelial cells. GRP blockade abrogates GRP receptor serine-phosphorylation induced by ozone exposure. In summary, GRP blockade prevents asthma-like responses in mice exposed to air pollution or allergens, and may be useful for preventing acute and chronic inflammatory lung diseases, especially asthma, which is a growing medical problem.

RECENT ADVANCES IN CHOLINERGIC MECHANISM WITH SPECIAL REFERENCE TO THE IDENTIFICATION OF CHOLINE ACETYLTRANSFERASE OF THE PERIPHERAL TYPE (PCHAT)

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Acetylcholine (ACh) seems the most phylogenetically ancient chemical transmitter in vertebrates and invertebrates. Cholinergic neurons, which use ACh as a neurotransmitter, play key roles in CNS and PNS. However, histochemical detection of cholinergic structures has been hampered by the lack of suitable methods. No good histochemical technique exists for detecting ACh. Although acetylcholinesterase, the degrading enzyme of ACh, has been identified histochemically owing to its presence also in noncholinergic neurons it is not accepted as a selective marker for ACh. Conversely, choline acetyltransferase (ChAT), the synthetic enzyme of ACh, has been used as a reliable marker for detecting cholinergic neurons by immunohistochemistry. However, it has often been difficult to label peripheral cholinergic cells with most antibodies against ChAT that clearly label central ones. We then hypothesized that the protein structure of ChAT in PNS may differ from that in CNS. In 2000 we isolated a novel form of ChAT from rat parasympathetic ganglia. Because of its predominant distribution in PNS the new form was termed ChAT of the peripheral type (pChAT), and the previous one was renamed ChAT of the common type (cChAT). pChAT is a splice variant of cChAT, with pChAT mRNA lacking exons 6-9 of rat cChAT gene. Since the amino acid sequence of cChAT (67 kDa) encompasses that of pChAT (49 kDa), certain ChAT antibodies may recognize pChAT protein. To circumvent this problem and obtain an antiserum that did not react with rat pChAT, we produced an antiserum against the rat cChAT peptide encoded by exons 7-8. Interestingly, the cChAT-specific antiserum allowed firstly labeling cholinergic nerves in octopus. We showed that an octopus ChAT-like immunoreactive protein was capable of producing ACh. Our study further showed that octopus neural tissues possess pChAT-like immunoreactivity, implying the omnipresence of cChAT and pChAT among the animal kingdom from vertebrates to invertebrates

NORMAL EXPRESSION AND BIOLOGICAL FUNCTIONS OF α-SYNUCLEIN

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Abnormal aggregation of α-synuclein (α-Syn) is linked to the PD pathogenesis. To understand the pathological role of α-Syn, a very essential work is to clarify its normal expression and potential biological functions. We show that α-Syn is mainly localized in the presynaptic terminals and nuclei of many neurons throughout the brain and spinal cord. α-Syn is also observed in axons, cytoplasm and mitochondria of brain neurons. α-Syn in mitochondria is usually richer in the brain regions sensitive to neurodegeneration. We found several potential biological functions of α-Syn. α-Syn can inhibit the expression and activity of tyrosine hydroxylase (TH). We suggest that inhibition of DA synthesis is a normal function of α-Syn and the aggregation and mutations of α-Syn will lead to the disruption of DA metabolism and increased ROS. α-Syn can inhibit the complex I activity. We speculate that proper inhibition of complex I activity by α-Syn may help suppress free radical production, but over-accumulation of α-Syn in mitochondria will reduce ATP production and increase ROS. α-Syn can promote neurite outgrowth of neurons, possibly by polymerizing tubulins into microtubules. Mutations or aggregation of α-Syn may cause disrupted axonal transport and neurodegeneration.

ROLE OF α SYNUCLEIN IN NEURODEGENERATION

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Occurrence of inclusions bodies is quite common in neurodegenerative diseases, but the significance of such a cell response (neuroprotective vs neurotoxic) as well as the mechanisms responsible for inclusions formation remain under investigation. It is widely established that formation of intracellular aggregates is triggered by misfolded proteins. α-synuclein belongs to the class of synucleins and it is involved in a variety of neurological disorders ranging from Parkinson's disease to amyotrophic lateral sclerosis. Synucleins become aggregate-prone as a consequence of several events; namely, gene mutations, defects in protein translation, and post-translational modifications, leading *wrong* molecular conformation. In inherited forms, structural alterations or overexpression of the α-synuclein, caused by point mutations or duplication of the coding gene, are responsible for neurodegeneration. In these conditions, production of excessive amount or structurally altered α-synuclein results in a *toxic gain of function*, consisting in the formation of oligomeric protofibrils, which progress to insoluble α-synuclein fibrils and precipitate in the cytosol. This seems to be facilitated by the interaction with dopamine, however the pathogenic mechanisms remains still unknown. For instance it remains unclear to which extent an increase of α synuclein should always produce toxicity or, vice versa, it might also be connected with protective effects. This presentation is an overview on the role of α synuclein in connection with degrading pathways and neurotoxicity emphasizing novel findings about the autophagy pathway.

THE RELEVANCE OF BASIC NEUROSCIENCE IN THE SEARCH FOR NEW DRUGS AND THERAPIES INVOLVED IN PARKINSON'S DISEASE, MEMORY FUNCTIONING, AND DEPRESSION

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Neurotransmitters interact in the CNS through an intricate network of efferent and afferent projections. Alterations in neurotransmitters like GABA, serotonin, dopamine, opiate, acetyl choline and nitric oxide (NO) have been implicated in the etiology and progression of many neuropsychiatric and neurological diseases, such as Parkinson's disease. NO is the first of a new family of rare neurotransmitter molecules. The dorsal raphe nucleus (DR) is known to contain dopaminergic neurons, which send efferent projections to the Caudate-Putamen. More recently, the influence of serotonin on midbrain DA systems has received significant attention. While many of studies have presented a complicated picture in which serotonin exerts a modulatory influence on DA function, one promising line of research has significant clinical relevance: the observation that atypical antipsychotics often share both DA and 5-HT antagonist properties. Notwithstanding, in Parkinson's disease (PD), the most dramatic changes occur in the dopaminergic nigrostriatal system, and probably in other dopaminergic systems, significant changes have also been observed in several non-dopaminergic systems, e.g., the serotonergic system. Extensive studies using a rich variety of neuroanatomical, neurophysiological and behavioral techniques suggest that 5-HT inhibitory or excitatory role in modulating striatal DA remains controversial. There is considerable neuroanatomical evidence that supports a direct connection between 5-HT and DA containing neurons. 5-HT/DA interactions are clinically relevant for the treatment of various neurodegenerative diseases. The rate of progress in the dynamic field of neuroscience is impressive and further major advances in our understanding of the relationship between gene mutations, Lewy-body formation, disturbance of internal cellular metabolism and selective nigro-striatal cell death appear likely, within the near future.

THE AMPHIBIAN MODEL FOR THE STUDY OF INNATE IMMUNITY

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Amphibia represent an easy to hand source of bioactive molecules as well as antimicrobial peptides (AP). The latter are widespread in all eukaryotes of both vegetal and animal kingdoms, from invertebrates to humans. AP have their homologues in different and less accessible districts of higher vertebrates, even though they are present in small quantities. In frogs AP are present in skin secretion, and their emission can be artificially stimulated by a mild electric shock; the sample is thus easily collected by washing the skin. This secretion is of holocrine type, it can be considered as a dilute total cell extract and used in *in vitro* assays. Being vertebrates, amphibia posses an adaptive immunity containing lymphocytes, antibodies, class I and II MHC, antigen and complement, but this system is not much efficient and the innate immune system, acting by releasing AP in skin secretion, is prevalent in host defence. The signalling pathways used by amphibia to regulate their innate immune system are the same used by mammals for regulating adaptive immune genes. Therefore, amphibia are a suitable *in vivo* model system for functional studies on the mechanisms underlying the immune response in higher vertebrates as mammals. A common transcriptional regulation system is responsible for the expression of most of such molecules. This mechanism clearly resembles the one involved in the control of genes coding for immunoglobulins and acute phase response (APRs) proteins in vertebrates, and is mainly based on the Rel family (NF-κB). Glucocorticoids (GC) are known to suppress immune response by inducing the synthesis of the NF-κB inhibitor, IκBα. Treatment of frog skin with a GC cream determines an almost complete block of peptide synthesis and of antibacterial activity in skin secretions. Cloning of antimicrobial peptides genes and functional studies on their promoters confirm the central role played by Rel factors in inducing these genes.

Oral Communications Symposium I

DOPAMINE RECEPTOR SUBTYPES EXPRESSION AND LOCALIZATION IN THE RAT GASTROINTESTINAL TRACT MICROVASCULATURE

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The expression of dopamine (DA) D1-D5 receptor subtypes and their relationships with sympathetic neuroeffector plexus were investigated in muscular coat, submucous coat and mucous arteries of rat gastrointestinal tract by immunohistochemical techniques. Male Sprague-Dawley rats were fasted for 24 h, anesthetized and killed. Stomach, duodenum, jejunum, ileum and colon were removed, put in a 30% sucrose solution and frozen. Serial sections obtained using a microtome cryostat were processed for tyrosine-hydroxylase (TH) and DA receptor protein immunohistochemistry DA D1-like (D1-D5) receptors were located in smooth muscle of muscular coat, submucous coat and mucous arteries of stomach, duodenum, jejunum and ileum. The D1 receptor was apparently more expressed than the D5 receptor. DA D2-like (D2, D3 and D4) receptor immunoreactivity was located in the adventitia and adventitia-media border of muscular coat, submucous coat and mucous arteries of stomach, duodenum, jejunum and ileum. The intensity of D3 receptor immunostaining as well as of TH immunoreactivity was inversely proportional to the decrease of arterial diameter. No DA receptor immunostaining was observed in colonic microvasculature. An association between TH and DA D2-like receptor was found in double immune staining experiments. The above findings indicate that rat gastric and small intestine arteries express the different subtypes of DA receptors. Both D1 and D5 receptors are postjunctional while the demonstration of the co-expression of the sympathetic marker TH and DA D2-like receptor protein immunoreactivity, indicates the prejunctional localization of these receptors. The particular microanatomical localization of DA receptors in rat gastrointestinal microcirculation suggests that their stimulation or inhibition may cause different hemodynamic consequences on gastrointestinal circulation.

GD3 GANGLIOSIDE AND NG2 PROTEOGLYCAN EXPRESSION IN GLIOBLASTOMA MULTIFORME AND PERITUMOR TISSUE

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Invasion is a defining hallmark of glioblastoma multiforme (GBM). This infiltrative capacity is supported by the presence of tumor cells at a distance greater than 4 cm from the tumor edge. Moreover, several works suggest that the space demanding growth, the production of soluble factors and the peculiar vasculature of the tumor induce changes in surroundings. We previously reported that the presence of activated MAP kinases and stem cell marker nestin in peritumor tissue of GBM, even in the absence of tumor cells, carries a prognostic significance. We also demonstrated that neoangiogenesis occurs in the peritumor areas, being nestin and endoglin expressed in endothelial cells of hyperplastic microvessels. GD3 ganglioside is associated with malignant transformation and it has been found to promote migration, invasion and neoangiogenesis. Neuro-glial proteoglycan 2 (NG2) is involved in the invasive process as well as in promoting angiogenesis. In order to establish the role of GD3 and NG2 in peritumor tissue we investigated their expression in enhanced lesion compared to white matter at different distances from the tumor edge. Paraffin-embedded sections from 22 patients (pts) with GBM were immunostained with an anti-GD3 monoclonal antibody, and frozen sections, from 10 pts of the same group, were immunolabeled with an anti-NG2 polyclonal antibody. GD3 and NG2 are expressed not only in the enhanced lesion, but in the peritumor tissue also. GD3 has been detected in endothelial cells, tumor cells, reactive astrocytes and apparently normal cells. NG2 seems to be present not only in pericytes but in endothelial cells also. It is expressed by neoplastic cells in GBM. In surroundings, cells of variable morphology do express NG2, but further studies are warranted to clarify their nature. Our results confirm that peritumor tissue undergoes a transformation and they might be useful to develop more informed and unified approaches to diagnosis and therapy.

AUGURIN EXPRESSION IN RAT TISSUES THROUGH REAL TIME-POLYMERASE CHAIN REACTION AND IMMUNOHISTOCHEMISTRY

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Augurin (also called esophageal cancer-related gene 4) has first been cloned and identified from normal human esophageal epithelium. Apart from esophagus, it has been found to be expressed in intermediate lobe of the pituitary, glomerular layer of the adrenal cortex, choroid plexus and atrio-ventricular node of the heart by *in situ* hybridization and in bladder and brain by RT-PCR. Augurin expression is also down-regulated in esophageal and prostate tumors and tumor cell lines. The aim of the present work was analysis of augurin expression and location in a wide range of rat organs both by RT-PCR and immunohistochemistry. Comparative analysis of augurin expression in adrenocortical regeneration after enucleation in rats was also performed. RT-PCR identified augurin mRNA in all tissues examined, i.e., stomach ≈ uterus ≈ brain ≈ heart > esophagus ≈ thyroid gland > skeletal muscle ≈ bladder > ovary ≈ pancreas ≈ kidney ≈ lung ≈ adrenal gland ≈ hypophysis ≈ testis ≈ small bowel ≈ liver ≈ spleen. Immunohistochemical analysis revealed augurin expression in some epithelial (for instance, quite strong reaction in esophagus and oviduct), muscle (skeletal ≈ heart > smooth), endocrine (hypophysis, thyroid, adrenal, Leydig, ovary) cells. Augurin expression was found to be higher in zona glomerulosa and medulla than in zona fasciculata/reticularis, both by RT-PCR and immunohistochemistry. Augurin expression was also up-regulated in adrenocortical regeneration suggesting a probable role in the regulation of cell proliferation in the adrenal cortex. Augurin immunostaining was also detected in many different neuronal groups of central and peripheral (trigeminal and superior cervical ganglia) nervous systems, also suggesting a possible role as neurotransmitter/neuromodulator.

ROLE OF REACTIVE GLIOSIS AND ANALYSIS OF NERVE GROWTH FACTOR TREATMENT IN A NEUROPATHIC PAIN RAT MODEL

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Neuropathic pain is a chronic debilitating condition characterized by lancinating or continuous burning-type pain, and typically associated with the occurrence of allodynia and/or hyperalgesia. In this study, we investigated the activity of NGF on inflammatory and neuronal markers in the spinal cord and the behavior in a spared nerve injury (SNI), a model of neuropathic pain in the rat. Seven days after SNI, allodynic and hyperalgesic behavior was investigated respectively by Von Frey filament test and thermal Plantar test. Rat recombinant NGF (125 ng/μL/h) or artificial cerebro spinal fluid (controls) was administered by intrathecal continuous infusion device (Alzet pump) for 7 days on lumbar enlargement of spinal cord, a third group received recombinant NGF for 7 days followed by a 7 days CSF. NGF treated animals, 1 week later showed reduced allodynia and thermal hyperalgesia compared to control group. Molecular and morphological analysis were performed on lumbar spinal cord, and segments of the injured and naive sciatic nerve. Image computer assisted analysis of the expression of IBA-1 for microglial cells; S100β, GFAP as macroglial markers; NeuN and GAD75 as neuronal markers; GLT and Glyt1 as glial transporters markers, PARPP and Edu as markers of death and cell division respectively were measured in the spinal cord of treated and control animals. In NGF treated animals expression of both neuronal markers NeuN and GAD75 compared to controls revealed a net increase. A net reduction of microglial Iba1 and macroglial GFAP expression in NGF treated animals compared to controls. NGF treatment induced a net increase of both glial transporters GLT and Glyt1. The interruption of NGF treatment determined a revert of all values. We hypothesize that NGF results critical in maintaining neurochemical homeostasis in the spinal cord of nociceptive neurons, and that supplementation may be beneficial in restoring and/or maintaining analgesia in chronic pain conditions.

ROLE OF ANDROGENS IN THE DIFFERENTIATION OF RODENT ARGININE-VASOPRESSIN SYSTEM

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In rodents, the arginine vasopressin (AVP) system located in the bed nucleus of the stria terminalis (BST), medial amygdala (MeA) and lateral septum (LS) is sexually dimorphic, being AVP immunoreactive (AVP-ir) elements denser in male that in female. Both androgen (AR) and estrogen receptors are localized within BST and MeA and AVP expression in these nuclei is regulated by testosterone (T) and decreases after male castration. To study the effects of the chronic lack of estrogens or androgens on the differentiation of limbic AVP system, we used two experimental models: a) knock-out mice lacking a functional aromatase gene (ArKO) determining a chronic lack of estradiol (E2) during the whole life; b) Tfm rats, a strain with a spontaneous mutation of the AR gene that is insensible to androgens actions. Adult ArKO mice show a significant decrease of the AVP-ir elements in LS, BST and MeA; to determine whether this reduction is due to a lack of organizational or activational effects of E2, we investigated male ArKO and wild-type (WT) mice when treated with E2 in association with dihydrotestosterone in adulthood. This treatment restored AVP-ir elements in ArKO males to levels observed in intact WT males. This result suggests that the sexually dimorphic AVP system of the mouse forebrain is predominantly influenced by activational effects of the estrogenic metabolite of T and that the organization of the AVP neuropeptide system may depend on androgen or sex chromosomes rather than on estrogens. To confirm this hypothesis, we studied the AVP-ir system in Tfm rats, that, due to the mutation of AR, are not exposed to T during the whole life. In these animals we detected a significant decrease of the AVP-ir system in comparison to Wild-type males, thus confirming the central role played by androgens in the differentiation of this system.

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MODIFICATIONS OF THE VASOPRESSIN AND ANP IN THE RAT HYPOTHALAMIC SUPRAOPTIC NUCLEUS DURING AND AFTER THE PHYSICAL EXERCISE

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Recent studies showed that in sports the exercise-associated hyponatriemia occurs, that is a life-threatening complication that lead to fatal cerebral and pulmonary oedema due to inappropriate vasopressin (VP) secretion. Few studies on the neurosecretion of hypothalamic nuclei during the physical exercises carried out, we in this immunohistochemical study investigate the changes of the VP and ANP, antagonist hormones occurring during the physical exercises. Wistar rats were trained by a type *Power* exercise using a rung ladder varying the load-weight fastened to the rat-tail; the exercise last 20 min, everyday, for 15, 30, and 45 days. The trained and control rats sacrificed after each time and at 15 days after stopped exercises. In trained rats at 15th day of training the number of VP and ANP-immunopositive neurons is lower than the controls; from 30th to 60th days the number of VP-immunopositive neurons increases. The results show that from 15th days of training to 60th days the number of the VP and ANP immunopositive neurons linearly increases. The lower number of immunopositive neurons for either VP or ANP at 15th day of training, in respect to the successive 30 and 45 days, indicates that progressively the release of peptides decreases. The comparison between the VP/ANP-immunopositivity evidences that they have same activity and that, in respect to VP, at beginning of exercise the number of ANP-immunopositive neurons is lower than VP, while during the exercise the number of ANP immunopositive neurons progressively increases up to equal the number of VP-immunopositive neurons. Because VP plays antidiuretic effect at beginning of training its activity prevails on the diuretic activity of ANP with consequent retention of fluids. By progressing of exercise the antidiuretic effect of VP diminishes and is balanced by the diuretic effect of ANP, to restore the salt and water balance.

Symposium II
Signal Transduction Within Nuclear Domains

NUCLEAR PHOSPHOLIPASE C AND SIGNAL TRANSDUCTION

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In the new scenario of inositide dependent signal transduction in the nucleus, Phosphoinositide-specific phospholipase C (PI-PLC) β1 is a key enzyme, and it is involved in many cellular processes, such as differentiation and proliferation. Its activation and/or up-regulation is upon the control of type 1 insulin-like growth factor receptor (IGF-R) in both mouse fibroblasts and myoblasts, suggesting that its signaling activity is essential for the normal behavior of the cell. Besides the close link between PI-PLCβ1 and the highly conserved splicing regulator SRp20, PI-PLCβ1 also affects the expression of cyclin D3 targeting specific regions of cyclin D3 promoter. Because of the fact that CD24 gene, coding for an antigen involved in the very early stages of haematopoiesis, was up-regulated only by nuclear PI-PLCβ1, we explored the behaviour of this PI-PLC in patients affected by high-risk myelodysplastic syndrome (MDS). We have identified a cryptic mono-allelic deletion of PI-PLCβ1 gene only patients who progressed suddenly to acute myeloid leukaemia (AML). This constitutes a new marker for prognosis and further treatment. Interestingly in high risk MDS patients, even though without PI-PLCβ1 deletion, the expression of PI-PLCβ1 was reduced, but to a lower extent respect to patients bearing the deletion. Treatment with the demethylating drug 5′-Aza-2′-deoxycytidine induces a recovery of the expression of PI-PLCβ1 only in the responsive patients, who are characterised by the absence of the mono-allelic deletion.

NUCLEAR PHOSPHOINOSITIDES

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Phosphoinositides are a family of lipid second messengers interlinked by the activities of an extensive and highly regulated network of kinases and phosphatases that modulate phosphoinositide levels in response to environmental changes. Alterations in phosphoinositide levels can regulate many different cancer-relevant cellular pathways including survival, proliferation, migration, cell substratum interactions and transcription. While much is known about phosphoinositide regulation at the plasma membrane there is still a paucity of information concerning the nuclear phosphoinositides and what cellular functions they may regulate. Phosphoinositides within the nucleus are regulated distinctly from their plasma membrane counterparts and are able to engage with nuclear effector proteins to control specific nuclear functions. We will discuss phosphoinositide regulation in the context of nuclear PI5P which appears to be involved in regulating a subset of chromatin regulating proteins.

NUCLEAR ARCHITECTURE STUDIED BY MICROSCOPY: WHERE THE FIELD STANDS AND WHERE TO MOVE NEXT

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The dynamic, functional topography of nuclear components, such as chromosome territories, chromatin loops and domains, genes, nuclear bodies, splicing speckles, as well as molecular machineries for transcription, co-transcriptional splicing, DNA-replication and repair, is not well understood. Knowledge of evolutionary-conserved common principles of nuclear architecture shared by eukaryotes and species-specific peculiarities of the nuclear architecture is still in an unsatisfactory state. Although it is now well established that changes of nuclear phenotypes, including changes of higher order chromatin arrangements, take place during normal cell differentiation, functional implications of such changes are still speculative. Nor do we know which differences of the nuclear architecture may play an important role in the deregulation of genes in cancer cells. Profound differences between present models of nuclear architecture point to the huge gap of knowledge, which must still be bridged to achieve an integrated understanding of normal and pathological nuclear structures and functions from the molecular level to the level of higher order organization. Recent methodological breakthroughs, such as 4D (space and time) live cell imaging, 3D light optical nanoscopy and 3D electron microscopy, bear the promise to close this gap.

ROLE OF PRELAMIN A IN THE PATHOGENESIS OF LAMINOPATHIES

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The nuclear envelope is the nuclear domain which connects the nucleoplasm with the cytoplasm. It is composed by the nuclear membrane, the nuclear pore complex, and the nuclear lamina. Interest in the field has intensified after the discovery that mutations in proteins of the nuclear envelope give rise to a wide range of inherited diseases, collectively termed laminopathies. These diseases include striated muscle disorders, familial partial lipodystrophy, peripheral neuropathies and syndromic pathologies. The association of nuclear envelope proteins with such a wide range of diseases implies important functions in development and maintenance of tissue homeostasis. The localization of nuclear envelope proteins and their interactions with partner proteins can be detected by immunocytochemical methods; interestingly, altered localization and interactions occur in cells from laminopathic patients. A number of hypotheses link the pathogenesis of laminopathies to the physiological role of nuclear envelope proteins. They include failure of mutated lamins to maintain the mechanical integrity of the nucleus, to modulate transcription and cell signalling, and to control stem cell proliferation and differentiation. We analyze the pathogenic role of prelamin A in the most severe laminopathies, including premature ageing syndromes, and

further show a physiological role of prelamin A processing. In progeroid laminopathies, accumulation of prelamin A causes structural and functional nuclear defects which can be rescued by drug treatment. On the other hand, in control cell lines, prelamin A forms differently affect heterochromatin dynamics. Moreover, prelamin A is modulated during myoblast differentiation and it regulates caveolin 3 expression. These data provide evidence for a critical role of the lamin A precursor in myogenesis and suggest that an altered processing of lamin A could underlie muscle defects in syndromic laminopathies.

Oral Communications Symposium II

INNER NUCLEAR MEMBRANE PROTEIN EMERIN AND THE PROTEIN PRECURSOR OF LAMIN A, ARE ABLE TO INTERACT AND TO INFLUENCE EACH OTHER'S LOCALIZATION IN HUMAN FIBROBLASTS

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Emerin is a nuclear envelope protein that contributes to nuclear architecture, chromatin structure and gene expression through its interaction with various nuclear proteins. In particular, emerin is molecularly connected with the nuclear lamina, a protein meshwork composed of lamins and lamin binding proteins underlying the inner nuclear membrane. Among nuclear lamina components, lamin A is a major emerin partner. Lamin A, encoded by the LMNA gene, is produced as a precursor protein (prelamin A) which is post-translationally modified at its C-terminal region. The lamin A precursor plays a major role in nuclear behavior since it interacts with key constituents of the nuclear lamina including lamin B and LAP2a, the heterochromatin protein HP1a and the nuclear envelope protein Sun1. Impairment of the lamin A maturation pathway causing lamin A precursor accumulation is linked to the development of rare diseases such as familial partial lipodystrophy, mandibuloacral dysplasia, Werner syndrome, Hutchinson-Gilford progeria and Restrictive Dermophaty. Here we investigate the interplay between prelamin A and emerin in human skin fibroblasts. We show that the accumulation of non-farnesylated as well as farnesylated carboxymethylated lamin A precursors in human fibroblasts affects emerin localization. In Emery-Dreifuss cells, emerin absence from the nuclear membrane leads to unprocessed prelamin A aberrant localization. These effects are consistent with a potential binding of emerin to the lamin A precursor. In fact, emerin and prelamin A are efficiently coimmunoprecipitated from HEK293 cells co-expressing GFP-emerin and unprocessable prelamin A mutants. Moreover we observed that the restoration of emerin expression in Emery-Dreifuss cells induces the recovery of prelamin A localization. These results indicate that emerin and prelamin A are interacting proteins and that their interplay may be relevant for the understanding of laminopathies.

NUCLEAR PLC β 1 SIGNALING: eEF1A2 IS A NOVEL PHOSPHO-SUBSTRATE OF PKC β1 IN MYOBLASTS DIFFERENTIATION

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Phospholipase C β1 (PLC β1) is a key player in the regulation of nuclear inositol lipid signaling and of a wide range of cellular functions, such as proliferation and differentiation.[1,2,3] PLC β1 signaling depends on the cleavage of phosphatidylinositol 4,5-bisphosphate and the formation of the second messengers diacylglycerol and Inositol tris-phosphate which activate canonical protein kinase C (cPKC) isoforms. Here we describe a proteomic approach to find out a potential effector of nuclear PLC β1 dependent signaling during insulin stimulated myogenic differentiation. We have previously shown that PLC β1 is greatly increased at the nuclear level during insulin-induced myoblasts differentiation and that this nuclear localization is essential for induction of differentiation. Thus, nuclear proteins of insulin stimulated C2C12 myoblasts, were immunoprecipitated with an anti-phospho-substrate cPKC antibody. After Electrophoretic gel separation of proteins immunoprecipitated, several molecules were identified by LC-MS/MS. Among these most relevant and unexpected was eukaryotic elongation factor 1 α2 (eEF1α2). We found that eEF1α2 is phosphorylated by PKC β1 and that these two molecules co-immunolocalized at the nucleolar level. eEF1α2 could be phosphorylated in many sites among which both threonine and serine residues. By site direct mutagenesis we demonstrated that it is the serine residue of the motif recognized by the antibody that is specifically phosphorylated by PKC β1. The silencing of PLC β1 gives rise to a reduction of expression and phosphorylation levels of eEF1α2 indicating this molecule as a target of nuclear PLC β1 regulatory network during myoblasts differentiation.

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INVOLVEMENT OF NUCLEAR PLCβ1 IN LAMIN B1 PHOSPHORYLATION AND G2/M CELL CYCLE PROGRESSION

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Inositide-specific phospholipase Cβ1 (PLC-β1) signaling in cell proliferation has been investigated thoroughly in the G1 cell cycle phase. However, little is known about its involvement in G2/M progression. We used murine erythroleukemia cells to investigate the role of PLC-β1 in G2/M cell cycle progression and screened a number of candidate intermediate players, particularly mitogen-activated protein kinase (MAPK) and protein kinase C (PKC), which can, potentially, transduce serum mitogenic stimulus and induce lamin B1 phosphorylation, leading to G2/M progression. We report that PLC-β1 colocalizes and physically interacts with lamin B1. Studies of the effects of inhibitors and selective si-RNA mediated silencing showed a role of JNK, PKC-α, PKC-β1, and the β1 isoform of PI-PLC in cell accumulation in G2/M (as observed by FACS). To shed light on the mechanism, we considered that the final signaling target was lamin B1 phosphorylation. When JNK, PKC-α, or PLC-β1 were silenced, lamin B1 exhibited a lower extent of phosphorylation, as compared to control. The salient features to emerge from these studies are a common pathway in which JNK is likely to represent a link between mitogenic stimulus and activation of PLCβ1, and, foremost, the finding that the PLC-β1-mediated pathway represents a functional nuclear inositide signaling in the G2/M transition.

IDENTIFICATION OF A NOVEL NUCLEAR AKT SUBSTRATE: MATRIN 3

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In this study we attempted to identify novel Akt substrates, Matrin 3, using a proteomic approach. Matrin 3 is a nuclear matrix protein of 125 kDa that has been implicated in interacting with other nuclear proteins to anchor hyperedited RNAs to the nuclear matrix, in modulating the activity of proximal promoters, in mRNA's splicing and as the main PKA substrate following NMDA receptor activation. Protein extracts from pure nuclei were separated by 2D-E/MS, then blotted onto PVDF and revealed using an antibody directed to the Akt consensus motif RxRxxpS/T. Next, the proteins recognized by the antibody were excised and analyzed by mass spectrometry. Consequently, the nuclear matrix protein Matrin 3 was identified and regarded as a putative substrate of Akt. To confirm that Akt phosphorylates Matrin 3 in physiological conditions, we asked whether the two proteins associates *in vivo*. Accordingly, co-immunoprecipitation experiments showed a direct interaction between Matrin 3 and Akt. Remarkably, Matrin phosphorylation by Akt is a physiological event since it can be triggered by the well-known Akt activator insulin in C2C12 cells. MS analysis showed that, in response to insulin, matrin 3 is phosphorylated by Akt at Ser596, in a conserved Akt motif. Furthermore, co-immunoprecipitation experiments showed an interaction between Matrin 3 and PI3K regulatory subunit, p85, though insulin was unable to modulate this event. All together, these observations support the hypothesis of Matrin 3 behaving as a nuclear platform which can recruit signaling molecules of the PI3K/Akt pathway in proximity of a particular sub-nuclear domain.

INTERPLAY OF PRELAMIN A FORMS ACCUMULATED IN MANDIBULOACRAL DYSPLASIA WITH NUCLEAR ENVELOPE PARTNERS DIRECTS CHROMATIN REORGANIZATION

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Mandibuloacral dysplasia is a rare autosomal recessive disorder characterized by craniofacial defects, skeletal manifestations including clavicular hypoplasia, acroosteolysis and mandibular bone resorption, cutaneous changes and partial or generalized lipodystrophy. The mandibuloacral dysplasia form linked to mutations in the LMNA gene encoding lamin A/C has been called MADA, while the form linked to mutations of the prelamin A endoprotease ZMPSTE24 is referred to as MADB. Both MADA and MADB are characterized by accumulation of

prelamin A, which is detected at the nuclear rim and in intranuclear invaginations. Prelamin A is a 74 κDa protein which undergoes subsequent post-translational modifications leading to transient formation of at least four intermediates and ultimately yielding mature lamin A. The post-translational modifications of prelamin A in MADA had not been so far determined. Here we report the characterization of prelamin A in MADA cells. We show accumulation of different prelamin A processing intermediates depending on the passage number, suggesting the onset of a feedback mechanism. Moreover, we show that accumulation of farnesylated prelamin A causes nuclear enlargement, while further accumulation of full-length prelamin A mislocalizes the nuclear envelope protein SUN1 to intranuclear prelamin A-labeled structures and SUN 2 to an honeycomb structure in the nuclear rim. Interestingly, the lamin A/C and DNA-binding protein BAF is also mislocalized to prelamin A - containing intranuclear structures overlapping to densely stained chromatin domains. Since SUN1 is known to interact with prelamin A, we suggest that a complex formed by SUN1, prelamin A and BAF may mediate the reorganization of chromatin domains reported in Mandibuloacral Dysplasia.

