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SOCIETY FOR HISTOCHEMISTRY

GESELLSCHAFT FÜR HISTOCHEM

48th Symposium of the Society for Histochemistry

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Palazzo dei Congressi
Stresa - Lake Maggiore (Italy)
7-10 September, 2006

HISTOCHEMISTRY OF CELL DAMAGE AND DEATH

Organizer
Marco Biggiogera
University of Pavia - Italy

PROGRAM

September 7

10.00-15.30 REGISTRATION

15.30-16.00 OPENING CEREMONY

16.00-17.00 **Robert Feulgen Lecture**
D. Hernandez-Verdun: *The happy story of the nucleolus*

17.00-17.30 *Coffee break*

17.30-18.30 **Session 1. Cell cycle alteration and death**
Chair: D. Hernandez-Verdun, B. Mignotte

B. Mignotte: *p53 and Retinoblastoma protein (pRb) at the interface between cell cycle and cell death* **(European Journal of Histochemistry Lecture)**
A. Krumbholz, R. Zell, A. Eitner, H. Oehring, R. Krieg, K.J. Halbhuber, P. Wutzler: *Mitochondrial localisation of pro-apoptotic PB1-F2 of porcine influenza A viruses*
O. Cazzalini, P. Perucca, M. Savio, L.A. Stivala, A.I. Scovassi, E. Prosperi: *DNA damage response: multiple roles for p21CDKN1A protein*

20.00-22.00 WELCOME PARTY & DINNER

September 8

9.00-10.45 **Session 2. Cell damage induction and repair: nucleus**
Chair: K. Smetana, A. von Mikecz

A. Von Mikecz: *The nuclear response to damage: DNA repair and quality control by the ubiquitin-proteasome system*
L. Janderová-Rossmeislová, Z. Nováková, J. Vlasáková, P. Hozák, Z. Hodný: *PML protein association with specific nucleolar structures differs in normal, tumor and senescent human cells*
T.D. Rockel, A. von Mikecz: *DNA double-strand break repair and the involvement of the nuclear ubiquitin-proteasome-system (nUPS)*
A.L. Kleschyov, S. Strand, S. Schmitt, D. Gottfried, M. Skatchkov, N. Sjakste, A. Daiber, V. Umansky, T. Münzel: *Dinitrosyl-iron triggered apoptosis in Jurkat cells is insensitive to overexpression of Bcl-2*
B. Püschel, T. Reupke, P. Schwartz, C. Viebahn: *Live cell imaging and single cell ablation in living rabbit blastocysts using two-photon laser microscopy*
M.J. Coronado, N. Chakrabarti, J. Cortés-Eslava, M.C. Risueño, P.S. Testillano: *Stress-induced PCD in plant cells involves apoptotic nuclear domain rearrangements and expression of caspase 3-like proteins*

10.45-11.15 *Coffee break*

11.15-12.30 **Session 3. Cell damage induction and repair: cell membrane and cytoplasm**
Chair: C. van Noorden, A. Pompella

A. Pompella: *Production, detection and interpretation of cell injury data*
P. de Lanerolle, L. Gu, W.Y. Hu: *Inhibiting myosin light chain kinase induces apoptosis and synergizes with etoposide to inhibit the growth of breast tumors in mice*
W. Widlak, N. Vydra, E. Malusecka, B. Winiarski, P. Widlak: *Active HSF1 down-regulates spermatocyte-specific HSP70.2 protein and induces HSP70i-resistant apoptosis in male germ cells*
K. van Nierop, F.J. Muller, C.J.F. Van Noorden, M.C. van Eijk, C. de Groot: *Lysosomal destabilization contributes to apoptosis of germinal center B-lymphocytes*

13.00-14.30 *Lunch*

14.30-16.00 **POSTER SESSION**

16.00-16.30 *Coffee break*

16.30-17.45 **Lectures of the Robert Feulgen Prize Winners**
Chair: F. Wachtler

M. Nilsson: *Genotyping single DNA molecules in situ*
P.J. Verschure, J. Mateos-Langerak, M.C. Brink, W. de Leeuw, S. Fakan, A.S. Belmont, R. van Driel: *Functional chromosome organization in the interphase nucleus: epigenetic gene control*

18.00 SOCIETY MEETING

September 9

9.00-10.30 **Session 4. Molecular tracing in dying cells**
Chair: P. de Lanerolle, M.R. Duchen

M.R. Duchen: *Illuminating routes to cell death with live cell imaging* **(Apoptosis Lecture)**
S. Burattini, P. Ferri, M. Battistelli, A. D'Emilio, L. Biagiotti, E. Falcieri: *DNA fragmentation patterns during differently triggered apoptosis*
I. Paschkunova-Martic, B. Stark, M. Stollenwerk, C. Kremser, H. Talasz, E. Soelder, B. Hugl, H. Dietrich, K. Albrecht, F. Föger, B. Keppler, W. Jaschke, R. Prassl, P. Debbage: *Two nanoparticulate contrast agents for magnetic resonance imaging: characterisation and evaluation*
M.T. Olea, T. Nagata: *Radioautographic study on the localization of ^{3}H-thymidine in bone marrow cells*
A.C. Croce, U. De Simone, A. Ferrigno, M.P. Vairetti, I. Freitas, G. Bottiroli: *Autofluorescence as a parameter for metabolism impairment evaluation in liver tissue*

10.30-11.00 *Coffee break*

11.00-13.00 **Session 5. Phagocytosis of apoptotic cells**
Chair: A.A. Manfredi, C. Godson

C. Godson: *Taking insult from injury: Phagocytosis of apoptotic cells and the resolution of inflammation*
L. Ballarin: *Recognition and clearance of apoptotic cells in colonial ascidians*
D. Krysko*: Mechanisms of recognition and internalization of apoptotic and necrotic cells*
L. Dini: *Environment: adding further complexity to the apoptotic cell recognition scenario*

13.00-14.30 *Lunch*

14.30-16.15 **Session 6. Cell damage and death in pathology**
Chair: G. Losa, S. Muller

S. Muller: *Post-translational modifications, apoptosis and autoimmunity*
A.A. Manfredi, P. Rovere-Querini*: Immune outcomes of apoptotic cell clearance in health and disease*
I. Mylonas, J. Vogl, C. Kuhn, S. Schulze, U. Jeschke, K. Friese: *Inhibin/activin subunits are immunohistochemically expressed in complete and partial hydatidiform moles*
I. Freitas, S. Fracchiolla, R. Vaccarone, G. Milanesi, G.F. Baronzio, S. Barni, G. Gerzeli: *Lobular-zone dependent expression of alpha-fetoprotein in the liver of MMTV-neu (erbB-2) mice*
A. Tinari, E. Mormone P. Matarrese, M.G. Farrace, M. Piacentini, W. Malorni: *Self- and xeno-cannibalism in heterozygous and homozygous lymphoblasts from patients with Huntington disease*

16.15-16.45 *Coffee break*

16.45-18.15 **Session 7. Life needs death during development and differentiation**
Chair: I. Mylonas, Z. Zakeri

Z. Zakeri: *Cell death in shaping the embryo: a role for cell cycle genes* **(Histochemistry and Cell Biology Lecture)**
P. González-Melendi, M.J. Coronado, C. López-Iglesias, P.S. Testillano, M. C. Risueño: *Epigenetic marks of chromatin remodelling in microspore-derived embryogenesis: reprogramming vs. death*
F. Cima: *Cyclic apoptosis in the digestive tract of a protochordate*
A. Erman, K. Jezernik: *Urothelial cell desquamation during the mouse urinary bladder development*
D. Märker, C. Tag, M. Wimmer: *PTP interacting protein 51 in eye development during mouse embryogenesis*

18.30 CLOSING CEREMONY

September 10

Breakfast and departure

ORAL PRESENTATIONS

Robert Feulgen Lecture 2006

The happy story of the nucleolus

D. Hernandez-Verdun
Nuclei and cell cycle - Institut Jacques Monod, CNRS, Université Paris VI et VII, Paris, France.

First observed in the 19th century, the nucleolus has been the object of intense investigations. It is the ribosome factory of the cell and its size and organization in three components [fibrillar center (FC), dense fibrillar component (DFC) and granular component GC)] reveal its activity. Recently, the role of the nucleolus in cell cycle, apoptosis, and pathology was demonstrated. It is therefore crucial to know how nucleolar activity is assembled and maintained during the cell cycle. The nucleolus is the first active domain to be assembled after mitosis. Its functions depend on recruitment of the nucleolar machineries involved in processing of ribosomal RNAs (rRNAs) as well as rDNA transcription activation. During mitosis, the RNA polymerase I transcription machinery is assembled on ribosomal genes and repressed by the CDK1-cyclin B pathway. The proteins involved in rRNA processing are distributed around the chromosomes. The translocation of processing machineries on transcription sites will be presented as well as the discovery of stable mitotic pre-rRNAs participating in nucleolar assembly. Upon migration of the processing machinery from chromosome periphery to sites of rDNA transcription, prenucleolar bodies (PNBs) are formed. Interactions between processing proteins occur in PNBs before being recruited in nucleoli suggesting that PNBs are pre-assembly platforms for rRNA processing complexes. During interphase, the dynamics of compartmentation of the nucleolar machineries in DFC and GC demonstrate that the connection between DFC and GC is ATP/GTP-dependent, sensitive to temperature, and CK2-driven. In conclusion, the nucleolus is an excellent model to decipher the functional organization of the nucleus along the cell cycle.

Session 1

Cell cycle alteration and death

p53 and Retinoblastoma protein (pRb) at the interface between cell cycle and cell death

B. Mignotte, N. Godefroy, C. Lemaire, F. Renaud, I. Costina-Parvu, A. Rincheval, V. Rincheval, I. Guénal, J.L. Vayssière
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p53 and the Retinoblastoma protein (pRb) are two oncosuppressive proteins whose loss of function is linked to the development of most human cancers. These two proteins are able to inhibit cell cycle progression and to regulate apoptosis. We have shown that, in rat fibroblasts, caspase-9 is able to modulate the activity of p53 by antagonizing, in a paradoxical manner, its apoptotic properties (Lemaire et al. 2005; Oncogene, 24:3297-3308). We have also showed that p53 is able to induce a new apoptotic pathway which is both Bcl-2 and caspase-independent (Godefroy et al. 2004; Cell Death Differ, 11:785-787). This cell death pathway involves the transcriptional repressor activity of p53 and seems involve histone désacétylases (Godefroy et al. 2004; Nucleic Acids Res, 32:4480-4490) Last, we have identified a new truncated form of pRb ($p76^{Rb}$) which accumulates specifically in cells which cease proliferating without undergoing apoptosis following p53 activation. Unlike what is observed with full length pRb, overproduction of $p76^{Rb}$ permits cell survival and reduces the pro-apoptotic effect of the inhibition of the caspase-9. This suggests that this form could be the mediator of the action of caspase-9. On the basis of these data, since the caspase cleavage site generating $p76^{Rb}$ is conserved in the Drosophila ortholog of pRb (called RBF), we have undertaken a study of the role of the truncated form *in vivo* in the fly. Our results show that $p76^{RBF}$, contrary to full length RBF, does not exhibit a pro-apoptotic activity in imaginal wing disc. In contrast, in differentiated post-mitotic neuronal cells $p76^{RBF}$ and RBF both have an anti-apoptotic effect.

Mitochondrial localisation of pro-apoptotic PB1-F2 of porcine influenza A viruses

A. Krumbholz[(1)], R. Zell[(1)], A. Eitner[(2)], H. Oehring[(2)], R. Krieg[(2)], K.J. Halbhuber[(2)], P. Wutzler[(1)]
[(1)]Institute of Virology and Antiviral Therapy and [(2)]Institute of Anatomy II, Medical Center at the Friedrich Schiller University, Jena, Germany.

PB1-F2 is a pro-apoptotic polypeptide encoded by an alternative open reading frame (ORF) of segment 2 of influenza A virus (FLUAV) isolates. This protein has several unusual features as it is absent in many virus isolates and appears not to be essential for viral replication *in vitro*. Furthermore, a predominant mitochondrial localisation in appr. 50 percent of the PB1-F2 expressing cells was demonstrated. Several authors proposed that PB1-F2 interacts with mitochondrial membrane proteins. Cell type-dependent induction of apoptosis was observed in infected host immune cells. However, the absence of PB1-F2 in FLUAV isolates particular of porcine origin was postulated. In contrast, this study describes the expression of a full-length PB1-F2 ORF in many porcine and avian isolates. The PB1-F2 polypeptide of FLUAVs isolated from European pigs are detectable in the host cell as demonstrated by immunohistochemistry. In order to assess patterns of subcellular distribution of various porcine PB1-F2-EGFP constructs, transfected cells were labelled *in vivo* with the mitochondrium-specific fluorescence marker JenMitoStain-1 and analyzed by confocal laser scanning microscopy. After transfection, impaired mitochondrial bioenergetics was detected. A mitochondrial localisation was demonstrated for those PB1-F2 polypeptides which are greater than 78 amino acids while a truncated version shows a diffuse cytoplas-matic distribution. This indicates similar properties and function of human and porcine FLUAV PB1-F2. First preliminary ultra-structural studies on transfected cells revealed significant structurally damaged mitochondria and diminished cytochrome C oxidase activity.

DNA damage response: multiple roles for p21CDKN1A protein

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The cyclin-dependent kinase inhibitor p21*CDKN1A* plays a fundamental role in the DNA damage response by inducing cell cycle arrest, and by inhibiting DNA replication through association with the proliferating cell nuclear antigen (PCNA). Such an interaction occurs also during DNA repair, yet its role is still poorly understood. Here, we provide evidence that p21 protein is involved in nucleotide excision repair (NER), since both endogenous p21, and a chimeric p21-GFP protein, co-localise and interact with PCNA and NER proteins, like XPG, at UV-irradiated sites. In addition, we show that p21 appears to be also involved in base excision repair (BER), being p21-null fibroblasts more sensitive to treatment with the base alkylating drug, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). In BER pathway, an important player is the enzyme PARP1, which interacts with PCNA and p21. The kinetics of recruitment of PCNA, PARP1 and p21 to DNA damage sites, have been investigated in both wt- and p21-null fibroblasts after DNA damage. The results have suggested that in the absence of p21, an accumulation of PARP1 occurs at DNA damaged sites, as shown by fluorescence confocal microscopy and western blot analysis of chromatin-bound proteins. Interestingly, in normal fibroblasts, p21 co-localised with PARP1 at the nucleolar level, suggesting a specific interaction at this site. These results indicate that in addition to participate in checkpoint response, p21 protein is directly involved in different DNA repair pathways through its interaction with PCNA and PCNA-interacting proteins.

Session 2

Cell damage induction and repair: nucleus

The nuclear response to damage: DNA repair and quality control by the ubiquitin-proteasome system

A. von Mikecz
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The cell nucleus is compartmentalized and highly dynamic. Nuclear proteins cluster in distinct subnuclear domains such as nucleoli, speckles and other nuclear bodies that form in response to the requirements of gene expression. However, the dynamic properties of the cell nucleus may come at a price. Environmental changes stress cells and induce alterations of nuclear structure and function that eventually may cause generation of diseases. Since such postsynthetic damage is inflicted on both, DNA and nuclear proteins, this lecture will address quality control systems that enable repair of DNA and selective degradation of misfolded or misplaced proteins. Special emphasis will be laid on the nuclear ubiquitin-proteasome system (nUPS) that plays a major role in damage responses. In eukaryotic cells, components of the nUPS are essential for DNA repair, and the proteolytic activity of the 26S proteasome prevents the accumulation of non-functional, potentially toxic proteins.