TRANSCRIPTIONAL MODULATION OF THE MYELOPOIESIS-REGULATOR MICRORNA-223 DURING HEMATOPOIETIC CELL LINEAGE SPECIFICATION

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MicroRNAs (MiRs) are tissue specific, genomically encoded negative regulators of the expression of genes involved in development, differentiation, proliferation and apoptosis. MiR223 is specifically expressed in human myeloid cells and plays a crucial role during myelopoiesis. By northern blot and qRT-PCR analyses, we found that miR223 expression is increased during monocytopoiesis, strongly up-regulated during granulopoiesis and negatively regulated during erythroid differentiation. MiR223 levels appear regulated by two putative promoter regions, both presenting DNA binding sites for hematopoietic lineage-specific transcription factors whose activity is required for the correct execution of the hematopoietic program. We investigated the transcriptional regulatory circuits modulating miR223 gene expression in relation to lineage specification and terminal differentiation of myeloid precursor cell lines into monocytic, granulocytic and erythroid lineages. We tested the expression levels of hematopoietic transcription factors as C/EBPa, NFI-A, PU.1, C/EBPb, TAL-1 GATA-1 and LMO2 and their presence and activity at their binding sites on the two upstream regulatory regions of the miR223 gene by immunoblot, ChIP and promoter assays. The recruitment of C/EBPb and PU.1 to the distal promoter region appears to drive miR223 expression during monocytic differentiation. Conversely, the recruitment of C/EBPa to the proximal regulatory region of miR223 is related to its strong induction during granulocytopoiesis, while the binding of transcription factors TAL-1-LMO2 and GATA-1 at their sites on this region repressed the expression of miR223 during erythroid differentiation. These results suggest miR223 activity is finely modulated by the alternative usage of two promoters and the coordinate and consequent function of lineage specific transcription factors. These findings further underline miR223 as a key player for the correct execution of the hematopoietic cell program.

Symposium III: Tissue Development, Renewal and Regeneration

VESSEL ASSOCIATED PROGENITORS AND THEIR MYOGENIC FATE

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Mesoangioblasts were identified and characterized as vessel associated progenitors, associated to the wall of blood vessels. When derived from the mouse dorsal aorta they express early endothelial markers and differentiate to a low extent into most cell types of solid mesoderm. However when derived from postnatal tissues, they express mainly pericyte markers and differentiate mainly in the cell type typical of the tissue where they are resident. *In vitro* experiments will be presented addressing the possible lineage relationship of these progenitors with skeletal myoblasts during skeletal muscle histogenesis. *In vivo* lineage tracing experiments will be presented investigating their fate during unperturbed development of the mouse.

PW1 INTERSTITIAL CELLS (PICS): A NON-SATELLITE CELL SOURCE OF MYOGENIC PROGENITORS

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Non-satellite cells participate in muscle regeneration, however their precise anatomical location and level of contribution is unclear. PW1 is expressed in satellite cells (SCs) and a sub-population of interstitial cells, termed PICs (PW1 interstitial cells), which do not express other known lineage markers. PICs can be FACs-isolated from muscle using stem cell-surface markers to obtain a population distinct from SCs. PICs generate smooth and skeletal muscle *in vitro* and skeletal muscle *in vivo*. Most notably, PICs are highly myogenic *in vivo* and also generate numerous PICs after a single round of regeneration following injection into damaged muscle. In the presence of SCs, PICs convert readily to the skeletal myoblasts (PW1+/Pax7+/MyoD+), and fuse with primary satellite cells and myotubes. Pax7 mutant PICs cannot generate skeletal muscle whereas Pax7 mutant SCs show pronounced myogenicity revealing that PICs require Pax7 to enter the skeletal muscle lineage. We observe that PICs are not derived from a Pax3 lineage using a Pax3Cre x Rosa lacz cross, however, PICs initiate Pax3 expression upon skeletal muscle conversion. Affymetrix-based profile comparisons of PICs and SCs reveal that PICs express genes involved in embryonic development as well as many stem cell-related markers. In addition, several growth factors and their receptors are differentially expressed including many members of the TGF/BMP/fst and Wnt pathway. A PW1 reporter line has been generated which reveals that PW1 not only marks progenitors in skeletal muscle, but labels stem cells in a wide variety of adult tissues suggesting a common regulatory pathway in stem cells. This work was supported by the NIH, MDA, and a strategic plan support from the AFM.

GROWTH FACTOR ENHANCEMENT OF MUSCLE REGENERATION

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The adult mammalian body does retain the robust repair capacity of other species and gradually loses its regenerative potential with age. Our approach has been to intervene in the mechanisms at work in the mammalian response to damage or disease by reducing the impediments to effective regeneration of skeletal and cardiac muscle. In one intervention, transgenic supplementation of a locally acting Insulin-like Growth Factor 1 isoform (mIGF-1) promotes efficient tissue repair of damaged skeletal and cardiac muscle without scar formation, and prevents muscle atrophy in heart failure. In a second intervention, repression of the NFκB inflammatory pathway by mIGF-1 in damaged muscle has prompted studies in which mice lacking functional NFκB signaling specifically in skeletal muscle exhibit increased muscle regenerative capacity, whereas mice lacking NFκB signaling specifically in cardiac muscle progressed to failure. In a third intervention, the importance of inflammation to tissue regeneration was explored in a mouse model of impaired macrophage polarization. Taken together, these observations highlight the complexity of recapturing embryonic regenerative capacity by modulating key signaling pathways or cell-tissue interactions in the adult to restore injured or degenerating tissues.

Oral Communications Symposium III

TRANSCRIPTION FACTOR NFI-A DIRECTS THE FATE OF HEMATOPOIETIC PROGENITORS TO THE ERYTHROID OR GRANULOCYTIC LINEAGE

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Hematopoietic lineage fate choice is governed largely by the intrinsic combination of lineage restricted transcription factors (TFs). The TF Nuclear Factor I-A (NFI-A) is a member of the Nuclear Factor I (NFI) TF family that are known for their positive or negative transcriptional regulatory roles in a cell type and promoter specific context. NFI-A was previously noted by our group as a relevant target of the myeloid regulator microRNA-223 in myeloid leukemia cells and suppresses monocytic differentiation of HPCs whereas nothing is known on its role in normal erythro-granulopoiesis. Here we have identified NFI-A as being necessary for directing the fates of HPCs to the erythroid (E) or granulocytic (G) lineage. In cord blood $CD34^+$ HPCs placed in hematopoietic unilineage culture differentiation systems, we demonstrated a lineage specific expression pattern of NFI-A: during E differentiation it is strongly upregulated whereas during G differentiation is markedly downregulated. Using lentiviral vectors encoding NFI-A and siNFI-A for expression in myeloid cell lines and in $CD34^+$ HPCs, we showed that NFI-A is required for erythroid differentiation and its overexpression enhances E differentiation under suboptimal erythropoietin concentrations. Conversely, the silencing of NFI-A during unilineage G differentiation is required as its overexpression blocks G differentiation. Using an erythroid/granulocytic (E+G) bilineage culture system exogenous manipulation of NFI-A was found to direct HPCs to the E or G fate. Finally, a dual and opposite transcriptional mechanism for lineage choice was discovered whereby NFI-A directly transcriptionally activates the β globin promoter while repressing the granulocyte colony stimulating factor receptor (G-CSFR) promoter. Altogether, these results indicate that in early hematopoiesis the NFI-A expression level acts as a novel factor directing HPCs into either the E or G lineage.

SPHINGOLIPID PATHWAY AS POTENTIAL THERAPEUTICAL TARGET IN MUSCLE ATROPHY

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One of the important signaling pathways triggered by TNFα, a proinflammatory cytokine that seems to be crucial for muscle wasting, is the production of ceramide. The accumulation of this sphingolipid mediator has been shown to affect all the factors involved in the etiology of muscle atrophy. We considered the possibility that ceramide, or one of its metabolite, plays a pivotal role in muscle wasting. *In vitro*, we have treated large myotubes with drugs such as TNFα or short-chain ceramides, and we have quantified their surface after PAS staining. TNFα and all tested ceramides had a clear atrophic effect on C2C12 myotubes, supporting the idea that ceramide participates in TNFα-induced atrophy. To evaluate the *in vivo* role of sphingolipids in the occurrence of muscle atrophy, we have determined the effects of Myriocin, an inhibitor of the de novo pathway of ceramide synthesis, using a well-established model of muscle atrophy, BalbC mice carrying C26 adenocarcinoma. We observed that Myriocin tended to protect animals against tumor-induced loss of body weight and of muscle weight. We have also quantified the cross-sectional area of Tibialis muscle and we observed that Myriocin treatment significantly reverted the decrease in myofiber size associated with tumor development, and was thus able to protect muscle against atrophy. This strongly suggests that ceramide, or a sphingolipid metabolite, is involved in tumor-induced atrophy. The blockade of the ceramide signaling pathway might thus be taken into consideration as a possible target of pharmacological approaches aimed at favoring muscle regeneration or counteracting muscular atrophy.

HISTOCHEMICAL ANALYSIS OF DAMAGE AND REGENERATION IN SKELETAL MUSCLE WASTING

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Cachexia is a severe form of muscle wasting, triggered by elevated levels of cytokines in chronic diseases, which interferes with the management of the primary disease and accounts for the death of a significant percentage of patients. In order to identify mechanisms underlying the loss of muscle homeostasis in cachexia, we analyzed the effects of cytokines on skeletal muscle damage and regenerative capacity. We exploited the properties of Evans Blue Dye to detect sarcolemmal damage in a murine model of cachexia. Fiber damage-associated inflammation was also revealed by immuno-histochemistry for activated macrophages invading muscle fibers. Muscles responded to damage and necrosis by activating a satellite cell-mediated response. The cachectic musculature is enriched in satellite cells expressing high levels of Pax7 and MyoD. However, a morphometric evaluation of the muscle regenerative potential indicated that cachectic muscles lack the ability to fully regenerate following satellite cell activation. The uncoupling between increased fiber necrosis and impaired regeneration resulted in a decreased muscle fiber number per cross-section in the cachectic musculature. Following experimentally-induced damage, intramuscular TNF injection significantly decreased the number and size of the regenerating fibers, an effect abolished by treatment with a caspase inhibitor. Active caspases were highlighted in interstitial cells expressing stem cell markers in the absence of apoptotic phenomena. TNF-dependent impairment of regeneration was rescued by muscle gene delivery of the dominant negative form of PW1 (a TNF effector) or the V1a receptor (to sensitize muscle to the myogenic factor Vasopressin). We conclude that both increased damage and decreased regeneration contribute to muscle wasting in cachexia, suggesting to counteract cytokine effects on muscle regeneration by pharmacological and gene therapy approaches.

IN VITRO ENGINEERING OF HUMAN CRYOPRESERVED VALVE LEAFLETS BY REPLACING ORIGINAL CELLS WITH HUMAN MESENCHYMAL STEM CELLS

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In spite of their limited durability, cryopreserved human homografts or glutaraldehyde-preserved porcine xenografts are still the mostly used substitutes in heart valve surgery. An innovative tool for autologous-like valve replacement could be represented by decellularization of native valves and subsequent *in vitro* repopulation with human bone marrow-mesenchymal stem cells (hBM-MSCs). Here, human cryopreserved pulmonary valve leaflets (hPVLs) underwent (i) decellularization with Triton X100 and sodium cholate (TRICOL) and nuclear fragment removal with endonuclease Benzonase®, (ii) disinfection protocol, (iii) coating with FBS and fibronectin, (iv) seeding with hBMMSCs ($2x10^6$ cells/cm^2), and (v) statical culture for 30 days. The used hBM-MSCs had been priorly isolated by density-gradient centrifugation from young volunteer donors' bone marrow, selected for plastic adherence, expanded and characterized with immunofluorescence and FACS analysis. The processed hBM-MSC-seeded hPVLs were found to be lined by a monolayer of endothelium-like cells (positivity for vWf and CD31), and to contain fibroblast-like, myofibroblast-like, and smooth-muscle-cell-like cells (positivity for vimentin, platelet myosin, smooth muscle actin, SM22 and smooth muscle myosin), so roughly resembling native leaflets. Consistently, the presence of these cell phenotypes, collagen neosynthesis as well as good preservation of the preexistent ECM was revealed by ultrastructural analysis. However, persistence of original stem cell antigens (SSEA4, OCT3/4) was also assessed for some of the seeded cells. These results show that after cryopreservation, thawing and the decellularization treatments here used, hPVLs (i) are permissive for *in vitro* repopulation by hBM-MSCs and their proper differentiation and (ii) potentially could overcome the limited durability of current bioprostheses, since the most frequent transplant failure depends on calcification primed by the dead cells of donor valves.

MAGIC FACTOR-1: A NEW ENGINEERED PROTEIN INVOLVED IN MUSCULAR HYPERTROPHY AND REGENERATION

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Hepatocyte Growth Factor (HGF) is a pleiotropic cytokine of mesenchymal origin that mediates cell proliferation, survival, motility and morphogenesis. Its high affinity receptor, the tyrosine kinase Met, is expressed by a wide range of tissues. Adult myogenic precursor cells (satellite cells) express both HGF and Met. Following muscle injury, autocrine HGF-Met stimulation plays a key role in promoting activation and early division of satellite cells. Magic-F1 (Met-Activating Genetically Improved Chimeric Factor-1) is an HGF-derived, engineered protein that contains two Met-binding domains. Hemizygous Magic-F1 transgenic mice displayed constitutive muscular hypertrophy, improved running performance and accelerated muscle regeneration following injury. We followed the transgene expression during embryogenesis (E8.5 to E17.5) by *in situ* hybridization and we found that Magic-F1 is expressed in bone cartilage primordium, somite dorsal side, tail and limb bud. These data confirm that Magic F1 is expressed in embryo muscle precursors, suggesting a role of this factor in muscle development possibly triggering the downregulation of Pax3 signal pathway in skeletal muscle precursor cells. Moreover, Magic-F1 ameliorates the dystrophic phenotype of α-sarcoglycan null mice, a model of muscular dystrophy, as measured by both anatomical/histological analysis and functional tests of mice subjected to adenovirus mediated Magic-F1 gene delivery. We analyzed also homozygous Magic-F1 mice by morphometric and physiological tests that demonstrate a higher size of the muscle fibers and a better running performance. Preliminary data show a possible implication of the engineered protein in development of the vascular network, increasing the capillary vessel number. Because of these features Magic-F1 represents a novel molecular tool to counteract muscle wasting in major muscular diseases such as cachexia or muscular dystrophy.

PROTEIN KINASE C τ IS REQUIRED FOR CARDIOMYOCYTES SURVIVAL AND CARDIAC REMODELLING

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Cardiac hypertrophy represents a compensatory or adaptational process in response to a variety of stimuli, associated with alterations in intracellular signal transduction pathways, including MAPK and PKC. PKCs constitute a family of ser/thr kinases which plays distinguished and specific role in regulating heart homeostasis and hypertrophic growth. To investigate the role of PKCτ in cardiac hypertrophy and re-modelling we employed PKCτ knock out mice. *In vivo* analysis indicates that, already at basal conditions, the lack of PKCτ expression leads to left ventricular dilation and reduced function, associated to cardiomyocytes hypertrophy and reduction in number, and cardiac fibrosis. Moreover, we observed activation of the p38 and JNK pathways, involved in promoting cell death in response to hypertrophic stimuli and stress. Accordingly, cultured PKCτ$^{-/-}$ cardiomyocytes undergo cell death upon α1-adrenergic agonists. Taken together, our results demonstrate that PKCτ is required to protect cardiomyocytes from *stress-induced* cell death, and to allow *adaptive* hypertrophic response to occur.

Symposium IV: Immunohistochemistry in Pathology From Theory to Practice

DIAGNOSTIC RELIABILITY OF IMMUNOHISTOCHEMISTRY. ROLE OF TECHNICAL FACTORS

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Immunophenotypic evaluation of histopathological lesions plays a pivotal role in diagnostic definition, in prognostic prediction and in drug tailoring assessment, so that a variable percentage of biopsies (from 5 up to 80 per cent, related to the type and origin) routinely undergo immunohistochemical staining. In order to assure the required reliability of the results it is mandatory to assess, a series of technical factors involving the organization of diagnostic services, a standardization of fixation and embedding procedures, planning of controls and knowledge of the possible pitfalls. The list of pitfalls (spanning from fixation and pre-analytical processing, down to interpretation artifacts) is very long.[1] Most technical steps have now been reliably encountered and standardized by commercial companies. However, selection and application of procedures for antigen retrieval still represent a source of discrepancies among laboratories, since a single universal process is lacking and the type and length of process for the retrieval of different antigens is still based on personal experience. Protocols of antigen retrieval of use in my laboratory are reported at the site: www.oncologiaumana.unito.it/treatments. A final, but not minor, item, is related to the cost and economic balance involved in the now widespread use of immunohistochemical tests, a topic where histopathologists should play an active role.

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A NOVEL TOOTH-SPECIFIC PROTEIN IDENTIFIED BY SECRETOME ANALYSIS IS HIGHLY-UPREGULATED IN NEOPLASMS OF EPITHELIAL ORIGIN

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As part of our efforts to identify the secretome of the cells that manufacture enamel, our group has found a novel gene encoding for a protein with unique biochemical characteristics, called APIN/ODAM. Broad expression profiling unexpectedly revealed that it is also strongly expressed in the junctional epithelium (JE). The JE is an incompletely differentiated epithelium which seals off the supporting tissues of the tooth from the aggressive oral environment, and represents the first line of defense against periodontal diseases. Immunolabeling further showed that APIN localizes to the specialized basal lamina which attaches the JE to the tooth surface and that it is also conspicuously found between cells of the JE. It has also been shown that it is highly expressed by a number of epithelial cells when they dedifferentiate to become cancerous, including tooth-associated tumors. While APIN is likely part of the molecular mechanism by which the JE attaches to the tooth surface, its expression by incompletely differentiated and dedifferentiated cells also suggests a relationship to differentiation status. While exciting in potential, it remains to be determined if overexpression of APIN in some cancers is causative or just coincidental, and if this has some prognostic or diagnostic value.

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MORPHOLOGICAL AND MOLECULAR ASSESSMENT OF APOPTOTIC MECHANISMS IN PERIPHERAL NEUROBLASTIC TUMORS

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Peripheral neuroblastic tumors (NTs) are embryonal tumors of the sympathetic nervous system in which multiple defects in apoptotic pathways have been described. Mitosis-karyorrhexis index (MKI) is a reliable morphological marker identifying favourable and unfavourable NTs. The extent to which apoptotic processes contribute to determine the prognostic significance of MKI, however, is still undefined. The relevance of apoptotic processes in a series of 110 peripheral NTs was investigated by comparing morphological MKI to apoptotic gene profile evaluated by immunohistochemistry and microarray analysis. High MKI was found in 55/110 NTs and was associated with advanced stage (p=0.007), NB histological category (p=0.024), MYCN amplification (p<0.001), and poor outcome (p=0.011). Overall survival probability was 45% in patients with high MKI compared to 73% in patients with low MKI. The expression of Bcl-2, Bcl-XL, Bax and Mcl-1 was studied by immunohistochemistry, but no significant associations were found with clinical, histological and prognostic features. Microarray analysis designed to profile the expression of apoptotic genes was performed in 40/110 representative tumors, 20 with low and 20 with high MKI. Thirty-eight representative genes were selected and grouped as belonging to the intrinsic route (n. 8), extrinsic route (n. 16) or to caspases (n. 14). No significant associations between the expression of these apoptotic genes and either MKI or clinical, histological and prognostic features were found. This study confirms that apoptosis is a significant prognostic parameter and that MKI is a reliable morphological marker of clinical-biological behavior in NTs. Apoptotic gene profiling rather than providing additional information for risk class categorization, can potentially contribute to identifying deregulated apoptotic pathways to target and further refining tailored treatment.

Oral Communications Symposium IV

A MULTIPLE TECHNICAL APPROACH TO HUMAN ARTICULAR CHONDROCYTE CELL DEATH

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Cartilage diseases and, in particular, osteoarthritis (OA) have been widely correlated to apoptosis,[1] but recently chondroptosis, a type of death with peculiar features typical of cartilage cells, has been reported.[2] Chondrocyte death is here investigate in a human experimental model. Cell death has been induced in chondrocyte micromasses[3,4] from 1 to 3 week with hyperthermia for 1 h at 43°C followed by 4 h recovery, UV-B for 30 min followed by 4 h recovery, 500 nM staurosporine for 24 h[5] all well known apoptotic triggers. Besides conventional electron microscopy (TEM),[6] TUNEL reaction was applied to investigate potential DNA fragmentation. A scarce positivity, in terms of reaction intensity and stained cell number appears in control, both at 1 and 3 week culture. This could be correlated to the well known cell death patterns, occasionally occurring along cartilage differentiation. After treatments a general increase of TUNEL positivity appears. Hyperthermia shows a diffuse and strong positivity, in particular at 3 weeks, in agreement with TEM observations, which suggest the presence of both chondroptosis and necrosis. UV-B show a strong positivity, particularly localized at nuclear periphery: this could be due to the impaired UV-B capability to fully penetrate micromass. Surface chondrocytes are, nevertheless, strongly affected by the treatment, in agreement with TEM data. Staurosporine evidences a diffuse, but significantly reduced, positivity, a behavior which is, again, supported by TEM. Therefore, DNA fragmentation is a common pattern in dying chondrocytes, both in apoptotic and chondroptotic cells.

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IMMUNOHISTOCHEMICAL IDENTIFICATION OF CELLULAR SUBPOPULATIONS AND CYTOKINES INVOLVED IN INFLAMMATORY PROCESSES UNDERLYING CAROTID ATHEROSCLEROSIS

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Atherosclerosis is a chronic inflammatory disease in which immune responses are key pathogenetic factors. The subintimal immune cell infiltration of atherosclerotic lesions is critical to pathological progression and include macrophages, T and B lymphocytes and mastocytes.[1] The emergence of dendritic cells (DCs) in atherosclerotic plaques has been associated with plaque destabilization and ischemic syndromes. Molecules that locally stimulate lymphocytes and attract DCs to the lesions and sustain the reduced DC emigration detected in progressive plaques are partly defined.[2] Multiple antigens have been implicated in immune-mediated processes related to atherosclerotic plaques and in particular a role has been hypothesized for several self proteins. Oxidative stress, increasingly reported in patients with atherosclerosis, is the major event causing protein structural modification, thus inducing the appearance of neo/cryptic epitopes on the molecule. Convincing evidence supports a determinant role of autoimmune responses to self-structures in shaping the progression of atherosclerosis. Elevated levels of antibodies against oxidized low-density lipoproteins, β2-glycoprotein I, and various heat shock proteins have been associated with the presence of atherosclerotic disease.[3,4] Self-structures were also demonstrated to be target autoantigens of T cells derived from atherosclerotic plaques. Our study aimed to i) identify altered self-proteins eliciting a specific immune response in patients with carotid atherosclerosis; ii) characterize cell populations infiltrating the plaque, recognizing macrophage sub-populations present in different regions of the lesions; iii) evaluate cell activation and the release of specific new cytokines at the level of the plaques.

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NEUROMUSCULAR EXPRESSION OF CYCLO-OXYGENASES IN ULCERATIVE COLITIS

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Ulcerative colitis (UC) is an inflammatory bowel disease associated with dysfunctional colonic motility which is responsible for abdominal pain and diarrhoea. These changes may be mediated by prostaglandins which are increased in this condition. Increased expression of the constitutive and inducible isoform of cyclo-oxygenases (COX-1 and COX-2, respectively) has been found in active inflammatory bowel disease although their cellular distribution remains uncertain at the level of the colonic neuromuscular compartment. The aim was to evaluate the cellular distribution of COX-1 and COX-2 in the neuromuscular tissues from patients with UC. Using immunohistochemistry, COX-1 and COX-2 expression was evaluated in 8 full-thickness colectomy specimens from patients with UC who had failed medical therapy. Histologically normal colon full-thickness specimens was obtained from 10 patients having resection for colorectal neoplasia and evaluated as above. All specimens expressed both COX isoforms although with different cellular distribution and intensity degrees. In control tissues COX-1 was detected in myenteric neurons and circular layer myocytes; COX-2 was expressed mainly in longitudinal layer with little immunoreactivity in neurons. Although a pronounced cell depletion was observed in myenteric ganglia in all inflamed specimens, neurons maintained appreciable COX-1 labelling; COX-2 was significantly upregulated in UC, being localized in cells infiltrating the myenteric plexus and both muscle layers. These findings provide the first evidence that COX-1 and COX-

2 are expressed in myenteric neural cells and muscle layers of colon in UC and may contribute to the colonic dysmotility associated with this pathological condition.

ALTERED SREBP-1 LOCALIZATION IN LAMINOPATHIC FIBROBLASTS WITH PRELAMIN A ACCUMULATION

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Sterol regulatory element binding protein 1 (SREBP-1) is synthesized as a precursor that is attached to the endoplasmic reticulum. When cellular sterol stores are depleted, sterol regulatory element is activated by cleavage which generates a soluble NH2-terminal fragment of 68 kDa that translocates to the nucleus. This process activates transcription of specific genes during adipocyte differentiation. When prelamin A is accumulated in cells, it localizes at the nuclear envelope and colocalizes with SREBP1. Coimmunoprecipitation experiments demonstrated an *in vivo* interaction between SREBP1 and prelamin A.[1] Binding of SREBP1 to the lamin A precursor was detected in fibroblasts from familial partial lipodystrophy, Mandybuloacral Dysplasia patients, these cell lines bear mutations of lamin A/C at amino acids R482, R527 , respectively, and accumulate partially processed prelamin A. G608 mutation of lamin A/C in Hutchinson-Gilford Progeria cell lines and mutations in the prelamin A endoprotease ZPMSTE 24 in Mandibuloacral dysplasia and Restrictive dermopathy cell lines, lead to accumulation of prelamin A. We overexpressed a GFP-SREBP1 fusion protein construct encoding active cleaved form[2] in laminopathic cells and evaluated localization of the transcription factor. In laminopathic cell lines SREBP1 was unevenly distributed and/or its amount was reduced with respect to control cell lines. These data may in part explain the lipodystrophy phenotype associated with familial partial lipodystrophy, Mandibuloacral dysplasia, Hutchinson Gilford progeria and Restrictive dermopathy.

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Posters Symposia I-II

EXPRESSION OF VAMP-2 AND SNAP-25 IMMUNOREACTIVITY IN GABAERGIC AND GLUTAMATERGIC AXON TERMINALS OF THE RAT CEREBELLAR CORTEX

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GABAergic and glutamatergic terminals are widely distributed in the mammalian cerebellar cortex, displaying layer specific patterns of distribution. Aim of this study was to analyze the immunoreactivity (IR) for the vesicle associated membrane protein-2 (VAMP-2) and synaptosomal associated protein of 25 kDa (SNAP-25) within the GABAergic and glutamatergic terminals of the rat cerebellar cortex. The GABAergic and glutamatergic terminals were revealed by imunohistochemistry for glutamic acid decarboxylase 65/67 (GAD-65/67) and vesicular glutamate transporters 1 and 2 (VGLUT-1 and 2), respectively. GAD65/67-positive terminals were observed throughout the cortex. Terminals showing IRs for both GAD-65/67 and VAMP-2 were never observed. Terminals which co-localize GAD-65/67 and SNAP-25 were only occasionally observed, surrounding the Purkinje neuron bodies. Terminals which co-localize VGLUT-1 and VAMP-2/SNAP-25 were observed diffusely in the cortex layers. Terminals showing a co-localization of VGLUT-2 with VAMP-2/SNAP-25 were observed only in the granular layer; VGLUT-2-positive terminals in the inner zone of the molecular layer appeared VAMP-2/SNAP-25-negative. Our results show that VAMP-2 and SNAP-25 are differently expressed in the 2 mayor types of terminals present in the cerebellar cortex. They seem not to be present in the GABAergic terminals (with the exception of some terminals of basket neurons). On the contrary, VAMP-2 and SNAP-25 IRs are expressed in VGLUT-1-reactive terminals. These co-localizations characterize the terminals of parallel and mossy fibres. VAMP-2/SNAP-25 IRs are not detectable in the terminals of climbing fibres, which end mainly in the internal zone of the molecular layer and display IR for VGLUT-2.

IMMUNOHISTOCHEMICAL AND RT-PCR PROFILE OF NEUROTROPHINS IN HUMAN NORMAL DURA MATER AND MENINGIOMA

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The dura mater shows a high density of sympathetic nerve fibers and an impressive population of mast cells, mainly perivascular. It also receives significant sensory projections from the trigeminal system. The presence of these three elements in the meningeal layer suggests a relevant functional interaction between the nervous and the immune system, both mediated by neurotrophic factors. The immunohistochemical profile of neurotrophins and their receptors in the human cranial dura mater has been studied by examining some dural zones in specimens, obtained during neurosurgical operations,

harvested from different regions (frontal, temporal, parietal and occipital). Neurotrophins (NTs), also known as neurotrophic factors, constitute a family of dimeric proteins working as polypeptidic growth factors and acting like extracellular ligands. Neurotrophins, including nerve growth factors (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), are involved in vertebrate neuronal cell development, differentiation, survival and functional activities. The concrete role played by these neurotrophic factors in general regulation, vascular permeability, algic responsivity and release of locally active substances in the human dura mater is still controversial. Our study revealed a general structural alteration of dural tissue due to the possible invasiveness of meningiomatous lesions, together with an improved expression of BDNF in highly proliferating neoplastic cells and an evident production of NGF in inflammatory cells. Our experimental data suggest that BDNF has a role in supporting the proliferation rate of neoplastic cells, while NGF is involved in the activation of a chronic inflammatory response in neoplastic areas. RT-PCR analysis confirmed the immunohistochemical results.

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF THE DISTRIBUTION OF GLUTAMATE RECEPTORS, *HIGH AFFINITY* MEMBRANE BOUND GLUTAMATE TRANSPORTERS AND OF GLUTAMATE CARBOXIPEPTIDASE II (GCPII) IN *IN VITRO* CULTURES OF EMBRYONIC DORSAL ROOT GANGLIA

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Glutamate (*Glu*) is the major mediator of excitatory signalling the mammalian central nervous system, but it has recently been shown to play a role also in the transduction of sensory input at the periphery and in peripheral neuropathies.[1,2] New advances in research have demonstrated that rat peripheral sensory terminals and Dorsal Root Ganglia (DRG) express molecules involved in *Glu* signalling, including *High-affinity* membrane bound *Glu* transporters EAAT1, 2 and 3, *Glu* receptors NMDA, AMPA and mGluR3 and glutamate carboxypeptidase II (GCPII), the enzyme that produces *Glu.*[3,4] Moreover, defects in *Glu* signalling, due to alterations in the function and/or in the expression of Glu transporters, receptors and GCPII have been implicated in several models of peripheral neuropathy,[5,6] neuropathic pain[7] and hyperalgesia.[8] We have also demonstrated a strong activity of *Glu uptake* in *in vitro* models of peripheral nervous system. Here we describe, through multiple labelling immunofluorescence analysis, the distribution of the glutamatergic molecules EAATs, NMDA, mGluR3, and GCPII in *in vitro* cultures of embryonic DRG neurons. The targets were localized in neurons with rare labelling also of satellite cells. The labelling was observed in the cytoplasm of cells with a strong positivity underneath the membranes. These results will be useful to test if the distribution and the function of the glutamatergic molecules should be altered in *in vitro* models of peripheral nervous system damage.

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IMMUNOLOCALIZATION OF CHOLINE ACETYLTRANSFERASE OF COMMON TYPE (CCHAT) IN THE NERVOUS SYSTEM OF OCTOPUS VULGARIS

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A lot of previous studies indicated that acetylcholine (ACh), the first neurotransmitter to be identified in the vertebrate frog and widely distributed in the animal kingdom, may also be a neurotransmitter in invertebrate cephalopod. The presence of a large amount of acetylcholine in the nervous system of cephalopods is well known from several biochemical and experimental studies, but little is known about the precise distribution of cholinergic structures in the octopus because no good histochemical technique exists for detecting Ach. The most reliable method to visualize the cholinergic neurons is the localization by immunohistochemistry of enzyme choline acetyltransferase (ChAT), the synthetic enzyme of ACh, that has been widely used as a specific marker for cholinergic neurons. Some specific commercial ChAT antibodies (against the vertebrate ChAT proteins) are able to map cholinergic systems mainly in mammals and in some non- mammalian vertebrates. Other ChAT antibodies (against the invertebrate protein) have been applied to study ChAT presence in some invertebrates but no data is available on cholinergic structures labelled by ChAT immunohistochemistry in Octopus vulgaris, probably because of their different epitopes. In 2000 a novel form of ChAT was isolated and two polyclonal antisera were raised in rabbit: anti cChAT (is widely present in the central nervous system of all animal species of the zoological chain) and pChAT (has been prevalently demonstrated in mammalian peripheral nervous elements). Various parts of the nervous system of Octopus Vulgaris were analyzed in this study for their Choline Acetyltransferase immunolocalization. We observed cChAT containing neurons and fibers localized in the octopus lobes, with variations of intensity and density of immunoreactive cells. Our findings clearly indicated that the different parts of the octopus nervous system show large differences with regard to their cChAT immunoreactivity.

INCREASED cCHAT IMMUNOREACTIVITY IN BASAL FOREBRAIN OF HEROIN SENSITIZED RATS

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Numerous biochemical and clinical studies suggest that heroin addiction and sensitization could affect the memory function and could promote cognitive impairment, especially acting on cholinergic neurons. However, very few data are currently available concerning the anatomical pathways involved in these processes. Furthermore, is still poorly known if the cholinergic system's modifications incur also while the acute heroin addiction or, if they necessary need a chronic exposure to the toxic. To investigate the cholinergic projections of the basal forebrain, we used two different rats' models of drug addiction: an acute model that received a single injection of heroin after a saline solution (SH) and a chronic model that received two heroin injections (HH). After heroin treatment all

the rats' brain were submitted to the same immunohistochemical protocol, using a non commercial antibody (Kimura's property) specifically recognize the choline acetyl-transferase of the common type (cChat). We found an increase of cChat positive neurons in the septal nuclei, in the cortex, and in numerous other formations of basal forebrain, like accumbens nucleus, amygdala and bed nucleus of stria terminalis, in both chronic and acute models, with a particular intensity in chronic models, confronting with controls. Furthermore, in the hippocampus, we detected an increased immunostaining for cholinergic fibers, in the heroin treated rats. These results support the hypothesis that an hyper cholinergic innervation of the hippocampus follow the heroin addiction and could represent a compensatory mechanism to the reduced sensitivity of the mossy cells to acetylcholine. Thus, our findings could help to explain the cognitive and memory impairment related to heroin exposure and indicate that also in acute assumption the cholinergic system is affected.

CATECHOLAMINERGIC AND CHOLINERGIC NERVOUS FIBRES IN HUMAN LYMPHATIC VESSELS

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The aim of the present study is to analyze the innervation of lymphatic human vessels, using classic histochemical techniques as well as fluorescence microscopy in order to identify the localization of catecholaminergic and cholinergic nervous fibres within the wall of lymphatic vessels. Small fragments of lymphatic vessels were drawn from human mesenteric organs during autopsies and treated with histochemical methods. The demonstration of Ach.E. activity by histochemistry allows us to identify the distribution of peripheral cholinergic nervous fibers. The catecholaminergic nervous fibres can be shown by histochemical techniques specific for catecholamines and observed at microscopic level under fluorescence microscopy. Our results demonstrated that human mesenteric lymphatic vessels are richly innervated by both catecholaminergic and cholinergic nervous fibres. The nervous fibres of the lymphatic vessels, such as arteries and veins , arise from the vegetative system. The distinction between sympathetic and parasympathetic systems is no longer valid because, for example, there are sympathetic cholinergic fibres, once considered only of parasympathetic origin. An improvement of the morphological and functional knowledge's concerning the neural control mechanisms of the lymphatic system could help us to clarify also some pathological aspects of the lymphatic circulation.

PACAP AND VIP AFFECTS NF1 EXPRESSION IN SCHWANNOMA CELLS

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Neurofibromatosis type I is the most common inherited cancer predisposition syndrome and it is caused by mutations within the gene encoding the protein neurofibromin (NF1). This protein acts as a tumor suppressor by inhibiting cellular proliferation. Patients affected by this disease are at high risk of developing certain tumors, most notably optic pathway gliomas and neurofibromas. The primary neoplastic element in neurofibromas is derived from Schwann cell lineage. Stimulation of these cells by some growth factors or aberrant expression of their receptors contributes to neurofibromas growth. In a previous study we have demonstrated the expression of pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) as well as their receptors in a Schwann cell line.[1] To establish whether these two peptides could have a role in neurofibromas growth we investigated their effects on NF1 expression in an immortalized schwannoma cell line. Results showed that cell proliferation is significantly increased in cells treated with 100 nM PACAP or 100 nM VIP. This finding was also correlated to a notable reduction of NF1 protein expression after 24 h and 48 h, as evidenced by western blotting. Immunofluorescent staining of paraformaldehyde fixed cells using an anti-NF1 monoclonal antibody revealed that neuropeptides treatment induce a significant signal attenuation. Furthermore, PACAP or VIP treatment decreased NF1 cytoplasmic localization at both times considered. The results suggest that both neuropeptides may act as regulators of NF1 expression, supporting the hypothesis that PACAP and VIP autocrine/paracrine release might also be involved in aberrant cellular growth in schwannoma cells.

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PROTEASE-ACTIVATED RECEPTOR 1 UPREGULATION IN RAT MICROGLIA FOLLOWING TRIMETHYLTIN TREATMENT: AN *IN VIVO* AND *IN VITRO* STUDY

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Protease-activated receptors (PARs) are a unique family of G-protein-coupled receptors. The PAR family consists of four members: PAR1, 2, 3 and 4, and all members are widely expressed in the brain, including neurons, microglia, astrocytes, and oligodendrocytes. Recent evidence shows that PARs contribute to neuroprotection and/or neurodegeneration in the brain under pathological conditions. We have previously shown that PAR1 is increased in astrocytes both *in vivo* and *in vitro* after treatment with trimethyltin (TMT).[1,2] This neurotoxicant produces a selective neuronal degeneration in the hippocampus which is also associated to early response of microglia and prolonged activation of astrocytes.[3-5] In the present study we analyzed the expression of PAR1 in the hippocampus of TMT-treated rats at early time points of treatment. Double labelling with antibodies against PAR1 and the microglial marker OX42 revealed PAR1 expression in microglial cells after 7 days of TMT administration. In order to gain further insights into this matter, we also investigated PAR1 expression in microglia primary cultures treated with TMT. Western blot and immunohistochemical analysis showed that, as observed *in vivo* after TMT treatment, PAR1 was also upregulated in primary cultures of rat microglia after exposure to TMT, suggesting a direct effect of TMT on microglia.

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AUTOCRINE ACTION OF GDNF IN THE PURKINJE CELLS OF ADULT ZEBRAFISH?

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The cerebellum of teleosts is composed of three parts: the valvula cerebelli (Va), the corpus cerebelli (CCe), and the crista cerebellaris (CC).[1] In higher vertebrates, the molecular layer is located on the surface of the cerebellum and the granular layer is beneath this. Large drop-shaped Purkinje neurons are present between the two layers. These basic features of the cerebellum are not present in teleosts. The boundary between the molecular layer and the granular layer is very complex. Some of the granular cells are located on the surface of the Va and CCe.[2] Moreover, the axons of Purkinje cells do not exit from the cortex but instead terminate locally on nearby efferent neurons (eurydendroid cells) that are equivalent to cerebellar nuclear neurons in mammals. Finally, in teleosts, the cerebellum shows a considerable regenerative capabilities. Glial cell line-derived neurotrophic factor (GDNF) is a growth factor that acts through RET receptor tyrosine kinase and its co-receptor GFRα1.[3] In mammals GDNF and its receptors expression have been described, but no data exist regarding the presence of this neurotrophic factor and its receptors in lower vertebrates. The cerebellum of teleosts, particular of zebrafish, a widely used model species for genetic and development studies, could lead to interesting results for the peculiarities above described. Double immunocytochemical staining showed a co-localization of GDNF, GFRα1 and RET in Purkinje cells, identified morphologically and with a specific marker for these cells, the Aldolase C enzyme. Proliferating cell nuclear antigen (PCNA)-immunohistochemistry, used for demonstrating the spatiotemporal course of proliferation in the cerebellum, showed a proliferative activity. In conclusion, the pattern of co-localization of GDNF, GFRα1 and RET in the cerebellum seems to suggest an autocrine action of GDNF in the Purkinje cells and suggest an involvement of this neurotrophic factor in the cerebellar function and in the peculiar neurogenesis of adult zebrafish.

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GFRα1 RECEPTOR IN THE BRAIN OF ADULT ZEBRAFISH

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Glial cell line-derived neurotrophic factor (GDNF) is a potent trophic factor for many different types of CNS and PNS neurons. The biological activity of the GDNF is mediated through a multicomponent receptor complex that consists of a common transmembrane signaling component, the tyrosine kinase RET and a high-affinity GPI-linked binding component, the GDNF family receptor α1: (GFRα1) subunit. There are four GFRα1; subunits: GFRα1, 2, 3, 4. GFRα1 preferentially binds to GDNF.[1] In adult zebrafish, the central nervous system shows a considerable regenerative capabilities, thus a study regarding neurotrophic factor receptor expression could lead to interesting results. In zebrafish embryos GFRα1 gene has been sequenced and its expression has been described.[2] However, in adult zebrafish, no data exist regarding the expression of this receptor in the brain. Thus, the aim of the present research was to investigate the expression of GFRα1 in the brain of adult zebrafish (*Danio rerio*), a widely used model species for genetic and development studies. Transcripts of GFRα1 mRNA were observed in brain extracts by a standard RT-PCR. Single immunocytochemical staining and *in situ* whole-brain hybridization experiments showed that GFRa1 protein and mRNA were localized in various nuclei of the telencephalon, diencephalon, mesencephalon, cerebellum and medulla oblongata of the adult zebrafish brain. In conclusion, this study demonstrates that the presence of GFRα1 is not limited to developmental periods. Moreover the pattern of GFRα1 expression in the adult brain suggests an involvement of this receptor in the reception of the olfactory and gustatory stimuli and also in the premotor and associative functions.

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PRELIMINARY DATA ON THE PRESENCE AND DISTRIBUTION OF IMMUNOREACTIVITY TO PEPTIDES IMPLICATED IN THE CONTROL OF FOOD INTAKE IN THE STOMACH AND SMALL INTESTINE OF MARINE MAMMALS

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For some years, our interest has turned toward the immunohistochemical identification of peptides involved in the control of food intake of various Vertebrates. In this study we analyzed gastroenteric tissues of *Otaria flavescens* and *Zalophus californianus*, supplied by the *Banca Tessuti Cetacei del Mediterraneo* (University of Padua) about the presence of immunoreactivity to ghrelin and leptin, two peptides with an opposite activity in appetite modulation. Cromogranin A was utilized as marker of neuroendocrine cells and PGP 9.5 was employed to identify neuronal structures of enteric nervous system. The localizations obtained by means of Avidin/Biotin method displayed numerous ghrelin-ir cells and some leptin-ir cells in the gastric glandular epithelium. The cells, rounded in shape, were morphologically similar to cromogranin-ir cells and showed a distribution similar to that reported in other vertebrates. Some nervous fibres of the circular musculature and various gangliar cells and nervous fibres in the myenteric and submucous plexuses were immunoreactive to ghrelin, leptin and PGP 9.5 in the stomach and small intestine. Our findings could not only indicate the phylogenetic conservation of ghrelin and leptin like-peptides in these marine mammals showing anatomo-physiological aspects not completely understood, but also suggest the existence of mechanisms regulating food intake like in other vertebrates.

SOME PEPTIDES INVOLVED IN FOOD INTAKE IN THE INTESTINE AND PANCREAS OF BUFFALO: IMMUNOHISTOCHEMICAL STUDY

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In animals there is a complex network of neuro-humoral interactions linking Central Nervous System (SNC) and peripheral organs that regulates food intake, energetic homeostasis and body weight. Among hormonal factors involved in this mechanism, a fundamental role is performed by ghrelin[1] orexins A and B[2] and leptin.[3] In this study the presence of immunoreactive (ir) cells to these substances in digestive apparatus of buffalo (Bubalus bubalis) is reported. Samples of pancreas and gastrointestinal segments were collected from 5 adult buffaloes. Single immunohistochemical stainings were carried out by avidin-biotin technique. Through quali-quantitative morphological analysis ghrelin ir cells, localized in mucosa of pylorus, duodenum, ileum, caecum, can be divided into two different cytotypes: endocrine and epithelial cells. Endocrine cells mainly localized at the base of villi contain immunopositive granules in the whole cytoplasm; epithelial ones are present quite uniformly on the villi and show positivity in the apical portion. Orexins ir cells belong to the only epithelial cytotypes and show features and localizations as well as ghrelin ir cells. Leptin ir endocrine cells are found only in the epithelium of abomasal mucosa and in some pancreatic isles. The obtained results represent the first indication of the presence of these substances in buffalo digestive apparatus. Thus, they are the starting point to investigate the mechanisms involved in the food intake of this species of increased cattle breeding interest.

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IMMUNOCYTOCHEMICAL STUDY ON THE ENDOCANNABINOID AND OREXIN-1 INTERACTIONS IN THE HYPOTHALAMUS

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Endocannabinoids and CB_1 receptors stimulate food deprivation by acting on hypothalamic neurons.[1] We have focused our study on the endocannabinoid/orexin-1 interactions in *ob/ob* mice and littermates in order to investigate, in obesity, the inhibitory role of cannabinoids on orexinergic neurons as yet suggested in physiological conditions.[2] The immunodetection of orexin-1 (OX-1), CB_1, DAGL-α and NAPE-PLD (the endocannabinoid biosynthesising enzymes) and vescicular GABA or glutamate transporters as markers of GABAergic or glutamatergic fibers was performed in single and double immunostaining by immunoperoxidase, immunofluorescence and immunogold techniques. The immunoperoxidase staining was followed by quantitative analysis of each immunoreactive signal using a digital densitometric system. Lateral hypothalamus orexinergic neurons exhibited DAGL-α immunoreactivity (IR) in the somatodendritic compartment and were immersed in a meshwork of CB1-positive fibers. Increased DAGL-α, but not NAPE-PLD, was observed both in the LH and arcuate nucleus of obese vs. lean mice. CB_1-IR was localized on both glutamatergic and GABAergic axon terminals surrounding orexinergic neurons. A significant increase of 2-AG, but not of AEA, levels was also found in the hypothalamus of obese *vs.* lean mice. These results suggest a model of retrograde control by CB1 over OX-1 release. In obese mice, the increase of DAGL-α and 2-AG in orexinergic neurons might cause a post-synaptic disinhibition of orexin release by 2-AG acting on presynaptic CB_1-expressing GABAergic neurons and contribute to hyperphagia.

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EFFECTS OF A CHRONIC EXPOSURE TO CADMIUM ON THE GLIAL ARCHITECTURE IN THE LIZARD BRAIN: AN IMMUNOCYTOCHEMICAL STUDY

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The astroglial cells are positioned to be the first cells of the brain parenchyma to encounter metals crossing the blood-brain barrier.[1] For this they have also a neuroprotective action for the encephalon from cytotoxic action of heavy metals like cadmium. Numerous studies provide evidence that cadmium induces neuronal toxicity and damage to the brain. In this work we have studied the effects of this metal on the glial architecture in the brain of the lizard *Podarcis sicula* treated for four months with CdCl2 at dose of 1 mg/kg-BW in the drinking water. The study was performed by the immunodetection of GFAP by ABC technique on serial sections of brains at 10, 30, 60, 90 and 120 days of treatment. After 10 days the encephalon of treated lizards showed a decrement of GFAP-immunoreactivity only in grey matter of the cerebellum. After 30 days a reduction of expression and distribution of GFAP-immunopositive structures was observed in the telencephalon, mesencephalon and medulla. After 60 days only few GFAP-immunopositive structures were revealed and the absence of the radial glia was observed. Indeed at 90 days the radial glia was observed again in the telencephalon and at 120 days there was a return of occurrence of GFAP structures similar to that of control lizards. This study confirms the cytotoxic effect of cadmium on the lizard glial cells like we have just observed in lizard exposed to an acute treatment.[2] However a chronic exposure to cadmium involved a maximum reduction of the glial cells at 60 days.

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DIFFERENTIAL IMMUNOHISTOCHEMICAL DETECTION OF S100 AND GFAP IN FISH OLFACTORY ENSHEATHING CELLS

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The unmyelinated axons of olfactory neurons are supported by glial cells specific to the olfactory system, the olfactory ensheathing cells (OECs). They promote axon guidance both during ontogeny of the primary olfactory pathway and throughout adulthood, during regeneration of the olfactory epithelium after damage or for periodical turnover. A lack of knowledge about their molecular characteristics in fish olfactory system exists. Through immunohistochemical investiga-

tion, we compared the labelling patterns of two markers of mammalian OECs, S100 and glial fibrillary acidic protein (GFAP), in three species of fish: *Carassius auratus* (goldfish), *Poecilia reticulata* (guppy) and *Tilapia mariae*. In the goldfish immunoreactivity for GFAP showed positive cells in the epithelial lamina propria, along the olfactory nerve and in the peripheral fibre layer of the olfactory bulb. Instead, S100-positivity was restricted to the cranial cavity, while the glial cells in the olfactory chamber, from the lamellar basal lamina to the ethmoid bone, remained negative. We could suggest that OECs in goldfish are divided in subpopulations with specific antigenic properties that could reflect different functions, as already observed in the olfactory nerve layer of mammals. However, in mammals S100 is reported to be expressed by all OECs, both in the lamina propria and in the bulb. As a consequence we could also hypothesize that the goldfish GFAP+/S100- glial cells are not OECs. In both guppy and Tilapia anti-GFAP showed no immunostaining (this protein seems to be unexpressed in their olfactory pathway), while S100 appeared to be expressed all along the olfactory nerve pathway, from the bundles in the lamina propria to the bulbar fibre layer. Guppy and Tilapia possess sessile olfactory bulbs, while those of goldfish are pedunculated, so that the length of the nerve is extremely reduced. This anatomical aspect may affect OECs functions in fish, and, consequently, their antigenic properties.

DISTRIBUTION OF GLIAL FIBRILLARY ACIDIC PROTEIN- AND VIMENTIN-IMMUNOPOSITIVE STRUCTURES IN THE CENTRAL NERVOUS SYSTEM OF THE LEOPARD GECKO, EUBLEPHARIS MACULARIUS

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The distribution of intermediate filament molecular markers, glial fibrillary acidic protein (GFAP) and vimentin, in the different astroglial cell types, as well as the relative proportion and the regional distribution of these astrocytic subtypes are very important in studies on brain evolution. The immunoperoxidase technique has been applied on paraffin embedded sections to study these glial markers in the central nervous system (CNS) of the leopard gecko, *Eublepharis macularius*, as a representative of primitive saurians. In the gecko, GFAP immunopositive elements were observed throughout the CNS in both the gray and the white matters. The pattern of GFAP immunoreactivity mainly consisted of long fibres originating from cell bodies located in the ependymal layer. The apical pole of these cells delimited the ventricular surface and the abluminal pole gave rise to fibres radially oriented to the meningeal surface. The endfeet of these fibres constituted the submeningeal and the perivascular glial layers. The radial ependymoglia showed regional specialization in relation to the different size and immunocytochemical staining intensities of both their cell bodies and cytoplasmic processes. GFAP immunopositive star-shaped astrocytes were observed in various areas of the CNS, mingled with predominant radial ependymal elements. The vimentin-immunopositivity was scarce and associated with elements belonging to the radial glia in the telencephalon, diencephalon and mesencephalon. This immunohistochemical study points out the presence of different astroglial cell types in the CNS of *Eublepharis macularius*. In the different nervous regions, the staining intensity appears not to be identical even in the same cellular type. These observations result in a heterogeneous feature of the astroglial pattern of gecko. This may suggest that different brain regions need different glial functions and the glial pattern fits the requirements of the different nervous areas.

CHANGES INDUCED BY AN ACUTE TOXIC DOSE (ATD) OF METHAMPHETAMINE (METH) ON CANNABINOID CB1 RECEPTORS: AN IMMUNOCYTOCHEMISTRY STUDY

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Biochemical and behavioral effects of both cannabis and METH are well described, but their effects when given in combination are largely unknown. Exposure to an ATD of METH induces damages to the dopaminergic and serotoninergic terminals in some brain areas, including the caudate putamen, nucleus accumbens shell and the prefrontal cortex, which are involved in cognitive and motivational processes as well as adaptation to environmental stimuli. Exposure to high doses of cannabis produces persistent alterations in mnemonic functions in humans and rats, yet the underlying neurobiological mechanisms are still elusive. This study aimed to measure by semi-quantitative immunohistochemistry in selective rat brain areas, the extent of the alterations produced by an ATD dose (4.0 mg/kg x 4, at 2h intervals) of METH on the CB1 receptor density. In addition, by means of a battery of behavioral tasks covering the main brain functioning domains, we estimated the effect of an acute dose (0.5, 1, 2 mg/kg/2mL) of the synthetic cannabinoid receptor agonist WIN 55,212 (WIN) in combination with an ATD of METH. Immunohistochemical analyses (Saline, METH) and behavioral (Saline, METH, METH+Saline, METH+WIN) tests were performed three weeks after ATD administration. We observed that CB1 immunoreactivity (IR) was significantly increased in the cingulate cortex 1(Cg1, +35.26%), CA1 field (+25.19%) and dentate gyrus of hippocampus (DG, +79.08%), amygdaloid nucleus (+29%) and perirhinal cortex (PRh, +37%). Behavioral data of METH-WIN combination are currently in progress. Our immunohistochemical findings suggest that an ATD of METH increases CB1 receptor IR in brain areas crucial for attentive-cognitive and emotional functions. As behavioral data will be obtained, is our intention to look for their potential correlation with neurochemical alterations caused by an ATD of METH.

FINE DISTRIBUTION OF THE EPILEPSY-RELATED LGI1 PROTEIN IN RAT CORTICAL NEURONS

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The Lgi1 protein is involved in the pathogenesis of autosomal dominant lateral temporal epilepsy because mutations in the leucine-rich, glioma-inactivated 1 (LGI1) gene have been found in affected subjects and families; however, the function of Lgi1 is still unclear. Definition of the fine intracellular distribution of Lgi1 in normal cortical tissue would add to understanding its role and envisage pathogenetic mechanisms. Therefore, we analyzed normal rat cortex for Lgi1 by combining biochemistry, immunohistochemistry and immunoelectron microscopy. Our results demonstrate that Lgi1 is a cytoplasmic protein distributed both in the soma and in the processes of neurons: it occurs on the rough endoplasmic reticulum, the site

of synthesis, in the Golgi complex, where it undergoes glycosylation, and in close proximity to neurotubules and neurofilaments, especially in the axons, but it is scarce or absent at synapses and the neurilemma. Lgi1 association with axonal cytoskeletal structures would imply Lgi1 is either transported along axons by motor proteins, or playing some role as a carrier in the axonal flux, or it could be involved in the regulation of cytoskeletal organization. At variance with current functional hypotheses, which favor an involvement of Lgi1 in the control of neuronal current transmission at synapses, our data provide evidence for cytoplasmic localization and function of Lgi1 in cortical neurons.

DOPAMINERGIC MARKERS AND DOPAMINE RECEPTOR LOCALIZATION IN RAT THYMUS, SPLEEN AND PERIPHERAL BLOOD LYMPHOCYTES

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The possibility that thymus expresses a dopaminergic system was first suggested by studies identifying dopamine receptors in it. Dopamine stores, vesicular monoamine transporter (VMAT) type-1 and 2, and dopamine D1-like and D2-like receptor subtypes localization were investigated in rat thymus, spleen and in ficoll-isolated thymocytes, splenocytes and lymphocytes of 1-month-old male Wistar rats. Thymus, spleen and lymphocytes were processed by Western blot analysis, RT-PCR and immunohistochemistry analysis for dopaminergic markers. In the thymus dopamine immunoreactivity was developed only in the cortico-medullary junction and in the medulla. In the spleen, dopamine stores were found in reticular structures in the white pulp border and in the white pulp. Both thymus and spleen expressed VMAT-1, VMAT-2 and dopamine D1- and D2-like receptors immunoreactivity. Immunohistochemistry revealed VMAT-1, VMAT-2 and dopamine receptors immunoreactivity primarily in the thymic cortical-medulla transitional zone and to a lesser extent in the medulla but not in the cortex. In the spleen, the dopaminergic immunoreactivity was located primarily in the white pulp border and to a lesser extent in the white pulp. Immunochemical analysis revealed the expression on dopaminergic markers in isolated thymocytes, splenocytes and lymphocytes. These findings indicate that both thymus and spleen express a dopaminergic system. The presence of these dopaminergic markers suggests that dopamine likely originating from immune cells and/or from sympathetic neuroeffector plexus is released in the lymphoid microenvironment. Based on the microanatomical localization of dopaminergic markers investigated, a role of dopamine in maturation and selection of lymphocytes and activation of immune responses is suggested.

IMMUNOHISTOCHEMICAL EVIDENCE THAT MPTP-INDUCED PARKINSONISM AFFECTS DOPAMINERGIC NEURONS IN MOUSE ENTERIC NERVOUS SYSTEM

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Parkinson's disease (PD) is a degenerative pathology which affects dopaminergic neurons of the substantia nigra, leading to a movement disorder. Non motor symptoms commonly involve the muscles and glands of the gut. To clarify this point, in the present study we tried to reproduce digestive dysfunctions by using the parkinsonism-inducing neurotoxin 1-methyl, 4-phenyl, 1,2,3,6,-tetrahydropyridine (MPTP) in 9-week old C57BL mice. One week after treatment with MPTP (i.p. 20 mg/kg x3, 2 h apart) we analyzed morphological and biochemical changes on the nervous network of the gut: immunostaining for tyrosine hydroxylase (TH), dopamine transporter (DAT) and noradrenaline transporter (NET); fluorescent immunostaining for α-synuclein; monamine level measurement by HPLC-ED. In controls, TH immunopositivity was well evident in both myenteric and submucous plexuses, appearing as continuous markedly stained rings. From the submucous plexus nervous fibres and neurons extended to the mucosa up to the axes of the villi. DAT and NET immunopositivity was also well evident as stained rings. In MPTP-treated mice, both TH and DAT, but not NET, immunopositive neurons were reduced in both plexuses and the continuous ring-like staining was no longer evident. Moreover, α-synuclein fluorescence was revealed in the submucous plexus. Consistently, while noradrenaline levels were unchanged, there was a severe dopamine depletion. Finally, these morphological and biochemical features were accompanied by a functional impairment which was reminiscent of constipation occurring in PD. Our data provide a novel and reliable model to study the altered digestive function in PD, while offer the basis to interpret the digestive dysfunction in PD as a consequence of a selective dopaminergic impairment of the gut.

EXPRESSION OF TOLL-LIKE RECEPTORS 3, 4 AND 7 IN MURINE PERIPHERAL NERVOUS SYSTEM DEVELOPMENT

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Based on our previous findings showing that innate immunity receptors of the Toll-like family (TLRs) are expressed in adult murine enteric nervous system (ENS) and dorsal root ganglia (DRGs), the aim of the present study was to evaluate if such expression could also be detected during embryonic development. Embryos from stages E12, E14, E16 and E18 were excised, fixed in paraformaldehyde and paraffin embedded. Sections were processed for immunohistochemistry and immunofluorescence with antibodies against TLR3 and TLR7 (recognizing viral RNA), TLR4 (recognizing LPS, membrane component of gram-negative bacteria), β3 tubulin, PGP 9.5 and PCNA (proliferating cell nuclear antigen). Intestines from E13 and E14 embryos were also processed as whole-mount preparations. In ENS, weak TLR7 immunoreactivity was already present at E12 in the region of the myenteric plexus and by E14 had sensibly increased in intensity and was accompanied by the appearance of TLR3 immunolabelling. Conversely, TLR4-positive cells could be clearly detected in enteric plexuses only starting from E17. TLR3, TLR4 and TLR7 expression was also visible in other neural crest derivatives (DRGs, sympathetic ganglia, vagus nerve, small ganglia of the air pathways, and in visceral and somatic peripheral nerve fibres). Also in this case, expression of TLR7 and TLR3 preceded the expression of TLR4. TLRs are the mammalian homologues of the Toll protein. Toll was first identified in Drosophila melanogaster where it fulfils both an early role in different aspects of morphogenesis and a late function in innate immunity responses. The present results seem to indicate that the same pattern is also present during the development of mammalian neural crest derivatives. The late expres-

sion of TLR4 compared to TLR7 and TLR3, most notably in ENS, suggests that the expression of the former is probably related to the forthcoming colonization by commensal microorganisms at the moment of delivery.

SPEXIN EXPRESSION IN RAT TISSUES THROUGH REAL TIME-POLYMERASE CHAIN REACTION AND IMMUNOHISTOCHEMISTRY

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Spexin is a highly conserved peptide which was recently identified through bioinformatics approach and has been found to be processed and secreted in transfected rat pancreatic cells. Spexin has been localized in brain and submucosal layer of esophagus and stomach by *in situ* hybridization and mainly in kidney, brain, and pancreas through Northern blot analysis. Immunohistochemical studies have not yet been performed. Thus, in the present work, we examined spexin expression and location in a wide range of rat organs both by RT-PCR and immunohistochemistry. RT-PCR identified spexin mRNA in all tissues examined, i.e., thyroid gland ≈ ovary ≈ pancreas ≈ brain > esophagus ≈ kidney > lung ≈ uterus ≈ stomach ≈ adrenal gland ≈ hypophysis ≈ testis > bladder ≈ small bowel ≈ heart ≈ skeletal muscle ≈ liver ≈ spleen. Spexin immunoreaction was cytoplasmic. Spexin was immunohistochemically detected, although with different staining intensities, in epithelia and glands of skin, respiratory, digestive, urinary and reproductive systems. Smooth muscle cells showed only weak immunostaining and connective structures were negative. In the central nervous system, different neuronal groups showed different intensities and percentages of immunoreactivity. Immunoreaction was also found in ganglionic cells of both trigeminal and superior cervical ganglia and in photoreceptor, inner nuclear and ganglionic layers of the retina. In the endocrine system, spexin immunoreaction was detected in the hypothalamic paraventricular and supraoptic nuclei, adenohypophysis, thyroid and parathyroid glands, adrenal cortex and medulla (mainly ganglionic cells), Leydig cells, thecal, luteal and interstitial gland cells. On the basis of its biochemical characterization and widespread expression, spexin may be included in the neuropeptide family. It is probably involved in many different physiological functions, in particular its location in neurons suggests a role as neurotransmitter/neuromodulator.

UPTAKE AND RECYCLING OF BRAIN-DERIVED NEUROTROPHIC FACTOR BY CORTICAL ASTROCYTES AND HIPPOCAMPAL NEURONS

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Regulation of brain-derived neurotrophic factor (BDNF) secretion plays a critical role in long-term potentiation (LTP). It is generally thought that the supply for this secretion is newly synthesized BDNF targeted to the synapse. Here we provide evidence that hippocampal neurons additionally recycle BDNF for activity-dependent secretion. Exogenously applied BDNF is internalized by cultured neurons and rapidly becomes available for activity-dependent secretion, which is controlled by the same mechanisms that regulate the secretion of newly synthesized BDNF. Moreover, BDNF recycling replaced the new synthesis pathway in mediating the maintenance of LTP in hippocampal slices: the late phase LTP, which is abolished by protein synthesis inhibition, was rescued in slices preincubated with BDNF. Thus, endocytosed BDNF is fed back to the activity-dependent releasable pool required for LTP maintenance.[1] Furthermore, we show that BDNF is secreted by astrocytes in its precursor form (pro-BDNF) and is then cleared from the extracellular space through rapid uptake by nearby astrocytes after τ-burst stimulation in layer II/III of cortical slices, a paradigm resulting in LTP of synaptic transmission. Internalization of pro-BDNF occurs via the formation of a complex with the pan neurotrophin receptor p75 and subsequent clathrin-dependent endocytosis. Fluorescence-tagged pro-BDNF and real-time total internal reflection fluorescence microscopy in cultured astrocytes is used to monitor single endocytic vesicles in response to the neurotransmitter glutamate.[2] We find that endocytosed pro-BDNF is routed into a fast recycling pathway for subsequent soluble NSF attachment protein receptor-dependent secretion. Thus, astrocytes contain an endocytic compartment competent for pro-BDNF recycling, suggesting a specialized form of bidirectional communication between neurons and glia.

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IMMUNOCYTOCHEMICAL CHARACTERIZATION OF MESENCHYMAL STEM CELLS EFFECT OF DRG NEURONS APOPTOTIC DEATH

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The positive effect of adult undifferentiated Mesenchymal Stem Cells (MSC) on nerve regeneration, axonal growth and neuronal survival has already been reported,[1] although the mechanisms by which MSCs exert their effects are still unclear. We have previously demonstrated that the direct co-culture between adult undifferentiated MSC and rat DRG dissociated neurons allows neuronal long-lasting survival and maturation.[2] Here we studied by confocal microscopy the mechanisms leading to neuronal death and how the co-culture with MSC inhibit such mechanisms, thus resulting in neuronal long term survival. We demonstrated that DRG neurons die after 2 weeks of culture through an apoptotic mechanism involving caspase 3 activation, but the co-culture with MSC is able to block the apoptotic death. Moreover, by using specific antibodies we shed light on the proteins mediating the death pathway thus demonstrating the involvement of Metalloproteinases family members (MMPs) and of their inhibitors, as well as of some antiadhesive proteins. In particular we observed that metalloproteinases inhibitor Timp1 is present only in co-cultured neurons, both into the nucleus and in cytoplasm, while it is absent in neurons alone. On the contrary the anti-adhesive protein Sparc (able to activate MMPs) is present into the nucleus and the cytoplasm of neurons alone but not in co-cultured neurons. By immunofluorescence we demonstrated that MSC are able to inhibit the modifications occurring in DRG neurons, thus allowing a long term survival. MSC are also able to inhibit MMPs activation in DRG neurons, as demonstrated by zymography.

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IMMUNOHISTOCHEMISTRY OF THE SENSORY NERVE ENDINGS LOCATED IN THE HUMAN SKIN

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The sensory nerve endings are special structures differentiated in transforming mechanical into nervous inputs. These structures are widely distributed in the human tissues and organs, especially in the human skin and connective tissue. We have carried out some immunohistochemical studies using specific cytoskeletal protein-markers on sensory formations of the human skin. Most of our data confirm the origin of the different parts of sensory formations, but the functional significance of these cytoskeletal proteins in the inner core cells remains unclear. 1. S-100 protein. It is a soluble acidic calcium-binding protein and a marker for glial cells *in situ*, and its presence in the inner core of sensory corpuscles strongly support the glial origin of these structures. 2. Glial fibrillary acidic protein. In Pacinian corpuscles of the human skin GFAP-like immunoreactivity has been found in the inner-most layers of the inner core, and could be speculated that a closely relation between axon and epithelial cells is necessary for the expression of GFAP in the human skin. 3. Vimentin. Vimentin is a cytoskeletal protein characteristic of mesenchymal cells, specially fibroblast but also was demonstrated by us in the human skin. The inner core but not the capsule of sensory corpuscles display a vimentin-like immunoreaction . The lamellae forming the inner core contain numerous filaments which presumably are responsible of the immunohistochemical reaction. On the other hand, since the capsule and intermediate layer participate in the formation of inner core it could be hypothesized that the immunoreaction is developed in these components. 4. Neurofilament protein (NFP) and neuro-specific enolase (NSE). These neuronal markers have been demonstrated in sensory axons of the human skin. The presence of NFP confirm the neuronal nature of the central axon in sensory corpuscles.