PML protein association with specific nucleolar structures differs in normal, tumor and senescent human cells

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The nucleolus is the site of rRNA synthesis as well as a structure actively involved in the regulation of cellular proliferation and senescence. Promyelocytic leukemia protein (PML), a tumor suppressor crucial for induction of growth arrest, senescence or apoptosis, forms in most human cell types discrete multiprotein complexes termed PML

nuclear bodies. Functional link between PML and nucleolus upon extensive DNA damage has been recently uncovered. However, little is known about PML nucleolar localization under normal growth conditions or after inhibition of nucleolar transcription. We report that under standard growth conditions PML protein encircled nucleolus-derived structures (NDS) in human mesenchymal stem cells (hMSC) and skin fibroblasts but not in several immortal cell lines with defects in p53 and pRb pathways. In addition, shut-off of rRNA synthesis induced by actinomycin D caused PML translocation to the surface of segregated nucleoli. This translocation was dynamic and reversible, following changes in nucleolar activity. Intriguingly, PML binding to NDS and to the surface of segregated nucleolus was in HeLa restored by treatment causing premature senescence. Together, our data suggest that PML may be involved in nucleolar functions of normal non-transformed or senescent cells. The lack of PML nucleolar association in several rapidly growing tumor-derived cell lines and its restoration in prematurely senescent cells indicates the functional link between PML and nucleolus necessary for cell cycle regulation.

DNA double-strand break repair and the involvement of the nuclear ubiquitin-proteasome-system (nUPS)

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DNA in a living cell is often subject to chemical alterations. If the genetic information encoded is to remain uncorrupted, these DNA modifications must be corrected. DNA repair events split into a damage detection event followed by the recruitment of multi-protein machines containing the repair capacity. A defined maintenance system is the cells' repair mechanism for DNA double-strand breaks (DSB): The breakage of the chromatin results in a specific phosphorylation of H2AX yielding a modified histone variant form named γH2AX. γH2AX reacts as a damage marker and attracts the multi-protein complex MRN (assembled from MRE11, RAD50 and NBS1). MRN sticks to the open DNA ends, keeps them in closed proximity and recruits additional repair factors. Whereas the assembling processes of DSB repair is well described, little is known about the disassembling and termination mechanisms. Since we have shown recently that the nucleus posses its own functional ubiquitin-proteasome-system (nUPS), we are investigating the role of proteasomal proteolysis in DNA repair. Here we show that DNA damaging agents (UV, γ-radiation, etoposide) induce different DNA damage patterns, which are typical for the specific agent. In time kinetic studies we followed the appearance, recruitment and localization of γH2AX, MRE11 and areas of proteasomal activity (proteolytic foci). We report that sites of DNA damage/repair primarily localize to euchromatic regions of the cell nucleus and partially colocalize with proteolytic active proteasomes.

Dinitrosyl-iron triggered apoptosis in Jurkat cells is insensitive to overexpression of Bcl-2

A.L. Kleschyov[(1)], S. Strand[(1)], S. Schmitt[(1)], D. Gottfried[(1)], M. Skatchkov[(1)], N. Sjakste[(2)], A. Daiber[(1)], V. Umansky[(3)], T. Münzel[(1)]
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Cells expressing the cytokine-inducible NO synthase are known to trigger apoptosis in neighbouring cells. Paramagnetic dinitrosyl nonheme iron complexes (DNIC) were found in tumour tisuues about 40 years ago; however, the role of these NO+-bearing species is not completely understood. In the human Jurkat leukaemia cell line, the application of the model complex DNIC-thiosulfate (50-200 μM) induced apoptosis defined by phosphoserine externalization and chromatin fragmentation in a concentration- and time-dependent manner. In Jurkat cells, the pan-caspase inhibitor zVADfmk (50 μM) and/or stable transfection of antiapoptotic protein, Bcl-2, was unable to protect against apoptosis. The membrane-impermeable iron chelator, *N*-methyl-D-glucamine dithiocarbamate (MGD; 200 μM), in the presence of DNIC significantly increased apoptosis, but had no effect on its own. Electron paramagnetic resonance studies showed that MGD led to rapid transformation of the extracellular DNIC into stable imperemeable NO-fe-MGD complex and to a burst-type release of nitrosonium (NO+) equivalents in the extracellular space. These results suggest that in Jurkat cells, DNIC-thiosulfate induces Bcl-2- and caspase-independent apoptosis, which is probably secondary to local nitrosative

stress at the cell surface. We suggest that the local realease of non-heme Fe-NO species by activated macrophages may play a role in killing of malignant cells that have high Bcl-2 levels.

Live cell imaging and single cell ablation in living rabbit blastocysts using two-photon laser microscopy

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The anterior marginal crescent (AMC) is an area of high cell density in the hypoblast emerging immediately prior to the onset of gastrulation in the mammalian embryo and represents the first morphological sign of the anterior-posterior axis. However gene expression analysis for signalling molecules such as Dkk1 or Cer1 revealed that anterior-posterior axis is formed as a molecular prepattern in the hypoblast prior to AMC formation. Experiments manipulating the hypoblast (visceral endoderm) in the mouse suggest that this prepatterning is accompanied by movements and proliferation of hypoblast cells. To approach the question as to whether a causal relationship exists between the pattern of signalling molecules and cellular movements we decided to use two-photon laser microscopy on DAPI stained rabbit blastocyst for time lapse recording. Using high intensity infrared laser pulses confined to one cell nucleus in the focal plane we were also able to destroy single hypoblast cells. Continuing live cell imaging produced several fold higher fluorescence intensity in the irradiated nucleus indicating cell damage while surrounding nuclei showed normal fluorescence levels and normal behaviour suggesting undisturbed surrounding embryonic tissue. For ultrastructural examination embryos were fixed either immediately after the irradiation procedure or after a maximum culture period of 5h. Control embryos examined in a conventional confocal setting (405 nm laser) using minimum intensities, regularly developed high cell death in both epiblast and hypoblast. In contrast, two-photon microscopy produced less cell death even after longer examination times thus emphasizing a clear benefit over conventional (UV) laser imaging in examining living embryos.

Stress-induced PCD in plant cells involves apoptotic nuclear domain rearrangements and expression of caspase 3-like proteins

M.J. Coronado, N. Chakrabarti, J. Cortés-Eslava, M.C. Risueño, P.S. Testillano
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Programmed cell death (PCD) is a fundamental physiological process characterized by a series of cellular events which have been described in great detail in animal cells, but in plants the different ways of death, as well as, their corresponding normal features, are not well established. In plants and animals, under the influence of variuos enviromental factors (pathogens, chemical and physical agents), some varieties of programmed cell death along with apoptosis exist as a result of activation of specific internal triggers of cell suicide. In this work, a PCD process was induced in root tip meristems by salt-stress. Ultrastructural cytochemical and immunocytochemical analysis showed structural changes similar to those established in animal cell apoptosis. Sequential events occurring from the very early to the final death stages have been characterized in the nucleus. Different nuclear markers were used to identify the changes in nuclear domains and the formation of apoptotic bodies. Results showed that chromatin, interchromatin and nucleolar components become segregated at late stages, and interchromatin RNPs rearranged in HERDs-like bodies, new structures in plants which can also be considered markers of transcriptional arrest, as proposed in mammalians. In situ cleaved-caspase 3 has been localized by immunofluorescence for the first time in plants and also, specific protease activities have been detected by biochemical caspase essays in stress-induced apoptotis. The observations of this cell death pathway defined apoptotic features, and showed new evidences of caspase-like expression and activity in plants.

Supported by Projects BFU2005-01094 and AGL2005-05104.

Session 3

Cell damage induction and repair: cell membrane and cytoplasm

Production, detection and interpretation of cell injury data

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Ever since the seminal perception by R. Virchow, indicating the living cell as the "physiological and pathological unit of the body", the investigation of biochemical changes occurring at the single cell level has represented the most fruitful approach for the comprehension of mechanisms of disease. With special reference to cell membrane and cytoplasm, the most accurate information has been provided by studies on toxic cell injury, i.e. damage produced by a wide variety of chemical agents. Oxidation/reduction (redox) processes are usually involved, which can eventually produce major modifications in the structure of membrane phospholipids (lipid peroxidation and fragmentation, with production of reactive aldehydes and carbonyls) and/or proteins (loss of reduced sulfhydryls, direct aminoacid oxidation, binding of lipid peroxidation products). Such pathological changes can be exploited as reliable histo- and cytochemical end-points, using the sensitive and useful techniques described by Frank et al. (2000; Histol Histopathol, 15:1731-1784; Free Rad Biol Med, 29:1096-103). Care must be taken however in the interpretation of results, bearing in mind that the sequence of events observed in trials in vitro may not reflect the real situation in vivo, where several additional factors can affect the development of phenomena. This concept is illustrated by the cell-bound enzyme activity gamma-glutamyltransferase, which can display both antioxidant and prooxidant effects depending on the experimental conditions used (Dominici et al. 2005; Meth Enzymol, 401:484-501). Thus, in some instances the defensive advantage offered by expression of this enzyme will be apparent, while in other circumstances the same activity can be exploited in order to produce site-specific cell damage in tissues.

Inhibiting myosin light chain kinase induces apoptosis and synergizes with etoposide to inhibit the growth of breast tumors in mice

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Increased cell proliferation and motility, signature features of metastatic cancers, require profound changes in the cytoskeleton. The organization of the cytoskeleton is determined primarily by actin and myosin II. The actin-myosin II interaction in smooth muscle and nonmuscle cells is regulated by the phosphorylation of the 20 kD light chain of myosin II by myosin light chain kinase (MLCK). Myosin phosphorylation (MLC-P) and de-phosphorylation (MLC-DP) are required for smooth muscle contraction/relaxation, cell motility and cytokinesis. We have also shown that inhibiting MLCK is sufficient to induce apoptosis of smooth muscle cells. Therefore, we tested the effects of inhibiting MLCK on breast cancer cells using ML-7, a potent and selective inhibitor of MLCK (Ki = 0.3 μM). *In vitro,* ML-7 treatment resulted in a dosedependent increase in apoptosis in murine mammary tumor cells and 10 mM ML-7 potentiated the ability of etoposide to induce apoptosis (IC50 = 25.4 and 572 mM etoposide +/- ML-7). To test for *in vivo* effects, adult female mice were injected with 106 cells subcutaneously and treated with vehicle, ML-7, etoposide or ML-7 plus etoposide. Drugs were started 1 week after the cells were injected and the mice were sacrificed after 4 weeks of drug treatment. ML-7 and etoposide both decreased tumor growth. Importantly, the combination of ML-7 and etoposide dramatically reduced tumor growth compared to mice receiving vehicle (88.5% inhibition of tumor growth, $P<0.001$) and to mice receiving etoposide alone ($P<0.05$). Thus, inhibiting MLCK is sufficient to induce apoptosis in cancer cells and that ML-7 is an effective adjuvant in preventing tumor growth *in vivo*.

Active HSF1 down-regulates spermatocyte-specific HSP70.2 protein and induces HSP70i-resistant apoptosis in male germ cells

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Apoptosis of male germ cells can be induced by different types of environmental insults, including heat shock. In somatic cells an elevated temperature activates heat shock transcription factor 1 (HSF1) that activates expression of inducible heat shock proteins (HSP), mostly HSP70i, which have cytoprotective and anti-apoptotic functions. In contrast, highly thermosensitive male germ cells – spermatocytes – do not induce HSPs in response to hyperthermia. Expression of constitutively active HSF1 in spermatocytes of transgenic mice does not lead to activation of inducible *Hsp70* genes, but induces caspase-dependent apoptosis that leads to male infertility. We have found that both mitochondria-dependent and death receptor-dependent pathways are involved in such HSF1-induced apoptosis. Additionally, the constitutive spermatocyte-specific expression of HSP70i in transgenic males does not protect against apoptosis and degeneration induced by either active HSF1 or hyperthermia resulting from experimental cryptorchidism. However, we have found a marked down-regulation of endogenous spermatocyte-specific *Hsp70.2* gene prior to the onset of HSF1-induced apoptosis. During later developmental stages germ cells undergoing HSF1-induced apoptosis are essentially lacking the HSP70.2 protein, which suggests a functional relationship between down-regulation of the HSP70.2 and the degeneration of a seminiferous epithelium. The DNA sequences responding to such repression were mapped to the immediate promoter region of the *Hsp70.2* gene, but none of them was directly associated with HSF1. The down-regulation of *Hsp70.2* gene expression by HSF1 is paradoxical because HSF1 is the prototypical activator of HSP genes.

Lysosomal destabilization contributes to apoptosis of germinal center B-lymphocytes

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Germinal centers (GCs) are specialized structures in lymphoid follicles of secondary lymphoid organs where B-lymphocytes undergo affinity maturation of their B-cell receptors (BCRs) as well as immunoglobulin (Ig) isotype switching. This results in the formation of plasma cells and high-affinity memory B-cells. During a GC reaction, B-cells are selected and their antigen specificity is checked at different levels. Anti-apoptotic signals provided by follicular dendritic cells (FDCs) are crucial in this selection process, as FDCs present native antigens as immune complexes to the B-cells. B-cells with high affinity BCRs bind to these antigens on the basis of competition and receive survival signals from the FDCs. Low-affinity, non-binding B-cells rapidly die by apoptosis. The mechanisms by which FDCs silence apoptosis in GC B-cells have been clarified only partially. Apoptosis in GC B-cells is mainly induced via the death receptor pathway. We have shown that apoptotic DNA fragmentation in GC B-lymphocytes cannot only be blocked by inhibition of caspase-8 or caspase-3, but also by inhibition of cathepsins (Van Eijk and De Groot 1999; J Immunol, 163:2478-2482; Van Eijk et al. 2003; Int Rev Cytol, 222:197-236). Since cathepsins are mainly lysosomal enzymes, we decided to investigate in more detail a possible role of lysosomes in GC B-cell apoptosis. Lysosomal destabilization has been implied in apoptosis under influence of several stimuli. As a result, lysosomal proteases such as cathepsins translocate from the lysosomes to the cytosol. It has been shown that several cathepsins, especially the cysteine cathepsins B and L and the aspartyl cathepsin D, participate in apoptosis in caspase-dependent and caspase-independent manners. One of the possible mechanisms in which cathepsins trigger apoptosis is cleavage of the Bcl-2 family member Bid resulting in mitochondrial inactivation. We applied the lysosomotropic drug O-methyl-L-serine dodecylamide hydrochloride (MSDH), which specifically induces lysosomal destabilization. MSDH accumulates in the lysosomes by protonation and induces lysosomal leakage and translocation of cathepsins into the cytosol, resulting in apoptotic features. These included phosphatidyl serine exposure, mitochondrial inactivation and the formation of DNA strand breaks, in the absence of caspase-3 activation. The features were visualized and quantified in

individual GC B-lymphocytes using confocal scanning laser microscopy and flow cytometry. FDCs protect GC B-lymphocytes that are bound from lysosomal destabilization, both in the absence and presence of MSDH. Our study demonstrates that lysosomal leakage is an early event in apoptosis of GC B-cells and high-affinity binding to FDCs prevents lysosomal leakage and apoptosis in GC B-cells.

Lectures of the Robert Feulgen Prize Winners

Genotyping single DNA molecules *in situ*

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Techniques will be described that allow analysis at the ultimate level of single nucleic acid molecules in individual cells. Single-copy gene specificity is obtained by applying padlock probes that become circularized through DNA ligation upon identification of a specific DNA (Nilsson et al. 1994; Science, 265:2085-2088) or RNA (Nilsson et al. 2000; Nat Biotechnol, 18:791-793) sequence. To obtain robust single-molecule detection, circularized probes are amplified in a rolling-circle amplification reaction, producing tandem repeated copies of the probe sequence. Rolling-circle products spontaneously form micron-sized coils that can be observed and enumerated using microscopy by hybridization of fluorescence labeled tag-oligonucleotides (Blab et al. 2004; Anal Chem, 76:495-498). This digital detection format allows multiplexed quantification of specific DNA sequences with high dynamic range and precision (Jarvius et al. submitted). The technique can also be applied for detection of individual target DNA molecules *in situ*. This was demonstrated in a study where point mutations in mitochondrial DNA were genotyped *in situ* (Larsson et al. 2004; Nat Meth, 1:227-232). We are now extending the technology to analyses of single-copy nuclear genes (Henriksson et al. in preparation), and their transcripts (Larsson et al. in preparation) in fixed cells and tissue sections.