DOPAMINERGIC MARKERS IN THE GUT ASSOCIATED LYMPHOID TISSUE (GALT) OF THE RAT

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The sympathetic nervous system supplies both vasculature and parenchymal fields of lymphocytes and associated cells in lymphoid organs such as thymus, spleen, lymph nodes, gut-associated lymphoid tissue (GALT), and bone marrow. GALT is composed of discrete inductive and effectors sites, discriminating between harmful and harmless antigens. Inductive sites are organized into specialized aggregations of lymphoid follicles called Peyer's patches, while effector sites are more diffusely dispersed. Dopamine as well as other catecholamines contribute to modulate immune functions through the interaction with specific receptors expressed on the surface of immunological target cells. To clarify the possible role of dopamine in immune modulation, we have investigated the expression and the anatomical localization of markers of dopaminergic function such as the plasma membrane dopamine transporter (DAT) and vesicular monoamine transporter (VMAT)-1 and -2 in lymphoid system associated with jejunum and ileum. DAT is the plasmalemmal protein responsible for the reuptake of released dopamine, whereas VMATs are responsible for the translocation of monoamines from the cytoplasm into synaptic vesicles using a proton electrochemical gradient. Analysis was accomplished using immunochemical and immunohistochemical techniques. Western blot analysis revealed the expression of DAT, VMAT-1 and VMAT-2 protein immunoreactivity in preparations of rat jejunum and ileum. Immunohistochemistry demonstrated the expression of DAT, VMAT-1 and VMAT-2 immunoreactivity in grouped lymphoid nodules associated with gut (Peyer's patches). Immune reaction was located primarily in the internodular zones and in the nodule-associated epithelium. These data suggest an interaction between dopamine and GALT the functional relevance of which is under evaluation.

IMMUNOLOCALIZATION OF α-SYNUCLEIN IN THE C57 BL\6J MOUSE CENTRAL NERVOUS SYSTEM BY TWO NOVEL MONOCLONAL ANTIBODIES: A PRELIMINARY STUDY TO INVESTIGATE THE α-SYNUCLEIN PATHOLOGY IN THE MPTP MODELS OF PARKINSON'S DISEASE

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α-synuclein, a 140 amino acids' protein, richly expressed in the central and peripheral nervous system, is a protein closely related with Parkinson's disease and numerous others neurodegenerative disorders. However, although the pathological involvement of this protein is widely reported in the literature, its physiological function in the healthy neurons remain unclear, as the mechanism with witch this protein contribute to neurodegeneration. Since we dispose of two novel homemade monoclonal antibodies, able to detect α-synuclein in different compartments of the nerve cells, we aimed to create an anatomical map of the protein's distribution in the CNS of C57 bl\6J mouse. We choose this particular mouse strain because it is the most sensitive to the lesion of MPTP, thus, the most used in toxic models of Parkinson's disease. The two monoclonal antibodies, we used, confirm their ability to visualize the protein in different compartments of the neurons, since 2E3 detected α-synuclein in the nerve cells' fibers and 3D5 in the nuclei. Both antibodies, however, are able to show the synaptic α-synuclein. The protein seems ubiquitary expressed either in the brain, as in the spinal cord, with some particular organization patterns detectable in some specific region, such as habenular nuclei, dorsal Hippocampus, cerebellar cortex, dorsal motor nucleus of vagus and cochlear nucleus. These particular patterns could be related to a specific function of the protein in these regions of the CNS or to their particular cytoarchitecture. Although our study is only an immunohistochemical study, our findings provides the first map of the α-synuclein expression in the C57 bl\6j mouse CNS and they will be very useful to better analyze the α-synuclein pathology in the mice experimental models of Parkinson' disease.

IDENTIFICATION OF ANKRD2/ARPP AS AN AKT SUBSTRATE IN C2C12 MUSCLE CELLS

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Ankyrin-repeat protein with a PEST motif and a proline-rich region (Arpp), also designated as Ankrd2, is a member of the muscle ankyrin repeat proteins (MARPs), which are involved in muscle stress response pathways, occurring after eccentric contraction (EC), in the response to acute exercise, or during work overload hypertrophy. Also pathological conditions, as tumors and some muscular dystrophies see the involvement of these proteins.[1] Akt/PKB is a serine/threonine kinase activated downstream of multiple cellular signals. In muscle cells, activation of Akt is crucial for survival, differentiation and regeneration of muscle cells. By phosphorylation of Lamin A/C, recently we demonstrated the involvement of Akt in the correct function of nuclear lamina of muscle cells.[2] In this work we describe the involvement of Ankrd2 in response to Reactive Oxygen Species (ROS) in C2C12 differentiating myotubes. By immunocytochemical study, we found that Ankrd2 localization is prominently cytosolic; however ROS arrival strongly induces Ankrd2 nuclear accumulation. More, we show that after 5 minutes from the arrival of the stress stimulus, Ankrd2 binds to Akt. The Akt-Ankrd2 association leads to the phosphorylation of Ankrd2, peaking maximum levels after 30 minutes of stimulation. In particular, both Akt-Ankrd2 binding and Akt-mediated Ankrd2 phosphorylation reflect ROS-induced Akt activation. Possible roles for Akt-dependent Ankrd2 phosphorylation are described.

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CATALYTIC ACTIVITY OF NUCLEAR PLC-β 1 IS REQUIRED FOR ITS SIGNALLING FUNCTION DURING C2C12 DIFFERENTIATION

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Here we report that PLCβ1 catalytic activity plays a role in the increase of cyclin D3 levels and induces the differentiation of C2C12 skeletal muscle cells. PLCβ1 mutational analysis revealed the importance of His331 e His378 for the catalysis. The expression of PLCβ1 and cyclin D3 proteins is highly induced during the process of skeletal myoblast differentiation. We have previously shown that PLCβ1 activates cyclin D3 promoter during the differentiation of myoblasts to myotubes, indicating that PLCβ1 is a crucial regulator of the mouse cyclin D3 gene. We show that after insulin treatment cyclin D3 mRNA levels are lower in cells overexpressing the PLCβ1 catalytically inactive form in comparison to wild type cells. We describe a novel signalling pathway elicited by PLC-β1 that modulates AP-1 activity. Gel mobility shift assay and supershift performed with specific antibodies indicate that the c-jun binding site is located in a cyclin D3 promoter region specifically regulated by PLCβ1 and that c-Jun binding activity is significantly increased by insulin and PLCβ1 overexpression. Mutation of AP-1 site decreased the basal cyclin D3 promoter activity and eliminated its induction by insulin and PLCβ1. These results hint at the fact that PLCβ1 catalytic activity signals a c-jun/AP-1 target gene, i.e. cyclin D3, during myogenic differentiation.

FAS AND STAUROSPORINE EFFECTS IN APOPTOSIS LINKED TO TROGOCYTOSIS

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Trogocytosis corresponds to an active transfer phenomenon triggered specifically by antigen receptor signaling. It occurs within minutes of conjugate formation between two live cells and therefore cannot be the result of phagocytosis of apoptotic bodies. Key events of T and B cell biology are regulated through direct interaction with APC or target cells. Trogocytosis is a process whereby $CD4^+$ T, $CD8^+$ T and B cell capture their specific membrane-bound Ag through the acquisition of plasma membrane fragments from their cellular targets. Fas (CD95/APO-1) belongs to the TNF receptor superfamily and mediates programmed cell death upon Fas ligand binding. The Fas surface receptor mediates rapid death of various cell types, including autoreactive T cells. We have evaluated trogocytosis in primary activated $CD4^+$ T cells from healthy donor and in Jurkat T cells. Both cell lines were treated with FasL and staurosporine to study the intrinsic and extrinsic pathway. Jurkat and $CD4^+$ T cells were analyzed by flow cytometry: the treatment with FasL and staurosporine promotes not only membrane exchange, as the lipophylic dyes PKH26 (red) and 67 (green) demonstrate, but also a considerable cytoplasmic exchange, as shown by CFSE labelling. PKH dyes and CFSE staining were evaluated also for detection of spontaneous transfer on both Jurkat and $CD4^+$ primary T cells. Both membrane and cytoplasmic exchange were stronger in Jurkat and in stimulated $CD4^+$ cells, without pretreatment with latrunculin B (to prevent actin polymerization): in fact this pretreatment deeply reduced transfer of lipophylic dye, which is consistent with trogocytosis, but it didn't substantially reduce CFSE transfer. T cells are known to take up plasma membrane fragment from antigen presenting cells during immunological synapse contact, our results suggest that this behavior may be linked with the apoptotic phenomenon, involving also a cytoplasmic exchange in both Jurkat and $CD4^+$ primary T cells.

EXPRESSION OF NUCLEAR FACTOR-κB AND ITS RELATIONSHIP WITH P53 AND SURVIVIN IN CUTANEOUS MELANOMA

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Nuclear Factor-κB (NF-κB) is a transcription factor that plays a crucial role in inflammatory events, apoptosis, cell cycle control, and several other cellular processes. It is expressed in most cells but kept inactive in the cytoplasm by interaction with IκB inhibitors. After pro-inflammatory, mutagenic, and pro-apoptotic stimuli, NF-κB translocates to the nucleus and activates the expression of various genes. Several studies have recently found that NF-κB expression may be involved in the development and progression of various tumors. In melanoma, NF-κB has been suggested to activate the expression of anti-

apoptotic proteins, such as survivin, thus allowing the escape of tumor cells from apoptosis and enhancing their metastatic potential. Survivin is a member of the inhibitor of apoptosis protein family, undetectable in most differentiated normal tissues, but strongly expressed in embryonic and fetal organs. It is implicated in cell division, prevention of apoptosis, cellular stress response, and checkpoint mechanisms of genomic integrity. It is overexpressed in many human malignancies and such overexpression is associated with poor prognosis. Transcription of the survivin gene has been shown to be repressed by p53, the product of the tumor suppressor gene p53, essential in the regulation of cellular response to DNA damage. Up-regulation of wild-type p53 results in a transient G1 arrest, allowing cells to repair the DNA damage before resuming cell cycle. Moreover, the ability of p53 to induce apoptosis has been considered primary to its role in tumor suppression. Loss or mutation of p53, in addition to be a possible mechanism responsible for survivin overexpression, seems to directly or indirectly lead to NF-κB activation in melanoma cells. In this study, the expression of NF-κB, survivin, and p53 was immunohistochemically examined to investigate the possible relationship between these factors in primary cutaneous melanoma. The results will be discussed.

MAJOR ROLE OF THE NF-KB/TOLL LIKE RECEPTOR PATHWAY IN THE EMBRYONIC BRAIN INFLAMMATORY RESPONSES INDUCED BY LIPOPOLYSACCHARIDE

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Endotoxin interaction with cells involves specific membrane receptors, including Toll-like receptor (TLR)-4. TLR-4 acts as the signal-transducing receptor for the lipopolysaccharide (LPS), a component of the outer cell wall of Gram-negative bacteria. Recently, a relationship between systemic infection and worsening of several diseases of the central nervous system has been documented, although the mechanisms inducing the neuronal loss are not yet well established. In this work we examine the expression of TLR-4 in chick embryo neurons and glial cells and its role in cell responses after LPS treatment in order to understand how downstream signaling pathway is engaged by TLR-4. Primary glial cell or neuron-enriched cultures, obtained from 10-day chick embryo brains, were maintained in MEM supplemented with antibiotics and fetal calf serum or in Neurobasal medium supplemented with B27, respectively. Cultures were treated with LPS of *Salmonella typhimurium* for different times. The surface expression of TLR-4 was determined using an anti-human TLR-4 monoclonal Ab in presence of specific Abs directed against cellular proteins of neurons or glial cells. Nitric oxide (NO) and tumor necrosis factor (TNF)-α releases were evaluated by the Griess reaction and ELISA assay, respectively. The iNOS and NF-κB expressions were evaluated by immunoblotting. LPS treatment for 72 h caused a dramatic cell loss in both glial and nerve cell cultures, NF-κB activation, iNOS expression, NO and TNF-α release, thus identifying NO and TNF-α as major effector molecules responsible for the LPS-induced cell damage. Toxic effects elicited by endotoxin appear to be mediated by the activation of the nuclear factor NF-κB, since its inhibition brought about a reduction in cell loss. Furthermore, NF-κB activation is dependent on the engagement of TLR-4 (expressed in both glial and neuronal cells), since treatment with a specific Ab against TLR-4 abrogates the LPS-induced toxic effects.

SEROTONIN AND NEUROTENSIN CELLS IN ATRESIC CHICK EMBRYO INTESTINE: IMMUNOHISTOCHEMICAL STUDY

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Intestinal motility disorder is an important problem in the postoperative managements of patients with intestinal atresia. Intestinal motility could be initiated by luminal factors that activate intrinsic and extrinsic primary afferent nerves involved in the peristaltic reflex. Endocrine cells act as a key point, because they transfer information regarding the intestinal contents and intraluminal pressure to nerve fibers lying in close proximity to the basolateral surface of the epithelium. In chick embryo, experimental intestinal atresia is associated with disorders in the development of the enteric nervous system, related to the severity of intestinal dilation. Our aim was to investigate the distribution pattern of endocrine cells in the developing endocrine system of chick embryo small intestine with experimentally-induced atresia on day 12 (D 12) and D 16. Changes in enteroendocrine population were examined in gut specimens (excised proximal and distal to the atresia) from experimental D 19 embryo and in control sham-operated chick embryos at the same age. Sections from proximal and distal bowel and control bowel were stained with Grimelius silver stain, a valuable histochemical method for detecting the argyrophil and argentophilic cells, and with an immunohistochemical procedure for detecting 5HT and neurotensin immunoreactive cells. In chick embryo proximal bowel, intestinal dilation differed in the various embryos. We found significantly higher enteroendocrine cell counts in proximal bowel than in distal and control bowel. The differences depended on the precociousness of surgery and the severity of dilation. Considering the major contribution of enteroendocrine cells to the peristaltic reflex, our data may help to explain the pathogenesis of motility disorders related to intestinal atresia.

Posters Symposia III-IV

ROLE OF INSULIN-LIKE GROWTH FACTOR 1 AXIS IN THE MODULATION OF HEPATIC PROGENITOR CELLS COMPARTMENT IN NORMAL LIVER AND DURING PRIMARY BILIARY CIRRHOSIS

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Hepatic progenitor cells (HPCs) are resident stem cells capable to differentiate towards hepatocytes and cholangiocytes. HPCs are recognized thanks to the expression of several markers such as cytokeratin (CK)7 and CK19.[1] Primary biliary cirrhosis (PBC) is characterized by the progressive disappearance of interlobular bile ducts leading to ductopenia mainly caused by apoptosis of cholangiocytes. The insulin-like growth factor 1 (IGF1) axis has a key role in the surviving of cholangiocytes during PBC evolution.[2] We studied the role of IGF1 in the modulation of HPCs compartment of the liver during PBC. Biopsies of PBC patients (n=6) and normal subjects (n=6) were investigated by immunohistochemistry and by double immunofluorescence for CK7, CK19, IGF1 and IGF1-R. In normal livers, HPCs reside in canals of Hering; they are $CK7^+/CK19^+$ but almost negative for IGF1 and IGF1-R. In PBC, HPCs highly proliferate in correlation with disease progression. Some HPCs penetrate deeply into the cirrhotic nodules and some of them are CK19 negative indicating a differentiation towards hepatocytes. In PBC, confocal microscopy clearly showed the expression of IGF1 and IGF1-R by HPCs and their correlation with disease's evolution. In conclusion, HPCs are activated during the progression of PBC. IGF1 axis could play a key role for HPCs proliferation and niche expansion. The pharmacological modulation of IGF1 axis and liver stem cells compartment could represent a new approach for delaying the progression of PBC.

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A BIOCHEMICAL AND MOLECULAR STUDY OF STEM CELL ADHESION AND DIFFERENTIATION ON POSITIVELY CHARGED SURFACES

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Biomaterials are very important for many applications; in fact, from industry to regenerative medicine they are widely diffused. Three-dimensional (3D) Titanium (Ti6Al4V) and bioactive glass represent very good promises for orthopaedic prosthesis and dental implants. However, 3D Ti6Al4V shows some advantages with respect to glass like very high mechanical strength and resistance to corrosion. Human bone marrow derived stem cells (hMSCs) are pluripotent cells that can differentiate in many mesenchymal lineages: osteogenic, chondrogenic, cardiogenic, depending by multiple environmental factors. Dental Pulp Stem Cells (DPSCs) show stem cell properties and represent a promising tool for bone regeneration. Both hMSCs and DPSCs are able to express osteogenic markers *in vitro* when cultured on Ti6Al4V but their ability to self-renew on this biomaterial is quite low in comparison with tissue culture treated plastic. On the other side bioactive glass represent a good tool for studying cell adhesion and differentiation. In fact, glass slides have been used for tissue section adhesion for long time. Positively charged glass electrostatically attracts tissue sections and cytological preparations that can better adhere on this substrate in comparison to normal glass. We have characterized adhesion and differentiation of hMSCs and hDPSCs on positively charged glass, normal glass, plastic and 3D Ti6Al4V. Our results suggest that deposition of a positively charged biomolecule on titanium could increase adhesion and differentiation of stem cells on this substrate, ameliorating its suitability as biomaterial for bone prosthesis and dental implants.

TRAIL PROMOTES MIGRATION OF HUMAN BONE MARROW MULTIPOTENT STROMAL CELLS

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Adult multipotent stromal cells (MSC) represent an important source of cells for the repair of a number of damaged tissues. The aim of this study was to characterize the presence of TRAIL-receptors and to evaluate the potential biological activity of soluble TRAIL in terms of apoptotic, proliferation, differentiation and migratory responses on human bone marrow MSC. Surface TRAIL-receptor expression in MSC cultures was analyzed by flow cytometry. Apoptosis after treatments with recombinant TRAIL (rTRAIL) or infection with an adenovirus expressing TRAIL (AdTRAIL) was evaluated by Annexin-V/PI and DAPI staining. Osteogenic and adipogenic differentiation, induced using specific differentiation medium, was monitored by osteocalcin and adiponectin ELISA, as well as by von Kossa and Oil O Red staining, respectively. The migration response of MSC toward rTRAIL was investigated by measuring the transfilter migration. MSC expressed detectable surface levels of two (TRAIL-R2 and TRAIL-R4) out of four transmembrane TRAIL receptors. Although the best-characterized activity of TRAIL-R2 is the transduction of apoptotic signal, neither rTRAIL nor infection with AdTRAIL induced cytotoxic effects on MSC. Moreover, while rTRAIL did not affect proliferation or differentiation of MSC along the osteogenic and adipogenic lineages, it significantly promoted the migration of MSC in range of concentrations detectable in human plasma. In addition rTRAIL induced the rapid phosphorylation of ERK1/2 in MSC cultures, and pretreatment with pharmacological inhibitors of this pathway efficiently counteracted the rTRAIL-induced MSC migration. These data indicate that rTRAIL is able to stimulate MSC migration, through activation of ERK1/2 pathway. Taking into consideration that the soluble factors able to induce MSC migration have not been extensively characterized, this study indicate that the TRAIL/TRAIL-R system might play an important role in the biology of MSC.

OSTEOGENIC DIFFERENTIATION OF DENTAL PULP STEM CELLS (DPSC) IN 3D-MATRICES TO USE IN REGENERATIVE MEDICINE

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Our recent investigations concern the selection of mesenchymal stem cells, derived from adult human dental pulp (DPSC), to commit towards osteogenic differentiation in order to perform new strategies for the regenerative medicine. Niches of adult stem cells were obtained from dental pulp, extracted from the second or third permanent human molars of adult patients aged between 18 and 25 years. After a selection based on the identification of staminal and mesenchymal markers, i.e. c-kit, CD34 and STRO-1, by means of the immuno MAgneto Cell Sorting (MACS) method, the cells were cultured *in vitro* using a medium enriched with factors conditioning for osteogenic differentiation. Different types of cultures were performed, in order to obtain an osteoblast-like system capable to mime the osteogenic cell system devoted *in vivo* to form bone tissue, using differently shaped biomaterials. DPSC cultured in 2D surfaces and in 3-D matrices were analyzed by western blot, immunofluorescence and ultrastructural techniques in order to verify the expression of specific osteogenic markers. Several protocols were planned to implant, in animal models, the more suitable 3-D/DPSC complexes, namely those showing the best three-dimensional cytoplasmic network of osteoblast-like cells. The final aim of this study is to use DPSC in regenerative medicine approaches for recovering wide gaps of bone tissue due to post-traumatic locomotor apparatus damages.

3D MODEL OF HUMAN PREADIPOCYTE CULTURE BASED ON SELF-ASSEMBLING PEPTIDE NANOFIBER

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The *in vivo* implant of preadipocytes is proposed for the treatment of soft tissue congenital deformities and post traumatic repair. Experimental evidences show that the traditional bi-dimensional (2D) *in vitro* systems are not idoneous of *ex vivo* expansion of adipose tissue derived cells without alterations of phenotype and differentiation ability. Therefore, a three-dimensional (3D) synthetic matrix, named Puramatrix Peptide Hydrogel,[1] is proposed as the ideal 3D culture model to overcome the limits of the *ex vivo* cellular culturing. Human preadipocytes ($1x10^6$ cells/mL) were encapsulated into 0,25 peptide scaffold solution and then placed at a multiwell culture insert plate for assembling. Cellular proliferation was tested by MTT assay. The assessment of phenotypical/morphological modifications at different time points of sample culturing/differentiation were studied by histochemical assays, immunofluorescence staining and electron microscopy (TEM). Terminal adipogenic differentiation of hormonally stimulated cells was evaluated by leptin and glucose transporter-4 (GLUT-4) expression. After an instant strong cytotoxic effect induced by encapsulation of human preadipocytes, *in vitro* 3D model seemed to stimulate cell proliferation and organization into lobular-like structures. TEM analysis, histochemical assays and immunofluorescence staining confirmed terminal differentiation of human preadipocytes embedded into PuraMatrix Peptide Hydrogel. Leptin production, GLUT-4 expression, synthesis of collagen IV and laminin were observed after 9-12 days of hormonal stimulation versus 21 days required for terminal differentiation of 2D cultures. In conclusion, the 3D model proposed optimizes *ex vivo* expansion of human preadipocytes mimicking native microenvironment and therefore could be useful for systematic investigations of adipogenesis.

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DIFFERENT GROWTH FACTORS *IN VITRO* EFFECT ON MESENCHYMAL STEM CELLS AND ON CELLS FROM NONUNION SITES

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At a cellular level, wound healing and regeneration involves a complex cascade of events, including cell proliferation and differentiation. These processes are known to be modulated by growth factors. Consequently, over the past two decades, the potential use of growth factors in bone regeneration has been investigated and in principle viability has been demonstrated. Aim of the present study was to assess the effects of recombinant human bone morphogenetic protein 7 (rhBMP-7) and platelet rich plasma (PRP) on mesenchymal stem cells (MSCs) and on cells localised at nonunion sites. Five patients (mean age 35 years) affected by nonunion were selected and during surgery samples of cancellous bone, fibrous tissue and bone marrow were harvested to obtain cultures of different cell cytotypes (osteoblast, fibroblast-like and MSCs). Cells were incubated with rhBMP-7 (1 μg/mL) and PRP (10% and 20%) obtained from venous blood of 5 healthy volunteers. MTT viability test and evaluation of alkaline phosphatase (ALP) activity were performed at 3, 7, 14 and 21 days. Histochemical detection of ALP and calcium deposits (von Kossa staining) as well as immunohistochemistry for collagen I, osteonectin and osteocalcin were also performed. The addition of RhBMP-7 to culture medium did not induce higher proliferative rate, while it allowed the maintenance of the osteoblast phenotype. Autologous PRP stimulated cell proliferation in a dose-dependent manner. These data underline that, in clinical practice, the efficiency of both rhBMP-7 and PRP is enhanced by the presence of autologous cancellous bone as it provides a sufficient number of target cells in the graft site.

IMMUNOMAGNETIC SEPARATION AND BIOLOGICAL CHARACTERIZATION OF A PUTATIVE MESENCHYMAL STEM CELLS SUBPOPULATION

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Mesenchymal stem cells (MSCs) have the capability for self-renewal and differentiation into cells with the phenotypes of bone, cartilage, neurons and fat cells.[1] These features have driven investigators for using MSCs for cell-based therapies to treat several diseases. The most common source of MSCs has been bone marrow, but alternative sources have been

explored.[2,3] Our previous data demonstrate the presence of putative MSCs isolated from ovarian follicular liquid.[4] To confirm these preliminary results we have performed new experiments based on a novel immunomagnetic procedure to isolate rare cells in suspension, using Dynal microbeads and a dedicated multiwells magnetic device.[5] Cells were isolated from human follicular liquid as a whole samples or nucleated cell fraction separated by density gradient. The experiments were done in parallel on human MSC cells as positive control. For the first experiments we focus on CD44, a specific surface marker on MSCs. Results obtained in few cases (10) allowed to have a purified $CD44^+$ cell subpopulation that can be checked directly by microscope (conventional and fluorescence) at the bottom of the wells. In the whole samples there were less labelled cells as compare to fractioned ones. The possibility to recover the cells onto *coverslips* (posed of the bottom of the wells) is an important advantage for the next steps of immunostaining and/or biological characterization of the recovered cells. Experiments will be soon designed to verify the stemness of these cells, seeding them in culture and inducing differentiation into other cell lineages to assess *in vitro* the plasticity of these putative MSCs.

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LIPOPOLYSACCHARIDE-INDUCED AIRWAY INFLAMMATION: BEHAVIOR OF ADHESION MOLECULES EXPRESSION IN MURINE HEMATOPOIETIC STEM AND PROGENITOR CELLS

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The adhesion molecules and mechanisms regulating migration of hematopoietic stem/progenitor cells (HSPC) from and toward the bone marrow during lung inflammation are not well understood. It has been shown that β1 and β2-integrins are involved in the cellular interactions between HSPC, stromal cells and extracellular matrix, in particular VLA-4 (CD49d/CD29), LFA-1 (CD11a/CD18) and L-selectin (CD62) also seem play a key role in this process. The aim of this study is to assess the degree of inflammation and the production of proinflammatory cytokine/chemokine in the injured tissue in order to correlate these factors to adhesion molecules expression in HSPC in a murine model. C57BI/6 mice (8 week-old male) were intratracheally administered with *Pseudomonas aeruginosa* LPS. Pathological changes in the lung were evaluated at 24, 48, and 72 h after LPS stimulus. ELISA multiarray was performed to analyze the production of inflammatory cytokines (IL-1β, IL-6, TNF-β) and chemokines (KC, SDF-1) in the bronchoalveolar lavage (BAL), infiltration of inflammatory cells was also examined in BAL and lung tissue. Bone marrow-derived HSPC were stained with monoclonal antibodies against CD49d, CD11a, and CD62L and analyzed by cytofluorimetry. Mice LPS-injected lungs showed a severe, persisting neutrophil infiltration and altered cytokine/chemokine production. HSPC isolated from LPS-treated mice showed at 48 h a significative increase of CD49d, CD11a and CD62L expression compared to control mice (p<0,05). Moreover, at 48 h LPS-treated mice has a maximal expression of CD49d respect to the other analyzed times (p<0,05) and a significative increase of CD62L respect to 72 h (p<0,05). In summary, these data indicate that in lung inflammation, the adhesion molecule expression in HSPC is involved in their recruitment into injured tissue, suggesting a possible mechanism in order to repair the tissue damage.

EXTENDED CHARACTERIZATION OF HUMAN UMBILICAL CORD MATRIX MESENCHYMAL STEM CELLS: EXPRESSION OF NOVEL MARKERS, IMMUNOREGULATORY MOLECULES, AND DIFFERENTIATING POTENCY

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Mesenchymal stem cells (MSC) are emerging as a promising tool in regenerative medicine applications. Nevertheless, their extended characterization is being increasingly viewed as a needed feature, in order to avoid contrasting results when translating *in vitro* experiments to *in vivo* approaches. We recently demonstrated in human MSC isolated from the umbilical cord matrix (HEMSC) the expression of novel markers indicative of their stemness, as well as differentiation and immune properties.[1] HEMSC were cultured and subject to immunocytochemistry and RT-PCR for the analysis of expression of markers of interest. Undifferentiated HEMSC resulted positive for the expression of immunoregulatory molecules, both surface antigens and secreted cytokines. In addition to the differential expression of B7 co-stimulators (CD80+/CD86-), we showed that HEMSC expressed pregnancy-specific immunomodulatory molecules, together with non-classical type Ib MHC antigens. In fact, besides HLA-A and HLA-G, HEMSC expressed both HLA-E and HLA-F, two tolerance-promoting HLAs, involved in attenuating NK- and T-cytotoxic responses. In the continued characterization of these cells, we investigated the expression of endoderm-related molecules, suggestive of their possible trans-differentiation towards hepatocytes. We previously demonstrated that HEMSC expressed HNF4α, together with the endoderm-related GATA factors (GATA-4,-5,-6). Further results indicated that undifferentiated cells expressed also albumin and further hepatocyte-enriched transcription factors (HNF1α HNF1β), thus suggesting the possibility to be differentiated towards hepatocytes. The immunological and differentiation properties of human MSC are under intense investigation. Our data suggest that HEMSC bear new markers and have a hypoimmunogenic phenotype which can be a favoring factor to ensure the engraftment of *in vivo* transplanted cells.

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MORPHO-FUNCTIONAL INTERRELATIONS BETWEEN HYPOPHYSIS, BURSA AND THYMUS ON EMBRYONIC THYMIC DEVELOPMENT: A COMPARATIVE EVALUATION

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In previous experiments we provided evidence of the reduction of the total size in 18-day-embryo's thymus and of a less differentiation of cortical and medullary epithelial cells induced by hypophysectomy, performed on chick embryos at 36-40 h of incubation,[1] partially recovered after a hypophyseal

graft.[2] Moreover, different expressions of T-cell markers (PCNA, CD3, CD4, CD8) were evidenced in the thymus of bursectomized embryos.[3] In the present study we performed again a hypophysectomy at 36-40 h of incubation followed by hypophyseal or thymic allograft at the 12th day of incubation onto the chorio-allantoic membrane from 18 day-old donor embryos to verify the influence of the hypophysis or the thymus on the expression of the T-cell specific markers (PCNA, CD3, CD4, CD8). Experimental and control thymuses were collected at the 18th day of incubation and tested by anti-markers immune reactions. The immune reactive thymocytes were evaluated by computer-assisted statistical analysis. As concerns PCNA immune reactive cortical thymocytes, we observed a significant reduction ($p<0,05$) in hypophysectomized embryos and no improvement after the hypophyseal allograft, but a very significant recovery ($p<0,001$) after the thymic allograft. As concerns the CD3 marker, there was a reduction of the number of the reactive thymocytes both in cortex and medulla, the hypophyseal allograft allowed an increase of the number of the immune reactive thymocytes. The thymic allograft let a very good recovery ($p<0,001$). The expression of CD4 and CD8 was similar to that described for CD3. These findings show that the lack of hypophysis decreases the cortical thymocytes proliferation and the CD markers expression, as found also in bursectomized embryos, and that the hypophyseal allograft may influence the recovery of the differentiation, but the thymic allograft allows the best recovery, probably due also by an emigration of thymocytes from the thymic graft.

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TESTIS ATROPHY AND REDUCED SPERM MOTILITY IN TRANSGENIC MICE OVEREXPRESSING C-FLIP

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Loss of germ cells through programmed cell death is a common feature during normal spermatogenesis and the death receptor FAS is one of the key molecules triggering germ cell apoptosis. c-FLIP is a close homologue of caspase-8 and modulates FAS signaling. c-FLIP role in the control of mouse germ cell apoptosis and caspase activity has been previously established.[1,2] In the present study, the effects of c-FLIP overexpression in testicular germ cells have been investigated. To this aim, morphological and functional analyses were carried out on the testes of transgenic mice overexpressing the c-FLIP long isoform (c-FLIPL) under the transcriptional control of a 400 bp long regulatory region of the Stra8 promoter. c-FLIPL was found to be ectopically overexpressed in round and elongated spermatids and to induce dramatic loss of germ cells resulting in testicular atrophy associated with reduced sperm motility. The data show that c-FLIPL forced expression in haploid male germ cells has detrimental effects on spermatogenesis and sperm quality and reveal a possible mechanism underlying the onset of testicular atrophy.

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LOCALIZATION AND INTERACTION OF MATER PROTEIN IN HUMAN OOCYTES AND CUMULUS CELLS

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Mater (Maternal Antigen That Embryos Require) is an oocyte-specific protein dependent on the maternal genome, required for early embryonic development. The gene products expressed in oocytes play important roles in folliculogenesis, fertilization and pre-implantation development. Mater protein contains an Amino-terminal pyrine domain (PYD) and Carboxy-terminal leucine-rich repeats (LRRs). Proteins carrying a PYD are mainly involved in inflammation, apoptosis and NF-κB signalling. Moreover, Mater contains the LRR domain, known to be involved in protein-protein interactions that regulate different cellular functions. The aim of this study is to characterise the expression pattern and subcellular partitioning of the human Mater protein in oocytes and follicular cells. Besides we intend to get more insights on its functional role using the ectopically expressed protein. We performed immunocytochemistry experiments coupled with confocal and electron microscopy analysis to define the ultrastructural localization of Mater in human ovarian tissue and in isolated oocytes and cumulus cells, obtained during *in vitro* fertilization procedures. Moreover human cumulus cells were cultured with or without cycloheximide to confirm endogenous biosynthesis of Mater. To investigate the physiological role of Mater we performed immunoprecipitation experiments of HEK293 cells overexpressing human Mater; a similar approach was then followed in cultured cumulus cells. Our data show that human Mater localizes at specific domains of oocytes and cumulus cells. In HEK293 cells overexpressing human Mater we observed an interaction of this protein with PKC-ε. Western blot experiments indicate that PKC-ε is expressed in human cumulus cells unlike in oocytes. Immunoprecipitation experiments and confocal analysis performed in cumulus cells suggest that the interaction, observed in HEK293 cells, occurs in physiological conditions.

ROLE OF eNOS IN ACQUISITION OF OOCYTE DEVELOPMENTAL COMPETENCE IN LOW ANTRAL FOLLICLE COUNT BOVINE OVARIES

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Numerous angiogenic factors, including endothelial nitric oxide synthase (eNOS), are present in ovarian follicles of several species. Their role is to enhance the recruitment of vascular supply, to keep follicles healthy and to improve oocyte quality. Recently, it has been demonstrated that in single ovulating species, like humans and cattle, the amount of antral follicles is positively associated to the number of morphologically healthy oocytes and follicles in ovaries. In cow, we previously established that ovaries with low antral follicle count (<10) generate oocytes with poor developmental competence. Aim of this work is to test the hypothesis that follicles depletion and reduced oocyte quality in low antral follicle count ovaries can be related to a reduced expression of eNOS in bovine ovary. RT-PCR analysis show that eNOS is present in all the compartments of bovine antral follicles. Immunohistochemistry reveals

that the protein is produced during all the stages of folliculogenesis. However, in healthy medium antral follicles, eNOS concentration, as quantified by image analysis, is significantly lower ($p<0.05$, Student's t-test) in ovaries with low antral follicle count. This different concentration suggests that eNOS may be involved in the faulty developmental competence of oocyte isolated from low antral follicle count ovaries. To test this hypothesis, S-nitroso acetyl penicillamine, a Nitric Oxide donor, was added during oocyte *in vitro* maturation. However this molecule does lack in significantly improving embryo developmental capability. Advanced studies are in progress to assess the biological role of eNOS as angiogenic factor during the acquisition of oocyte developmental competence in low antral follicle count ovaries. Low follicle vascularity may reduce exchange of nutrients, gonadotropins and growth factors and this can account for a decrease in oocyte quality. Our findings further support the use of cow as animal model to study human fertility.

ISOLATION, STORAGE AND GRAFTING OF OVARIAN TISSUE FOR FERTILITY PRESERVATION: A MORPHOLOGICAL STUDY IN HUMANS

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Cryostorage and grafting of frozen-thawed (F/T) isolated ovarian follicles, ovarian tissue fragments or whole ovaries are all possible therapeutic approaches now available to preserve fertility in young patients undergoing cytotoxic treatments for cancer. Our aim was to evaluate through a morphological approach the impact of enzymatic isolation, cryopreservation and grafting on ovarian tissue integrity and viability. Human ovarian tissue was analyzed after the following treatments: 1- Follicle isolation by collagenase or Liberase enzymatic digestion; 2 - Xenotransplantation and orthotopic autotransplantation of F/T ovarian cortical strips; 3 - Cryopreservation of intact ovaries with their vascular pedicles. Observations were carried out by transmission electron microscopy, vital fluorescent staining, immunohistochemistry and DNA strand breaks analysis by TUNEL. In treatment 1, a higher proportion of follicles were viable after Liberase isolation in respect to those isolated with collagenase, and most of Liberase-treated follicles were of good ultrastructural morphology. These results suggest that the treatment with Liberase - a purified, endotoxin-free enzyme blend - is a promising alternative to impure collagenase preparation for the isolation of intact ovarian follicles for culture and grafting purposes. In treatment 2, cryopreservation and transplantation do not appear to greatly affect the morphology of human primordial/primary follicles, which even grow in the grafts; however, follicular density was reduced after transplantation and follicular development appeared initially impaired. Thus, further studies are needed to extend follicular life span and to improve follicular growth in the graft. In treatment 3, cryopreservation was not associated with any particular sign of apoptosis or ultrastructural alteration in all ovarian compartments, suggesting that whole-organ transplantation may be a viable option in the future.

IMMUNOHISTOCHEMICAL AND NUCLEOTIDE SEQUENCING ANALYSIS OF SARCOGLYCANS IN EPITHELIUM

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The sarcoglycan complex is a component of dystrophin-associated glycoprotein complex (DGC), which links the cytoskeleton to extracellular matrix in skeletal muscle fibers. The sarcoglycans are a complex of six glycosylated transmenbrane proteins (α, β, δ, γ, ε, ζ). The exameric sarcoglycans structure, primarily expressed in skeletal muscle fibers, is present in cardiac and smooth muscle fibers too. Our previous studies, lead on human biopsies obtained from respiratory, gastrointestinal and urinary tract, demonstrated also the presence of α-sarcoglycan in smooth muscle tissue. This indicates that sarcoglycans are not tetrameric complex. Further our research have documented the presence of sarcoglycans in the epitethelium of respiratory, gastrointestinal and urothelial tract. In this research we wanted to verify if the sarcoglycans epithelium positivity may be in relation with the presence of different isoforms. Therefore, we have correlated immunofluorescence study to nucleotide sequencing analysis. Our results have confirmed that the sarcoglycans positivity in smooth muscle, respiratory epithelium such as myocardium are not due to different isoforms. Since it is known that the dystroglycans are present in respiratory epithelium such as other epithelia, we can assume that the DGC exist also in the epithelium besides striated and smooth muscle. Thus, the different function role of these districts, may be due to the different signalling proteins belonging to the system, rather than the nucleotide sequencing.

MICRORNAS' ROLE IN DUCHENNE MUSCULAR DYSTROPHY

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In mdx mice the absence of dystrophin leads to a deficiency in all of the components of the dystrophin-glycoprotein complex, suggesting that lack in DAPC may render skeletal muscle fibers more susceptible to muscle cell necrosis. The mechanisms involved in the disappearance of members of the DAPC are not completely understood. In muscle of defective mice, the presence of normal amounts of mRNAs for the different components of the DAPC, and the absence of the correspondent proteins suggest a post-transcriptional regulation. The mRNA and protein expression of three proteins of the DAPC (α1 and β1 synthrophin and dystroglycans) was analyzed by qPCR, western blot analysis and immunolocalization. Since the β1-syntrophin protein reduction in dystrophic muscle was not caused by mRNA decrease we decided to verify the hypothesis that the reduction of the DAPC could be associated with the microRNAs system. Some microRNAs (let7i, mir 22, mir 222 and mir 339) were found to be upregulated in skeletal muscle tissue of mdx compared to wt mice. It was demonstrated that, among these microRNAs which could putatively target the mRNAs of the DAP proteins (α1, β1 synthrophin and dystroglycaβ1 synthrophin by luciferase assay. To investigate the possible molecular mechanism regulating the 3'UTR of the mRNAs target *in vivo*, the mRNA β1-syntrophin-3'UTR was subcloned in a pEGFP-C1 vector to perform gene delivery *in vivo*, by electroporation in wt and mdx posterior limb mice

muscles. The GFP protein expression was blocked in the mdx muscle in the presence of β1-syntrophin 3'UTR suggesting the possibility of a *in vivo* regulation of pEGFP-3'UTRSnt β1 translation by microRNAs. Taken together these results show the importance of microRNAs system in the regulation of DAPC components, occurring in dystrophic muscle suggesting that miRs may have a potential role in the pathophysiology of primary muscle diseases.

RIBONUCLEAR INCLUSIONS AS BIOMARKER OF DM2 EVEN IN IMPROPERLY FROZEN OR DEFROST SKELETAL MUSCLE BIOPSIES

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Myotonic dystrophy type 2 (DM2) is a dominantly inherited disorder caused by a CCTG repeat expansion in intron 1 of *ZNF9* gene. The repeat's size and somatic instability of DM2 expansion complicate the molecular diagnosis of DM2. However *in situ* hybridization allows the direct visualization on skeletal muscle sections of mutant transcripts which accumulate in cell nuclei as ribonuclear inclusions (RI). RI represent an important biomarker of this pathology making *in situ* hybridization a rapid and sensitive method to obtain a definitive diagnosis in few hours. To date we have screened by fluorescence *in situ* hybridization (FISH) more than 50 patients suspected of having DM2 based on clinical criteria. FISH has been often combined with immunofluorescence staining of MBNL1 which it has been demonstrate to colocalize with RI in myonuclei and to represent a further pathological marker for DM pathologies. This approach makes the muscle biopsy an important tool for definitive diagnosis of DM2. Consequently, a rapid freezing at ultracold temperature and a good storage of specimens are essential to avoid morphologic alterations and nucleic acids degradation. However frequently we receive specimens from outside hospitals either submitted incorrectly or incorrectly frozen at the point of origin. Moreover, an accidental tissue thawed and refrozen may occur (power failure of the freezer) causing severe tissue damages and RNA degradation. We have revaluated old DM2 biopsies (n=11), previously diagnosed by FISH, that underwent to accidental tissue thawed and refrozen. The application of FISH on these muscle sections shows that RI are still well detectable in muscle sections no more useful for histopathological evaluation. Our results demonstrate that in presence of biopsy improperly frozen, defrost or incorrectly submitted from other hospitals, a second biopsy might not be necessary to obtain a definitive DM2 diagnosis or to screen patients with myotonic disorders not well defined.

AGE-RELATED EFFECTS ON APOPTOSIS OF HUMAN SATELLITE CELLS

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Human satellite cells (HSC) are small mononuclear stem cells. HSC are quiescent in undamaged skeletal muscle, but in response to muscle damage are activated to proliferate as skeletal myoblasts or myogenic precursor cells.[1] During the ageing process HSC display a reduced capability to undergo the differentiation process into myoblasts.[2] Apoptosis may be a mechanism responsible for the decreased differentiation ability of these cells in the elderly population. This study was aimed at evaluating the incidence of apoptotic features in ageing HSC. Cells were obtained from Vastus lateralis of 5 young (28.8±4.7 years old) and 5 old (74.0±4.6 years old) subjects and cultured in complete or serum-free medium to be collected at 4-24-48, and 72 h. Apoptosis was assessed with Annexin V/PI staining, terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labelling (TUNEL) technique and flow cytometry analysis of Caspase-8 activity. Annexin V/PI staining revealed a time-dependent and significantly higher percentage of apoptotic HSC from old subjects (35.3±7.0 vs 17.7±13.5, old vs young at 72 h). TUNEL technique demonstrated the presence of atypical features of apoptotic nuclei that appeared as a dot-like labelling finely dispersed in the cytoplasm of apoptotic cells and particularly evident after 48-72 h in culture. Caspase-8 assay displayed an early (4 h) and sustained (up to 48 h) activation of this enzyme in the elderly. The parallel analysis with microarrays showed an age-related altered expression of some genes involved in HSC antioxidant and repair activity (Polymerase K, SHC1 and FOXO1A).[3] These results suggest that ageing enhance HSC susceptibility to apoptosis that could be responsible for a scanty response to muscle damage.

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ROLE OF PKCθ SKELETAL MUSCLE REGENERATION

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Skeletal muscle is a dynamic tissue able to respond to several stimuli, such as atrophy and hypertrophy and to regenerate following muscle damage. Satellite cells (the muscle stem cells, SCs) are involved in muscle regeneration and growth. Following muscle damage, SCs are able to give rise to myoblasts, which then merge together to form new muscle fiber or fuse to pre-existing myofibers. An initial immunological response is required to remove damaged fibers and to contribute to a favourable microenvironment for SCs activation/differentiation; finally, extra-cellular matrix remodeling is required to restore muscle functionality. PKC θ; is the predominantly expressed Protein kinase C isoform in skeletal muscle. It has been shown that PKC θ; is involved in myofiber development, neuromuscular cross talk and metabolic homeostasis (lack of PKC θ; induces insulin resistance). In this work we investigate the role of PKC θ; in skeletal muscle regeneration, comparing WT C57BL6 mice with PKC θ; KO C57BL6 mice, following Tibialis anterior (TA) freeze injury at different periods of time after damage. Histological analysis of hematoxylin/eosin-stained TA transverse sections, showed an impaired reorganization of regenerating muscle at 4 and 7 day post injury, and a reduced cross-sectional area (CSA) of regenerating, eMyHC (embryonic myosin) positive, fibers in PKC θ; KO mice, as compared to WT. Accordingly, immunofluorescence and Western blot analyses showed a reduction in eMyHC expression in mice lacking of PKC θ. Semi-quantitative RT-PCR analysis showed a delay in the expression of SCs activated markers (MyoD, myogenin, Myf5) but not of the early marker, Pax7. These data demonstrate that lack of PKC θ impairs skeletal muscle regeneration. Whether PKC θ; activity is required for SCs activation/differentiation, for the inflammatory response or for extra-cellular matrix remodeling, is still to be investigated.

EFFECT OF AGEING ON MORPHO-FUNCTIONAL FEATURES OF SKELETAL MUSCLE CELL NUCLEI

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Ageing is associated with a progressive decline of muscle mass, strength and quality: this condition is known as sarcopenia, and entails a loss of muscle fibres and a decrease in total cross-sectional area of the remaining myofibres, especially in those muscles which mostly contain type II fibres. The contributing mechanism(s) leading to sarcopenia remain to be fully elucidated; they are probably multifactorial, including among others denervation and reinnervation of motor units, decline in anabolic hormone concentrations, decrements in microvascular function, loss of satellite cells, loss of myonuclei possibly through apoptotic mechanisms (in fact, the role of apoptosis in sarcopenic muscle fibre loss is still debated). Despite the extensive literature on sarcopenia, no data on the morpho-functional features of myonuclei from sarcopenic skeletal muscles have so far been reported. In this study, we used ultrastructural morphometry and immunocytochemistry to investigate the fine structure of myonuclei as well as the distribution and amount of transcription, splicing and cleavage factors, and of polyadenylated RNA in the biceps and quadriceps muscles of adult and old rats. In both muscles of old rats, we observed significant changes in several nuclear parameters, and quantitative alterations of most of the factors analysed. Taken together, these data suggest a decrease in pre-mRNA transcription and alterations of the mRNA processing and intranuclear transport during ageing.

IMMUNOCYTOCHEMICAL CHARACTERISATION OF MBNL1-CONTAINING FOCI IN MYOBLAST NUCLEI FROM PATIENTS AFFECTED BY MYOTONIC DYSTROPHY TYPE 2

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Myotonic dystrophy type 2 (DM2) is a dominantly inherited disorder with multisystemic clinical features, caused by a CCTG repeat expansion in intron 1 of the zinc finger protein 9 (ZNF9) gene. The mutant transcripts are retained in the cell nucleus forming multiple discrete ribonucleoprotein (RNP)-containing foci. Among the proteins able to interact with CCTG repeats, the splicing factor Muscleblind-like 1 (MBNL1) proved to be a good marker for nuclear RNP foci typical of DM2. It is likely that other pre-mRNA processing factors might be sequestered in the RNP foci, thus inducing a general alteration in the expression of mRNAs, which could lead to the multiple pathological dysfunctions observed in dystrophic patients. To test this hypothesis, we analysed *in situ* the molecular composition of RNP foci in cultured myoblasts from DM2 patients by using immunocytochemistry at both confocal and electron microscopy. A panel of antibodies directed against transcription, splicing and cleavage factors were tested to verify the possible co-localization with MBNL1-containing foci. Moreover, observations at the electron microscope revealed the fine intranuclear distribution of MBNL1 and the ultrastructural morphology of the RNP domains accumulating MBNL1.

ROLE OF INTERLEUKIN-6 (IL-6) IN DUCHENNE MUSCULAR DYSTROPHY

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Chronic muscle inflammation, a predominant feature of dystrophic muscles, promotes muscle atrophy and inhibits regeneration, thus contributing to the progressive loss of function. The aim of the project is to prove that the inflammatory cytokine IL-6 is a key mediator of muscle inflammation, damage and degeneration. IL-6 is overexpressed in skeletal muscles of dystrophin deficient mice (mdx) and of DMD patients, especially in infiltrating inflammatory cells. To evaluate the impact of IL-6 on the disease of mdx mice, we crossed mdx mice with IL-6 transgenic mice over-expressing IL-6 systemically (mdx/IL6) and in mdx mice we neutralized IL-6 activities with an IL-6 receptor neutralizing antibody. The skeletal muscle of mdx/IL6 mice showed larger area of damage, with a higher percentage of necrotic fibers in diaphragms, compared to mdx littermates, and diffuse presence of centrally located nuclei, suggesting defective maturation of regenerating fibers. This is probably due to more severe inflammatory response, as indicated by increased accumulation of $CD45^+$ cells and of the chemokine MCP1 that plays a pivotal role in recruiting mononuclear cells. Muscle functional performance was more compromised compared to mdx littermates. Mdx/IL6 mice showed shortened survival. In summary, overexpression of IL-6 exacerbates the dystrophic phenotype. In mdx mice, in a therapeutic approach with administration of an anti-IL6 Receptor antibody from day 15 of age, we observed decreased myofiber necrosis and mononuclear cell infiltration, further supporting the pathogenic role of IL-6 in muscular dystrophy.

NON STEROIDAL ANTI-INFLAMMATORY THERAPY IN DUCHENNE MUSCULAR DYSTROPHY

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The only pharmacological treatment currently available for Duchenne muscular dystrophy (DMD) is the use of glucocorticoids, but very few data are available to assess whether their anti-inflammatory activity is the explanation of their efficacy. To address this issue and to study the role of inflammation in DMD we have compared the effects of glucocorticoids and non steroidal anti-inflammatory drugs (NSAIDs) in mdx mice in order to evaluate if the inhibition of ciclooxygenases (COX) activity could have a beneficial effect on the pathology. Mice were daily treated with: methylprednisolone (glucocorticoid), aspirin, ibuprofen (non selective COX inhibitors) and parecoxib (COX-2 selective inhibitor). Inflammation, necrosis and centronucleated fibres were evaluated in tibialis anterior muscle samples obtained from treated and untreated mdx mice at the age of 15 days, 30 days and 11 weeks. Macrophage infiltration was evaluated by non specific esterase staining and it was sig-

nificantly reduced by the administration both methylprednisolone and NSAIDs. Anti-inflammatory therapy also induced a drastic reduction of the necrosis in the muscles, in fact the number of necrotic myofibres stained by Evans blue dye was decreased in all groups of treated mice. The morphology of the muscles was evaluated by hematoxylin-eosin staining and it wa clearly ameliorated. The percentage of regenerating myofibres was not modified by the therapeutical treatments. These data suggest that chronic treatment with NSAIDs has a beneficial effect on skeletal muscle morphology of mdx mice, suggesting that inflammation has a crucial role in the progression of the disease.

VINCULIN-TALIN-INTEGRIN SYSTEM IN SMOOTH MUSCLE AFFECTED BY URETEROPELVIC JUNCTION OBSTRUCTION: AN IMMUNOHISTOCHEMICAL ANALYSIS

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In this report we investigated the vinculin-talin-integrin system arrangement in smooth muscle fibers of paediatric patients affected by ureteropelvic junction obstruction (UPJO). This disease is the most common cause of congenital hydronephrosis. Although it was suggested a key role in dearrangement of smooth fibers as primary anomaly in UPJO, the proteic organization has not been investigated. Then, we obtained tissue specimens from ten pyeloplasty divided into 3 sections: renal pelvis above the obstruction, UPJO, and ureter below the obstruction. In these biopsies we carried out single immunofluorescence reactions using antibodies anti-β1D-, anti-β1A-, anti-α7B-, anti-α7A-integrin, anti-vinculin and anti-talin. Section were observed by a CLSM Zeiss META. Our results showed in smooth muscle of renal pelvis above the obstruction and in UPJO, a significant loss of α7B-integrin, β1D-integrin, talin and vinculin, while a significative enhancement of fetal isoform α7A- and of β1A-integrin, in comparison to control muscle, has been shown. In smooth muscle of ureter below the obstruction, we observed a normal staining pattern for integrins, talin, and vinculin with a loss of α7A- and β1A-integrin. In our opinion, the loss of talin, a flexible cytoskeletal protein important in the assembly of cell-matrix interaction, and the lacking of vinculin, protein of control of apoptosis, could be able to modulate the expression of muscle-specific integrins, α7B-, and β1D-, and the consequent replacement with α7A-, and β1A-. This condition suggests that primary anomaly in UPJO might be attributed to a critical alteration of smooth muscle fiber cytoskeleton. The alteration could provoke, as in denervation of skeletal muscle, a reorganization of the protein arrangement, and the replacement of muscle-specific integrins with the fetal isoforms could be a decisive alternative for maintaining of muscular motility. In this way, the viability of smooth muscle fiber is maintained.

IN VIVO CELL REPOPULATION AND DIFFERENTIATION IN DECELLULARIZED AND ALLOGENICALLY TRANSPLANTED AORTIC VALVES

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The purpose was the attainment of acellular valve substitutes amenable to acquire postimplantation autogenic-like characters with concurrent non-thrombogenicity, anti-calcification and suitable mechanical properties, and growth capacity. Valved aortic conduits were excised from 3-month-old minipigs. Decellularization was performed with defined procedure including detergents Triton X-100 and Colate and endonuclease Benzonase®. Surgical replacements were performed at pulmonary position in three 12-month-old minipigs. Postoperative conditions were clinically monitored. Samples were explanted after 6 and 12 months and processed for (i) immuohistochemical detection of markers vWF, CD31, vimentin, α-SMA, and (ii) transmission electron microscopy. Besides favorable postoperative functional outcomes, proper compensatory implant growth occurred (30-35% increase in diameters). Both aortic wall and valve leaflet surfaces were completely coated by an adhering monolayer of vWF+/CD31+ endothelium-like cells with the presence of a weak basal lamina and early/mature intercellular tight junctions. At the valve aortic aspect, many of these cells were clearly involved in prominent ECM formation resulting in the appearance of a subendothelial layer with a distinct organized texture of fibrillin microfibrils and elaunin fibers. In addition, a lot of vimentin+ and/or α-SMA+ interstitial cells exhibiting fibroblast-like, myofibroblast-like or smooth myocyte-like features populated the leaflet stroma, most of them showing ultrastructural features consistent with prominent collagen fibrillogenesis (i.e.: fibril-forming-channels), elastogenesis and fibrillin microfibrillogenesis. In conclusion, this decellularization procedure allowed to produce promising bioprosthetic valved conduits, being glutaraldehyde-free and permitting *in vivo* spontaneous cell repopulation by cells expressing phenotypes mimicking those in native conditions and tissue growth/remodelling.

i-NOS ACTIVATED MITOCHONDRIAL APOPTOTIC PATHWAY IN HYPOXIC AND AGED RAT HEARTS

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The effects of hypoxia and ageing on mammalian cells are quite similar since both determine a stress response, which implies a modified production of oxidants at mitochondrial level. These oxidants, such as ROS (Reactive Oxygen Species) or RNS (Reactive Nitrogen Species), can interfere with cell signalling proteins leading to mitochondrial damage, apoptosis occurrence and functional consequences. Thus here we report the effects of hypoxia on the *in vivo* morphological and biochemical response of rat young and aged myocardial tissue. Besides the morphological modifications, which evidence mitochondrial suffering upon hypoxia exposure in both young and aged heart, the role played by PKCα in controlling NOS (Nitric Oxide Synthase) proteins level has been investigated. Downstream PKCα activation events show a dramatic increase of iNOS expression in the hypoxic young concomitant to high percentage of apoptotic cells and apaf-1/cyt c co-immunoprecipitation suggesting an iNOS-mediated activation of the mitochondrial apoptotic pathway. Moreover, overexpression of iNOS is parallel to VEGF (Vascular Endothelial Growth Factor) induced angiogenesis in the hypoxic young, suggesting that VEGF increased level may allow coordinated development of the lymphatic and blood vasculature, necessary for fluid

homeostasis and to counteract oxidative stress. Thus the inhibition of such growth factor proposes new therapeutic possibilities for diseases associated to vascular function and for solid tumours, which show pathological angiogenesis and lymphangiogenesis.

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CATIONIC LIPOSOMES AS A TOOL TO LABEL ACTIVATED ENDOTHELIAL CELLS *IN VIVO* DURING ACUTE CENTRAL NERVOUS SYSTEM INFLAMMATION

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Endothelial cells are highly active cells able to modify their characteristics in a variety of physiologic and pathologic conditions. These modification, which are at the basis of changes in the capacity of endothelial cells to interact with circulating cells and of differences in vessels permeability, are particularly relevant during angiogenesis and inflammation. Positively charged liposomes are known to target activated (angiogenic) endothelial cells in tumor growth and chronic inflammation. The mechanism of binding is independent form the presence of specific ligands, and therefore, this approach promises to be applicable for tumor vascular targeting in various indications and in pathological situations of chronic inflammation in general. Aim of the current study was to investigate the possibility to identify *in vivo* sites of acute neuroinflammation using rhodamine labeled electrostatically charged colloidal carriers (cationic, CL, and anionic, AL, liposomes) in Experimental Allergic Encephalomyelitis (EAE) rats. No CL or AL staining was found in the spinal cord of healthy rats at each time. By contrast, in the spinal cord of EAE rats, already on day 7 slight rhodamine signal could be detected, indicating that CL accumulation occurred. The extent of the rhodamine signal was definitely higher on day 14 pi, involving the white and grey matter and assuming on some occasion the shape of round spots. The signal was clearly evident still on day 21, although infiltrate was virtually no longer present in the spinal cord at this time point. Endovascular localization of CL was demonstrated by laminin staining of endoneural vessels. No AL staining was observed in the spinal cord of EAE rats at each time point. We conclude that activated endothelial cells *in vivo* identification can be effectively achieved through the use of labelled CL in acute neuroinflammation models.

IDENTIFICATION OF VAV1 TYROSINE RESIDUES INVOLVED IN ATRA-INDUCED DIFFERENTIATION OF APL-DERIVED CELLS

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Vav1 belongs to a family of multidomain signal transduction proteins involved in the regulation of diverse cellular responses such as proliferation, differentiation, survival and migration. In blasts derived from acute promyelocytic leukemia (APL) Vav1 plays a crucial role in the overcoming of the differentiation blockade. In particular, this protein promotes differentiation, potentiates ATRA-induced maturation and is involved in regulating expression of genes and proteins up-regulated by ATRA. It has also been demonstrated that in differentiating APL-derived cells Vav1 is tyrosine phosphorylated by Syk and his event is crucial for the maturation-related changes of cell morphology. Starting from these notions, we tried to identify tyrosine residue(s) of Vav1 phosphorylated during ATRA treatment and involved in the overcoming of the differentiation blockade of APL-derived cells. Vav1 was then immunoprecipitated from differentiating NB4 cells and subjected to mass spectra analysis, allowing to identify the Y745 residue phosphorylated after ATRA treatment. The mutation of the residue Y745 of Vav1 impaired the completion of maturation program of NB4 cells, in terms of CD11b expression and migration capability. Since a functional role for Y745 has not been described so far, the tyrosine phosphorylation of this residue could be at the basis of a role of Vav1 alternative to its best known activity as guanosine exchange factor. Since, in HL-60 cells, Vav1 interacts with Syk by means of its SH2 domain, where 4 tyrosines are present, we tried to assess if these residues are possible substrates of Syk during ATRA-induced granulocytic differentiation. With this aim, HL-60 cells were transfected with plasmids containing mutated forms of Vav1 and induced to differentiate with ATRA. The role of each tyrosine residue on the differentiation process was studied and the results indicate that both Y672 and Y711 are possible Syk substrates since their mutation, as well as Syk inhibition, have no effect on CD11b expression, but affect migration capability of ATRA-treated cells. Even though further analysis will be necessary to clearly establish its functional role, these data contribute to define the involvement of Vav1 in the maturation process of APL-derived cells.

EXPRESSION OF SUPER OXYDE DISMUTASE (SOD) AND APOPTOSIS IN HEART FAILURE

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The heart needs an aerobic metabolism to produce adequate energy, but oxygen participates in the production of NO, which is fundamental for vascularisation and myocardium contractility but also forms the reactive species of oxygen (ROS), involved in cell signalling and inducing irreversible damage and cell death. A balance between ROS and endogenous anti-oxidant substances normally exists. SOD family represents diffuse antioxidant enzymes with three forms, cytoplasmic SOD1, mitochondrial SOD2 and extracellular SOD3. An altered ROS/antioxydants rate causes oxidative stress and cardiovascular damage, increasing the production of ROS and activating Bcl-2, caspases and MAPKs pathways which result in apoptosis and altered cardiac function. We studied 132 human heart specimens from the Department of Pathology of the University of Basel. 122 were from ischemic patients (26 sub-acute, 37 acute, 59 hyper-acute) and 10 controls from subjects deceased independently from cardiovascular diseases. Age, sex and Body Mass Index (BMI) were considered for statistical analysis. SOD expression was demonstrated by immunohistochemistry on tissue microarray (TM) as well as by molecular biology with RT-PCR. Our hypothesis was the presence of a relation between SOD, heart failure and apoptosis. Our data suggest

that age and BMI are really a risk while sex does not seem a predisposing factor, as regards the population we studied. SOD1 and SOD3 expression are significantly greater in normal subjects but increase during ischemia and in the early stages after it. Apoptosis is significant, in the same way, during the early myocardial damage but decreases with the time, while necrotic tissue is removed and fibrosis begins to predominate. The activation of caspase3 positively relates with augmented SOD expression, probably due to the increased oxidative stress that plays a role in activating apoptotic processes.

HUMAN EPICARDIAL CELLS: *IN VIVO* AND *IN VITRO* MORPHOLOGICAL STUDY OF THEIR FATE IN THE NORMAL AND PATHOLOGICAL HEART

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The presence of stem cells able to give rise to the cells of cardiac lineages was observed in the adult human heart, raising questions concerning their origin and biology. The scope of the present study was to investigate whether an epithelial-mesenchymal transition, which contributes to heart development during organogenesis, takes place also in the adult human heart, generating the population of cardiac stem cells. To this aim, we examined by immunohistochemistry and immunofluorescence epicardium and subepicardium of human adult normal (n=11) and pathological hearts with chronic ischemic disease (n=22). Strikingly, only the normal hearts were layered with epicardial cells. On the contrary, cell nuclei were absent from the surface of the diseased hearts. While normal epicardium resulted positive for cytokeratin 5/6, E-cadherin and Bves, in the pathological hearts cells with epithelial markers were distributed in subepicardium. Considering the hypothesis that subepicardial cells originate from mesothelium, fragments of epicardium of adult human atria were cultured in the presence of extracellular matrix produced by cardiac fibroblasts. Only on this substrate it was possible to obtain the outgrowth of cells forming epithelial sheets, as confirmed by the positive immunolabeling of E-cadherin and β-catenin at the intercellular junctions and cytokeratin in the cytoplasm. When stimulated with TGFβ; and HGF, intercellular contacts were lost and cells acquired mesenchymal characteristics, with spindle-like shape and vimentin expression. The results indicate that epithelial-mesenchymal transition of epicardial cells takes place in the adult human heart, leading to the exhaustion of this cell population. Given the lack of mesothelial cells on the surface of pathological heart, it could be reasonably argued that epicardium-derived cells enrich the pool of cardiac primitive cells that contribute to the regenerative properties of the heart.

ULTRASTRUCTURAL IMMUNOLOCALIZATION OF TRANSFERRIN RECEPTOR IN CIRCULATING ERYTHROID CELLS: ITS ROLE AS BIOMARKER FOR X RAY DAMAGE IN ELASMOBRANCHS

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Transferrin receptor (CD71), a membrane glycoprotein involved in iron absorption, that plays a key role in metabolism and cell homeostasis, is a biomarker of X-ray damage in man and rat.[1] Following previous observations by FACS and immunocytochemical detection at light microscopy, that suggested an increased CD71 expression in the circulating erythroid cells (RBCs) of Torpedoes after X-ray exposure, the aim of this study was the ultrastructural localization of this marker before and after X-ray treatment of benthonic Elasmobranchs (*Torpedo marmorata* and *Torpedo ocellata*) in order to evaluate whether CD71 can be used as a biomarker of sea X-ray pollution. Ultrathin sections of RBCs, withdrawn from 3 specimens before and after irradiation (45, 80 and 90 Gy), were first incubated with polyclonal primary antibody anti-CD71, then with secondary antibody conjugated with colloidal gold and lastly contrasted and observed by transmission electron microscopy. Before irradiation, only few RBCs showed low CD71-immunoreactivity, mainly in plasma membrane but also cytoplasm. After irradiation, morphological apoptotic-like changes in the nucleus and cytoplasm vacuolization were observed and a more intense CD71-ir was found mainly into cytoplasmic vesicles, demonstrating an increased receptor endocytosis and occasionally in mitochondria, injured by irradiation. These preliminary data, showing an increased CD71 expression and internalization in circulating RBCs of Torpedoes after X-ray exposure may suggest if confirmed by further experiments, the role of CD71 as biomarker of X-ray sea pollution.