Functional chromosome organization in the interphase nucleus: epigenetic gene control

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Changes in chromatin structure are a key aspect of the epigenetic gene expression control. Little is known about in vivo mechanisms that establish and maintain functional genome organization. We studied the functional genome packaging using high resolution light microscopy in fixed and living cells, as well as electron microscopy approaches in combination with sophisticated 3-D image analysis and processing tools. Interphase chromosomes were found to represent open structures consisting of condensed chromatin fibers. Specific genomic loci were found to occur at condensed chromatin domain surfaces (Cmarko et al. 1999; Mol Biol Cell, 10:211-223; Verschure et al. 1999; J Cell Biol, 4:13-24; Verschure et al. 2002; J Histochem Cytochem, 50:1303-1312; Cmarko et al. 2003; J Cell Science, 116:335-343). Condensed chromatin was shown to be accessible for large macromolecules, demonstrating that other mechanisms than inaccessibility determine gene silencing in heterochromatin (Verschure et al. 2003; EMBO Rep, 4:861-866). Using a lac-operator-repressor system in living mammalian cells, we showed that HP1 targeting to amplified chromosome regions causes local heterochromatinization (Verschure et al. 2005; Mol Cell Biol, 25:4552-4564; Brink et al. 2006; Histochem Cell Biol, 125:53-61). The next step is to unravel complex molecular pathways of epigenetic gene control.

Session 4
Molecular tracing in dying cells

Illuminating routes to cell death with live cell imaging

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Loss of cell function, injury and ultimately cell death underpin the manifestation of many disease states. The development of physiological indicators for a wide range of variables and increasingly powerful fluorescence imaging techniques allow measurements of changing cell physiology and biochemistry and characterisation of the converging processes that result in cell injury. The technology can be applied to a range of preparations including isolated cells or to tissue slices. In order to illustrate the approach, I will focus on a study of the mechanisms generating reactive oxygen species (ROS) during the progression of ischaemia (deprivation of oxygen and glucose, OGD) and reoxygenation in neurons. We have used an array of fluorescent indicators to explore the relationships between free radical generation and the evolution of changing cellular biochemistry with time. At the onset of hypoxia or inhibition of respiration, mitochondria generate a burst of ROS which is curtailed once mitochondria depolarise. After a delay, a second phase of ROS generation begins, correlating with ATP consumption and prevented by xanthine oxidase inhibitors. The NADPH oxidase, apparently activated by an increase in calcium during anoxia, is the major contributor to ROS production on reoxygenation. Glutathione is depleted. Inhibition of either the NADPH oxidase or xanthine oxidase are significantly neuroprotective and conserve glutathione levels. Thus, oxidative stress contributes to cell death over and above the injury inevitably attributable to energy deprivation, justifying inclusion of antioxidant strategies in patient management.

DNA fragmentation patterns during differently triggered apoptosis

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Apoptosis is characterized by typical morphological changes and biochemical events. A common hallmark of apoptosis is considered DNA cleavage, initially producing large fragments (50-300 KBP). Later, nucleosomic/oligonucleosomic fragments appear, also identifiable as a ladder of approximately 180 BP and multimers, at the analysis by agarose gel electrophoresis. 3'-OH ends of DNA strand breaks can be detected in apoptotic nuclei *in situ* by TUNEL technique, since the terminal deoxynucleotidyl transferase is able to attach labelled nucleotides to these DNA break points. Previous investigations demonstrated that apoptosis is not always accompanied by DNA fragmentation even if chromatin condensation occurs (Renò et al. 1998; Histochem Cell Biol, 110:467-76). In order to investigate the relationship between these two processes, in the present study, we applied TUNEL technique to the ultrastructural analysis of apoptotic cells. Thus, a modified TUNEL technique, revealed by a gold-conjugated anti-digoxigenin antibody, as previously performed (Goping et al. 1999; J Histochem Cytochem, 47:561-68; Lossi et al. 2002; Neuroscience, 112:509-23), was carried out on U937 monocytoid cells, induced to apoptosis by staurosporine and UVB-treatment, in comparison to Molt-4 T-lymphoid cells. In apoptotic cells, gold labeling was more strongly localized in condensed chromatin than in the diffuse one; at late stages of apoptosis, micronuclei showed the most intense gold labeling. Anyway, TUNEL labeling was more intense in U937 than Molt-4. This technique demonstrates not only the presence of DNA fragmentation but also allows to detect a precise localization of DNA break points within the different chromatin domains.

Two nanoparticulate contrast agents for magnetic resonance imaging: characterisation and evaluation

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Nanoparticles are under investigation across the world, to identify those most appropriate for particular clinical applications. Our interdisciplinary project has prepared and characterised nanoparticles for use in magnetic resonance imaging (MRI). This report presents a liposomal and a coblock polymer formulation, and discusses their suitability for MRI. Nanoparticles bearing gadolinium were synthesized, and characterised by physical methods including SAXS and PCS, by chemical methods and by both TEM and SEM. Monodisperse suspensions were perfused into human vital blood vessels ex situ (V.-saphena magna, placental cotyledons) and into rats intravenously, and MR images obtained (field strength 1.5 Tesla). MR images ("blood pool" type) were obtained in human and rat vessels. Features of the images related to the properties of the nanoparticles. Evidently, nanoparticles of entirely different chemistries can be used as MRI contrast agents. Fine details of MR imaging may relate to the chemical nature of the particles.
The Nanohealth Consortium (Austrian Nano-Initiative) and the Austrian FWF and the Austrian National Bank supported this work.

Radioautographic study on the localization of 3H-thymidine in bone marrow cells

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The different types of blood cells are derived from a common pluripotent stems cells and generated chiefly in the bone marrow. At embryonic stage, pluripotent stem cells circulating in the blood stream are able to settle in the bone marrow, spleen and/or liver and establish new hematopoietic colonies in those organs. By radio-autographic approach using a radioisotope precursor one can elucidate the rate of cell proliferation and growth, and cell kinetics in a specific organ (Nagata 2001; Int Rev Cytol, 211:133-153). The present study was undertaken to identify differentiating cells by radioautographic localization of 3H-thymidine uptake in the mouse bone marrow and to determine the labeling index in the different ages of the animal from day one after birth up until the 10th month. The results of intracellular sites of radio-autographic localization of 3H-tymidine are shown in Figs 1-2. Silver grains due to the uptake of radiolabeled precursor were observed in the hematopoietic cells such as the erythroblasts, myeloblasts and monoblasts. The results of the quantitative analysis showed that DNA synthesis increased consistently from one day to 10 month old animals. An average of 10% labeled cells was recorded at one-day-old animals, increased slightly to about 15% in 7-day-old animals. At 14th day after birth, a labeling index of 25% was recorded. This was the highest activity recorded and thereafter, the number of labeled cells as well as the labeling index decreased. DNA synthetic activity recorded the least at 5% in almost a year old senescent animals. The number of silver grain per labeled cell was compared with the age of the animals. The average grain count showed a positive correlation with the labeling index ($p<0.05$). From the results of the present study, it could be inferred that DNA synthesis is highest among the younger animals and declined as animal ages. Furthermore, it could be inferred that the erythroblasts and some white blood cell precursors are actively undergoing DNA synthesis in bone marrow in young animals. Similar results were observed with mouse spleen cells undergoing DNA synthesis (Olea and Nagata 1992; Cell Mol Biol, 38:115-122) and protein synthesis (Olea and Nagata 2003; Ann Microsc, 3:70-84).

Autofluorescence as a parameter for metabolism impairment evaluation in liver tissue

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Autofluorescence is an intrinsic parameter of cells and tissues in close relationship with their morphology and metabolic engagement, its emission properties being dependent on nature, amount, physico-

chemical state, and distribution of endogenous fluorophores. In liver tissue, because of the multiple functions exerted, several fluorophores can be found: microspectrofluorometric analysis under excitation at 366 nm evidenced noticeable contributions by proteins, NAD(P)H, flavins, vitamin A, arachidonic acid, and lipopigments. Spectral fitting analysis of the overall autofluorescence emission allows to asses the contributions of the coenzymes strictly involved in energetic metabolism - bound and free NAD(P)H, flavins – thus providing a tool to monitor the liver functionality and investigate the redox reaction pathways. The rationale of the diagnostic approach consists in the evaluation of tissue autofluorescence response upon experimental stimuli (namely, hischemia/reoxygenation). Both time constant and signal amplitude associated to autofluorescene intensity decay upon reoxygenationare are expressive of the impairment of mitochondrial respiration pathway providing for NAD(P)H re-oxidation. The autofluorescence properties of livers submitted to treatments mimicking the organ transplantation phases (cold-ischemia, rewarming-reoxygenation), and of livers from eu- and hyper-thyroid rats were analyzed in situ via fiber optic probe spectrofluorometer. The dynamic of autofluorescence response was found to be strictly related to the extent of tissue metabolic alterations, as evaluated through histological and biochemical assays.
Supported by MIUR "COFIN 2004".

Session 5

Phagocytosis of apoptotic cells

Taking insult from injury : Phagocytosis of apoptotic cells and the resolution of inflammation.

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It is increasingly appreciated that the resolution of inflammation is a dynamically regulated process subverted in several chronic inflammatory conditions.
Phagocytosis of apoptotic cells plays a pivotal role by sparing injured tissue from exposure to the noxious and potentially immunogenic contents of necrotic cells by mechanisms coupled to the release of anti-infammatory cytokines.
Here we will describe molecules involved in the recognition and clearance of apoptotic cells including endogenously derived mediators of phagocytosis such the lipoxy-genase derived eicosanoids, lipoxins. Lipoxins stimulate phagocytic clearance of apoptotic leukocytes in vitro and in vivo through specific receptor-mediated actions on phagocytes coupled to rearrangement of the actin cytoskeleton.

Recognition and clearance of apoptotic cells in colonial ascidians

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The colonial ascidian *Botryllus schlosseri* forms new zooids by blastogenesis, through the formation of palleal buds which progressively grow and mature until an adult is formed. At a temperature of 19°C, adult zooids remain active for about one week; then they contract, close their siphons and are gradually resorbed, being replaced by buds which reach functional maturity, open their siphons and begin their filtering activity as adult zooids. This recurrent generation change, known as regression or take-over, is characterised by the occurrence of diffuse programmed cell death by apoptosis. During the take-over, circulating phagocytes infiltrate in zooid tissues and engulf apoptotic cells; in addition, the frequency of haemocytes showing nuclear condensation and annexin-V labelling significantly

increases. Moreover, the number of circulating phagocytes showing a globular morphology and containing ingested cells or cell debris significantly rises whereas the frequency of hyaline amoebocytes, which represent mobile, active phagocytes decreases. Phagocytes, both professional and occasional, actively recognise senescent cells and ingest them. As regards the eat-me signals, PS seems to be involved in the recognition of effete cells, as the addition of phospho-L-serine, a soluble analogue of PS, inhibits *in vitro* phagocytosis of apoptotic cells. CD36, a part of the receptorial complex binding thrombospondin which act as a bridging molecule between phagocyte surface and apoptotic cells, is expressed on *Botryllus* phagocytes: the frequency of cells recognised by anti-CD36 antibodies significantly increases during the take-over and the expression pattern changes from a patchy distribution to a uniform staining of the phagocyte surface during the take-over. Anti-CD36 antibodies significantly decreases the phagocytosis of effete cells suggesting that similarly to that described in Vertebrates the thrombospondin receptor play a role in apoptotic cell removal by phagocytes.

Mechanisms of recognition and internalization of apoptotic and necrotic cells

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The ultimate and most favourable fate of almost all dying cells is engulfment by neighbouring or specialized cells. Efficient clearance of cells undergoing apoptotic death is crucial for normal tissue homeostasis and for the modulation of immune responses. Engulfment of apoptotic cells is finely regulated by a highly redundant system of receptors and bridging molecules on phagocytic cells that detect molecules specific for dying cells. Among the changes on the surface of the apoptotic cells that facilitate their recognition, the best characterized is the loss of phospholipid asymmetry and the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the lipid bilayer, which occurs very early during the apoptotic process. Recognition of necrotic cells by phagocytes is less well understood than recognition of apoptotic cells, but an increasing number of recent studies, are highlighting its importance. Unexpectedly, it was shown that necrotic cells also externalize PS and that they can be recognized through a PS-dependent mechanism, although less effectively than apoptotic cells. New observations indicate that the interaction of macrophages with dying cells initiates internalization of the apoptotic or necrotic targets, and that internalization can be preceded by "zipper"-like and macropinocytotic mechanisms, respectively. Here we discuss recent findings on mechanisms of internalization, and molecules involved in engulfment of apoptotic versus necrotic cells, as well as their immunological consequences.

Environment: adding further complexity to the apoptotic cell recognition scenario

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Apoptotic cells are usually cleared by phagocytosis in vivo with a rapid and efficient process aimed to removed damaged cells and components and to inhibit inflammation. Indeed, phagocytosis of apoptotic cells is the last, but very important step of the apoptotic program. The removal of apoptotic cell is mediated by specific epitopes, externalized during the formation of the apoptotic cells, on the dead cells and by specific receptors on the phagocytes. Phagocytosis requires distinguishing features on the surface of apoptotic cells, recognition and tethering molecules, as well as the cytoskeletal and other cellular machinery involved in engulfment. If the details of the interaction between apoptotic cells and phagocytes are still far to be fully understood, even more obscure are the external and/or physiological factors able to modulate the process of phagocytosis of apoptotic cells. Since cell surface modifications are fundamental for a correct removal of apoptotic cells, it may be possible any factors influencing cell surface molecules expression (phagocyte and /or apoptotic cells) can in turn affect recognition of apoptotic cells. Increasing bulk of evidence are indicating biological effects of EMF (electromagnetic field) and MF (magnetic field), static as well as oscillating

on one side, while the number of machine and electronic device are increasing also in the medical practice. In particular, converging data indicate that the primary site of action of EMFs is the plasma membrane. Therefore, we studied the recognition of apoptotic lymphocytes by liver sinusoidal cells under exposure to 6 mT static magnetic field to define at which extent static magnetic fields are able to modulate this specific type of phagocytosis. Taking into account, that the physiological conditions and/or the developmental story of the cells affect the response to the static MFs exposure, in our study we investigated also the effect of the age to the exposure to static MF and their removal from liver circulation. It is worth noting that liver is the main site for apoptosis of blood cells, either as a battlefield or as a graveyard. We found that the recognition of apoptotic lymphocytes by the sinusoidal liver cells is affected by environmental stress, like exposure to static MF, as well as by the metabolic state , i.e. aging of the cells to be removed. In particular, the exposure to static MF has been found to be an extremely critical factor for the recognition of not only apoptotic lymphocytes, but especially for control ones.

Session 6

Cell damage and death in pathology

Post-translational modifications, apoptosis and autoimmunity

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Autoimmune diseases are characterized by antibodies that recognize a large array of self components. Recent studies have attributed an important role for apoptotic and non-apoptotic cell death in mediating tolerance breaking to these self autoantigens. These different forms of cell death occur in normal situations and generate new structures such as typical cleavage fragments or components containing post-translational modifications against which the immune system has been tolerized at the early stage of thymus development. However, deletion of autoreactive lymphocytes is not complete and under certain circumstances remaining lymphocyte subpopulations might be involved in the early stages of the autoimmune spiral. Thus, it is anticipated that any defect in apoptosis or non-apoptotic cell death can either generate fragments with uncommon modifications (generation of neo-epitopes) or delay elimination of modified/cleaved antigens, which might trigger the activation and proliferation of remaining autoreactive lymphocytes and potentially lead to an autoimmune disease. In our presentation, we will concentrate our discussion on the post-translational modifications, which intriguingly have been shown to be required for autoantibody recognition. We will also address the question of the potential role of such post-translational modifications that might trigger both a T- and a B-cell anti-self response at a very early stage of life, and lead after diversification of the immune response to a generalized response to unmodified self-antigens. Finally, we will discuss how modified self-antigens constitute a promising source of therapy to treat autoimmune diseases.

Decker et al. (2000 ; J Biol Chem, 275:9043-9046); Monneaux et al. (2003; Eur J Immunol, 33:287-296; 2004; Arthritis Rheum, 50:3232-3238; 2005; J Immunol, 175:5839-5847)

Immune outcomes of apoptotic cell clearance in health and disease

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The recognition of apoptotic cells plays a key role in tissue repair and possibly in establishing peripheral tolerance. In contrast, apoptosis leads to the activation of autoreactive T cells and the production of autoantibodies. We discuss the pathogenic potential of cells dying in vivo, dissecting the role of signals that favour immune responses (adjuvants) and the influence of genetic backgrounds. Diverse factors determine whether apoptosis leads or not to a self-sustaining, clinically apparent autoimmune disease. The *in vivo* accumulation of uncleared dying cells *per se* is not sufficient to cause disease. However, dying cells are antigenic and their complementation with immune adjuvants causes lethal diseases in predisposed lupus-prone animals (Manfredi et al. 2005; Arthritis Rheum, 52:11-15). At least some adjuvant signals directly target the function and the activation state of antigen presenting cells. Several laboratories are aggressively pursuing the molecular identification of endogenous adjuvants. The high mobility group B1 protein (HMGB1) is an intriguing candidate to bridge cell death and bystander inflammation (Dumitriu et al. 2005; Trends Immunol, 26:381-387). However, even the complementation of apoptotic cells with potent adjuvant signals fail to cause clinical autoimmunity in most strains: autoantibodies generated are transient, do not undergo to epitope/spreading and do not cause disease. Novel tools for drug development will derive from the molecular identification of the constraints that prevent autoimmunity in normal subjects (Baruah et al. 2006; Blood, 107:151-158).

Inhibin/activin subunits are immuno-histochemically expressed in complete and partial hydatidiform moles

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Inhibins are dimeric glycoproteins, belonging to the TGF-β, that are composed of an α-subunit (INH-α) and one of two possible β-subunits (βA or βB; INH-βA and INH-βB). Additionally two further β-subunits (βC or βE; INH-βC and INH-βE) have been cloned, although their function remain still quite unclear. The detection by immuno-histochemistry of inhibin/activin subunits has been recently proposed as a useful marker of trophoblastic diseases. Interestingly, complete mole cannot be easily differentiated from partial mole. Therefore, the aim of this study was to determine expression changes of the five inhibin/activin subunits in partial and complete moles. Histologically diagnosed complete (n=6) and partial (n=3) hydatidiform moles were immuno-histochemical analyzed for INH-α, INH-βA, INH-βB, INH-βC and INH-βE subunits. The immunohistochemical reaction in intermediate trophoblast was analyzed with a semi-quantitative score (IRS) and statistical analysis was performed. Immunohistochemical reaction with INH-α, INH-βA, INH-βB, INH-βC and INH-βE subunits could be demonstrated in hydatidiform moles. The INH-βA and INH-βB expression was significantly higher in complete compared to partial moles ($p<0.05$ each), while INH-α, INH-βC and INH-βE did not demonstrate any statistically significant differences. We demonstrated an immunohistochemical expression of all five inhibin/activin subunits in partial and complete hydatidiform moles. The expression of INH-βA and INH-βB were immunohistochemical significantly up regulated in complete moles, suggesting the utilization of these antibodies as diagnostic differentiation markers between complete and partial moles.

Lobular-zone dependent expression of alpha-fetoprotein in the liver of MMTV-neu (erbB-2) mice

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Female MMTV-*neu* (*erbB*-2) transgenic mice are models of human breast cancer giving metastases in the lungs. We have reported alteration of liver differentiation without parenchyma destruction nor secondary tumor growth to the liver in these animals (Freitas et al. 2003; Anticancer Res, 23:3783-3794). The expression by liver cells of the oncofetal marker α-fetoprotein (AFP), which in the adult is an index of regeneration

and/of hepatocarcinogenesis (Abelev and Eraiser 1999; Semin Cancer Biol, 9:95-107), will be presented. AFP immunolabelling started in periportal hepatocytes in a stage where liver sinusoids contained abundant CD34+ cells; it hence extended to the mid-zone (but not to the centrolobular region) in a later hemopoietic phase. It was highest in a subsequent phase of ductular reaction, in which many cells displayed markers of oval cell (presumed liver stem cells) including AFP. In parallel, Large Cell Dysplasia (LCD) was seen in mid-zone and pericentral regions. This step corresponded to fully vascularized tumors and presence of lung metastases. Large dysplastic hepatocytes did not express AFP, that acts as a growth factor in tumor environments (Mizejewski 2003; Obstet Gynecol Rev, 58:804-826). LCD is a further index of hepatocarcinogenesis (Theise et al. 2002; Clin Liver Dis, 6:497-512). The probable high ploidy of dysplastic cells suggests progression towards terminal differentiation and cell senescence (Gupta 2000; Semin Cancer Biol, 10:161-171). In conclusion, a gradient of liver differentiation response to extra-hepatic tumor growth is demonstrated, with expression of both retro- and terminal differentiation phenotype of hepatocytes in the same lobulus.

Self- and xeno-cannibalism in heterozygous and homozygous lymphoblasts from patients with Huntington disease

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Huntington's Disease (HD) is one of the growing numbers of neurodegenerative diseases caused by the expansion of a trinucleotide repeat in IT15 gene. This event results in an exceedingly high number of contiguous glutamine residues in the translated protein huntingtin. Although the primary site of cell toxicity is the nucleus (DiFiglia et al. 1997; Science, 27:1990-1993), the key role of mitochondrial dysfunction, detectable in HD, is suggested by the evidence that mutant huntingtin fragments accumulate at the mitochondrial membrane (Panov et al. 2002; Nat Neurosci, 5:731-736; Yu et al. 2003; J Neurosci, 23:2193-2202). In the present work we studied the mitochondrial function and cell death pathway(s) in heterozygous and homozygous immortalized cell lines from patients with Huntington disease (HD). Heterozygosis was characterized by specific alterations of mitochondrial ultrastructure and of membrane potential (hyper-polarization). This was correlated with an increased susceptibility to apoptosis. Lymphoblasts from homozygous patients were characterized by a significant percentage of cells displaying autophagic and cannibalic activity. Considering the pathogenetic role of cell death in HD, this work provides new useful insights regarding the role of mitochondrial dysfunction, i.e. hyperpolarization, in hijacking i) HD heterozygous cells towards apoptosis and ii) HD homozygous cells towards a peculiar phenotype characterized by both self- and xeno-cannibalism.

Session 7
Life needs death during development

Cell death in shaping the embryo: a role for cell cycle genes

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Cell death plays an important role in the maintenance of normal homeostasis of an organism. In the past two decades there has been an explosion of attention and knowledge about cell death and the roles it plays in many situations in organisms from yeast to human. One very important implication has been in development. In mammalian as well as other embryos, cell death plays a major role in shaping and sculpting the embryo. Abnormal patterning of cell death can lead to major abnormalities in the developing embryo that can be rescued by the induction of cell death. Mutations leading to aberrant pattern of cell death, implies a genetic base for regulation of cell death. Many cellular pathways have been implicated in cell death. One such pathway deals with the role of cell cycle genes during cell death. The cyclin-dependent kinases (Cdks) play essential roles during the progression of cell cycle. Cdk5, a member of Cdk family, originally identified due to its structural homology to Cdc2, has been found to be expressed in a wide variety of tissues with its highest level of expression and activity during neuronal differentiation, embryonic development, and cell death. Accumulating findings including ours indicate a role for Cdk5 in cell death during development. We have shown that Cdk5 is detected most abundantly in correlation with dead cells in the interdigital region of the developing mouse embryonic limb. This high level of Cdk5 correlates with an increase in the kinase activity but does not correlate with an increase in the level of Cdk5 message. We have furthermore found that the expression of Cdk5 during cell death can be found in a variety of cell types of different tissues undergoing cell death in developing embryos, such as death in the developing lens fiber, which displays a unique type of apoptosis, nuclear apoptosis. Not only is Cdk5 expressed during physiological developmental cell death, we have also associated its expression and activation in cells dying because of any of several inducers such as retinoic acid and cyclophosphamide induced cell death in developing embryos. We have provided evidence that the upregulation of Cdk5 during cell death is independent of p53, apaf-1 and caspase-9. Basically we see a direct correlation of Cdk5 with the occurrence of cell death.

Epigenetic marks of chromatin remodelling in microspore-derived embryogenesis: reprogramming vs. death

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Microspore embryogenesis involves the stress-induced switch of the pollen developmental programme. The proliferation of microspore-derived multicellular structures under suitable *in vitro* culture conditions responds to a reprogramming event, which affects chromatin organisation. However, unbalanced early cell death can compromise the success of such reprogramming. In this work, we present a study of the nuclear architecture of microspores in culture and define hallmarks of early cell death. We have approached the study of the nuclear organisation of reprogrammed microspores by localising 5-methylcitosine and acetylated isoforms of histones, as epigenetic marks of the different functional states of chromatin. As reference patterns of differentiated and proliferating systems we have used the vegetative and generative nuclei of the pollen grain, zygotic embryos and roots. We have observed that specific antibodies to those epigenetic marks localise in defined nuclear areas and with a different pattern. Under the confocal microscope, the inactive (methylated) genes are preferentially located in peripheral areas whereas open (acetylated) chromatin is found as many small bright foci in central positions. These foci correlate at the electron microscope level on high pressure freezing specimens with clusters of gold particles having a size comparable to those of transcription and replication sites, as observed after BrUTP incorporation and PCNA labelling. Our

results suggest that microspore embryogenesis involves epigenetic reprogramming of the pollen developmental pathway.
Supported by Projects BFU2005-01094 and AGL2005-05104.

Cyclic apoptosis in the digestive tract of a protochordate

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Tissue degeneration which occurs during development of organisms is often of morphogenetic importance as well as proliferation and differentiation. Cyclic apoptosis of organs was progressively lost in Chordates. *Botryllus schlosseri* is a colonial ascidian continuously forming new zooids by blastogenesis, through the recurrent formation of palleal buds, which grow and mature until an adult is formed. Three blastogenic generations are commonly co-present: adult, filtering zooids, their buds and budlets on buds. At a temperature of 19°C, adult zooids remain active for about one week (mid-cycle stages); then they contract, close their siphons and are gradually resorbed, being replaced by a new generation of adult zooids, represented by buds which reach functional maturity, open their siphons and begin their filtering activity (regression or take-over stage). This stage is characterised by the occurrence of diffuse programmed cell death by apoptosis in zooid tissues, as evidenced by TUNEL reaction for chromatin fragmentation and annexin V labelling for detection of exposed phosphatidylserine, whereas infiltration of circulating phagocytes, which appear engulfed with apoptotic cells, is observed. With these characteristics, colonial tunicates are suitable subjects for studies on cyclical involution and resorption of tissues. In residual zooids remaining for a long time in the centre of each colony, melanin and lipofuscins accumulate as detected with Masson-Fontana, Ziehl-Nielsen and H2O2 bleaching methods. Immunocytochemical assays to detect pro- and antiapoptotic factors reveal an opposite expression which progressively extends in tissues of adult zooids with an organ gradient starting from the branchial basket. Results support the idea that fundamental mechanisms for the induction of apoptosis are well conserved throughout Chordate evolution.

Urothelial cell desquamation during the mouse urinary bladder development

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During mouse urinary bladder development, the urothelium underwent some cycles of intense cell desquamation (Ayres et al. 1985; J Urol, 133:506-512; Jezernik et al. 1997; Cell Biol Int, 21,1:1-6). Superficial cells desquamate due to the first urine accumulation in the urinary bladder at embryonic day 16, then in perinatal period, when the urothelium starts fully to function and again around a week after birth, when cell desquamation is a part of the urothelial remodelling, which leads into terminally differentiated tissue. In the present study, we observed the morphology of desquamating urothelial cells by transmission and scanning electron microscopy of mouse urinary bladders, taken from 16 days old embryos, newborn mice and 7 days old mice. Proliferative activity of superficial cells, which undergo desquamation, was analysed by imunolabbeling of proliferative marker Ki-67. Apoptotic activity of desquamating cells was examined by TUNEL assay and by immunolabelling of active caspase 3, which plays a key role in early apoptosis. During superficial cell detachment, large intercellular spaces appeared between the desquamating cell and its neighbour. Some of desquamating cells had normal morphology, while in other cells, condensed cytoplasm or condensed nuclear chromatin was observed. Results of anti-Ki-67 immunolabelling showed that desquamating cells were proliferatively quiescent. TUNEL assay and anti-active caspase 3 immunolabelling revealed positive reaction in desquamating cells. We assume that apoptosis starts in superficial cells but before apoptosis is completed, superficial cells desquamate into the lumen of the urinary bladder.

PTP interacting protein 51 in eye development during mouse embryogenesis

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Protein tyrosine phosphorylation is a central mechanism in the regulation of many cellular processes. The level of tyrosine

phosphorylation is kept in limits by the activity of tyrosine phosphatases and is crucial for the regulation of numerous cellular processes, e.g. control of cell-cell-interaction, signal transduction by growth factors, cytokines, hormones, cell-adhesion molecules. In a yeast two hybrid approach aimed to identify potential substrates of PTP1B and TCPTP by their ability to interact with these phosphatases a novel protein sequence named protein tyrosine phosphatase interacting protein 51 (PTPIP51: SwissProt Accession code Q96SD6) was identified.PTPIP51 could be found in every investigated mammalian species: guinea pig, rat, mouse, pig and human. Preliminary experiments suggested that the protein might be involved in the regulation of cellular processes associated with differentiation. To verify this hypothesis we studied the localisation of PTPIP51 during murine embryogenesis by immunocytochemistry and in situ hybridisation. By the 12th d.p.c. the optic cup has completely formed and further differentiation processes are initiated. The protein is seen in the cornea, the lens, the neuroretina and in the extraocular muscles. Further development leads to a reduction of the protein within the layers of the retina. By the 18th d.p.c. the reaction with the antibody is restricted to the cuboidal epithelium on the outer surface of the lens, to the lamina of nerve fibres, the ganglion cells and the internal plexiform lamina. Identical localisation of PTPIP51 during eye developoment were found in the epidermis of the eyelids, the conjunctival epithelium, the endohtelium of the anterior chamber and the extraocular muscles. These studies emphasize the importance of PTPIP51 in differentiation processes during embryo-genesis of the mouse.

POSTER SESSIONS

Session 2

Cell damage induction and repair: nucleus

Nucleoli in terminal stages of human nucleated blood cells

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Terminal stages of nucleated blood cells (NBCs) are convenient models for studies on nucleoli in preapoptotic or apoptotic states at the single cell level since the morphology of these cells is well known. They are characterized by a condensed chromatin structure and micronucleoli. In the light microscope such nucleoli are visible after visualization with cytochemical methods for demonstration of RNA or silver stained proteins. In terminal stages of granulocytes or monocytes, RNA containing structures of micronucleoli may be below visibility but these nucleoli may be detected using silver reaction or immunofluorescence of nucleolar proteins. It should be mentioned that micronucleoli were observed even in expulsed pycnotic nuclei of terminal erythroblasts. In the electron microscope, the structural organization of nucleoli in terminal stages of NBCs was variable. However, such variability was not related to any particular state of pathological erythro- or leukopoiesis. Micronucleoli in terminal stages of NBCs may exhibit segregation of nucleolar components, reduction of dense granular components and retraction of perinucleolar chromatin from the nucleolar body. In apoptotic leukemic myeloblasts without preceding terminal differentiation, nucleolar bodies possessed mostly dense granular components. In terminal erythroblasts of refractory anemia micronucleoli may participate in the formation of HERDS (heterogeneous ectopic ribonucleoprotein derived structures). Summarizing mentioned observations, it seems to be clear that the variability of the nucleolar structure in terminal stages of NBCs reflects various pathways which result in the cessation of nucleolar main functions and participate in terminal cell events.

Supported by Research Project MZO00023736 (Czech Ministry of Health).

Intranuclear microtubules and microfilaments are typical signs of an unusual form of cell death in C6 glioma cells after cisplatin treatment

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The study describes cells with intranuclear microtubules and microfilaments (CIMMs) in a subpopulation of C6 glioma astrocytes damaged by Cisplatin in cultures. The intranuclear microtubules were arranged in a festoon-like pattern placed in a lucid zone between an incompletely marginated condensed chromatin and the nuclear envelope; their ultrastructure and diameter were the same as those in the mitotic spindle in some rarely occurring proliferating cells. With respect to the G_2/M cell cycle arrest, we assume that the intranuclear microtubules may represent mitotic spindle fragments which were abortively formed or not disassembled in the cells which escaped the cell cycle blockade. In deeper nuclear areas of CIMMs, tufts of microfilaments also occurred among the chromatin patches. Many of CIMMs showed also other regressive nuclear changes, such as segregation of the nucleolar components and formation of *heterogeneous ectopic RNP-derived structures* (HERDS). On the contrary, most of the CIMMs had almost intact cytoplasmic organelles and their plasma membrane function was preserved, as they did not accumulate propidium iodide. The morphology of CIMMs and their time-limited occurrence suggest that they were lethally damaged and committed to an atypical form of non-accidental cell death resembling early apoptosis. This is supported by positive TUNEL reactions in a small number of these cells. We assume that the intranuclear microtubules, together with nuclear structure changes indicating disruption of the chromatin- and RNP-based transcription machinery, have affected the nucleus-to-cytoplasm signaling thus pre-venting completion of the execution phase of apoptosis.