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CO-LOCALIZATION OF RUNX2 AND DKK1 IN RELATION TO MEDIAL ARTERY CALCIFICATION OF UREMIC PATIENTS

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The arterial calcification of uremic patients has been compared to the ossification process that occurs in bone. To verify whether vascular smooth muscle cells (VSMCs) in calcified arteries undergo differentiation to osteoblast-like cells, the osteoblast differentiation factor Runx 2, and the Wnt antagonist DKK1, have been studied in specimens of the epigastric artery of 49 uremic patients undergoing kidney transplantation. By von Kossa staining, 38 of them showed medial calcification of various extents, whereas none showed ossified areas. Runx2 immunostaining was found in the cytoplasm and nucleus of most of the VSMCs in the uncalcified specimens; in the calcified specimens, the VSMCs near the calcified areas were lightly stained or unstained. Dkk1 immunostaining overlapped that of Runx2. In the cases with severe mineralization the calcified matrix, after decalcification, consisted of amorphous material that was not stained by Picrosirius red for collagen, PAS for glycoproteins, and Alcian blue for acid proteoglycans. Around these areas, a few DKK1 and Runx2 immunostained cells and numerous degenerated cells positive to the TUNEL method for apoptosis were found. At TEM, many of the degenerating cells were characterized by large and empty vacuoles in the cytoplasm. Around these cells, some collagen fibrils were associated with vesicular bodies similar to matrix vesicles and larger electron-dense masses of cell debris. These findings suggest that the mineral deposition in the medial artery of uremic patients does not mimic the ossification process and can be due to degenerative processes.

IMMUNOLOGICAL CHARACTERIZATION OF CHRONIC CAROTID GLOMITIS IN HEROIN ADDICTION

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The aim of the present work was to investigate the occurrence and immunological characteristics of chronic carotid glomitis in opiate addicts. Carotid bodies were sampled at autopsy from 50 subjects who died of heroin intoxication (mean age 28 years), and from 16 young (24 years) and 10 older subjects (66 years) who died of trauma. Sections were stained with haematoxylin-eosin and azan-Mallory, and immunohistochemistry was carried out with anti-CD45, -CD3, -CD8, -CD4, -CD20, -CD68, -CD56. Inflammatory aggregates were not observed in young cases, but were found in 21/50 (42%) opiate cases and in 4/10 (40%) older cases. Infiltrates were mainly located in subcapsular and interlobular positions, and were also found around nerve fibres. Inflammatory aggregates were mainly composed of T suppressor/cytotoxic lymphocytes (50-80%). Monocytic/macrophagic cells and B lymphocytes comprised about 10% and 5-20% of inflammatory cells, respectively. T helper lymphocytes were fewer and only rare Natural Killer cells were found. Chronic carotid glomitis must be included among the autopsy findings of opiate addiction, and may be ascribed to inflammatory reactions to exogenous immunogens or to responses to drug-induced degenerative changes of carotid body components.

MORPHOFUNCTIONAL ANALYSIS OF THE SPLEEN FROM PATIENTS WITH OR WITHOUT PMF

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In our previous study on emperipolesis we analyzed spleen parenchyma damage of myelofibrotic mice that presented diffuse extramedullary hemopoiesis, increased number of Mk, neutrophil emperipolesis in Mk and, in particular, increased number of fibroblasts. The aim of our study is to underline similarities between spleen damage in the mouse and in human. We studied 9 patients, 1 control and 8 affected by myelofibrosis. Both in mice and in humans we can observe increased number of Mk correlated with increased number of preapoptotic Mk. Then we made a classification based on Mks frequency: modest increase, average increase, and high increase. Contemporarily, we noted increased number of fibroblasts both in patiences and in mice. This increase is independent from the bild-up of Mks in the spleen. The IHC for fibronectin showed us that the Mks were positive. Instead the IHC for Gata-1 shows that Mks were positive (76%) because that is not present in the marrow. Then, the IHC for TGFα shows both a negative population (91%) and a positive ones (90%) in the patients. The histopathological picture of the spleen from PMF patients is more complex than predicted by Tefferi. The Mk abnormalities in the marrow of Gata-1low mice and spleen of PMF patients are similar (increased numbers, low expression of Gata-1). Patients' variegation has been instead observed for the positivity of MK to TGFβ Additional new abnormalities, found in MK from the marrow of Gata-1low mice, are represented by positivity to fibronectin and interaction with the fibroblasts. If these abnormalities are also present in the MK from the marrow of PMF patients is not known.

ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL STUDY OF ADVANCED ATHEROSCLEROTIC LESIONS IN HUMAN CAROTID WALL

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Atherosclerosis is a degenerative-inflammatory process that develops in the artery wall,[1] involving a progressive accumulation of lipids. Mechanisms underlying atherosclerosis development and progression are still unclear whereas, studies on humans that evaluate and correlate circulating and tissue markers of inflammation, vascular calcification and plaque instability are still lacking. Therefore, our work will focus on the ultrastructural characterization of plaque phenotype in patients with early chronic kidney disease, suffering from cardiovascular disease. Carotid endoartherectomy samples were collected from 10 diabetic and 5 nondiabetic patients presenting carotid stenosis, with or without history of cardiovascular events. Samples were fixed in glutaraldehyde and prepared for transmission and scanning electron microscopic-correlated analyses. A histopathologic control was performed on semithin sections. Immunohistochemistry for galectin-3 was also performed. The endothelial layer was generally absent, being usually replaced by a fibrous thin cap, in which macrophages and inflammatory cells were often found. Smooth muscle cells (SMC) switched into fibroblast-like actively-secreting cells or apoptotic cells, were observed in the degenerated tunica media. Micro-calcium deposits were seen among SMC cellular debris. Atherosclerotic lesions were defined as stable if the surface was smooth and plane, and unstable when the surface was rough, with fractures or ulceration and/or thrombosis. Galectin-3 was expressed predominantly in unstable lesions and staining was positive mostly in macrophages but also in SMC. In conclusion, inflammation is strictly associated with microcalcification in unstable atherosclerotic lesions.

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IMMUNOHISTOCHEMICAL EXPRESSION AND APOPTOTIC ROLE OF CASPASE 9 IN AORTIC ANEURYSMS

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Aortic aneurysms (AA) is a degenerative vascular disease characterized by localized dilatation of the aortic wall as a result of altered matrix composition (elastin and collagen degradation). However, the pathogenesis of the changes is elusive and unclear. Some experimental evidences suggest that iNOS (who synthesize a large amount of NO in inflammatory processes) and the metalloproteinases (MMP) are implicated in the pathogenesis of AA but the relationship between NO and MMP to aneurismal disease is currently unknown. Probably during clinical evolution of that diseases the degeneration of endothelial cells is caused by apoptotic processes. In our previous study we have hypothesized a possible role of MMP2 in apoptotic events in AA. The aim of this study is to investigate the immunohistochemical expression of caspase 9, well known apoptotic marker, in human aneurysmal tissues to confirm the

apoptotic events in the endothelial degeneration. Fragments of 10 human AA, 10 dilatative TAA and 10 dissecting TAA were obtained during surgical procedure. In addition, during surgical aorto-coronary bypass were obtained 10 punches of normal aorta in the side of joint of bypass, to use like normal control. All the specimens were fixed in Bouin's mixture and embedded in paraffin; obtained sections were processed with anti caspase 9 (Sigma) by EnVision+System HRP (AEC) (Dako Cytomation). All the samples have been studied with microscope Leica DM1000 and Nikon OPTIPHOT 2. Our results underline that endothelial cells of AA in comparison with control tissues show a significantly higher immunopositivity of caspase 9 in some nuclei. These studies provides a morpho-histochemical basis to further support the role of caspase 9 in the apoptotic events in AA.

VASOMEGALY: ROLE OF CGRP-LIKE NERVOUS FIBRES

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Vasomegaly is a disease characterized by the length, dilatation and tortuousness of the blood vessels, both arteries (*mega-arteries*) and veins (*mega-veins*). This disease is caused by an alteration of elastic fibres, which appear elongated and dilated with lateral thickenings resembling a *swelling of the elastic fibres*. Ultra-structural examination by transmission electron microscope of tissue blocks taken from mega-arteries showed changes of elastic material in the tunica media and tunica adventitia. In the tunica media three forms of elastic material were found. Slightly osmiophil amorphous elastic material was found near on the basement membrane of the myocytes. In the superficial parts of myocytes there occur a great number of pinocytotic vesicles pointing to a rich creation of the new elastic material. The second form of elastic material represents middle or highly osmiophil thick elastic fibers with irregular side protrusion and elastic membranes containing highly osmiophil thick elastic micro-fibrils. Finally, highly osmiophil elastic material was found among myocytes remembering the moth-eaten picture. In the tunica adventitia, highly osmiophil elastic fibers located among myocytes of this layer were observed. On the contrary in the tunica intima only slight structural changes were found. The internal elastic membrane was slightly osmiophil and only in some place it was disrupted or more osmiophil with an invasion of collagen micro-fibrils. No changes were found in the endothelial cells. In this study, the Calcitonin gene related peptide (CGRP) like immune-reactivity was tested in tissue sections of megadolichoarteries, treated with the reaction of Streptavidin Biotin Peroxidase. A strong immunoreactivity was demonstrated in experimental sections. These results suggest that the vascular wall in the case of vasomegaly has a characteristic structure and it shows a peculiar pattern of immune-reactivity when tested with anti-CGRP antibodies.

MICROVESSEL REMODELLING AND ENDOTHELIAL PHENOTYPE REGULATION IN THE EMBRYONIC BRAIN

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Because of its unique composition, the human cerebral cortex follows a development course substantially different from other species in terms of vascularization and differentiation. Immunolocalization and detailed confocal microscopy analysis of blood-brain barrier (BBB)- and angiogenesis-specific markers during normal human corticogenesis reveal that a constant, vivacious 'phenotype remodelling' takes place in the cell components of the neurovascular unit (NVU) to supply adequate nourishment and protection to the developing neuroblasts. In fact, tight junction-associated transmembrane proteins, such as occludin and claudin-5, are already expressed by endothelial cells at 12 weeks of gestation and at midgestation show a typical junctional, linear pattern together with the presence of BBB-specific transporters, such as GLUT-1 and P-glycoprotein.[1,2] At this time, the process of cortex vascularization is characterized by radial vessel branching and intense vascular sprouting. The angiogenically activated endothelial cells of the forming microvessels are guided by an intimate interplay with immature pericytes expressing the transmembrane proteoglycan NG2 and exhibit a heterogeneous phenotype characterized by the expression of growth factor receptors and basement-membrane degrading enzymes together with features of BBB.[3]

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HYDROGEN SULFIDE INDUCES FUNCTIONAL INHIBITION AND CELL DEATH OF CYTOTOXIC LYMPHOCYTES SUBSETS

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Hydrogen sulfide is now considered as a gasotransmitter with specific functional roles in different cell types, like neurons and vascular smooth muscle.[1] We have recently demonstrated that H_2S promoted the survival of cultured granulocytes delaying onset of apoptosis by the inhibition of caspase 3 cleavage and p38 MAPK phosphorylation and reduces clonal growth, cell proliferation and cell adhesion of human keratinocytes impairing mitogen-activated protein kinase signaling and interfering with cell surface expression of $\beta4$, $\alpha2$ and $\alpha6$ integrins.[2,3] The toxic effects of exogenous hydrogen sulfide on peripheral blood lymphocytes have been investigated in detail. We have purified and activated peripheral blood human lymphocyte subsets: T $CD4^+$, T $CD8^+$ and NK cells. Primary cell cultures were treated with NaHS salt as H_2S donor. Here we show that exogenous H_2S promotes a caspase-independent cell death of lymphocytes that depends on their intracellular glutathion levels, with a subset specificity for $CD8^+$ T cells and NK cells. Although cell activation does not affect their sensitivity to NaHS, after 24 h exposure to hydrogen sulfide surviving lymphocytes show a dramatically decreased proliferation in response to mitogens and a reduced IL-2 production. Our data demonstrate that H_2S impairs the cellular cytotoxic response of lymphocytes as well as their secretion of IL-2, therefore de-activating the major players of local inflammatory responses, adding new basic knowledge to the clinically well known anti-inflammatory effects of sulphur compounds.

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IDENTIFICATION OF A NCRS+/NKG2D+/LFA-1LOW/CD94-/CD159A- IMMATURE HUMAN NK CELL SUBSET

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CD56bright natural killer (NK) cells generated *in vitro* from CD34+ cells were characterized after 30-day culture with flt3 ligand plus IL-15. Virtually all CD56bright cells expressed: CD117, CD25, natural cytotoxicity receptors (NCRs), NKG2D, CD161, and CD244, while only a subset expressed: LFA-1, CD11b, CD11c and CD94-CD159a heterodimer. The majority of cells co-expressed CD18 antigen with CD11a, CD11b, CD11c as well as CD94-CD159a heterodimer, however a significative proportion of NK cells did not express these antigens, defining an immature CD56bright/NCRs+/NKG2D+/CD18-/CD11a,b,c-/CD94-/CD159a- subset. A minor subset of cells expressing CD94-CD159a but not CD18/CD11a (LFA-1) integrin was also identified, suggesting that during NK cell differentiation LFA-1 might be up-regulated later than CD94-CD159a. To verify this hypothesis *in vivo*, we look for discrete stages of NK cells based on LFA-1 density of expression in peripheral and umbilical cord blood samples. Interestingly, in these blood fluids we have identified a lineage negative CD34-/LFA-1low/NKp46dim/NKG2Ddim/CD94-/CD159a- subset that resembled an immature stage of NK cells present in lymph nodes. Altogether the results indicate that CD18/CD11a integrin, as well as CD11b in mice, may be a useful marker to identified immature stages of NK cell differentiation.

IDENTIFICATION OF C-KIT+/CD105+ AND ISL-1+ CELLS IN HUMAN FETAL AND INFANT HEARTS

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During embryogenesis mammalian heart develops from a primitive heart tube which derives from two bilateral primary heart fields located in the lateral plate mesoderm. Later on in the development process, the atrioventricular (A-V) canal and the sinu-atrial segment, at the venous pole, and the conotruncus, at the arterial pole, are added to the heart tube just prior to tube looping. Some authors[1,2] have demonstrated the presence of a secondary or anterior heart field in the ventral pharyngeal mesoderm. This region contains a pool of NKX2.5 and GATA-4 positive precardiac cells which migrate to the arterial pole of the primary heart tube. Isl-1 is a marker of the secondary heart field, and Isl-1+ fate-mapped cells can contribute to the right ventricle and outflow tract of the developing mouse heart. Most studies concerning the localization of cardiac precursor cells in the developing heart have been performed in mice or chicks, whereby the localization and identification of cardiac precursor cells in the human fetal and adult heart has been investigated only by Limana *et al.*[3] with their analysis limited to epicardium, and CD34+ or c-Kit+ cells. Isolation and characterization of adult human cardiac stem cells was characterized by the expression of c-Kit, the receptor for stem cell factor, and CD105, the regulatory component of the TGF-β receptor complex important in angiogenesis and hematopoiesis. In view of this, in the present study we analysed by immunohistochemistry the presence of c-Kit+/CD105+ and Isl-1+ cells in human normal hearts from infants and from fetuses at different gestational ages. We found that cells double positive for c-kit and CD105, and single positive for Isl-1 were present solely after the 18th week of gestation. Isl-1+ cells were localized also inside vessels, like non resident cells, in infant hearts.

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EFFECTS OF BISPHOSPHONATES IN HUMAN ORAL MUCOSA AND IN HUMAN MANDIBULAR BONE: IMMUNOHISTOCHEMICAL AND MORPHOLOGICAL ANALYSIS

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Bisphosphonates (BP) are most effective inhibitors of bone resorption and are chemioterapeutic molecules widely used as the first treatment for osteoporosis. As well known, the main effect of BPs is an avascular necrosis, or osteonecrosis, exclusively localized in the mandibular and maxillary bones (ONJ). In this study, we tested both the oral mucosa and the bone tissue of patients affected by ONJ. In particular, by immunohistochemistry, on oral mucosa, we tested α2-integrin, α4-integrin, important collagen receptors on epithelial cells, vinculin, talin, and, besides, collagen type III, and VEGF, in order to evidence their role during treatment with BPs; we tested these protein in control samples, in subjects treated with bisphosphonates without ONJ and in samples of patients treated with BPs and with ONJ. Moreover, by SEM, we analyzed the morphological modifications of BPs on bone. By immunohistochemistry technique, our results revealed, in oral mucosa without ONJ an increase of VEGF and integrins in epithelial cells and in vessels; the staining for collagen type III showed a weak decrease. In oral mucosa with ONJ we showed a decreased staining for VEGF and integrins in epithelium and a normal staining in vessels. Collagen type III shows a staining comparable with control mucosa. By SEM we showed a massive presence of bacterial population, lymphocytes and large zone of resorption. In our opinion the increase of tested proteins, in concomitance with treatment with BPs but without ONJ, could indicate an attempt of compensative behaviour in the remodelling of the oral mucosa in order to restore the epithelial architecture, and then restore the signalling of the cells. Moreover, our SEM results demonstrated that the mandibular bone, during treatment with BPs, could show a feed-back mechanism that provokes an increase of osteoclasts in order to compensate inhibition of osteoclasts by BPs.

EXPRESSION OF β–ENDORPHIN AND CASPASE-9 IN ORTHODONTICALLY TREATED DENTAL PULP

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For several years, our histology laboratory has worked in cooperation with the Winchmore Hill Dental Practice studying the activity of acetylcholinesterase and some neuropeptides (CGRP, substance P, enkephalins, endorphins)[1] in orthodontic treated human dental pulp to clarify force's action. It seems

that recent data do not yet, fully elucidate the exact effects of the force applied on teeth. For this reason we are investigating whether the traction is involved in some cases of pulpal death. In this study we focused on the expression of caspase-9 and β-endorphin. The expression of the two peptides has been identified through immunohistochemistry assay: not treated dental pulp and treated dental pulp (six months orthodontic traction, the force was applied using 0.12, 0.14, 0.16 NiTi archwire). Control pulps: weak positivity to β-endorphin of intraparenchymal and vascular nervous fibres. Treated pulps: the pulpal morphology is altered with oedema, inflammatory infiltrate and disorganized odontoblastic epithelium. Intense β-endorphin reactivity of intraparenchymal and perivascular nervous fibres; strong positivity of odontoblasts. Control pulps: caspase-9 positivity in some odontoblasts and pulpal parenchymal cells. Treated pulps: We identified a strong nuclear caspase-9 staining in many odontoblasts and some parenchymal cells. Vessels endothelium appears to be positive too. We observed weak changes in the expression of both β-endorphin and caspase-9 in orthodontically treated pulps compared to control pulps. The present result confirm our purpose to continue study the action of orthodontic traction for longer time to understand better the role of those proteins.

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USE OF RIBOFLAVIN-UVA TO INCREASE DENTIN COLLAGEN STIFFNESS AND TO IMPROVE DENTIN-RESIN BOND

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A new technique of collagen cross-linking by the photosensitizing riboflavin and UVA has been developed with significant increase in collagen stiffness. Since dentin bonding is related dentin collagen stiffness, the use of riboflavin on human dentin was challenged to increase collagen cross-link and to prevent bond degradation due to metalloproteases (MMPs). MMPs are calcium/zinc-dependent endoproteinases and operate a specific proteolytic activity on most constituents of the extracellular matrix. Non-carious human molar were selected and middle/deep dentin substrates were exposed and then assigned to Group 1: XP Bond was applied with a combined riboflavin-UVA; and Group 2: XP Bond was applied in accordance with manufactures' instructions. In Group 1, the dentin surface was treated with a water solution of riboflavin 0,1% and subjected to UVA-rays for 2 min at 1 cm from dentin surface. Composite build-ups were applied on the adhesive surface. Composite/dentin beams were obtained in accordance with the microtensile non-trimming technique and then pulled to failure after 24 h or 6 months artificial aging. Interfacial nanoleakage evaluation was performed on additional adhesive interfaces quantifying the amount of silver tracer along the interface. The role of riboflavin-UVA before XP application increased immediate bond strength of approx 16% compared to controls. After 6 months of artificial aging, bond strength of the groups decreased but the differences, related to 6-month period, are approx 38% for the riboflavin beams. Microtensile bond strength and interfacial nanoleakage expression has shown that this treatment increases bond strength of the adhesive interfaces created by XP Bond on human dentin. These data support the hypothesis that the use of riboflavin-UVA allows the collagen cross-linking also in dentin collagen.

NICOTINE MODULATES GELATINASE B (MMP-9) AND EPILYSIN (MMP-28) EXPRESSION IN RECONSTITUTED HUMAN ORAL EPITHELIUM

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Oral epithelial keratinocytes express nicotinic cholinergic receptors[1] which activation modulates keratinocyte differentiation[2] and migration[3] that depends on the modification of cell-cell and cell-extracellular matrix interaction and matrix metalloproteinases (MMPs) production. Gelatinase B (MMP-9)[4] and Epilysin (MMP-28)[5] are two MMPs expressed by human keratinocyte during both wound healing and proliferation. Their expression has been investigated in reconstituted human oral epithelium (HOE) exposed to nicotine (Nic). HOE reconstituted onto polycarbonate membrane (SkinEthic) was stimulated for 72 h by Nic both in the absence and presence of the nicotinic antagonist Mecamylamine (Mec), a PKC inhibitor (H7) and a MAPK inhibitor (PD98059). At the end of treatment MMP-28 expression has been analyzed in epithelium sections using an anti-MMP-28 antibody, while MMP-9 presence and activity in cell conditioned medium has been analyzed by gelatine zymography. Nic reduces in a dose-dependent fashion the expression of MMP-9 in the HOE. This effect was antagonized by Mec, H7 and PD. Nevertheless, Nic increased the expression of MMP-28, while Mec did not antagonize this effect but enforced it. MMP-28 overexpression induced by Nic was blocked both by H7 and PD. Nic affects the expression of MMP-9 in HOE through receptor activation and PKC-MAPK pathway. MMP-28 overexpression induced by Nic was not previously reported and it can be linked to a rearrangement of cytoskeleton as suggested by the lack of Mec antagonism.[6] These findings indicated MMPs as a target for Nic toxicity in oral keratinocytes.

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EFFECTS OF LOW PULSE ENERGY ND: YAG LASER IRRADIATION ON CELLS OF THE ORAL MICROENVIRONMENT: AN *IN VITRO* STUDY

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Low pulse energy laser irradiation represents a promising therapeutic approach for the treatment of periodontitis and peri-implantitis, based on the ability of this laser to eradicate bacteria from periodontal pockets, titanium implants and peri-implant tissues. Despite its known anti-inflammatory properties, there are several concerns regarding the use of this therapy in the dental practice, mostly due to the conflicting data concerning the treatment laser parameters and the lack of comprehension about its mechanisms of action. In the present study, we investigated the effects of low pulsed Neo-

dymium:Yttrium Aluminum Garnet (Nd:YAG) laser irradiation, on the viability, proliferation and differentiation of different cells representative of the oral microenvironment.. Saos-2 osteoblastic cells, H-end endothelial cells and NIH/3T3 fibroblasts were irradiated with appropriate laser parameters and then analyzed by confocal immunofluorescence and Real Time PCR. Analysis of intracellular calcium mobilization was also performed in osteoblasts loaded with Fluo3-AM. By MTS assay, it was found that Nd:YAG laser irradiation did not affect cell viability in all the tested cell types. With regards to cell proliferation, the laser treatment caused a significant increase of the cell number in osteoblastic Saos-2 cells, with a lower effect on endothelial and fibroblastic cells. Moreover, the laser irradiation increased the expression of several differentiation markers in the examined cell lines, including osteopontin, TRPC1, ALP and Runx2 in osteoblasts, collagen-type1 in fibroblasts and vinculin in endothelial cells. Notably, in the osteoblastic cells, the Nd:YAG irradiation induced a rapid intracellular calcium mobilization, suggesting an involvement of this ion in the regulation of the elicited cellular responses. In conclusion, low energy Nd:YAG laser therapy may represent a feasible and safe technique in the treatment of periodontal diseases and its stimulating effects may be crucial for the improvement of the healing process.

SURVIVIN AND P53 EXPRESSION IN PATIENTS WITH CROHN'S DISEASE: AN IMMUNOHISTOCHEMICAL STUDY

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Crohn's disease (CD) is characterized by acute phases of patchy transmural inflammation, involving any part of the intestinal tract with intervening periods of remission. It has been well established that patients with Crohn's disease are at increased risk of developing intestinal adenocarcinoma. The regulation of apoptotic cell death may have an important effect on the pathogenesis and progression of colon cancer. Disturbance of apoptosis is known to be very crucial in neoplastic progression and transformation. Mutations in the proapoptotic and tumor suppressor gene p53 are associated with neoplasia in ulcerative colitis, but little is understood of their significance in Crohn's disease. Members of the inhibitor of the apoptosis protein (IAP) family including survivin are expressed in many tumors. It is known that well differentiated adenocarcinoma tend to express higher level of survivin than normal mucosa. Expression of survivin was observed in the cytoplasm of adenoma with dysplasia and colonrectal carcinoma cells. In order to explore the role of p53 and survivin as marker of neoplasia in CD patient, the aim of this study was to evaluate, by immunohistochemical analysis, the expression and distribution of p53 and survivin immunoreactive cells in a series of Crohn's disease bioptic tissues with apparently normal adjacent tissues. The immunohistochemical study showed an increase in p53 and survivin expression. Our results suggest that p53 and survivin overexpression in CD patients may predispose the intestinal mucosa to dysplasia that may progress to a higher grade of neoplasia overtime.

THE O_2-SCAVENGING FLAVODIIRON PROTEIN IS DETECTABLE IN GIARDIA INTESTINALIS

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Giardia intestinalis is the amitochondriate O_2-susceptible protozoan pathogen causing the human giardiasis. The microorganism grows preferentially under microaerophylic conditions being highly sensitive to oxygen and reactive oxygen species. This notwithstanding, Giardia intestinalis colonizes the human small intestine, where up to 50 micromolar oxygen is present. Survival, and thus pathogenicity of Giardia, has been proposed by others[1] to rely on the FAD-containing NADH oxidase scavenging oxygen efficiently. Genomic analyses proved that Giardia codes for a Flavodiiron protein (FDP)[2,3] a prokaryotic enzyme that harbors a FMN and a non-heme diiron site reducing oxygen to water, or NO to N_2O. We have expressed the FDP from Giardia in *E. coli*; the protein was purified and structurally-functionally characterized.[(4)] The enzyme proved to be very reactive towards oxygen (Vmax >40s-1), produces water and is poorly reactive towards NO. Here we show that FDP can be detected in cultured Giardia by indirect immunofluorescence, using specific antibodies. Moreover, FDP is degraded by hydrogen peroxide and the reaction probably involves the proteasome. We speculate that the primary function of Giardia FDP is to allow the parasite to cope with oxygen and to survive in the duodenal mucosa.

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ROLE OF SMAD 3 LOSS IN RESISTANCE TO THE DEVELOPMENT OF EXPERIMENTAL COLORECTAL FIBROSIS INDUCED BY TRINITROBENZENE SULPHONIC ACID

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Transforming growth factor-β (TGF,)/Smad3 signalling plays a central role in tissue fibrogenesis, acting as potent stimulus of extracellular matrix (ECM) protein accumulation.[1,2] Scope of this study was to evaluate the potiantial role of Smad3 in the pathogenesis of colonic fibrosis induced by trinitrobenzene sulfonic acid (TNBS) in Smad3 null mice. Colon of TNBS mice was evaluated for macroscopic examination and histological, morphometric and immunohistochemical analysis (α-SMA, collagen, TGFβ CTGF, Smad3 and Smad7). At a macroscopic examination of the colon of Smad3 wild type mice appeared significantly harder, thicker and shorted than that of the Smad3 null mice. Of the wild type mice, 50% presented colonic adhesions and strictures. Histological and morphomet-

rical evaluation revealed a significantly higher degree of colonic fibrosis and accumulation of collagen in the Smad3 wild type compared to null mice, whereas the degree of colonic inflammation did not differ between the two groups of mice. Immunohistochemical evaluation showed a marked increased in CTGF, collagen, TGFβ, and Smad3 staining in the colon of Smad3 wild type compared to null mice, whereas Smad7 was increased only in null mice. In conclusion, targeted disruption of Smad3 confers resistance to the development of the TNBS-induced colonic fibrosis. The reduced fibrotic response appears to be due to a reduction both in fibrogenic mesenchimal cell activation and ECM production and accumulation. Smad3 could be a novel target for potential treatment of intestinal fibrosis expecially in inflammatory bowel diseases.

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DIFFERENT DIETS MODULATE THE GLYCOSYLATION PATTERN IN THE RAT COLONIC MUCOSA

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In studying the effect of dietary habits on the assessment and maintenance of health status, growing research interest is addressed to evaluate the influence of diet on the intestinal mucosa. In particular, it has been shown that dietary fiber and resistant starch have a great impact on the colonic tissue in terms of mucosal architecture, epithelial cell proliferation, and chemical characteristics of its secretory products, mainly consisting in glycoconjugates. Carbohydrates are known to regulate adhesion of bacteria to the intestinal cell surface. In addition, alterations of mucin expression and glycosylation have been observed in human colon cancer.[1] Thus, we evaluated the effects of selected dietary carbohydrates, a starch-based diet and a sucrose-based diet, on the glycosylation pattern of the rat colonic epithelium by using lectin binding procedures, added with chemical and enzymatic pretreatments for *in situ* characterization of sialic acids. The results indicate that the consumption of a high-resistant starch diet affects inversely the expression of GalNAc and sialic acid-D-GalNAc residues, thus suggesting a possible relation to modified activity of exogenous enzymes due to the altered composition of the intestinal microflora.[2] Similarly, the modulation of fucosylated glycocomponents seems to be consistent with the observed reduction of Bacteroides in the intestinal environment in both the experimental conditions.[3] Differential sialylation patterns as well as changes in acetylation degree and sites of sialic acid residues support a direct effect of diet on the cellular glycosylation pathway.

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IMMUNOHISTOCHEMICAL STUDY ON THE RESPONSE OF THE SURROUNDING LIVER TO COLORECTAL CARCINOMA LIVER METASTASIS

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The liver frequently hosts metastases in patients with colorectal carcinoma (CRC). Resident and tumor-infiltrating host inflammatory cells, such as Kupffer cells (KCs) and T cells (TCs) can regulate tumor growth and dissemination.[1,2] Hepatic stellate cells (HSCs) produce tumor-associated extracellular matrix and have been involved in the migration and growth of metastatic cells.[3] We investigated the activated KCs, HSCs and infiltrating TCs in the surrounding liver of CRC metastasis and their correlation with peritumoral fibrosis and angiogenesis, and the metastasis proliferation index. Using immunohistochemistry and a semi-quantitative scoring system, the expression of KCs markers (CD68 and COX-2), microvessels density (MVD, CD34), TCs marker (CD3), activated HSCs marker (α-SMA) and proliferation index of metastases (Ki67) were analysed in metastases and surrounding liver from patients with CRC. Fibrosis was assessed with Masson's trichrome staining and expressed with a semi-quantitative score. Statistics were performed by using non parametric tests. The number of KCs was higher in surrounding liver compared to liver at distance ($p<0.05$). COX-2 positive activated KCs positively correlated with peritumoral fibrosis score ($p<0.05$). COX-2 expression in the surrounding liver positively correlated with the infiltrating TCs ($p<0.001$). TCs positively correlated with peritumoral MVD ($p<0.05$) and peritumoral fibrosis score ($p<0.001$). The number of activated HSCs positively correlated with proliferation index of metastases ($p<0.05$). Liver proinflammatory response to CRC metastasis seemed to be associated with peritumoral fibrosis and angiogenesis. HSCs appeared related with proliferation index of CRC metastases. Different roles could be argued for the non parenchimal liver cells in response to CRC liver metastasis.

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TAUROCHOLATE PREVENTS CAFFEIC ACID-INDUCED BILE DUCT DAMAGE IN BILE DUCT-LIGATED RATS BY CHANGES IN CHOLANGIOCYTE VEGF EXPRESSION

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Vascular endothelial growth factor (VEGF) mediates the adaptive proliferative response of cholangiocytes to cholestasis.[1] Caffeic acid phenethyl ester (CAPE) induces growth inhibition in different cells.[2] Taurocholic acid (TC) protects cholangiocytes against injury induced by parasympathetic or sympathetic denervation.[3] Scope of the study was to determine if: (i) CAPE induces bile duct damage; and (ii) TC prevents CAPE-induced bile duct damage by increasing cholangiocyte VEGF expression. Normal and BDL rats were fed 1% TC or control diet in the absence/presence of daily IP injections of CAPE. One week later, we evaluated: (i) cholangiocyte apoptosis, proliferation and ductal mass, VEGF-A/C and VEGFR-2/R-3 expression in liver sections; (ii) functional activity by measuring secretin-stimulated bile and bicarbonate secretion. *In vitro*, BDL cholangiocytes were exposed to CAPE in the

absence/presence of TC with and without pretreatment with VEGF receptor inhibitors before evaluating cholangiocyte apoptosis and proliferation. Chronic CAPE administration to BDL rats increased cholangiocyte apoptosis and decreased ductal mass. This effect was associated with reduced expression of VEGF-A, VEGF-C and VEGFR-2, VEGFR-3. *In vivo* and *in vivo* TC feeding partly prevented CAPE-induced changes in cholangiocyte apoptosis and growth. The protective effect of TC was associated with enhanced VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3. In conclusion, our findings may provide novel perspectives in the regulation of cholangiocyte loss in chronic cholestatic liver diseases via manipulation of cholangiocyte VEGF expression.