Reactive astrocytes in cisplatin-treated cultures: damaged but functioning

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Majority of C6 glioma cells in culture treated with cisplatin die by apoptosis within 48 to 96hr. Here, we report cytochemical and ultrastructural analyses of the surviving cells. C6 glioma cells grown for 3 days were exposed to 1.66 x 10^{-5} M or 3.32 x 10^{-5} M cisplatin for 90min and then grown in a fresh medium for another 9-10 days. Propidium-iodide stained cells were measured by flow cytometry for DNA content. Most of the C6 glial cells surviving the cisplatin treatment were significantly hypertrophic and formed long radial processes. Their cytoplasm was voluminous and the nuclei were large and highly lobulated or fragmented into micro-nuclei. In the largest cells, the nuclear lobes, including micronuclei, were inter-connected with very narrow *nuclear bridges*. Their nucleoli were either active and reticulated or underwent segregation. Small micronuclei were often close at the periphery of largest nuclear lobes which contained either lose pale or dense chromatin without signs of its massive condensation or peripheral accumulation. Mitochondria were elongated with dense matrix and intact cristae. Vesiculated Golgi apparatus was surrounded with a wide network of smooth endoplasmic reticulum which propagated into cell processes. Large accumulations of lipid droplets and autophagic or heterophagic vacuoles often occurred in the cytoplasm of these hypertrophic cells. Flow cytometric evaluation of DNA showed that cell population was inhibited in cycling being blocked in late-S/G_2 phases. We conclude that the cisplatin treatment-surviving C6 glioma cells underwent hypertrophy and resembled reactive astrocytes in brain gliomas *in situ* (gemistocytes). The electron microscope analysis revealed complex regressive and metabolic-activation changes, dominated by a peculiar hyperlobulation of nuclei.

L-DNase II: a new PARP-1 partner

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Poly(ADP-ribosylation) consists in the conversion of NAD to ADP-ribose with liberation of nicotinamide and protons; this reaction is carried out mainly by PARP-1, which undergoes a rapid and transient activation during apoptosis and is the favorite target of caspases. A PARP-dependent/caspase-independent pathway of apoptosis, characterized by the release of AIF from mitochondria has been recently described. During apoptosis, PARP-1 regulates the activity of some nucleases responsible for DNA degradation, i.e. DNase1L3, CAD and DFF. We investigated a possible correlation between PARP-1 and L-DNase II, which derives from LEI (Leukocyte Elastase Inhibitor) and is involved in caspase-independent apoptosis. LEI is an ubiquitous cytoplasmic protein belonging to the serpin family, thus displaying an anti-protease activity; when apoptosis is triggered by certain inducers (long-term culture, Na^+/H^+ exchanger inhibition), LEI undergoes a cleavage mediated by serine proteases like elastase, is converted into the acid endonuclease L-DNase II, then translocated to the nucleus where it is responsible for DNA degradation into oligonucleosomes. That L-DNase II has a poly (ADP-ribose) sequence, prompted us to search for a possible interaction with PARP-1. *In vitro* experiments revealed that PARP-1 and poly(ADP-ribosylation) do not affect DNA degradation; on the contrary, the presence of both LEI and L-DNase II stimulates the autoribosylation of PARP-1. A physical association between these proteins, based on experiments of coimmuno-precipitation, was found, thus suggesting a functional correlation between LEI and PARP-1.

Effect of the topoisomerase II inhibitor Etoposide on H2AX deficient immortalized mouse embryonic fibroblasts

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In mammalian cells, the H2AX histone is rapidly phosphorylated upon the induction of DNA double strand breaks in the DNA

molecule. Cells deficient for H2AX are hypersensitive to ionizing radiation and show elevated levels of spontaneous genomic instability, suggesting that H2AX plays a role in the DNA damage response. Etoposide is a chemotherapeutic agent that causes DNA double strand breaks via inhibition of DNA topoisomerase II and induces H2AX phosphorylation. To check whether H2AX can modulate cellular sensitivity to Etoposide, we studied the response to this agent in immortalized embryonic fibroblasts derived from mice in which the *H2AX* gene had been inactivated by homologous recombination and in cells from wild type animals (a generous gift of A. Nussenzweig, NIH, Bethesda, USA). Etoposide treatment induces the appearance of micronuclei and nuclear fragmentation both in normal and mutant cells. Clonogenic assays in cells treated for 24 hours with increasing concentrations of the drug revealed a greater sensitivity of mutant cells compared to wild type cells (LD_{37} ~0.2 μM in mutant cells vs. ~1 μM etoposide in wild type cells). In wild type cells, flow cytometric analysis showed a slight accumulation of cells in G_2-M phase treated for 24 h with 1 μM Etoposide and a clear arrest in cells treated with a 10 μM concentration of the drug, with a recover of cell cycling after the removal of the drug. In contrast, in mutant cells, the lowest concentration of Etoposide was already associated with an evident G_2-M arrest, while the highest concentration led to a permanent perturbation of the cell cycle leading to the cell death. These observations suggest an increased sensitivity to Etoposide in cells deficient for H2AX.

Apoptosis without DNA fragmentation in HeLa cells after photosensitization with Rose Bengal acetate: involvement of mitochondria

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Photodynamic therapy (PDT) is a mode of cancer therapy based on the irreversible photodamage of tumor cells *via* photosensitizer-mediated oxidative cytotoxicity. Rose Bengal (RB) is a powerful photosensitizer due to its high efficiency of singlet-oxygen production, under suitable excitation. The addition of an acetate group (RB Acetate, RBAc) inactivates both fluorescence and the photosensitizing properties while improving the drug uptake. Inside the cell, specific esterases remove the acetate group thus restoring the native photoactive molecule. Previous experiments showed that, after RB-Ac administration, the restored RB molecules dynamically redistribute in the cytoplasm, first following the endosomal route and then localizing in the whole vacuolar system, to finally diffuse into the cytosol; following irradiation, RB-Ac treated cells undergo apoptosis. Here we demonstrate that mitochondrial photodamage occurs as an early event of RB-induced apoptosis which is not characterized by DNA degradation. HeLa cells incubated with RBAc for 60 min and irradiated with a light does of 1.6 J/cm2 were investigated after different post-treatment intervals in drug-free medium, up to 72hr. At confocal and electron microscopy, a progressive morphological damage of the mitochondria was observed. This was accompanied by the loss of the mitochondrial intermembrane potential and a reduction of the active mitochondrial mass, as revealed by fluorescence microscopy and cytofluorometry. Experiments of immunolabeling showed that these changes induced the activation of caspase 9 and 3 resulting in the triggering of the apoptotic program: this was demonstrated by fluorescence and electron microscopy, based on the nuclear morphological changes, and by cytofluorometry after straining cells by annexin V and propidiun iodide and by the immunolabeling for the p89 fragment of PARP-1. Interestingly, DNA fragmentation was never detected by the TUNEL procedure nor by the agarose gel electrophoresis of low molecular weight DNA

Supported by the Italian MURST (PRIN 2005, project nr. 2005058254)

Disassembly of the nucleolus during apoptosis: dynamic redistribution of the proteins phosphorylated-c-Myc, fibrillarin and Ki-67

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During apoptosis, a severe reorganization of all the nuclear ribonucleoprotein (RNP)-containing structures occurs, in parallel with chromatin margination and condensation. In particular the nucleolus has been described to segregate and disappear during apoptosis, although we have recently observed that the dense fibrillar component and the granular component can be still recognized in the interchromatin space until relatively late apoptotic stages. In addition, nucleolar remnants may also aggregate with other nucleoplasmic RNPs to form Heterogeneous Ectopic RNP-Derived Structures (HERDS). The aim of the present investigation was to track the fate of three representative nucleolar proteins, namely phosphorylated c-Myc (P-c-Myc), fibrillarin and Ki-67 during apoptosis and to compare their apoptotic relocation with their dynamic redistribution during mitosis. HeLa cells were driven to apoptosis by a 20 hr treatment with either 1 µg/ml actinomycin D or 10 µM etoposide. We found that P-c-Myc, fibrillarin and Ki-67 redistribute in distinct compartments during mitosis, whereas during apoptosis they may either be cleaved (Ki-67) or be extruded into the cytoplasm with a different kinetics and following an ordered, non chaotic program. The separation of these nucleolar proteins occurs in early apoptotic nuclei and continues also in the cytoplasm, where it finally leads to the formation of apoptotic blebs containing different nucleolar proteins: this evidence confirms that the apoptotic bodies may be variable in size and content, and include heterogeneous aggregates of nuclear proteins and/or nucleic acids. The extrusion from the nucleus of a wide and heterogeneous spectrum of proteins which survive in a partially degraded (or even in an undegraded) form during the late steps of apoptosis, legitimates the growing interest for those *novel* and ectopic molecular complexes which may play a role in the ethiology of autoimmune diseases.

Supported by the University of Pavia (Fondo di Ateneo per la Ricerca 2006)

Germ cell apoptosis and cell damage after gamma irradiation in the human fetal testis

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We investigated the effect of gamma irradiation on human fetal testis in organotypic culture. We tested three doses of ionizing radiations (0.2, 0.5 and 1,5 Gy) and studied gametogenesis three days after irradiation. We demonstrated, by light and electron microscopy, that:

- Irradiation reduced the number of germ cells in a dose-dependent manner.
- This effect was accompanied by an increase in apoptosis, as shown both immunologically (presence of cleaved caspase - 3 positive cells) and morpho-logically (light and electron microscopy).

In conclusion, human fetal gonocytes appeared to be highly radiosensitive. We are currently investigating wether the effects of irradiation on germ cells are direct or indirect.

Early epidermal response to gamma irradiation in organotypic cultures of normal human breast skin

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Early epidermal effects induced by ionising rays still remain difficult to study in human volunteers, therefore organotypic cultures of normal human breast skin can greatly help in defining the radiation response. Using this experimental model, the early epidermal radiation response was investigated by light/electron microscopy and RT-PCR. Bioptic fragments (n=7) were obtained from cosmetic surgery of young healthy women and cultured as previously described by Donetti et al (2005; Br J Dermatol, 153:881-886). Samples were harvested 24 hrs after exposure to a single clinical dose of gamma rays (2 Gy). We analysed keratinocyte proliferation after 5-Bromo-2'-deoxyuridine incorporation and keratinocyte terminal differentiation based on gene and protein expression of the biomarkers keratin 10,

involucrin, desmocollin 1, and desmoglein 1. Apoptosis was investigated by transmission electron microscopy in parallel samples. After irradiation, the mean percentage inhibition of keratinocyte proliferation was 53.7% (Student's t-test for paired samples, $p< 0.01$), whilst gene and protein expression of the considered biomarkers was unaffected. Ultrastructural analysis showed that the basal layer was undamaged, but a condensation of intermediate filaments and apoptotic keratinocytes were evident in the upper spinous layers of irradiated group. In our experimental conditions, a single dose of gamma rays on normal human skin inhibits keratinocyte proliferation within the first 24 hours, induces apoptosis, but does not have any evident effect on terminal differentiation. The characterization of the cellular and molecular mechanisms underlying the early radiation epidermal response can contribute in better defining potential non invasive interventions.

DNA breakage as common feature of apoptotic and differentiated cells

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The presentation will summarize own and literature data on accumulation of DNA breaks in differentiating cells. Large 50 Kbp DNA fragments were observed by several research teams in non-apoptotic insect, mammal and plant cells. A more intensive DNA breakage was observed during maturation of spermatides, embryo development, and differentiation of myotubes, epidermal cells, lymphocytes and neutrophils. In general accumulation of DNA breaks in differentiating cells cannot be attributed to decrease of the DNA repair efficiency. We hypothesize that DNA fragmentation is an epigenetic tool for regulation of the differentiation process. Scarce data on localization of the differentiation-associated DNA breaks indicate their preferable accumulation in the nuclear matrix attachment sites, the same sites are degraded on the first apoptosis stages. Using an original method we have observed DNA incisions in the nuclear matrix attachment sites in several mammalian cell cultures, avian and amphibian erythrocytes, developing barley shoots. We have also observed individual patterns of the large-scale DNA fragmentation in the Xenopus erythrocytes and specific distribution of single-strand breaks in the satellite 1 sequences in these cells. During induction of differentiation of the HD3 cell culture (AEV-transformed chicken erythroblasts) onset of the haemoglobin biosynthesis is coupled to intensive chromatin fragmentation. In the case when HD3 cell apoptosis is induced by serum deprivation, a 5 Kbp fragment around the alphaA globin gene is protected against the chromatin fragmentation. Recent data on non-apoptotic functions of caspases provide more evidence for our hypothesis and indicates elucidation of mechanisms of differentiation-induced DNA breaks as prospective research direction.

Transcription and splicing alterations during ageing

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Ageing implies a progressive deterioration of the concerted functions of molecular components, needed for the cell viability and proliferation. In particular, ageing involves alterations in the pathways of gene expression, with impairments in pre-mRNA transcription and/or splicing. The events leading to the formation of mature mRNA occur mostly cotranscriptionally and imply the co-presence of numerous molecules. The structural counterpart of such macromolecular complexes is represented by the perichromatin fibrils (PFs), fine fibrillar structures mainly distributed along the borders of the condensed chromatin, which represent the in situ form of nascent transcripts as well as of their splicing and 3' end processing. We previously described an unusual accumulation of PFs in hepatocyte nuclei of old rats, suggesting altered pre-mRNA pathways. In this study we investigated, by means of immunoelectron microscopy, the presence of different pre-mRNA processing factors and the incorporation of bromouridine (BrU) in the hepatocytes of adult and old animals, in order to elucidate the nature of the PFs stored during ageing. Our observations revealed lower amounts of transcription and

splicing factors as well as lower BrU incorporation in newly transcribed RNA in old rats, suggestive of a decrease in pre-mRNA transcription and a slow down of PF processing and/or transport during ageing. Such altered mRNA pathways would explain the persistence in the nucleoplasm of pre-mRNA and/or mRNA as PFs in hepatocyte nuclei of old rats.

A diet containing genetically modified soybean affects cell nucleus ageing in mice

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Previous studies demonstrated several modifications of cell nuclear structural constituents in hepatocytes of mice fed on a genetically modified (GM), herbicide-tolerant soybean from their weaning until 1, 2 , 5 and 8 months of age. In detail, hepatocyte nuclei of GM-fed mice showed larger size with more irregular shapes, a higher number of nuclear pores and larger amounts of nucleolar dense fibrillar component, all suggestive of increased metabolic activity. It is known that cell nuclear components undergo alterations during ageing, probably due to changes in the pathways of gene expression, such as impairments in pre-mRNA transcription and/or splicing. In order to elucidate the possible effect of a GM soybean-containing diet on the cell nuclear modifications occurring physiologically during ageing, in this study we analysed the ultrastructural features of hepatocyte nuclei of mice fed on GM or non-GM soybean for 24 months. Our data revealed that the hepatocytes of old GM-fed mice are characterised by roundish nuclei with a lower nuclear pore number and higher amounts of condensed chromatin, perichromatin granules and nucleolar granular component. This suggests a decrease and/or impairment of nuclear activities in GM-fed old animals. However, it should be underlined that this phenomenon was not accompanied by higher mortality or incidence of pathologies in the GM-fed group in comparison to the control.
Supported by the Agenzia Servizi Settore Agroalimentare delle Marche (ASSAM), Regione Marche.

Ageing alters the distribution of the circadian protein CLOCK in neurons of the reticular formation

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Several biochemical, physiological and behavioral processes exhibit cyclic oscillations of about 24 hours, defined as circadian rhythms. In mammals, the primary circadian pacemaker resides in the suprachiasmatic nuclei, although it has been demonstrated that cell-autonomous circadian oscillators occur in many tissues. CLOCK protein is a transcription factor essential for normal circadian rhythms and we recently demonstrated that it undergoes intranuclear redistribution in hepatocytes along the circadian cycle and this distribution is affected by ageing. It is known that aging leads to a progressive deterioration of the circadian rhythm at the behavioral, physiological and cellular levels; in particular, the rest-activity cycle undergo important alterations in old age. In this view, we carried out ultrastructural immuno-cytochemical analyses on neurons of the reticular formation - known to be involved in the control of many physical behaviours such as sleep - of adult and old rats, in order to investigate possible quali-quantitative modifications of CLOCK protein related to the aging process. Our observations demonstrated that most CLOCK protein was located in the cell nucleus, where it accumulated on perichromatin fibrils (the sites of pre-mRNA transcription and early splicing); in addition, CLOCK showed circadian oscillations in the different nuclear compartments, with significant differences between adult and old rats. This unusual distribution of CLOCK protein during aging could be related to altered nuclear pathways which could in turn influence the functions of the reticular formation.

Induction of reversible hibernation-like status in culture cells with delta opioids

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DADLE [D-Ala(2)-D-Leu(5)-enkephalin] can induce hibernation when injected into ground squirrels in summer and is able to increase the survival time of explanted organs such as liver and lung. Since cell metabolism is a target of this peptide (Su 2000; J Biomed Sci, 7:195-199), we have treated HeLa cells with DADLE (Vecchio et al. 2006; Histochem Cell Biol, 125:193-201; Baldelli et al. 2006; Histochem Cell Biol, in press) and other opioids and investigated its possible effect on transcription and proliferation as well as the resumption of metabolic activity after the treatment. DADLE decreases both pre-mRNA and pre-rRNA transcription and induces a marked reduction of RNA export. Interestingly, these modifications in cell metabolism are reversible, and do not induce any increase in cell death rate. Here, we have followed the effects of another enkephalin, DALE, under the same conditions, by means of immunocytochemistry and hybridization in situ at EM level. A panel of antibodies has been tested (Pol I, Pol II, snRNPs, SC35, cleavage factors) and we have labelled the fine distribution of polyA. Our data point out that most of the action of the enkephalins is localized on the perichromatin fibrils, where the opioids can be traced by immuno-labelling.

Temporary depression of trans-cription in mouse pre-implantion embryos from mice fed on genetically modified soybean

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Genetically modified (GM) soybean, resistant to the pesticide Round up, is a widely spread crop for animal and human consumption. We have focused our attention on embryos at 4-8 cells stage from mice fed on a diet containing 14% of GM soybean, analysing the possible alterations in transcription and splicing processes. We have evaluated, at electron microscopy, the nuclear distribution and the relative amount of some factors involved in transcriptional and processing mechanisms of mRNA and rRNA. We have observed in the nucleoplasm a significant decrease of the labelling for the active form of RNA polymerase II, associated with a reduction of labelling for splicing, polyadenilation and cleavage factors. As for the latter we have taken into account the cleavage factor CFI_m, found in the nucleoplasm and, surprisingly, in the nucleolus-like bodies which, in early embryos, likely represent nucleolar precursors. In embryos from GM-fed mice the nucleolar CFI_m also decreases, suggesting that the nucleolus may play a role in the stockage of such factors. On the basis of our results we could suggest that a temporary decrease in the transcription and maturation of mRNA exists in embryos from mice fed on GM soybean. Moreover, on the basis of the reduced presence of factors active in RNA maturation, we could hypothesize that an accumulation of immature RNA could be present in the nucleoplasm.
Supported by the Agenzia Servizi Settore Agroalimentare delle Marche (ASSAM), Regione Marche.

The role of PML in 5-BrdU-induced senescence

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Cellular senescence is proposed to be one of the safeguard mechanisms restraining uncontrolled proliferation of damaged cells and tumor promotion in organism. Replicative senescence usually occurs after exhausting replicative potential of normal dividing cells in vitro, whereas premature senescence can be induced by various stresses, for example by activated *ras* oncogene. This type of senescence is accompanied by up-regulation of tumor suppressor PML, and increase of PML nuclear bodies, multiprotein nuclear structures. Moreover, PML seems indispensable for *ras*-induced senescence. As reported earlier, 5-bromodeoxyuridine (5-BrdU) can trigger premature senescence-

like phenomenon not only in normal cells but also in various tumor-derived cell lines. We examined therefore the role of PML in 5-BrdU-induced senescence. We found that PML is up-regulated by 5-BrdU at both protein and mRNA levels with marked increase in number of PML bodies in HeLa cells. Moreover, the same effect was observed by distamycin A, an AT-binding ligand, which was reported to synergize the effect of 5-BrdU. Importantly, only cell lines bearing intact pRb tumor suppressor pathway responded by induction of PML and senescent phenotype. This indicates that intact pRb pathway is necessary for 5-BrdU-induced senescence and that PML expression is linked to pRb pathway induction.

Session 3

Cell damage induction and repair: cell membrane and cytoplasm

Effect of cisplatin on neuronal cells B50: involvement of mitochondria in apoptosis induction

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The antitumor drug, Cisplatin is cytotoxic during the postnatal development of rat cerebellar cortex where cell death occurs in proliferating granule cells (as demonstrated in cultured cells too), and alteration (or even degeneration) is induced in differentiating Purkinje cells. Cisplatin interferes with the morphological and molecular events of the postnatal cerebellar development and, early after treatment, changes have been found to occur in the proliferating activity of the external granular layer and in cell migration; alterations in the immunoreactivity for glutamate receptors have also been observed in Purkinje cells. In order to elucidate the mechanisms involved in cisplatin-induced cytotoxicity and apoptosis, the rat neuronal cell line, B50 has been used as a model system for neurotoxicity *in vivo*. Flow cytometric analyses showed that, after cisplatin treatment, there were changes in the cell cycle progress and an increase of apoptotic cells as revealed by the annexin V positivity: this suggests that cell cycle perturbation and apoptosis are inextricably linked in this system too. We also found that the morphology and intracellular distribution of mitochondria are affected; in parallel, we detected by immunocytochemical techniques a significant increase in caspase-9 positivity which indicates that the mitochondrial apoptotic pathway in activated in B50 cells as a consequence of cisplatin exposure. Consistently, the cells entry into the execution phase of apoptosis in cisplatin-treated B50 cells was confirmed by caspase-3 activation and DNA degradation, as shown by the increase in TUNEL positivity.

Hypocrellin-B acetate: a new fluorogenic substrate for enzyme-assisted cell photosensitization

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Photosensitizers preferentially accumulate in tumor cells, where they become cytoctoxic, upon irradiation by light at the proper wavelength. Unlike the fluorochromes commonly used in cytochemistry and cytometry, photosensitizers have a high efficiency of *intersystem crossing* (i.e., of passage to the triplet state), in which the absorbed energy is mostly dissipated through photochemical processes, rather than through fluorescence emission; these processes generate unstable chemical species (singlet oxigen, free radicals and other reactive oxigen species) which can damage biomolecules by photo-oxydation, evetually leading to cell death. Photo-sensitizers may be modified by addition of chemical groups (e.g., acetate) which improves their intracellular accumulation, while quenching their fluorescence and photosensitizing properties. Once inside the cells, these modified compounds behave as *fluorogenic substrates*, since the added chemical groups are removed by cellular enzymes, so that the native photoactive structure is restored. Here, we investigated the photophysical and photodynamic characteristics, and the cytotoxic action of a new fluorogenic substrate, Hypocrellin-B

acetate (HypB-Ac) which has been obtained by chemically modifying HypB: in fact, this is a powerful photosensitizer whose wide absorption spectrum extends above 600nm thus making it very promising for an application *in vivo*. Conventional and confocal fluorescence microscopy showed that, in HeLa cells, the photosensitizing molecules first accumulates in lysosomes; upon irradiation, dramatic changes occur in both the cytoplasm and nucleus, with prominent disruption of the cytoskeleton and chromatin organization and massive induction of cell death.

Supported by the Italian MURST (PRIN 2005, project nr. 2005058254)

Reactive astrocytes in the substantia nigra of chronic alcoholics

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Our previous findings in postmortem tissue of chronic alcoholics revealed immuno-reactivity of the stress protein ubiquitin (UBQ) in dopamine (DA) neurons of substantia nigra (SN), localized in cytoplasm, dendrites and along cellular membranes (Chrysanthou and Issidorides 1999; Neurol Research, 21:426-443), but not in astrocytes. Alcohol, as a stressor, is known to disturb the lipoprotein composition of membranes, causing fluidization by release of cholesterol. Is this the metabolic disturbance signaled by the presence of UBQ? Astrogliosis is the most common reaction in CNS damage. In the present study we applied, to SN of 27 alcoholics and 10 controls, the phosphotungstic acid-hematoxylin method for gliosis (PTAH-G), which enhances the *protein staining* of astrocytes and their fibrous branches by obliterating myelin staining (Manlow and Munoz 1992; J Neuropathol Exp Neurol, 51:298-302): groups of glia stand out against a pale background. Our results revealed “gliosis” not only numerically, but also as profuse fibrous astrocytic extensions which invest closely the surface of the DA neurons and their dendrites. Given the role of astrocytes in neuronal repair and protection following injury (Sofroniew 2005; Neuroscientist, 11:400-407), the expression of UBQ and the investment of neurons and dendrites by astrocytic extensions support the formation of a glial *shield of repair*. We interpret our findings as showing that astrocytes can, actually, protect neurons from the alcohol-related disruption of cholesterol homeostasis, by synthesizing cholesterol (Guizzetti and Costa 2005; Med Hypothesis, 65:563-567), which is released via basic apolipoprotein E, outside the cell and imported by the neurons for maintenance and repair.

Degeneration and regeneration of mouse olfactory epithelium after exposure to the olfactory toxicant dichlobenil

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The olfactory neurons are directly exposed to a large number of environmental factors and are continuously replaced throughout life. An increasing number of chemicals are known to induce extensive morphological changes in the olfactory epithelium preferentially, but not only, following inhalation. In mice, a single intraperitoneal injection of dichlobenil, an herbicide, causes necrosis of the dorsomedial part of the neuroepithelium and underlying lamina propria, whereas the lateral part of the olfactory mucosa remains undamaged. The necrosis is not followed by regeneration and six months after exposure to dichlobenil the olfactory dorsomedial region shows a respiratory metaplasia with invaginations and fibrotic lamina propria. In this study we have investigated the ability of human umbilical cord blood-derived CD133+ cells in the regeneration of damaged mouse olfactory epithelium after intraperitoneal injection of dichlobenil. One week after the infusion of CD133+ cells, numerous cells were intensely labelled by lectin binding, in particular after SBA, BSA-I-B4 and UEA-I binding. These new differentiating elements were immature neurons since they expressed beta-III-tubulin and the neural growth-associated phosphoprotein GAP-43. After one month of recovery it was possible to observe a certain degree of neuronal differentiation, in fact in some districts of the damaged olfactory epithelium the lectin-stained cells were arranged to form a typical pseudostratified epithelium. The present results suggest that the infusion of human umbilical cord blood-derived stem cells may play an important role in the regeneration of the olfactory epithelium.

Calbindin D28k over-expression in hybrid mice might protect male germ cells from apoptosis

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t is well known that mammalian germ cells with structural rearrangements of karyotype show an increase in the death rate. Calbindin D28k (CB), a cytosolic protein which acts as a calcium buffer, has cellular protective functions in many tissues. In this work we evaluated the calbindin D28k (CB) capacity to protect male germinal cells from apoptosis in mice with Robertsonian (Rb) rearrangements. Testis from adult male mice with standard telocentric chromosomes (2n = 40, CD1), homozygous (2n = 24, Milan II) and heterozygous (2n = 24x40, hybrid) for Rb rearrangements were analyzed at different ages. CB expression was determined on transverse sections of the seminiferous tubules, employing immuno-peroxidase technique with policlonal antibody against CB. Apoptosis was studied via DNA fragmentation by the TUNEL assay. Hybrid mice have a larger number of apoptotic cells than the other two groups of mice. Apoptosis was mainly observed in cells from the stage XII. CB expression was also more intense in meiotic germ cells from the same stage in the hybrid mice, specially at 5 month old. Only 10% of germinal cells showed double staining of CB and TUNEL positive at 3 and 5 months old. CB expression in the germinal cells from CD1 or Milan II mice was absent in the stage XII and it was very low (1-5 cells/ seminiferous tubule) in other stages from these mice. The results suggest that CB over-expression in the meiotic germinal cells from hybrid mice might occur to buffer intracellular calcium increase and to protect cells from apoptosis.

Improved preservation of fatty liver by machine perfusion at 20°C respect to conventional cold storage: preliminary observations

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Donor scarcity stimulated the development of strategies to increase the organ pool for transplantation by accepting fatty livers which are usually rejected (Angulo 2006; Liver Transpl, 12:523-534). Zucker rats (ZR) are a model of hepatic steatosis (Sun et al. 2003; Gastroenterology 125:202-215). A machine perfusion (MP) system with recirculation of an oxygenated medium at 20°C was developed that proved to afford a good protection of normal rat liver (Ferrigno et al. 2006; Dig Liver Dis, 38:A58); the device was tested on fat Zucker rat liver. Preliminary biochemical analyses indicated that MP provided a better fatty liver preservation than traditional cold storage (CS) with Celsior solution, in terms of tissue ATP levels and enzyme release. These data are substantiated by better morphology and preservation of macrosteatotic cells (neutral lipid revealed with Nile Red), higher glycogen content (PAS reaction) and lower reactive oxygen species (ROS) production especially by sinusoidal cells (Mn-Co-DAB reaction) in liver submitted to MP, respect to CS. Cell damage induced by CS to fatty liver was better appraised by electron microscopy which showed intense autophagocytosis in hepatocytes, dilated bile canaliculi, frequent apoptosis of sinusoidal cells, collagen deposition in the space of Disse, and electron-dense material in sinusoids. By contrast, when fatty liver was preserved by MP, the hepatocyte ultrastructure was less damaged, fewer apoptotic cells were observed and no fibrotic reaction was seen.
Supported by MIUR-COFIN2004 and FAR-UniPv funds.

Cav-3, a muscle-specific caveolin-isoform, is expressed in rat airway epithelial cells

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Although many receptors known to interact with caveolins are present in tracheal epithelial cells (TEC), there are no reports about the expression and distribution of caveolins (Cav) in these cells. We have shown in our previous work that Cav-1 and

Cav-2 are expressed in a subset of bronchial epithelial cells. Surprisingly, we also detected the muscle-specific Cav-3 mRNA in abraded epithelium. In order to exclude a contamination of the epithelial cell preparations with smooth muscle cells (SMC), we subjected abraded TEC to RT-PCR analysis for smooth muscle myosin heavy chain (smMhC) and α-smooth muscle actin (α-SMA). Unexpectedly, we found mRNAs coding for smMhC and α-SMA in the abraded TEC. Real time quantitative RT-PCR revealed that the proportion of the relative expression of Cav-3 to smMhC and to α-SMA and of smMhC to α-SMA in abraded TEC was markedly different to the tracheal muscle. Laser-assisted cell-picking provided further proof that mRNAs coding for Cav-3, smMhC and α-SMA are expressed by TEC. Using Western blotting and well characterized mouse monoclonal antibody raised against Cav-3, we also found an immunoreactive protein of appropriate size in abraded TEC. Immunohistochemistry on cryosections demonstrated Cav-3 immunoreactivity apically in the ciliated cells of the trachea, in the same region as its interacting protein eNOS. In this cellular compartment, no caveolae were found by electron microscopy. In addition, Cav-3 immuno-reactivity was observed also in SMC of the trachea and in chondrocytes. Our data indicate a functional role of Cav-3 in the regulation of NO synthesis in ciliated cells and, therefore, in ciliary function.
Supported by DFG, GK 534.