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ROLE OF FOLLICLE STIMULATING HORMONE IN THE REGULATION OF CHOLANGIOCYTE PROLIFERATION OF NORMAL AND BILE DUCT LIGATED RATS

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Cholangiocyte proliferation is regulated by a number of factors including angiogenic growth factors and sex hormones such as estrogens and progesterone.[1,2] No information exists regarding the role of Follicle Stimulating Hormone (FSH) and its receptors in the regulation of cholangiocyte functions. We studied whether FSH regulates cholangiocyte proliferation. *In vivo*, normal female and male rats were treated with FSH, with Antide, (a gonadotropin releasing hormone antagonist) or a neutralizing FSH antibody for 1 week. We evaluated: (i) FSH receptor (FSHR) and FSH expression by immunohistochemistry, (ii) cholangiocyte proliferation by intrahepatic bile duct mass and PCNA expression; (iii) cholangiocytes apoptosis by TUNEL analysis, and (iv) changes in secretin-stimulated cAMP levels, and ERK1/2 and Elk-1 phosphorylation by immunoblots in cholangiocytes isolated. *In vitro*, cholangiocyte cultures (NRICC) were stimulated or not with FSH before evaluating: (i) FSHR and FSH expression by immunofluorescence, and (ii) changes in proliferation by immunoblots. Results: we found that cholangiocytes and NRICC express FSHR, FSH and secrete FSH. *In vivo* administration of FSH to normal rats increased, whereas administration of Antide and anti-FSH antibody to BDL rats decreased: (i) ductal mass and PCNA expression and (ii) secretin-stimulated cAMP levels and ERK1/2 and Elk-1 phosphorylation in cholangiocytes compared to controls. Our findings have important pathological implications since modulation of cholangiocyte expression and secretion of FSH may be important in the management of chronic cholestatic liver diseases regulating the balance between cholangiocyte growth/loss.

Acknowledgments: study supported by University funds from University of L'Aquila and Sapienza University of Rome to P0, AF, LP and EG.

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EXPRESSION OF P2X1 AND P2X3 PURINERGIC RECEPTORS IN BOVINE CARTILAGE

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Growing evidence suggests that extracellular nucleotides might play important roles in the regulation of cartilage metabolism. The aim of this study was to investigate the presence of P2X1 and P2X3 purinergic receptors in bovine chondrocytes, by using Western blotting, saturation binding assays and immunohistochemistry. The role of these receptors in modulating chondrocyte actvities was investigated by analyzing nitric oxide (NO) and prostaglandin E2 (PGE2) release. Chondrocytes were isolated from cartilage fragments and cultured *in vitro*. The maintenance of the chondrocyte phenotype was investigated by verifying the expression of aggrecan and type II collagen and the absence of type I collagen by immunocytochemistry. P2X1 and P2X3 purinergic receptors expression was analysed by western blotting, saturation and competition binding experiments, and immunocytochemistry. NO release was determined according to the Greiss method. PGE2 release was measured by a competitive enzyme immunoassay. Western blotting analysis showed that P2X1 and P2X3 purinergic receptors were expressed in chondrocytes cultured *in vitro*, although P2X1 receptor expression resulted lower than P2X3 receptor. Immunohistochemistry on cartilage slices confirmed the expression of P2X3 receptors in all zones of the intact cartilage. Typical purinergic agonists such as adenosine 5'-triphosphate (ATP) and α,β-methyleneATP were able to increase NO and PGE2 release. A P2X1 and P2X3 purinergic antagonist (A317491) blocked the stimulatory effect mediated by the agonists. These data demonstrate for the first time the presence of functional P2X1 and P2X3 purinergic receptors in bovine cartilage. Agonists and antagonists are able to modulate functional responses such as NO and PGE2 release. These results suggest the potential role of novel purinergic antagonists in the treatment of articular cartilage diseases.

OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION OF MESENCHYMAL STROMAL CELLS ON POLY(VINYL ALCOHOL)/GELATIN SPONGY SCAFFOLDS

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A major issue in scaffolding is the fabrication of substrates with a highly porous architecture in order to offer high surface area/volume ratio necessary for cell growth and extracellular matrix (ECM) deposition. In this study highly porous spongy scaffolds based on poly(vinyl alcohol) and gelatin (PVA/G) were used as substrates for rat mesenchymal stromal cells (rMSCs) differentiation into both osteogenic and chondrogenic lineages. PVA/G scaffolds were prepared by emulsion and freeze-drying technique. Their morphological characterization was carried out with scanning electron microscopy (SEM) and

microCT. Scaffolds were finally cut into discs, sterilized and cultured with rMSCs. RMSCs were isolated from femora and tibiae of Wistar rats, expanded *in vitro* and their multipotency was assessed. Cell characterization was performed. RMSCs were seeded on PVA/G scaffolds and cultured for 10-21 days. Cell differentiations were performed using either osteogenic or chondrogenic media. Cell viability was monitored with Alamar Blue. At the endpoints constructs were analyzed with SEM, cytochemistry and immunocytochemistry. Moreover, specific biomolecules of MSC differentiation were quantified. Poral features of PVA/G sponges resulted suitable for scaffolding purposes. RMSCs as obtained from isolation procedure could be committed into multilineage phenotypes. Undifferentiated rMSCs resulted positive for ALP, but negative for OPN, OCN and calcium. Both osteo- and chondro-induced rMSCs on PVA/G scaffolds were viable and metabolically active till 21 days. Osteoblastic maturation occurred at day 10 as confirmed by both SEM and von Kossa staining. Chondrogenic maturation was also found at day 10, when sulphated GAGs were detected, as highlighted by Alcian Blue staining pH 1. Moreover high immunopositivity for Sox9 and aggrecan was revealed. PVA/G scaffolds resulted then suitable to support rMSC differentiation.

IMMUNOHISTOCHEMICAL EVIDENCE OF LACTOFERRIN IN HUMAN ADULT NORMAL AND NEOPLASTIC CARTILAGE

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Lactoferrin (Lf), an 80 kDa basic iron-binding glycoprotein, has been extensively investigated by immunohistochemistry in many human normal and neoplastic tissues. After a first report in which we documented the Lf expression in foetal osteoblasts as well as in bone forming tumours, we have investigated herein the Lf immunopattern in 30 human cartilage-forming tumours, surgically obtained from an equal number of patients (18 males, 12 females; age range: 9-72 years; mean age: 34.56 years). The histopathological diagnosis was enchondroma (15), osteochondroma (6), chondroblastoma (CBL, 3), chondrosarcoma (3) and chondromyxoid fibroma (CMF, 3); human normal bone and cartilagineous tissues, obtained at autopsy from 5 adults (age range 50-75 years; mean age: 64 years) were also analyzed. Quantification of Lf immunoreactivity was performed by using an intensity distribution (ID) score, elsewhere previously applied. An immunohistochemical evidence of Lf, with a variable ID score, was encountered in all cases of CBL and CMF, with a constant immunonegativity in all other neoplastic cartilagineous samples. Therefore, on the basis of the Lf immunopositive phenotype, we suggest a different histogenesis for the above mentioned tumours. We conclude that the presence of Lf in neoplastic cells of chondroblastomas and chondromyxoid fibromas may be related to the production of this iron-binding protein by the neoplastic cells themselves.

IMMUNOHISTOCHEMICAL APPEARANCE OF LACTOFERRIN IN THE BONE GROWTH AND DEVELOPMENT OF EMBRYONIC AND FOETAL SKELETON

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The embryonic skeleton is first modeled in the cartilage and is covered by a condensation of mesenchymal cells; moreover, the endochondral ossification occurs as a process through which the cartilage is transformed into bone, with a deposition of bony matrix and vascular invasion. In the foetus, the process of endochondral ossification continues and only the ends of the bone are still formed of cartilage. By immunohistochemistry, we have investigated the appearance of lactoferrin (Lf), an 80 kDa basic iron-binding glycoprotein, in embryonic and foetal skeleton; we utilized 25 human bone and cartilagineous tissues from long and flat bones, taken at autopsy from an equal number of subjects (13 males, 12 females), aged from 8 to 34 weeks of gestation. Moreover, on same tissue blocks, haematoxylin-eosin, Perls' Prussian Blue and von Kossa stainings were also performed. Lf cytoplasmic immunoreactivity, but sometimes nuclear, was encountered in mesenchymal cells of periosteum as well as in chondroblasts present in foetuses from eighth to twentieth weeks. An intense cytoplasmic Lf immunostaining was also evident in plumped osteoblastic elements localized in the cores of dark blue calcified cartilage, that represents the newly formed bone, as revealed by von Kossa method. This peculiar pattern of Lf immunoexpression appears and increases from the tenth to twenty-sixth weeks, with a decreased Lf intensity towards the end of pregnancy. We suggest that Lf acts as an anabolic factor in embryonic and foetal skeletal growth, stimulating mesenchymal periosteal cells, cartilage elements and osteoblasts, similarly to that elsewhere documented in *in vitro* previous studies.

THE OVERLAPPING BETWEEN TRAIL AND OPG COULD CONTRIBUTE TO AN ENHANCEMENT OF THE EROSIVE PROCESSES INDUCED BY HUMAN SYNOVIAL CELLS

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Rheumatoid arthritis (RA) is a chronic inflammatory disease for which the etiology is unknown. It is now clear that osteoclast formation and activation at the cartilage-pannus junction is an essential step in the destruction of bone matrix in RA patients.[1,2] A number of inflammatory cytokines found in the RA synovial tissue [interleukin (IL)-1, 1β and 6, tumour necrosis factor (TNF-α) and macrophage colony-stimulating factor] have the potential to promote osteoclast formation and bone resorption. Cells within RA synovial fibroblasts also are substantial sources of sRANKL and OPG that could establish the contribution of these cytokines to the process of erosion. Bone erosion depends mainly on the synergic action of these cytokines, where receptor activator of NF-κB ligand (RANKL), produced by osteoblasts, fibroblasts and T cells and receptor activator of nuclear factor B (RANK) which is mainly expressed on pre-osteoclasts, possibly of the macrophage lineage. Another member of the TNF family, that could be a key factor in this process, is TRAIL (TNF-related apoptosis-inducing ligand), which shares homology with RANK and RANKL, which are also members of the TNF family of proteins. Therefore, to further elucidate the important relationship between RANKL, TRAIL and OPG in human RA synovial fibroblasts we have analyzed RANKL and OPG expression after administration of 100 ng/mL of human recombinant TRAIL. Based on our study we suggest that TNF-related apoptosis inducing ligand (TRAIL) is not suitable for therapeutic implications in the treatment of rheumatoid arthritis.

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EXPRESSION OF GROWTH FACTORS AND PROTEINS MODULATING BONE FORMATION IN HUMAN OSTEOBLASTS SEEDED ON ANORGANIC BOVINE BONE BIOMATERIAL

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Bone tissue engineering has emerged as a promising strategy in the effort to regenerate and repair diseased or damaged bone. The basic aspects in bone tissue engineering include chemical composition and geometry of the scaffold design since is very important to improve not only cells attachement and growth but especially osteodifferentiation, bone tissue formation, and vascularization. The Bio-Oss® is a xenograft biomaterial consisting of deproteinized, sterilized bovine bone, chemically and physically identical to the mineral phase of human bone. In this work, the ability to proliferate and adhere to substrate and the expression of bone specific proteins and growth factors as: type I collagen, OPN, BSP, BMP-2, BMP-7 in human osteoblasts (NHOst) seeded on xenogenic Bio-Oss® are evaluated. The structure of newly synthesized bone tissues was observed with optical microscope and scanning electron microscope and the biological properties of the engineered bone were detected by alizarin red staining and energy dispersive X-ray microanalysis. The biochemical study displays an increase of type I collagen, BSP, OPN, BMP-2 and BMP-7 in NHOst seeded with Bio-Oss® liken to samples without biomaterials. As verified in mesenchymal cells from periodontal ligament,[1] the Bio-Oss® do not interfere with the growth, the migration and the differentiation of the cells but on the contrary acts as inductor for a significant osteogenic differentiation. In conclusion, the *in vitro* study show that the Bioss xenogenic biomaterials, designs a harbour great potential for hard tissue engineering purposes, and future studies will be necessary to delineate the time course of other different specific growth factors and proteins in the process of bone formation.

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CHARACTERIZATION AND LOCALIZATION OF WOLFRAMIN, A TRANSMEMBRANE GLYCOPROTEIN, IN HUMAN TISSUES AND ORGANS

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The WFS1 gene, encoding wolframin, a transmembrane glycoprotein of endoplasmic reticulum (ER) consisting of 890 amino acids, is mutated in the Wolfram syndrome, also called DIDMOAD, an autosomal recessive disorder defined by the association of diabetes mellitus, optic atrophy, and further organ abnormalities. It has been demonstrated that disruption of the WFS1 gene in mice causes progressive beta-cell loss in the pancreas and impaired stimulus-secretion coupling in insulin secretion. However the physiological function of this protein remains totally unknown. To gain further insight into the pathogenesis of diseases associated with WFS1 mutations, we conducted a study to investigate the developmental patterns of localization of wolframin in a panel of different human tissues by immunohistochemistry using both light and fluorescence microscopy. First, we have characterized wolframin antibody in order to demonstrate its specificity. Then, we have observed that wolframin was ubiquitously expressed in many organs although with different tissue distribution and expression levels. During human foetal development, wolframin expression was faint at the 14-16th week and progressively increased when development proceeded in almost all systems. In human adult tissues, a variable positive staining was observed in both simple and stratified epithelia, with a slight wolframin expression in the basal layer of the skin compared to a more intense immunopositivity for this protein in other stratified epithelia, such as oesophagus and cervix. Moreover, we observed that in human placenta there was a modulation of wolframin throughout pregnancy with a strong level of expression during the first trimester and a moderate level in the third trimester of gestation. In conclusion, on the basis of wolframin distribution in several human systems, we may hypothesize that this protein may have important roles in cell proliferation, differentiation and in the maintenance of cellular homeostasis.

AN ALTERNATIVE FIXATION

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Fixation is the first step and the foundation in a sequence of events that culminates in the final examination of a tissue section. After fixation, the four classical tissue processing stages in the paraffin method are alcohol dehydration, xylene clearing, infiltration, and embedding. The purpose of this was to determine the effect of the new fixative Greenfix vs Formalin or Holland of biopsy specimens, and to determine the method that was most consistently associated with good nuclear and cytoplasmic details, safe contrast and absence of background. Three samples of the same biopsy were fixed with Formalin, Greenfix, or Holland. Sections stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) were scored for histopathologic criteria by a pathologist. Moreover molecular methods (FISH, PCR) were applied. The quality levels of the cellular features was determined by good resolution of the nuclear chromatin; reliable histochemical and immunohistochemical results, with more contrast and less diffusion of chromatic positivity, comparable with Holland; excellent contrast ephitelium-stroma and good results in molecular biology. Formalin-fixed paraffin-embedded archival clinical specimens were invaluable in discovery of prognostic and therapeutic targets for diseases such as cancer. However, formalin is a very toxic fixative. We suggest that the implementation of new, multipurpose fixatives may further improve the quality and suitability of histochemistry, immunohistochemistry and molecular analysis from fixed tissue specimens, and reduce the toxicity for operators. The alternative fixative Greenfix has shown minimal impact of nucleic acids equivalent Holland maintaining tissue morphology for diagnosis. Preparations were of high quality, with the cellular structure comparable to formalin fixation.

THE DOUBLE LABELLING TECHNIQUE FOR COMBINED HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY ANALYSIS

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Today the histochemistry methods are not very interesting for young pathologists and technicians like the molecular methods; these have new prognostic and therapeutic applications.

The aim of this study is to develop a double staining technique for simultaneous demonstration of chemical features and cells antigenic markers in histological sections. We remember that the double labelling is more informative of the biological features of the specimens and offers a more pathological diagnosis. First, the antibodies specific for CK, CD31, AML, were used for immunohistochemical analysis of skin pathology; LCA, and CD20, CD31, MIB for colorectal adenocarcinomas and LCA, CD31 for hepatic steatosis. In a second step, the Masson staining was applied to skin biopsies, and Alcian-blue or PAS to gastrointestinal tissue. The final visualization of the staining products of both reactions had been performed. Using this protocol, we show that there is not background, and the reaction products can be easily distinguished. In particular, the Masson staining, the classical method for detection of melanin, shows a higher efficiency and selectivity in detecting the granule's pigments also in the double staining method. Histochemistry deserves attention and consideration. It is the expression of basic diagnostic methods correlated to experimentation and to educational planning. The results of classical diagnostic methods for general functions of tissue (secretion, the pigments, for example), are too actual. We think that the double staining methods promise a good chance for methodological training of young technicians. This method can assure in a right way the evaluation of diagnostic cases with uniformity of views and judgement.

SUBCELLULAR LOCALIZATION OF STATHERIN IN HUMAN MALE UROGENITAL TRACT

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The human male urethra, in particular the urethral meatus, is continually subject to aggression by pathogens present in the external environment, but infections are surprisingly infrequent. This is due to the features of the urogenital epithelia, which release antimicrobial peptides such as α- and β-defensins and other substances, that prevent adhesion and survival of bacteria, virus, and fungi. In this research, we wanted to investigate if male accessory sex glands are source of statherin, a phosphoprotein involved in the control of microbial adhesion in several mucosal surfaces and chiefly studied as a salivary component. By applying an immunogold staining method, we showed at the ultrastructural level the presence of immunoreactive statherin in normal human prostate and seminal vesicles. In principal cells of seminal vesicles, the reactivity for statherin was detected within the secretory granules, while in the prostate secretory cells, labeling appeared confined to the cytoplasm. For the first time, this study suggests that human male genital organs produce statherin, which could play a role in the protection of urogenital mucosa together with other antimicrobial peptides. In addition, the observed distribution pattern of statherin suggests the existence of peculiar secretory pathways for this peptide in the different glands.

CYTOKERATINS IDENTIFICATION IN HUMAN UMBILICAL CORD AT TERM

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Cytokeratins (Cks) are the proteins that constitute intermediate filaments. The pattern of expression of cytokeratins is frequently organ or tissue specific and depends mainly on the type of epithelium, the level of differentiation and the stage of development. With this study we tried to make light on the pattern of expression of a wide range of CKs in human umbilical cord at term. We investigated the expression of CKs through immunohistochemistry and RT-PCR assay. AE1/AE3 antibody reacts with the basic CKs 1, 2, 3, 4, 5, 6, 7 and 8, and with the acidic CKs 10, 13, 14, 15, 16 and 19. The reactivity for this antibody is localized in the amniotic epithelium. Fibroblasts of the Wharton's jelly did not react with this antibody. Endothelial cells and some cells of the vessel wall seem to express CKs at low levels. Reactivity for 34βE12 antibody (that recognizes CKs 1, 5, 10 and 14) is localized in the cells of the umbilical epithelium, while all the other regions of the umbilical cord did not react with the antibody. The immunoreactivity for MNF116 antibody (that recognizes CKs 5, 6, 8, 17 and 19) is restricted to the cells of the umbilical epithelium while all the other cells of the umbilical cord seems not to express these types of Cks. RT-PCR analysis revealed gene expression for CKs 5, 6, 8, 10, 14 and at a lower level also cytokeratins 1 and 17. We identified the expression of several types of CKs in human umbilical cord at term both at protein and RNA level.

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APPLICATION OF A MODIFIED STAINING METHOD FOR EPOXY-EMBEDDED SEMITHIN SECTIONS ON HUMAN PATHOLOGICAL TISSUES

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A fast and simple staining method recently reported[1] which considers the use of two solutions, methylene blue-Azur B (MB-AzB) and basic fuchsine (BS), allows a better observation of semithin sections in comparison to methylene blue (MB) or toluidine blue (TB). Our aim was to modify this procedure in order to adapt it for the staining of human pathological testis and human atherosclerotic carotids. Samples were obtained after patients' informed consent and consisted of 12 testicular biopsies of subfertile men and 10 human carotids obtained after endoarteriectomy. Samples were prepared for standard transmission electron microscopy. Both solutions, MB-AzB and BS, were prepared as reported[(1)] with some modifications. In particular, semithin sections were thicker (0.8-1.0 μm) and were stained for a longer time (10-15 sec) than the original report. In addition, we applied a coverslip before the observation by light microscopy (LM). This staining allows to recognize nuclei in blue and cytoplasm in lighter blue; collagen and elastin from pink to purplish-red. Fat, intracytoplasmic lipid droplets, Charcot-Böttcher crystals (Sertoli cells) and Reinke crystals (Leydig cells) stain gray-green, and cholesterol crystals in atherosclerotic carotids appear white. The present method is easy to use and is qualitatively superior to procedures using MB. In particular, it is very informative when observing pathological samples. A remarkable difference is its stronger staining and greater contrast of the extracellular matrix. Moreover, it produces less precipitates than methods employing MB or TB. Finally, reproducibility and technical simplicity recommend this method for a better and more accurate observation of semithin sections by LM.

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DIFFERENT DYNAMICS OF NEURONAL CELLS ADHESION ON DIFFERENT SUBSTRATES

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Initial attachment of cells to the extracellular matrix proteins is mainly mediated by integrins, heterodimeric transmembrane receptors, which connect the extracellular matrix to the cellular actin cytoskeleton. The cell-matrix interactions have an important role in regulating several intracellular pathways. Many of the integrin effectors reside in the specific cellular membrane sites named *focal adhesions*. Extracellular matrix proteins such as collagen, laminin and fibronectin represent the most important factors in interaction between integrins and extracellular matrix in neuronal cells. In particular, collagen I represents a fundamental surviving factor. Laminin can regulate neurite outgrowth and extension, while fibronectin promotes cell differentiation. In the present work, neuroblastoma SKNBE cells and retinoic acid differentiated-SKNBE cells have been used. Cells have been seeded onto collagen I or laminin or fibronectin for 30 min, 1, 3, 6 and 24 h. Then, using immunofluorescence staining analyses, we have analysed adhesion dynamics and number, length and distribution of focal adhesion pattern expressed by cells. The focal adhesion pattern distributes along cell profile in according with well-known action of different protein tested. Collagen I induces mainly the occurring of focal adhesion in cell membrane pointed out its action as surviving factor. Laminin induces mainly the occurring of focal adhesion in cell axonal-like extension, remarking its action of neurite outgrowth and extension inductor. Fibronectin induces the occurring of focal adhesion without any preferential localization, pointed out its action as cell differentiation promoting factors. Our results indicate a compartimentation of different biological pathways mediated by interaction between focal adhesion and specific extracellular matrix proteins. The present work wants to be a further step in the comprehension of the adhesion dynamics concerning neuronal cells.

EGG-JELLY STRUCTURE, ULTRASTRUCTURE AND LECTIN-BINDING SITES IN THE APENNINE YELLOW-BELLIED TOAD, BOMBINA PACHYPUS (ANURA: BOMBINATORIDAE)

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Amphibian eggs are surrounded by jelly layers whose number and composition are species-specific and involved in many functions. We studied the organization of the jelly layers of the eggs of an Amphibian by multiple techniques to characterise each single layer, rather than the whole envelope. Freshly-layed eggs of *B. pachypus* were included in paraffin and technovit for light-microscopical observations and resin for ultrastructural observations. Staining techniques included PAS, Alcian Blue pH 2.5 and 1.0, iron-diamine-Alcian Blue pH 2.5, periodic acid-paradiamine, and binding with eight different lectins. The eggs of *B. pachypus* revealed 10 layers not observed *in vivo*, differing for histochemical staining, lectin binding and macromolecular texture. The layers consist of mucins, mostly with O-linked oligosaccharidic residuals. Most layers are acidophylic, with sulphation increasing towards the outer layers. Fucosylated residuals predominate in the inner layers. Ultrastructural observations show a mesh of fibres and granules whose organization differs between the layers. The outermost layer has a loose texture hosting a number of microorganisms. Sulphate and sialylated glycoconjugates can have an osmotic function and protect against pathogens, as well as fucosylated residuals. The outermost layer can be important for symbiotic interactions with oxygen-supplying algae. Our study indicates that a complete characterization of egg jelly layers can be obtained by comparing results from multiple technical approaches.

EXPRESSION OF REMODELLING ENZYMES MMPS IN HERNIA FORMATION

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Abdominal wall hernias occur when tissue structure and function are lost at the load-bearing muscle, tendon and fascial layer. Much evidence suggest that hernia formation and recurrence depends in part on a systemic predisposition due to an abnormal metabolism of connective tissue and in part on other risk factors,[1] surgical as well as non surgical. In particular, it has been believed that remodelling enzymes as metalloproteinases (MMPs) can determine the hernia formation and relapse of hernia. For this reason, our group has been studyng the expression of some MMPs (MMP-2, 3, 9, 10, 13) in healthy and pathological abdominal wall through immunohistochemistry assay that we will confirme by the molecular investigation with RT-PCR to investigate the entity of the remodelling process in the different cases. In this work we are investigating four pathological samples (laparocele). Four samples, as control, are collected from same patients affected by laparocele but far away from hernial area. In all pathological samples we identified an expression of MMPs, except for MMP-3; we identified same results in equivalent normal samples. Our data suggest that the formation of abdominal wall hernias has been associated with defects of abdominal wall due to action of these remodelling enzymes, infact, they change the molecular and metabolic environmental of connectival structure of abdominal wall by making both the hernia formation both recurrence.

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A POSSIBLE APPROACH TO THE EARLY DIAGNOSIS OF ALZHEIMER'S DISEASE BY FLOW CYTOMETRIC ANALYSIS OF T LYMPHOCYTES

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Alzheimer disease (AD) is an age-related neurological disorder that leads to progressive dementia and is characterized by

the presence of β-Amyloid (Aβ) plaques in the brain. The pathogenesis of AD is unclear, and it is controversial whether AD results from a primary abnormality in amyloid precursor protein (APP) or deregulation of the inflammatory system, although these two possibilities are not mutually exclusive. The definitive diagnosis of AD is based on both the observation of senile plaques and neurofibrillary tangles. The objective of the present study was to analyze Aβ-specific T-cell responses in patients suffering from AD. T-cell infiltrates in AD lesions have been described and mechanisms of T cell transendothelial migration have been suggested. A role of T-cells in progressing AD is being discussed. Here we describe for the first time the detection and functional analysis of Aβ-specific T-cell in blood from Alzheimer patients. Using 9-colour flow-cytometry we were able to detect Aβ-specific T-cell responses in peripheral blood of AD patients but not age-matched controls. Aβ-specific T-cells were analysed in regards of T-cell lineage, cytokine production and expression of P-PKCs. They were found to show an activation pattern characterized by bright levels of P-PKC-δ and P-PKC-ζ and a skewed profile of cytokine production. While these results are a major advance in understanding the T-cell response to Aβ, the exact role of T-cells in AD will have to be addressed in future studies.

TGF-β/SMAD SIGNALING PATHWAY AS TISSUE REMODELING MECHANISM IN IMMUNOALLERGICAL PATHOLOGIES THAT LEADS TO FIBROSIS

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Vernal keratocongiuntivitis (VKC) and bronchial asthma are both associated to an allergic inflammatory response and tissue remodelling. We investigated the immunoexpression of Smad2, 3, 7 and TGF-β in VKC and asthma tissues. In addition, the TGFβ/Smad and mitogen-activated protein kinase pathways expression were evaluated in fibroblast cultures exposed to histamine, Th1- and Th2-type cytokines. Smad2, 3, 7 and TGF-β were evaluated in conjunctiva from 9 VKC patients and compared with 5 bronchial biopsies from asthmatics by immunohistochemistry and a group of control subjects The mRNA expression of Smads, TGF-β1, TGF-β2, mitogen-activated protein kinase (p38/MAPK), c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2) were also determined in conjunctival fibroblast cultures exposed to histamine, IL-4, IL-13, IFN-γ and TNF-α, Smad2, Smad3 and TGF-β1 immunostaining scores were significantly increased in the stroma of VKC patients compared to controls. Smad7 did not change significantly. In the submucosa of asthmatic patients Smad2, Smad3, Smad7 and TGF-β1 scores did not change significantly. In conjunctival fibroblast cultures, histamine, IL-4 and TNF-α increased the expression of Smad3 and 7 by 2 or 3 folds, TGF-β1 expression was increased by IL-4, TGF-β2 by histamine and both TGF-β by TNF-α On the contrary, IFN-γ reduced the expression of Smad3 and TGF-β1. In addition, histamine, IL-4 and TNF-γ increased JNK and ERK1/2 expression by 3 folds. The TGF-β/Smad signaling pathway is over-expressed in VKC tissues and stimulated in conjunctival fibroblasts by histamine, IL-4 and TNF-α. These mechanisms may be involved in the tissue remodelling. The lack of significant changes of Smads expression in asthma may be due to the presence of a mild intermittent disease state of these patients or to the prevalence of concomitant different profibrotic mechanisms in the bronchi.

THE PARACRINE LOOPS OF KERATINOCYTE STIMULATION IN CHOLESTEATOMA TISSUE SHOWN BY QUANTITATIVE IMMUNOFLUORESCENCE AND MOLECULAR ANALYSIS COMBINED WITH TRANSMISSION ELECTRON MICROSCOPY

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Cholesteatoma is a temporal bone pathology characterized by active proliferation of epithelial cells with progressive growth and involvement of the neighbouring middle/inner ear structures. The pathogenic mechanism underlying the hyperproliferation of keratinocytes is not yet completely clarified. It has been suggested that keratinocyte proliferation and migration could be is mediated by several autocrine and paracrine growth factors and their receptors. We have previously reported that the expression of keratinocyte growth factors receptor (KGFR) is increased in more differentiated areas of the cholesteatoma tissue, while the expression of the epidermal growth factor receptor (EGFR) is associated with proliferative and migratory portions of the lesion. The aim of this study was to investigate the relationship between KGF expression and KGFR distribution in cholesteatoma tissue and the possible role of inflammatory infiltrate in the paracrine stimulation of keratinocytes. Fresh cholesteatoma tissues were collected after surgical procedure. Serial cryosections were examined by conventional haematoxylin and eosin or toluidine-blue staining. Quantitative immunofluorescence for KGFR and pan-cytokeratin was performed. The expression of KGF, K1 and vimentin in the sample was evaluated by qRT-PCR. The ultrastructural features were analyzed by transmission electron microscopy. We found that in the proliferative stage of cholesteatoma, the downmodulation of KGFR in suprabasal keratinocytes is associated with an overexpression of mRNA KGF and the presence of a strong inflammatory dermal infiltrate. These results suggest that KGF upmodulation is a consequence of fibroblast stimulation by inflammatory cells and this paracrine loop might be responsible for the hyperproliferation of keratinocytes in colesteatoma tissue.

USE OF PGP 9.5 TO REVEAL SMALL FIBERS NEUROPATHY: PRELIMINARY RESULTS

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Intraepidermal nerve fibers (IENF) have been identified in the basal layers of the epidermis in the human skin. Small fibers neuropathy is a relatively common disorder often associated with systemic conditions and neurological disorders. Despite good deal of symptoms, the diagnosis may escape detection by standard electrophysiology tests; therefore quantitative sensory tests for cold and warm sensations and sural nerve biopsy have been used to determine the damage to small nerve fibres (caliber C and Aδ). Recently, fiber loss and degeneration have been readily identified and quantified with a neuronal antibody to protein gene product 9.5 (PGP 9.5) at the dermal-epidermal interface.[1,2] The present study shows preliminary experience with PGP 9.5 as a marker of peripheral small

fibers neuropathy. Punch skin biopsies were performed on ten patients referring for peripheral neuropathies and from five control patients referring for genetic counselling with no evidence of peripheral neuropathy. Nerve fibers were revealed using immunoperoxidase staining with panaxonal antibody PGP 9.5. Patients with clinical features of small fibers damage showed denervation of the dermal-epidermal interface when compared to normal control subjects. Skin biopsy is an easy, painless, repeatable procedure for the diagnosis and follow up of small fibers neuropathy. PGP 9.5 assessment is a promising tool for patients in whom electrophysiology tests have resulted negative as an alternative to sural nerve biopsy.

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SKIN IRON DEPOSITION CHARACTERIZES LIPODERMATOSCLEROSIS AND ULCER

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The skin content of iron increases in legs afflicted with chronic venous disease (CVD). Excessive iron is stored in hemosiderin, which proinflammatory properties have been correlated to ulcer development. We have histochemically evaluated the presence of hemosiderin in relation to the absence, presence and severity of skin changes in CVD-legs designated according to CEAP classification. Hemosiderin deposition was evaluated by routine and Perl's Prussian Blue (PPB) stains in 81 biopsies from 47 legs with CVD and 4 samples from control legs. Hemosiderin was absent in control specimens, in C2 and C3 legs, in less severe pigmentations and in part of more severe ones. Hemosiderin was in all biopsies from LDS skin, from ulcers and from part of more severe pigmentations. Finally, hemosiderin was seldom found in the apparently normal skin of C4b and C6 legs surrounding lipodermatosclerotic plaques or ulcers borders. Our findings suggest that hemosiderin deposition is necessary to the worsening of CVD-related skin damaging. In fact, PPB-positive ferric ions are absent in C2 and C3 legs but characterize histologically lipodermatosclerosis and ulcers. Our data also support previous studies which described a skin iron reduction in healing ulcers. Finally, our results are in agreement with current theories suggesting that a genetic disorder regarding skin iron metabolism is essential to develop skin changes in CVD-legs.