Cell death in skeletal muscle cells

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Several muscular disorders are characterized by muscle cell death, which causes progressive muscle atrophy (Querfurth et al. 2001; Mol Cell Neurosc, 17:793). Our intent is to highlight apoptosis potentially correlated to myopathies. In this study muscle death was investigated in C2C12 murine myoblast cell line by UVB irradiation and staurosporine treatment. Monolayers were grown as previously described (Burattini et al. 2004; Eur J Histochem, 48:223), and observed at reverted microscope (RM) in undifferentiated and differentiated condition. For apoptosis triggering, they were subjected to UVB irradiation for 30 min and postincubated for 4h in 5%CO2 at 37°C. They were also treated with 0.5μM staurosporine for 24h in incubator. Cytotoxicity was determined with the trypan blue exclusion test and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetra-zolium bromide) assay (Sestili et al. 2006; Free Radic Biol Med, 40:837). Morphological apoptotic patterns were investigated with RM, transmission and scanning electron microscopy (Falcieri et al. 2000; Histochem Cell Biol, 113:134). Cytotoxicity analysis in UVB irradiated cells shows that viability considerably decreases, morphological investigation reveals apoptotic features and, in undifferentiated condition, bleb formation with an intriguing increase of substrate anchorage. In staurosporine treated cells viability again decreases both in undifferentiated and differentiated condition, showing apoptotic and necrotic features. Further studies are in progress to highlight the modified expression of cytoskeletal muscle proteins during apoptotic phenomena and to study a protective effect of melatonin as a free radical scavenger (Luchetti et al. 2006; J Pineal Res, 40:158).

Effects of melatonin on PC12 cells triggered to apoptosis by rotenone and UV-B irradiation

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In Parkinson's disease there's a dopaminergic neurodegeneration that involves both mitochondrial complex I inhibition and oxidative stress (Seaton et al. 1997; Brain Res, 777:110-118). PC12 is a rat adrenal phaeochromocytoma cell line, utilized as a model to study Parkinson's molecular mechanisms and prevention. Rotenone is a mitochondrial complex I inhibitor and causes apoptosis and dopamine release from these cells (Yang et al. 2004; Neurosci Lett, 366:53-57). UV-B irradiation alters mitochondrial function causing release of pro-apoptotic molecules, like cytochrome c, which acts as a trigger for the formation of APAF-1 and procaspase-9 (Li et al. 1997; Cell, 91:479-489). This mechanism is mediated by reactive oxygen species (ROS) that modify mitochondrial

membrane permeability (Luchetti et al. 2006; J Pineal Res, 40:158-167). Melatonin (Mel) is produced principally by the pineal gland and acts as a ROS scavenger. The aim of our work is to investigate the effect of Mel in rotenone and UV-B treated PC12 cells. In our model we have given 1mM Mel 24h before 20μM 24h rotenone treatment. It was also additioned immediately before 30 min UV-B irradiation and the cells have been analyzed after 4h post-incubation. We have investigated Mel effects by means of reverted microscope observations, transmission electron microscopy and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Sestili et al. 2006; Free Radic Biol Med, 40:837-849). Preliminary data show a protective effect of Mel on PC12 apoptotic cell death.

Cellular and subcellular localization of Na+/Ca2+ exchanger protein isoforms, NCX1-3 in cerebral cortex and hippocampus of adult rat

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Na+/Ca2+ exchanger (NCX) controls cytosolic Ca2+ and Na+ concentrations ([Ca2+]i and [Na+]i) in eukaryotic cells in normal and pathological conditions i.e. during excitotoxicity (Annunziato et al. 2004; Pharmacol Rev, 56:633-654). Here we investigated by immunocytochemistry the cellular and subcellular localization of the three NCX isoforms, NCX1, NCX2 and NCX3, in adult rat neocortex and hippocampus. NCX1-3 were widely expressed in both brain areas: NCX1 immunoreactivity (ir) was exclusively associated to neuropilar puncta, while NCX2-3 were also detected in neuronal somata and dendrites. NCX1-3 ir was often identified around blood vessels. In both neocortex and hippocampus, NCX1-3 were prominently expressed in dendrites and dendritic spines contacted by asymmetric axon terminals, whereas they were poorly expressed in presynaptic boutons. In addition, NCX1-3 ir was detected in astrocytes, notably in distal processes ensheathing excitatory synapses. All NCXs were expressed in perivascular astrocytic endfeet and endothelial cells.The intense expression of NCX1-3 in heterogeneous cell types in the brain *in situ* emphasizes their role in handling Ca2+ and Na+ in both excitable and non-excitable cells. Perisynaptic localization of NCX1-3 in dendrites and spines indicates that all isoforms are favourably located for buffering [Ca2+]i in excitatory postsynaptic sites. NCX1-3 expressed in perisynaptic glial processes may participate in shaping astrocytic [Ca2+]i transients evoked by ongoing synaptic activity.

Cytoskeleton alteration in programmed cell death induced by destabilizing/stabilizing antimicrotubular agents in different lines of NIH/3T3 cells

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We have analyzed cell alterations induced by Taxol (TAX) and Vinblastine (VBL) on NIH/3T3 cell line, in basal growth condition (NIHb) and after selection of a subpopulation (NIHs) obtained after cell stress induced by serum deprivation and low pH medium. The changes detected, by immunofluorescence and ultrastructure, were related to the onset and progression of apoptosis (PCD) at both nuclear and cytoplasmic levels. Variable responses to the drugs in the different lines of NIH/3T3 cells were observed. In NIHb culture, VBL induced major effects on depolymerization and the appearance of tubulin paracrystals, accompanied by intense micronucleation phenomena. In NIHs culture, VBL induced a low depolymerization and a high presence of macrotubule aggregates; in comparison with NIHb cells, these cytoskeletal changes were related to a lower incidence of aneugenic micronucleations and an increase of the typical apoptosis. TAX induced the appearance of thick microtubule bundles in both NIHb and NIHs cells. Nevertheless, the prevalence of microtubular bundles with circular arrangement was found in NIHb cells, while a higher number of linear bundles was shown in NIHs cells. In NIHb cells, circular microtubular bundles were related to a higher induction of canonic and alternative phenomena of PCD. Moreover, in these cells, ultrastructural analysis showed that the presence of microtubular bundles was associated with changes of

intermediate filaments, which appeared aggregated around the nucleus. This cytoskeletal feature could play a role in the induction and progression of nuclear fragmentation/micronucleation phenomena.

Dose-dependent effects on micro-filament network are related to cell death induced by cis-platinum in NIH/3T3 cell line

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The action of different concentrations (50, 75, 100 μM) of the cytostatic drug cis-platinum (cDDP) on microfilament network of NIH/3T3 cell line was evaluated. The variability of the effects found was related to cytokinetic activity changes and dynamics of nuclear fragmentation during cell death phenomena. The analysis performed after actin fluorochromization showed different effects on cytoskeleton that seemed to be related to the drug concentration: incubation with 50 and 75 μM cDDP for 48 h induced, in a dose-dependent manner, a stress fiber progressive decrease accompanied by an intense actin depolymerization. These aspects were often associated with classical apoptosis. Instead, 100 μM cDDP induced different effects: there were not evident phenomena of actin depolymerization, but rather the appearance of a reduced number of microfilament thick bundles and actin reorganization at the perinuclear area. This redistribution of the cytoskeletal protein appeared to be correlated with both cell shape changes and drastic nuclear alteration, such as incisure/lobulation of the nuclear envelope and micronuclei formation with heterogeneous size. TEM showed that these nuclear aspects represented the onset of an alternative form of cell death in which nuclear fragmentation was not associated with chromatin hypercondensation. Nuclear involution seems to be dependent on a partial persistence of microfilament network that could play a role in generating contractile forces applied on the nucleus. Therefore, variable concentrations of cDDP can induce, in NIH/3T3 line, different mechanisms involved in alternative forms of cell death.

Vitamin regulated expression of the novel protein PTPIP51 in epidermal cells

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The novel protein tyrosine phosphatase interacting protein 51 (PTPIP51), which has been found to interact with protein tyrosine phosphatases of the PTP1B/TcPTP subfamily is restricted in its expression to the suprabasal layers of epidermis. To study the underlying regulatory mechanism, a human keratinocyte cell line (HaCaT) was used as a model system. These keratinocytes were submitted to different hormonal agents inducing either proliferation or differentiation, Results were obtained by immunocytochemistry and subsequent statistical analysis. Additionally, immuno-blotting was performed to detect the possible occurrence of distinct molecular weight forms. HaCaT cells were subjected to treatment with factors that are crucial among others for the regulation of proliferation and differentiation of keratinocytes in human epidermis: epidermal growth factor (EGF), retinoic acid (RA) and 1,25-dihydroxyvitamin D3 (1,25 (OH)2D3). Epidermal growth factor receptor (EGFR) expressed in HaCat cells was inhibited by PD153035. Without any additional hormonal treatment, PTPIP51 protein could only be detected in a third of HaCaT cells. Whereas cells treated with increasing concentrations of 1,25 (OH)2D3 showed a stepwise numerical increase of PTPIP51 positive cells, treatment with RA did not influence the number of PTPIP51 positive cells except for application of supra-physiological concentrations. Concentration dependent increase of cells staining positive for PTPIP51 was also observed under EGF treatment. Application of PD153035 led to a statistically insignificant decrease of PTPIP51 positive cells.

Influence of staurosporine and protein kinase C on the expression of the novel protein PTPIP51 in keratinocytes

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The novel protein PTPIP51 (protein tyrosine phosphatase interacting protein 51: SwissProt Accession code: Q96SD6, EMBL: HAS 24271), which is known to interact with protein tyrosine phosphatases, namely PTP1B and TcPTP, is expressed in all suprabasal layers of human epidermis (Stenzinger 2005; Histochem Cell Biol, 123:19-28). Hence, a human keratinocyte cell line (HaCaT) was used as an in vitro system to study the regulation of PTPIP51 expression under treatment with staurosporine (Stsp) chelerythrine and phorbol-12-myristate-13-acetate (PMA). Application of Stsp, which is known to induce a programmed pattern of keratinocyte differentiation similar to that seen in vivo, led to an increase of 70% of cells being positive for PTPIP51 in comparison to controls (35% positive cells). Since Stsp is proposed to act as a protein kinase C (PKC) activator in keratinocytes eventually leading to sustained phospholipase D activation, it was of interest whether PTPIP51 is influenced by PKC. Interestingly, chelerythrine mediated inhibition of PKC led to a concentration-dependent and step-wise increase in the number of PTPIP51 positive keratinocytes up to 90%. This in accordance with results showing a significant decrease of PTPIP51 positive cells when submitted to PMA. However, pulldown experiments ruled out a direct interaction of PTPIP51 with PKC α. Stsp-induced upregulation of PTIP51 positive cells supports the hypothesis that PTPIP51 is involved in differentiation of cells in vitro and in vivo. The upregulation of PTPIP51 in keratinocytes under inhibition of PKC activity may be due to an unknown compensatory mechanism that is activated, if PKC activation and subsequent differentiation is down-regulated by Chelerythrine.

Effects of esomeprazole sodium on glutathione levels in the gastric mucosa of rats treated with indomethacin

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Proton pump inhibitors (PPIs) are effective in preventing and healing non steroidal anti-inflammatory drug-induced gastroduodenal lesions by acting as antisecretory agents. It has been proposed that PPIs may exert their protective action also through antioxidant mechanisms. To examine the effects of esomeprazole sodium on the levels of glutathione in the gastric mucosa of rats treated with indomethacin. Male albino Wistar rats, 200-225 g body weight (n=8/group), received one of six treatments by intragastric gavage: 1% methocel as vehicle (controls), esomeprazole 30 or 60 μmol/kg, indomethacin 100 μmol/kg, esomeprazole 30 or 60 μmol/kg and indomethacin 100 μmol/kg. In rats treated with esomeprazole and indomethacin, esomeprazole was administered 30 minutes before indomethacin. The animals were sacrificed 4 hours after indomethacin administration, and the stomachs processed for the evaluation of mucosal levels of total (tGSH), reduced (GSH) and oxidized (GSSG) glutathione. An histomorphometric evaluation revealed that esomeprazole markedly reduced mucosal injuries induced by indomethacin. Esomeprazole alone did not significantly affect the mucosal concentration of tGSH, GSH and GSSG whereas indomethacin caused a significant reduction of tGSH and GSH contents and a significant increase in GSSG levels compared to controls. Esomeprazole 30 μmol/kg partly reversed the effect of indomethacin on tGSH, GSH and GSSG levels. Esomeprazole 60 μmol/kg reversed the effects of indomethacin on glutathione levels up to control values. Our findings indicate that esomeprazole dose-dependently counteracts the effects of indomethacin on gastric mucosal glutathione levels.

Session 4

Molecular tracing in dying cells

Fractal statistical image analysis of cellular components in early death of human breast cancer cells

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An analytical strategy combining *fractal geometry* and *grey level co-occurrence matrix (GLCM)* statistics was devised to investigate ultrastructural changes in oestrogen-insensitive SK-BR3 human breast cancer cells undergoing apoptosis *in vitro*. Apoptosis induced by 1 µM calcimycin (A23187 Ca2+ ionophore) was assessed by measuring conventional cellular parameters, namely phospatidylserine - annexine V labelling, membrane permeability to propidium iodide, TdT nick end labelling DNA and cell cycle sub-Go/G1 peak, during all the culture period. SK-BR3 cells entered the early stage of apoptosis within 12-24h of treatment and calcimycin-induced changes were detectable in plasma membrane and nuclear components only by recording increased values of most GLCM parameters and a general reduction of the fractal dimensions. In these latter cells, morphonuclear traits were accompanied by a reduced expression of distinct gangliosides and a loss of unidentifiable glycolipid molecules at the cell surface. All these changes were shown to occur in apoptosis well before the detection of conventional markers mentioned above which were only measurable during the active phases of cell death (Castelli and Losa 2001; Anal Cell Path, 23:1-9). In overtly apoptotic cells treated with 1 µM calcimycin for 72h, most nuclear components underwent irreversible ultra-structural changes, including marginalisation and condensation of chromatin, as reflected in a significant reduction of their fractal dimensions. Hence, both fractal and GLCM analyses confirmed that the morpho-ultrastructural re-organization of nuclei and cell membranes was imputable to a loss of structural complexity that occurs early in apoptosis. Moreover, they may prove reliable for detecting early or initial cell death in cancer tissues where active apoptosis is a residual and rather scarce event (Losa and Graber 1998; Anal Cell Path, 16:1-10).

The HOPE (*H*epes Glutamic acid buffer mediated *O*rganic solvent *P*rotection *E*ffect) technique offers a complete panel of molecular applications in paraffin-embedded materials

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Asservation of materials for pathologic diagnostics is mostly achieved by formalin-fixation and paraffin embedding. This results in good preservation of histologic details, but to a large degree prevents the application of modern molecular techniques due to the bad condition of nucleic acids and proteins if compared to fresh or frozen specimens, which show a comparably low preservation of histomorphologic details. The purpose of this investigation was to establish an alternative fixation technique to overcome with these problems. We present protocols for In situ hybridization, Immuno-histochemistry, Western-blot, Northern-blot and Transcription-microarrays. We have shown that the novel HOPE-technique allows for both well preserved 'formalin-like' morphology and excellent preservation of nucleic acids and proteins. All of these methods can be unambiguously performed with HOPE-fixed materials. In conclusion, these results open up new perspectives for molecular approaches in paraffin-embedded specimens providing a novel powerful tool for modern pathology.

The use of halogenated precursors for DNA and RNA detection by means of immunoelectron and immuno-fluorescence microscopy

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We have developed a novel approach for labeling and detection of nucleic acids in cultured cells. It is based on *in vivo* incorporation of ClU and IdU into CHO cells with the aim of labeling RNA or DNA, respectively. The halogenated nucleotides are then immunolabeled on ultrathin sections with anti-BrdU monoclonal antibodies that specifically react with either IdU or ClU. Br-UTP microinjection was carried out in order to compare the efficiency of RNA incorporation with respect to ClU labeling. Furthermore, we combined ClU and IdU incubation to label simultaneously RNA and DNA. Both were visualized by means of anti-BrdU antibodies exhibiting strong affinity for one of the two halogenated epitopes. Confocal imaging of interphase nuclei as well as electron microscopic analysis put in evidence a partial colocalization of newly synthesized DNA and RNA inside the cell nucleus. RNase and DNase digestion of ultrathin sections after formaldehyde fixation and acrylic resin embedding confirmed the specifity of incorporation. Although statistical analysis performed on electron micrographs reveals a more efficient labelling with Br-labelled precursors, this method allows us to differentially label DNA and RNA on the same section. Using short pulse with the precursors we could show that newly synthesized DNA and RNA both occur preferentially within the perichromatin region at the border of condensed chromatin domains.

The effects of anticancer agents upon proliferation and apoptosis in human Non-Small Cell Lung Cancer (NSCLC) specimens using an *ex vivo* tissue culture model

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Inhibition of tumor growth and induction of apoptosis in lung tumors are two major goals of anticancer treatment in humans. However, especially NSCLC is highly resistant to the widely used chemo-therapeutical agents including carboplatin, vinorelbine and gemcitabine by mechanisms that are not well understood. Thus, in this investigation we used a recently established tissue culture model named STST (Short-Term Stimulation of Tissues) in combination with the novel HOPE-(*H*epes-Glutamic acid buffer mediated *O*rganic solvent *P*rotection *E*ffect) fixation and paraffin embedding method to examine the specific responsiveness of human NSCLC tissue specimens to direct treatment with the above indicated individual cytotoxic drugs. Expression of Ki 67 protein and of BrdU uptake, of caspase-3 and of apoptosis-regulators bax and bcl-2 were assessed by immunohistochemistry. Proliferation was clearly reduced by all 3 anticancer agents in a similar manner. In contrast, apoptosis was induced less effectively in response to the cytotoxic drugs, since the alterations in all 3 apoptosis-related proteins were hetero-geneous. In conclusion, although there was a high heterogeneity among the individual tumor responses as expected, we clearly demonstrate specific multiple drug-induced effects simultaneously. Thus, this novel *ex vivo* model is suggested to provide a novel tool to further study numerous aspects of mechanisms underlying tumor responsiveness towards enhanced anticancer treatment.

Session 6

Cell damage and death in pathology

Phagocytosis of apoptotic neutrophils in *Helicobacter pylori* gastritis and in gastric adenocarcinomas: an immuno-histochemical and electron microscopy study

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Phagocytosis of senescent neutrophils has been described in the late nineteenth century by Metchnikoff, although the significance of these observations has only become apparent in the last few decades. It has now been established that macrophages are the principally responsible cells for clearance of apoptotic cells in mammals, although cells with the potential for phagocytosis e.g. endothelial cells, fibroblasts, vascular smooth cells, epithelial cells may remove apoptotic cells in certain circumstances. Recently, we also showed phagocytosis of apoptotic neutrophils in gastric adenocarcinomas (cannibalism). In

an attempt to verify the specificity of cannibalism for tumour, the relationship between neutrophils and epithelial cells in human gastric adenocarcinoma and in control cases of Helicobacter (H) pylori gastritis were studied using immuno-cytochemistry for caspase-3 and conventional electron microscopy. A light microscopy review of 200 cases of resected gastric carcinomas identified 22 cases (11%) that were characterized by neutrophil-tumour cell cannibalism. Immuno-histochemical staining for caspase-3 and electron microscopy confirmed the presence of apoptotic neutrophils within the cytoplasm of the tumour cells. In 12 out of 52 cases of H. pylori gastritis, caspase-3 positive apoptotic neutrophils were found in the cytoplasm of non-neoplastic epithelial cells, as also confirmed in selected cases by electron microscopy. This study shows that the phenomenon of neutrophil cannibalism is not specific for tumours. To the best of our knowledge, it is the first report that provides light and electron microscopic evidences of phagocytosis of apoptotic neutrophils in non-neoplastic epithelial cells during H. pylori infection.

The possible role of nitric oxide in rat liver damage induced by reperfusion after ischemia *in vivo*

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The role of NO in cell damage induced by reperfusion after ischemia in rat liver was studied by the localization of eNOS, iNOS, Nitrotyrosine (NT) and ROS-producing cells in tissue sections. *In vivo* ischemia was induced in rat liver followed by 1, 5 and 24 hr reperfusion. Damaged areas in liver parenchyma were detected with LDH activity. Infiltration of monocytes and macrophages was based on the localization of G6PD activity, whereas PMNs were established with the demonstration of alkaline phosphatase activity. The proteins eNOS, iNOS and NT were demonstrated using immunofluorescence. ROS-producing cells were detected with Co-Mn-DAB. In control liver eNOS was found in endothelial cells of large vessels and in sinusoidal endothelial cells. INOS was localized in a rim of hepatocytes surrounding central veins. NT was found in epithelial cells of bile ducts, whereas ROS-positivity was only present in some cells, possibly PMNs. The localization pattern of eNOS changed in damaged areas at all time points of reperfusion. The numbers largely decreased and the shape of the cells was altered. INOS-positive cells appeared everywhere in liver parenchyma at all time points. ROS-positive cells increased slightly at 1 hr reperfusion everywhere in parenchyma, whereas at later time points many cells infiltrated damaged areas. NT-positivity was observed in some individual cells in damaged areas and in nuclei of damaged hepatocytes. It can be concluded that eNOS is not a likely candidate for the appearance of cell damage by NO. However, iNOS present in macrophages and ROS produced by macrophages and PMNs may affect liver cell damage.

Loss of histone H1 labeling from decondensed chromatin of leukocytes in bipolar disorder

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In view of reports suggesting immunological abnormalities in affective disorders, we studied by electron microscopy (EM) the relation between decondensation of the chromatin, which results in lymphocyte proliferation, and the distribution in the nucleus of histone H1, which is responsible for maintaining the chromatin in the condensed state. The latter is best studied by phosphotungstic acid hematoxylin (PTAH) with *en-block* staining of leukocyte pellets revealing, in the EM, the condensed chromatin as electron-lucent, and the decondensed-relaxed as electron-dense. Blood was obtained from 15 bipolar patients, and 7 controls. Leukocyte pellets were stained by PTAH and embedded in epoxy resins. Histone H1 labeling was studied on epoxy-thin sections of leukocyte pellets with the immunogold method and on formalin-fixed blood smears with Fast Red. By PTAH, condensed electron-lucent chromatin was revealed in the leukocyte nuclei of controls and normothymic patients, while relaxed electron-dense chromatin was evident in both manic and depressive patients. High density of H1 immunogold labeling was associated with condensed chromatin areas

in controls and normothymic patients, while low density of H1 was observed in the nuclei of both manic and depressive patients. H1 labeling, in the smears was highly variable in mononuclear and polymorphonuclear leukocytes of patients compared to controls. The fact that the ultrastructural conformations of the chromatin revealed by PTAH, correspond to H1 labeling in the three phases of the illness, i.e. depression, normothymia and mania, supports the use of PTAH as a screening method of choice in investigating blood markers in mental illness.

Chromatin alterations in leukocytes of first episode schizophrenics

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Studies of peripheral blood leukocytes of schizophrenics have shown in electron microscopy (EM), that decondensation of the chromatin constitutes a biological marker indicating increase in genomic expression. Since, this increase depends upon chromatin relaxation by dissociation of lysine-rich histone H1 from nucleosomes, with exposure of arginine residues of core histones, the ratio of arginine to lysine residues in each nucleus represents a reliable measure of activation. Lysine- and arginine-rich proteins are demonstrable in light microscopy (LM), differentially, in yellow and black color respectively with the Ammoniacal Silver Reaction (ASR). Application of ASR on leukocyte pellets before dehydration and embedding in epoxy resins, gives reliable results in semithin sections. Leukocytes of 15 first episode schizophrenics and 8 controls were used. Light micrographs of the semithin sections were inserted in a PC. We measured percentage of lysine and arginine in 300 nuclei per subject. In addition, histone H1 immunoreaction was studied a)in blood smears with Fast Red in LM and b)on thin sections of the pellets, visualized by immunogold in EM. Morphometry showed that lymphocytes of schizophrenics have increased ratios of arginine to lysine, compared to controls, indicating activation; neutrophils of the patients have even a higher ratio indicating an abnormal condition of the genome. Reduced H1 immuno-reactivity in leukocytes supports the results from ASR-morphometry. The efficacy of ASR to quantify decondensed chromatin, offers a tool for early diagnosis, since first episode schizophrenics have a better prognosis when treatment is started promptly at the beginning of the disease.

Macrophagic induction of injectable hydrogels: from the *in vitro* to the *in vivo* study

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Biocompatibility of implanted materials depends on the host foreign-body response that ensues immediately after implantation of biomedical devices. This response progresses through stages of inflammation and wound healing with different cell types as hallmark indicators of the particular stage of the reaction. Chronic inflammation is characterized by monocyte population adhesion to the surface of the implant and their differentiation into macrophages. These adherent cells may fuse into foreign-body giant cells (FBGCs) in consequence of different stimuli. FBGCs have degradative and phagocytic properties that can lead to both structural and chemical damages of the implant and, in some case, to an abnormal chronic inflammation responsible to serious diseases. The aim of our research was to evaluate the effects of synthetic hydrogels used as fillers in Plastic and Reconstructive Surgery for soft tissue augmentation, poly-Alchyl-Imide (pAI) and poly-Vinyl-Alcohol (pVOH), on the *in vitro* differentiation of monocytes in macrophages. In addition, the presence and distribution of differentiated macrophages in tissues were studied in an *in vivo* model, after subcutaneous injection of materials. We studied an *in vitro* model represented by U937 promonocytes cell line, able to differentiate in mature macrophages and morphological and functional parameters, characteristic of differentiated cells, were considered. In particular, cell adhesion onto the substrate and cells morphology were investigated by means of light and scanning electron microscopy; the reactive oxygen species production through Nitro Blue tetrazolium dye reduction; phagocytic activity by means of phago-

cytosis assay with latex particles. In order to simulate the *in vivo* cells behaviour grown with artificial hydrogels, we have also performed *in vitro* experiments in presence of fibronectin, a substrate similar to the natural tissues. The hydrogels (0.1 ml) were also injected in the subcutaneous tissue of male Swiss mice and the macrophages localization at regular intervals (1, 7, 15, 30, 60, 90, 120 days) after implantation was individuated by immune labelling with monoclonal antibody against CD68+ (macrophages surface antigen). Pyrogen free water was considered as a negative control. *In vitro* results showed that the monocytes differentiation in macrophages depends on the chemical nature of polymers. Moreover, the direct contact between cells and materials causes the induction of apoptotic cells. Immuno-histochemistry assays showed that CD68 positive cells appear localized 7 days after the implantation and diffuse in the derma at 15 days. The macrophages become occasional when the pAI is inoculated at 30, 60 and 90 days, while they are present near the pVOH in the same period investigated. However, all tissues result negative at 120 days from polymer implantation. In conclusion, the results indicated that synthetic hydrogels pAI and pVOH do not cause an abnormal activation of monocytes and macrophages population. The hydrophilic nature of these material surfaces could be a possible reason for the decrease of monocytes adhesion onto the implant while to the increase of apoptotic cells could be due the reduction of the implant damage. Then, the study of dynamic phenomena in the implant/tissue interface can provide an evaluation on lifetime of biomedical device and some important indications on chronic inflammation nature.

Diagnostic use of c-erbB-2 proto-oncogene expression in mammary gland neoplasia of young Arab women

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Breast cancer is characterized by a long history and marked heterogeneity in growth rates and clinical manifestations. In Europe and USA it occurs at peak between the age of 60-65 years, with only 14% occurring below the age of 40 years. In contrast, in Gulf Arab women the mean age is 48.7 years and the peak occurrence of breast cancer is at the age of 45-50 years. A significant proportion of 26% of cancers occur below the age of 30 years and the incidence is increasing. Only 14.1% of breast cancer in women occur above the age of 60 years. Breast cancer patients younger than 30 years have a worse prognosis than older patients. There are highly significant trends for the prevalence of poor prognostic features (Grade 3) histology, extensive intraduct component, lymphatic and vascular invasion to decrease with increasing age. In this study we evaluate the expression of c-erbB-2 oncoprotein in breast cancer of young Arab women. The c-erbB-2 gene codes for a putative transmembrane protein, similar in structure to the epidermal growth factor receptor (EGFR). A total of 122 patients were selected. The average size of the tumours was 5.6 cm, the majority of cases (76.2%) are invasive duct carcinoma. The amplification/overexpression of c-erbB-2 from paraffin wax embedded breast tumour tissues was analyzed by immuno-histochemistry using antiserum for presence of the c-erbB-2 protein. A chemical detection system was applied to identify the antibody. A high degree of immunoreactivity indicates a large amount of protein is present and no staining indicates the protein is absent. From the 122 patients, 70 cases were positive with different degrees of intensity ranging from weak (27 cases), moderate (23 cases) to strong (19 cases). The most intense immunoreactive of the 70 total positive cases were found in ductal carcinomas (16 cases) and infiltrating ductal carcinomas (14 cases). Some cases (n = 8) showed a correlation between the strongly positive c-erbB-2 protein expression and lymph node involvement. Only 6.8% are Grade I well differentiated tumours, 41% are Grade II moderately differentiated and 22% are Grade III. In conclusion, this study shows that the detection of c-erbB-2 oncoprotein in tumour sections may proof to be an additional prognostic marker of breast cancer, particularly in young women, in the histological diagnosis of ductal and infiltrating ductal carcinomas, followed by lobular, intraductal and infiltrating lobular carcinomas. The majority of tumour etiologies were c-erbB-2 oncoprotein positive.

Session 7
Life needs death during development

Biotic or abiotic stress elicits PCD in kiwifruit pollen grains

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In plants, programmed cell death (PCD) is often correlated with developmental processes (i.e., xylogenesis, senescence, ripening, or sexual reproduction); climatic changes, oxidative stress, and interaction with pathogens also generate reactions involving PCD. The present study deals with PCD induction in a relatively poorly studied system under this regard, such as the haploid male generation of higher plants, the pollen grain. Here we present evidence that PCD features are triggered in pollen by application of either chemical or biochemical stimulus, as a response to the toxicity of different agent. Pollen of kiwifruit was germinated in the presence of either trivalent/hexavalent chromium or, separately, of the α-helical peptide magainin 1, produced by the granular skin glands of the frog *Xenopus laevis* (Zasloff 1987; Nature, 415:389-395). Chromium is a dangerous environmental pollutant known to inhibit plant growth and development (Shanker et al. 2005; Environ Int, 31:739-753); magainin interacts with the lipid matrix of membranes, forming trans-membrane pores which severely damage cells (Lee et al. 2004; Biochem, 43:3590-3599). In both cases, a strong dose-dependent inhibitory effect on pollen germination was observed. Dramatic ultrastructural alterations in the treated pollen included chromatin margination, cytoplasmic vacuolization, dilatation of endoplasmic reticulum (ER) cysternae, and swelling of mitochondria. Similar symptoms have also been observed in other plant cell types undergoing PCD (Geitmann et al. 2004; Cell Death Diff, 11:812-822).

Presence, dynamics and role of caspase 6 in meiotic resumption in mouse oocytes

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Oocytes spontaneously resume meiosis after release from their follicles. As these oocytes have a low capacity for fertilization, we investigated the possible involvement of an apoptotic process. It has been suggested that apoptosis presents certain unusual features in mammalian oocytes. Firstly apoptosis begins with the resumption of meiosis. It is difficult to differentiate between a real resumption of meiosis and the first step of apoptosis. Meiosis resumption begins with nuclear breakdown. This process involves the modifications of lamins which are components of the nuclear envelope. We investigated the possible presence of the executive caspase 6, which is known to cleave these lamins specifically. The subcellular distribution and dynamics of caspase 6, lamin B and lamin A/C were studied by confocal microscopy and western blotting. Oocytes were incubated in the presence of an apoptotic inducer, staurosporin. Staurosporin treatment did not affect the distribution of caspase 6. Finally, the function of this executive protease in the oocyte was investigated, using the specific inhibitor VEID-CHO. Our findings demonstrate that caspase 6 is present in the mouse oocyte and suggests a role for this protein in meiotic resumption. It remains unclear whether caspase 6 is present in oocytes only at the time of release from their follicles or whether it is also present in oocytes during folliculogenesis *in vivo.*

AUTHOR INDEX

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