ROLE OF THE LIGHT EXPOSURE ON THE CYTOTOXICITY OF GUAIAZULENE ON HUMAN FIBROBLASTS: PHOTOPROTECTION EFFECTS

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Guaiazulene I (1,4-dimethyl-7-isopropylazulene; GA) is widely used as a natural popular component in health care products (emollient creams, toothpaste) and solutions (eye drops). Although it has been reported to have interesting biological effects, such as anti-inflammatory, anti-spasmodic, antimicrobial activities and relaxant properties, GA and azulene derivatives have been reported to be cytotoxic against normal human cells and human tumor cells, moreover guaiazulene showed photomutagenic properties in Salmonella typhimurium bacteria strains. The purpose of the present study was to evaluate and compare the cytotoxicity of GA on human gingival fibroblast cell culture (HGFs) in normal conditions and under photo-irradiation (using a solar simulator lamp as light source). HGFs have been exposed to different concentrations of GA, in the presence or in the absence of UV radiation, and cell viability, protein expression, cell morphology and DNA fragmentation have been evaluated. GA reduced significantly the number of cultured cells (dose-depending trend) and interfered with the protein procollagen $\alpha 1$ type I, chosen as marker for HGF protein synthesis. Transmission electron microscopy and DNA fragmentation studies has been performed to exclude apoptosis as mechanism of cell death. Unexpectedly, while the cytotoxicity was confirmed, guaiazulene was found not to be phototoxic, on the contrary, the irradiation of cell culture strongly decrease GA toxicity.

PATHOGENIC AUTOANTIBODIES MODULATE EXTRACELLULAR MATRIX PROTEIN EXPRESSION

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The Fibulins are a seven-member protein family hypothesized to function as intermolecular bridges that stabilize the organization of extracellular matrix (ECM) structures. Fibulins are involved in a variety of cellular functions including proliferation, migration, differentiation and survival. Pronounced ECM remodeling is detectable in the labial salivary glands of Sjögren's syndrome (SS) patients. Here we investigate the ability of anti-Ro autoantibodies (Abs), characterizing the SS syndrome, to modulate fibulin-3 and fibulin-4 expression in human salivary gland epithelial cells (SGEC). Real time-PCR, flow cytometric analysis and immunohistochemistry were used to analyze the fibulin-3 and fibulin-4 expression in primary human SGEC cultures, established from biopsies of labial minor salivary glands of healthy donors, both in untreated control cells and in anti-Ro/SSA Abs-treated cells. The methods used show a down-modulation of fibulin-3 and -4 mRNAs and protein expression after treatment of the cells with anti-Ro Abs. No differences in fibulin-3 and -4 expressions were observed in cells treated with IgG obtained from healthy donors in comparison with untreated control cells. Anti-Ro Abs dysregulate the expression of fibulins 3 and 4 in SGEC in culture, possibly altering the cellular ECM environment. This action might ultimately be responsible, at least in part, for the anatomical and functional damages of the salivary glands in SS patients.

OPSIN EXPRESSION IN THE ZEBRAFISH PINEAL GLAND FORM LARVAL TO ADULT STAGE

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The pineal gland of many non-mammalian vertebrates is a photoreceptive organ, containing photoreceptor cells sensitive to light. The pineal organ of these species is considered an extraocular tissue that plays an important role in the neuroendocrine system. In the last decade the zebrafish has become a useful model for the genetic and development biology analysis and it has been used in many studies concerning the pineal gland functions. These studies demonstrated that the zebrafish pineal gland is made up of circadian pacemakers and photoreceptive molecules responsible for the circadian clock. Some authors demonstrated the expression of the opsin, a specific

protein present in the pinealocytes and in the retina photoreceptor cells of different teleosts. Particularly, some data focused on the involvement of pinealocytes in the regulation of the circadian rhythms in the zebrafish through melatonin secretion but the data about the expression of the opsin in the extraretinal photoreceptors during development are scarce. Therefore, this study was undertaken to analyze the spatio-temporal expression of the opsin in the pineal gland of zebrafish from larval to adult stage to verify the involvement of this protein in the regulation of the circadian clock during zebrafish development. Our immunohistochemical study, using an anti-opsin monoclonal antibody, revealed the occurrence of opsin in the pineal photoreceptors of zebrafish from larval to adult stage. The electron transmission study was utilized to identify the cellular types expressing opsin and moreover revealed the occurrence of different cellular types like the interstitial cells in the pineal gland never previously reported in zebrafish. Taken together our results could demonstrate the involvement of opsin in the morpho-physiological maintenance of the pinealocytes throughout the zebrafish whole life span.

IMMUNOLOCALIZATION OF ANP AND INSULIN IN PANCREAS OF HUMAN FOETUSES FROM DIABETIC MOTHERS

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It is known that ANP peptides are synthesized by the atrial and ventricular myocardiocytes but they are synthesized also in different other organs. It has been demonstrated that in rat pancreatic islets ANP is present in α glucagon-synthesizing cells only; in the man the β insulin- synthesizing cells present binding sites for ANP; moreover it has been demonstrated that ANP plays a role on the activity of the plasma insulin levels because it inhibits the hepatic and/or renal degradation of insulin. To date no effect of ANP on human pancreatic islets in diabetic patients has bee showed. Aim of present work is study the ANP presence in β cells and its role in the insulin release in foetal pancreatic islets from the diabetic mothers. The observations were carried out in the immunostained sections for ANP in foetal pancreas from diabetic mothers and showed that the β cells present a ANP-immunoreactivity. Moreover, the double immunostaining for ANP and insulin showed a colocalization of the two peptides. The sections of pancreas of human foetuses from the diabetic mothers evidence that the β-cells of the pancreatic islets are ANP immunopositive, differently than in the adult rat where only the glucagon-synthesizing cells are immunopositive. Indeed, a double immunostaining for ANP and insulin certainly demonstrated that the β cells are immunopositive for ANP and insulin. The presence of ANP in the β cells indicates a synthesis of ANP or internalization of ANP that synthesized in the α-cells, by paracrine mechanism, binds to ANP receptors present in β cells. In conclusion, we may affirm that the maternal diabetes influence the foetal pancreatic metabolism although it is demonstrated that the ANP does not across the placental barrier.

MELATONIN PREVENTS H_2O_2-INDUCED CELL DEATH

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Human leukemia monocytic U937 cells readily undergo apoptosis when exposed to UVB-induced oxidative stress, and this action is significantly prevented by melatonin (MEL) treatment,[1,2] a well known neuro-hormone produced by pineal gland with anti-oxidant properties. Hydrogen peroxide (H_2O_2) has been described to induce both apoptotic or necrotic death, dependently on concentration.[3,4] The aim of this study was to investigate the response of U937 cell line to H_2O_2-induced cell death and to highlight the possible role of MEL.[5] Cells were treated with 2 mM MEL before and after 0.5 mM H_2O_2 exposure. The majority of morphological and functional analysis experiments showed a H_2O_2-induced apoptotic death, with chromatin condensation, nuclear breakdown, cell shrinkage, membrane blebbing and apoptotic bodies.[6] MEL incubation before and after H_2O_2 exposure protects U937 cells from reactive oxygen species (ROS) accumulation damage, reducing apoptotic cell number. MEL pre-treatment shows a more significant protection if compared to MEL incubation after H_2O_2 exposure. Our results evidenced that, even if in the presence of necrotic death, H_2O_2 is a powerful apoptotic trigger, apparently more effective than UVB. In addition, MEL appears, again, to significantly prevent apoptosis, in particular in pre-incubated cells. Further studies are in progress to understand underlying mechanism.

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IN VIVO STUDY OF NONYLPHENOL EFFECTS ON HYPOTHALAMUS-PITUITARY-ADRENAL GLAND AXIS OF THE BIOINDICATOR ORGANISM PODARCIS SICULA LIZARD

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Nonylphenol (NP), a critical metabolite of alkylphenol polyethoxylate detergents, is the most abundant environmental pollutant belonging to endocrine disrupting chemicals (EDC), substances that are able to change, mimic or antagonize the normal functioning of the endocrine system by interfering with the synthesis, metabolism, receptor binding and cellular responses of endogenous hormones. The aim of this study was to evaluate the *in vivo* effects of NP exposure on hypothalamus-pituitary-adrenal gland axis of bioindicator lizard Podarcis sicula by biochemical and histochemical approaches. Reptiles are particularly suitable as contaminant biomonitors due to their persistence in a variety of habitats, wide geographic distribution, longevity and, in many cases, site fidelity and to their ability to bioaccumulate. We demonstrated that prolonged exposure to NP induced a significant increase of CRF, ACTH and corticosterone plasma levels in a dose-dependent manner. Moreover, NP also induced a high increase of adrenaline plasma levels and a contemporary decrease of noradrenaline plasma levels. Accordingly to biochemical results, we observed an intense hypertrophy of steroidogenic cells together with a great increase of vasculogenesis of the whole gland. In addition, we also found an increase of adrenaline

cell number distributed in dorsal chromaffin ribbon of lizard adrenal gland and a complete degranulation of chromaffin cells. Furthermore, we observed a severe infiltration of macrophages especially close to connective capsule surrounding the gland. This study showed that NP caused a continuous stimulation of hypothalamus-pituitary-adrenal gland axis with a lack of negative feedback. Moreover, NP was able to induce histopathological alterations of lizard adrenal gland.

THE EFFECTS OF SOME ENDOCRINE DISRUPTORS ON THE SKIN OF THE ADULT NEWT, TRITURUS CARNIFEX (AMPHIBIA, URODELA)

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The endocrine disruptors are widespread in the aquatic environment where amphibian urodeles spend a great part of their life. Their naked skin is permeable and very sensitive to damages caused by these pollutants. Since skin performs several functions such as water absorption, respiration, osmoregulation and defence, the skin damage may compromise the same survival of the organism. We studied the skin of adult newts exposed to two endocrine disruptors, largely widespread in the water environment: 1) nonylphenol (NP), a ubiquitous biodegradation product of the nonylphenol ethoxylates 2) the fungicide thiophanate methyl (TM). Areas of dorsal skin from the trunks were studied with histological and histochemical methods. The integument of *T. carnifex* is composed of an epidermis, covered with a horny layer, and a dermis. Some keratinocytes show mitotic activity. The chromatophores form a thin and continuous layer. The cutaneous mucous and granular glands form a continuous layer; few intermediate, serous-mucous glands, are present. The surface of horny layer and the mucous glands are PAS positive. In TM exposed newts mitotic activity of keratinocytes increased. The skin of polluted newts had the epidermis flattened, keratinocytes gathered together; the dermis disorganized. The chromatophores formed a discontinuous, often sclerous layer. The serous secretory material strongly increased and accumulated on and under the horny layer and between the dermis and epidermis. Many serous and mucous glands were empty; the number of intermediate glands increased. Many mucous glands of TM exposed newts were stuffed with secretory granules. A strong PAS positivity was seen on the horny layer and in the superficial layers of the epidermis. The results show that both NP and TM altered the skin of *T. carnifex*, influencing its structure and secretory pattern, and possibly impairing its functions.

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EFFECT OF CHRONIC TREATMENT WITH CB1 RECEPTOR ANTAGONIST (RIMONABANT) ON B-CELLS APOPTOSIS IN THE PANCREAS OF OBESE ZUCKER RATS. AN IMMUNOHISTOCHEMICAL STUDY

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Recent reports suggest that there is an overactivation of the endocannabinoid system (EC) in obese humans and rats. In a previous publication we have shown that in obese and diabetic Zucker rats the pancreatic islets show B-cells apoptotic damage. In addition the CB1 receptor is overexpressed by all endocrine cells in comparison with normal rat islets in which CB1 immunoreactivity is restricted to A-cells. Also, other AA. have demonstrated that activation of cannabinoid CB1 receptor induces glucose intolerance in rats. Clinical trials in obese men with a cannabinoid CB1 receptor antagonist (Rimonabant) have shown a reduction of body weight and improvement of insulin resistance. With references to findings we have studied the effects of chronic treatment with Rimonabant on B-cells apoptosis in endocrine pancreas of the Zucker diabetic fatty rats (ZDF) underlining immunohistochemically caspase-9 as an indicator of apoptosis and nNOS/iNOS activities implicated in cellular damage. The treatment was performed for 4 weeks; specimens of ZDF rat pancreas and of normal rat were fixed in Bouin mixture and embedded in paraffin. Obtained sections were processed with anti caspase-9 (Sigma) and anti nNOs/iNOS (Transduction Laboratories) by Envision + System HRP (AEC) (DAKO Citomation). All the samples have been studied with photomicroscope Leica DM1000. Our results show that Rimonabant treatment significantly decreases the apoptotic incidence in the islets of ZDF rats compared with those of non-diabetic normal rats. These findings provide a morphohistochemical basis to support the use of this drug in metabolic syndrome associated with obesity.

FLUORESCENT *IN SITU* HYBRIDIZATION STUDY TO PREDICT CETUXIMAB RESPONSE IN METASTATIC COLORECTAL CANCER

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Colorectal cancer (CRC) is a common cause of death in the Western world.[1] Targeted therapies are a novel approach using agents that are directed against signalling molecules involved in tumor growth or progression. The monoclonal antibody cetuximab, approved for the treatment of advanced CRC, binds the epidermal growth factor receptor (EGFR), which is involved in cell proliferation, differentiation and survival. Overexpression of EGFR occurs commonly in human CRC,[2] although gene amplification is not commonly reported. Immunohistochemistry is often unable to predict the clinical response to cetuximab, while fluorescent *in situ* hybridisation (FISH) technique can be considered the method for primary assessment of EGFR status.[3] We used dualcolor FISH to evaluate EGFR gene amplification in metastatic colon cancer tissue sections of 38 patients. The deparaffinated, hydrated section was digested with pepsin, the probe mixture was added and denaturation and hybridization were performed. The slide was washed, dehydrated, air-dried and mounted. Evaluation was performed in fluorescence microscope, scoring 60 tumor cells. The ratio of the total number of EGFR signals to the total number of centromere-7 signals was calculated: a ratio of 2 or greater was recorded as positive for EGFR gene amplification. An increase of the DNA copy number of the EGFR gene was found in 6 of the 38 tumors, giving an amplification rate of 15,8 %. Our institution performs FISH assays on all 3+ and 2+ staining colon tumors and also on a number of negatively staining cases to confirm true EGFR negativity in patients with metastatic carcinomas.

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DECREASE OF APOPTOSIS IN PUTATIVE PREMALIGNANT HUMAN COLONIC LESIONS COMPARED TO NORMAL COLONIC MUCOSA

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Aberrant crypt foci (ACF) have been detected in humans with colon cancer and are thought to be precursors of adenomas and carcinomas. It may be speculated that disruption of apoptosis plays a permissive role respect to the subsequent inactivation of a DNA repair mechanism. Conceivably, loss of DNA repair might drive a rapid neoplastic progression. Caspases, a family of cysteine proteases, are among the essential components of the apoptotic machinery. Over-expression of any caspase family member can induce apoptosis in mammalian cells. Poly ADP-ribose polymerase-1, can be cleaved into 89 and 24 kDa fragments by caspase 3 and 7. The switching on and off of apoptosis is determined by the ratio of proapoptotic and antiapoptotic proteins. Bcl2 is an example of an antiapoptotic protein witch is over-expressed in many cancer cell types, colorectal adenocarcinomas as well as prostate and breast cancer. Overproduction of the Bcl2 protein also prevents cell death induced by almost all cytotoxic anticancer drugs and radiation, and contributes to drug resistance in patients with some types of cancer. The aim of the present study was to quantify the percentage of apoptotic cells in human ACF, respect with the apoptotic percentage of normal mucosa. One of the most important clinical applications of ACF endoscopic observation is its use as a target lesion for chemoprevention. Therefore, we set out to examine samples of dysplastic ACF and of normal mucosa by indirect confocal immunofluorescence techniques and immunoblot experiments. Using these techniques we have evaluated qualitatively and quantitatively the cleaved PARP-1, Caspase 3, 9 and Bcl2 proteins expression. Our results show that in normal mucosa the percentage of apoptosis is significantly higher than in ACF, 15,68% and 3% respectively. Western blot analysis also confirms these observations. We strongly suggest that ACF, particularly dysplastic ACF, are precursor lesions of the adenoma-carcinoma sequence in humans.

D2-40 MONOCLONAL ANTIBODY IS A SELECTIVE MARKER OF LYMPHATIC ENDOTHELIUM AND CANCER PROGRESSION

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The monoclonal antibody D2-40 is a new selective marker for lymphatic endothelium. In this study, we evaluated a cohort of Penile Squamous Cell Carcinoma (PSCC) to investigate the value of immunohistochemical D2-40 expression within tumoral, peritumoural and non-tumoural tissue as a prognostic marker. PSCC is rare but it is affected by high mortality. The presence of nodal metastases is determinant for disease specific survival. A number of studies are seeking for prognostic factors that will be able to distinguish those patients who may benefit for inguinal lymph node dissection. Lymphatic vessel density (LVD) was higher in peritumoral tissue compared to tumoral tissue and normal, and decreased in patients with lymph node metastasis respect to patients not affected. D2-40 showed also cancer cells intracytoplasmatic positivity, in particular, in tumoral tissue we observed that the cases negative for intracellular D2-40 showed low stage and grade and no lymph node metastases. The higher peritumoral LVD seems to lower the importance of lymphatic vessels in tumoral tissue, where they often are non functioning and with structural anomalies. In conclusion, LVD and D2-40 positive reaction in cancer cells were strongly associated with lymphatic vessel invasion and clinical parameters of worse prognosis, therefore D2-40 can be considered a useful marker to identify lymphatic vessels architecture and predict PSCC aggressiveness.

DEATH AND SURVIVAL OF C6 GLIOMA CELLS IN CULTURE AFTER CISPLATIN TREATMENT

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Astrocyte-like C6 glioma cells are a simple *in vitro* model for studying the response of astrocytes to cisplatin treatment. Three days old cultures were exposed to cisplatin (5 μg/mL for 90 min) and examined one to several days later by cytometry, immunofluorescence and electron microscopy. Since 24 h post-treatment (p.t.), the cells slowed down cycling and died by canonical apoptosis,[1] with nuclear condensation and cell shrinkage resulting in the formation of apoptotic bodies. At 48 h, some cells underwent an atypical form of regulated cell death characterized by the presence of intranuclear bundles of microtubules and microfilaments, without chromatin condensation and nuclear fragmentation.[2] By 72 h p.t., the cells with nuclear microtubules disappeared, and most of the surviving cells became hypertrophic and their large nuclei were highly-lobulated and surrounded by micronuclei; in their cytoplasm, hetero- and auto-phagosomes were found, suggesting the occurrence of autophagic cell death. More than 95% of these cells survived repeated Cisplatin treatment. These hypertrophic surviving cells up-regulated the activity of gamma-glutamyl-transpeptidase and were more positive for GFAP. We assume that similar mechanisms may lead to the origin of reactive astrocytes, including their resistance to cytostatic therapies, also in human glioma tumors *in situ*.

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PROGNOSTIC VALUE OF SURVIVIN PROTEIN EXPRESSION IN T4 BREAST CANCER PATIENTS

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Survivin, a member of the inhibitor of apoptosis protein family, is over-expressed in many tumors, including breast cancer, and is considered a promising molecular target for emerging therapies. Survivin over-expression is associated with poor clinical prognosis and chemo-resistance in different tumors. Survivin seems to be a strong predictor of poor survival in early-stage breast cancer patients (pts); nevertheless its role in predicting prognosis in locally advanced breast cancer (LABC)

pts is still unknown. Aim of the present study was to investigate the prognostic relevance of survivin on terms of survival in LABC T4 breast cancer. Survival was estimated by Kaplan-Meier method and differences between groups were tested by log-rank test. Survivin protein expression was screened in primary tumor by immunohistochemistry (samples with more than 10% of cells stained were considered to be positive); 53 out of 126 consecutive LABC T4 breast cancer pts observed between 1991 and 2001 were evaluated. Survivin protein was positive in 21 (40%), negative in 32 (60%) cases; median age was 51 years (32-67), clinical T4abc 38 pts (72%), T4d 15 pts (28%); N+ 50 pts (94%); ERneg 25 pts (47%); PRneg 36 pts (68%); HER2 status by IHC was negative in 43 pts (81%), positive in 10 pts (19%). All pts received anthracycline-based primary chemotherapy, surgery, radiation, adjuvant chemotherapy and hormone when indicated. At a median follow up of 125 months (70-182), 10y DFS and OS were 32,1% and 43,4% for the entire population, respectively. Patients with low survivin expression showed significantly better OS and a trend of improving DFS than patients with high survivin expression. Our data show that the over-expression of survivin protein is a negative prognostic factor in LABC pts, maintaining its relevance even at long term. Survivin protein should be considered a new marker for treatment decision making and a promising target for innovative therapies.

BIOLOGICAL EFFECTS OF NATURAL AND SYNTHETIC MINERAL FIBRES AND THERMALLY TRANSFORMED ASBESTOS WASTE. *IN VITRO* ASSESSMENT ON CELLULAR SYSTEMS

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Asbestos is the name of naturally occurring hydrated inorganic mineral silicates subdivided into serpentine and amphibole asbestos, based on their crystal structure and chemistry. Exposure to asbestos fibres, has long been known to cause lung cancer, asbestosis and mesothelioma, but their pathogenetic mechanisms are not entirely understood. Asbestos fibres can be transformed into non-hazardous silicate phases by high-temperature treatment. Elimination of cement-asbestos, Eternit, the main asbestos-containing manufactured product in Italy, is a priority in the prevention of pollution. We investigated the cytotoxic potential exerted *in vitro* by natural and synthetic asbestos fibres (morphologically and chemically characterized) and by raw and thermally treated cement-asbestos in standardized human cell lines representing the fibers' micro-environmental targets. Morpho-functional investigations were performed to assay cell viability, proliferation rate, senescence and apoptotic induction. Expression of Cdc42, β-catenin, and VEGF, considered potential risks for cancer development were also evaluated. Our studies indicate potential cellular toxicity of natural and synthetic fibres and a cytotoxic cell damage of more extended grade exerted by raw cement-asbestos compared to thermally treated material even from early culture time points.

LY294002, INHIBITOR OF PI3K, REGULATES SUPERFICIAL EXPRESSION OF NGF RECEPTORS IN HUMAN CANCER CELL LINES

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Several experimental data have shown a relevant involvement of Nerve Growth Factor (NGF) in neoplastic proliferation and survival. NGF acts through 2 receptors: high affinity TrKA receptor and low-affinity p75 receptor. The reciprocal interactions between their transduction pathways determine the final biological role of NGF. To better analyze these interactions, we have used LY294002, a specific inhibitor of PI3K, an intermediate step for TrKA transduction. Furthermore, studies reported that inhibition of PI3K induces activation of BAD, an intermediate step for p75. Our aim was to investigate the biological effects of this inhibitor by a cytofluorimetric quantification of the superficial levels of TrKA and p75 in some human cancer cell lines: HTB114, HTB82 (muscle sarcomas) and PC3 (prostatic adenocarcinoma), in basal conditions and following treatment with LY294002. Cytofluorimetric analysis shows that our cellular populations can be divided in three subsets: a) double negative for TrKA and p75; b) positive only for TrKA; c) double positive for TrKA and p75. At basal conditions in the latter subset we found a prevalence of TrKA on p75, with a TrKA/p75 ratio of about 3:1. In this group the treatment with LY294002 did not influence TrKA levels but induced an important increase in p75 levels, changing the TrKA/p75 ratio from 3:1 to 1:2. This treatment did not effect the double negative TrKA/p75 and the single TrKA positive subsets. This relevant modification in TrKA/p75 ratio following LY294002 treatment was associated to a decrease in proliferation and an increase in apoptosis. In conclusion, our study for the first time shows that an interaction on TrKA pathway is able to influence the superficial expression of p75 that, in turn, induces an increase in apoptosis. This effect has a preclinical importance, suggesting the relevance of TrKA/p75 targeting in oncology.

Acknowledgements: the project is supported by Fondazione Cassa di Risparmio di Perugia.

A NOVEL LOCALIZATION OF LOW AFFINITY NERVE GROWTH FACTOR RECEPTOR (P75) IN NORMAL AND NEOPLASTIC HUMAN PROSTATE. AN IMMUNOHISTOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDY

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The biological role of NGF and its p75 receptor in prostate cancer is still controversial. The aim of this work is to evaluate the localization of p75 in normal and neoplastic human prostate as prognostic marker. Human samples of normal and prostate cancer were analyzed at light and ultrastructural levels (TEM). At light microscopical level, p75 immunoreactivity (-IR) in normal human prostate was restricted to the basal cells of the acini, at epithelial-stromal junction. This result was confirmed by TEM. Normal prostatic stromal cells were p75 negative, except for nerves and blood vessels. Prostatic Intraepithelial Neoplasia (PIN) showed a relevant proliferation of the epithelial compartment, inclusive of the basal cells that remain p75-IR. However, samples of adenocarcinoma, medium

to high grade neoplasia, showed different patterns of p75 localization. In fact, while basal cells of the epithelial compartment became progressively p75 negative, a novel strong p75-IR was detected in stromal compartment, adjacent to the neoplastic acini. Ultrastructural analysis showed that the stromal p75-IR was localized on plasma membrane of smooth muscle cells. Other stromal cells were p75 negative. The amount of p75-IR in the stroma seems to be positively correlated to Gleason score. Our study shows a novel morphological localization of p75 into the stroma of prostate cancer. The positive correlation of this stromal localization with the cancer malignancy suggests a progressive dedifferentiation of the smooth muscle cells that normally are p75 negative. This dedifferentiation of neoplastic smooth muscle cells around the neoplastic acini could be relevant for metastatic invasion of the stroma. In conclusion, our study hypothesized that analysis of p75 in stromal compartment of prostate cancer could be a novel marker for a better definition of the prostatic cancer malignancy and prognosis.

Acknowledgments: The project is supported by Fondazione Cassa di Risparmio di Terni (CARIT)

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL CHARACTERIZATION OF U87 GLIOBLASTOMA XENOGRAFTS IN NUDE MICE

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Glioma cells have a remarkable capacity to infiltrate in the brain and migrate long distance from the tumor, making complete surgical resection impossible. Diffuse invasion of glioma is a major obstacle in the treatment and main cause for the poor survival of patients.[1] The use of model systems that mimic the biological characteristics of human brain tumors is critical for obtaining the knowledge necessary to develop successful therapeutic strategies.[2] Orthotopic xenografts were establish by injecting $5x10^5$ tumor cells from glioblastoma multiforme cell line U87 into the right striatum of nude mice. Tumor growth was monitored *in vivo* by serially sectioning the xenograft brains at 7, 21, and 28 days postinjection.[3] Immunohistochemistry was performed for detection of the expressions of nestin, vimentin, GFAP, PCNA and human mitochondria (as marker of cells of human origin). Beginning 14 days after U87 implantation, infiltrating tumor cells were detected as single cells and as cell clusters located near or around a blood vessel or as peripheral infiltrative zone migrated out of the main tumor mass. At all time intervals, primary tumor, peripheral infiltrative zones and tumor islands were surrounded by reactive gliosis developed at the xenograft-host interface, as revealed by GFAP immunostaining. U87 cells were negative for GFAP and positive for vimentin, nestin and PCNA at all time intervals. In conclusion, our present results demonstrate a stepwise progression of tumor in the brain of nude mice.

This work was supported by Romanian PN 2 Programme, Project No. 41-035/2007.

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HSP60 IS RELEASED FROM TUMOR CELLS BY VARIOUS PATHWAYS

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Heat shock protein (HSPs) are often overexpressed during carcinogenesis and recent studies show that, when released from cells, HSPs can mediate anticancer immune responses.[1-3] However, the mechanisms by which Hsp60, also called chaperonin, is released from tumor cells into the extracellular space are not fully understood. We are investigating the pathways involved in Hsp60 release, including Golgi's, exosomal and lipid-rafts one. For the present study, we examined NCI-H292 (mucoepidermoid carcinoma) and 16HBE (normal bronchial epithelial) cells. Both cell lines showed normally low levels of apoptosis (annexin V assessment) and NCI-H292 cells presented higher levels of Hsp60 and Hsp70 than 16HBE cells. We found Hsp60, as well as Hsp70 and alix [the latter are considered exosomal markers[4]], in the exosomal fractions of the NCI-H292 cells but only Hsp70 and alix were detected in the 16HBE-derived exosomes. Cells were therefore treated for 1 h with secretion inhibitors: brefeldin A (1 μg/mL), for Golgi's; dimetylamiloride (5 nM) for exosomes; methylcyclodextrin (1 mM) for lipid rafts. All compounds inhibited Hsp60 secretion ($p<0.05$). Elucidation of the secretory pathways for Hsp60, as well as for other HSPs, is fundamental for understanding their role in antitumor immune responses and tumor survival.[5]

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DISTRIBUTION AND MODULATION OF NEDD8 PROTEIN IN HUMAN PLACENTA THROUGHOUT PREGNANCY

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NEDD8 (neural precursor cell-expressed developmentally down-regulated) is an ubiquitin-like protein essential for protein processing and cell cycle progression. It is highly conserved in eukaryotes. Similar to ubiquitin, Nedd8 attaches to target proteins through an enzymatic cascade composed of Nedd8-specific E1 (activating)- and E2 (conjugating)-enzymes. The Nedd8 modification of Cullin (Cul) family proteins is evolutionarily conserved, and genetic analyses in various organisms suggest a positive role of the NEDD8 for the function of Cul family proteins. It has been demonstrated that the NEDD8 system is essential for both mitotic and the endoreduplicative cell

cycle progression. Moreover, NEDD8 system is essential for the regulation of protein degradation pathways involved in cell cycle progression and morphogenesis, possibly through the function of the Cul family proteins. In order to obtain further information on NEDD8 function, in the present study we have observed NEDD8 expression in human placenta during gestation by the use of immunohistochemistry. Human placental samples were obtained with informed consent during both the first trimester and the third trimester of gestation. We observed that in human placenta there was a modulation of NEDD8 throughout pregnancy with a strong level of expression during the first trimester and a weak level in the third trimester of gestation. Specifically, we observed a strong NEDD8 expression in the cytoplasm of cytotrophoblast close to the nucleus in the first trimester whereas a NEDD8 localization in the stroma of placental villi during the third trimester of gestation. Taken together, our results suggest an important role of NEDD8 in controlling proliferation and differentiation events in cytotrophoblast cells of human placenta. Further investigation will clarify which molecular and physiological mechanisms of NEDD8 in human placenta.

LATE ABSTRACTS

These abstracts arrived in print and were added at the end of the book.

AN IMMUNOHISTOCHEMICAL STUDY ON HUMAN FOETAL TOOTH GERM

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The aim of the study was to evaluate c-Met expression in human tooth germ development. An immunohistochemical study, on c-Met expression in tooth germs of 8 human foetus between the 7th and 9th week, was performed. In the bud stage c-Met moderately to strongly immunopositivity was detected in dental papilla, both in the inner and the outer epithelium of the enamel organ. In particular, moderate staining was detected in a specific portion of tooth germs that corresponds to the tip of the enamel organ. In the bell stage tooth germs were characterized by much stronger c-Met immunopositivity in the cytoplasm, inner enamel epithelium, bilateral cusps and above all in the plasma apical membrane on the mesenchymal side. In conclusion, because enamel organ cells can interact directly with mesenchymal cells, and c-Met is expressed in the stages at which mesenchymal induction is guided by the dental epithelium, it is conceivable that c-Met is related to tooth germ morphogenesis and cell differentiation.

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INVOLVEMENT OF RETINOBLASTOMA TUMOUR-SUPPRESSOR PROTEIN IN MINERAL LUNG EXPOSITION

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Recently studies have demonstrated a correlation between mesothelioma and fluoro-edenite fibers exposition. These fibers are very similar to asbestos' ones and induce functional modifications and biological cyto- and genotoxicity. However, the molecular mechanism involved in cells malignant transformations are still largely unknown. It has been shown recently that high level expression of the RB-family proteins as well as p53 have been detected in human mesothelioma. Retinoblastoma protein (RB) is a 928-amino acid nuclear phosphoprotein that plays a central role in the control of the cell cycle, cellular growth regulation, differentiation and apoptosis. pRB functions as a checkpoint during the G1 phase of the cell cycle; it is also involved in preventing cells from undergoing apoptosis. Phosphorylation of RB results in a consequent loss of its ability to inhibit cell cycle progression and S phase entry. Dephosphorylation of RB not only promotes cell cycle arrest and a return to the G1 phase, but also appears to be a key event in most instances of apoptosis.

In this study, we investigated for the first time in an ''in vivo'' sheep model the immunohistochemical expression of RB and pRB in lung tissues exposed to fluoro-edenite fibers.

The results showed an increased immunoexpression of pRB both in the alveolar epithelium and in the interstitium, above all localized in close contact to fluoro-edenite fibers. RB immunostaining was instead quite reduced or completely absent. Overexpression of pRB, therefore is suggested to be a programmed protective response of fluoro-edenite exposed lung, to uncontrolled proliferation.

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Acknowledgements will be placed at the end of the text.

References must be arranged in alphabetical order, as follows:

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

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