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XXVI SISC Meeting, Italian Society for the Study of Connective Tissues, Padova, Italy, 26–27 October 2006

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SESSION 1: BIO-PATHOLOGY OF ECM GLYCANS

The Tragic History of Keratan Sulphate, but What Does it Do in Cartilage and Cornea?

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Keratan sulphate (KS) and chondroitin sulphate (CS) are called after the Greek names for the tissues from which they were easily isolated (cornea and cartilage, respectively). That each occurs in both tissues is no help to the logically minded. Because sodium hydroxide extraction was routine, corneal KS was obtained as an alkali-resistant complex with protein, where CS was obtained protein-free because its link with protein was alkali-labile. M. Suzuki (1939) and Alan Woodin (1952, 1954) established the salient features of the KS proteoglycan before the term or the concept had been invented, but neither received the recognition he deserved and Woodin died in tragic circumstances. Karl Meyer regarded KS as a glycoprotein manqué, quite distinct from the well-behaved CS, an exasperating spectrum of molecular mass, sulphate content, sialic acid, mannose, and fucose. Greiling and Stuhlsatz simplified the picture by their demonstration of structural domains in KS (1987), using enzymes discovered in Suzuki's laboratory.

The fundamental unity of KS and CS emerged when I pointed out that the two polymer backbones were identical (i.e., polylactoses). What was the point of another form of the same polymer, decorated with extra sugars? A likely reason came with the realization by Stockwell (1967) that the different localizations of KS and CS in cartilage might be due to difficulties in biosynthesizing CS in anoxic parts of cartilage, since CS requires an oxidation in making the glucuronate portion, whereas in these regions KS could continue to be made, since it requires no oxidation step. I applied similar reasoning to cornea and intervertebral disc (1988), suggesting that the presence of KS was a sign of oxygen lack, caused by inefficient oxygen delivery by diffusion through the tissues.

If KS substitutes for CS in conditions of oxygen lack, what is the function they both can fulfill? Ultrastructural studies using Cupromeronic blue in electron histochemical methodology showed that the CS epimer, dermochondan sulphate (DS), forms bridges between collagen fibrils in tendon, skin, and sclera, which hold them in register and at critical separations, in "shape modules."

We found similar structures involving KS in corneas (1985) and thus, in that tissue at least, KS and CS (DS) have a common functionality. We now find (2006) similar structures in cartilage, in which a KS proteoglycan, probably fibromodulan, forms interfibrillar bridges in the same way as the KS PG (probably lumican) in cornea.

Because the amount of KS increases with age in human cartilage, is KS as efficient as DS? Is KS a risk factor in the age-dependant occurrence of osteoarthrosis? Is KS more or less sensitive to tissue enzyme degradation or even to free radical attack?

TGF-1β Induced Hyaluronan Production Inhibits NF-KB Translocation and Caspases Activation in Human Fibroblasts Exposed to Oxidative Stress

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Transforming growth factor-1β (TGF-1β) administration in human fibroblasts is able to stimulate hyaluronan synthases (HASs). AHAS rise in turn increases high molecular weight

hyaluronan (HA) levels. HA possesses several biological activities. Among them, during oxidative stress, it possesses antioxidant activity by chelating transition metal ions (Campo et al., *Free Radic Res*. 38:601–611). Nuclear factor kB (NF-kB), complexed with its inhibitory protein IkBα, is a response transcription factor involved in inflammation and exerts its action by expressing several detrimental molecules. Caspases instead are specific proteases responsible for the regulation and the execution of programmed cell death. It could be hypothesized that the damage exerted by free radicals may be strongly amplified by the activation of these key factors.

We investigated whether the protective effect exerted by HA, newly produced by TGF-1β treatment, in fibroblast cultures exposed to oxidative stress, may have any effect on NF-kB and caspases activation.

The exposure of fibroblasts to $FeSO_4$ and ascorbate caused cell death, a marked lipid peroxidation evaluated in terms of 4-hydroxyalkenals (HAE) level, NF-kB activation, cytoplasmic IkBα loss, increase in caspase-3 and caspase-7 expression and their related proteins, and depletion in the endogenous antioxidants CAT and GPx.

The fibroblast pretreatment with TGF-1β, 12 hr before oxidative stress induction, limited free radicals cell injury. This treatment, which increases HA levels, significantly inhibited NF-kB translocation, as confirmed by the normalization of IkBα protein, and reduced caspases activation, both at mRNA and protein level.

In light of previously findings reporting that antioxidants can work as inhibitors of NF-kB and apoptosis induction (Sarkar et al., 2004; Katunuma et al., 2006), a possible explanation of these results, since lipid peroxidation intermediates may induce NF-kB and caspase activation, could be that HA by inhibiting lipid peroxidation has indirectly blocked NF-kB translocation and apoptosis.

Vascular Smooth Muscle Cell Aging and Hyaluronan Metabolism

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Hyaluronan (HA) is involved not only in a variety of physiological processes, but also in several pathologies such as atherosclerosis. Aortic smooth muscle cells (AoSMCs) play a pivotal role in the formation of the atherosclerotic plaque (AP). Several studies have reported that the AP is constituted by a HA-rich extracellular matrix, a glycosaminoglycan (GAG) that is involved in the proliferation and migration of several cell types. As aging is one of the major risk factors for the insurgence of the pathology, we have established an *in vitro* aging model in order to investigate the HA content and metabolism in young and aged AoSMCs (Vigetti et al., 2006).

Culture medium of aged AoSMCs has been found to have HA increase in respect to younger cells without any change in smooth muscle cell differentiation. Gene expression experiments have shown an induction of the mRNA coding for the HA synthetic enzymes HAS2 and HAS3 in aged AoSMCs, whereas we did not detect any hyaluronidase activity. We found an augment of CD44 transcript, the HA receptor, in aged AoSMCs. As AoSMCs migration plays a critical role during vasculopathy onset, we assessed whether CD44, high molecular weight (HMW) HA, and HA oligosaccharides (LMW) could modulate cellular migration and the phosphorylation state of the MAP kinase ERK 1,2 We found that aged AoSMCs migrated to a higher extend and possessed higher level of phosphorylated ERK 1,2 than young cells. ERK 1,2 phosphorylation and migration could be enhanced by treating aged AoSMC cells with HMW HA, whereas young AoSMCs seemed to be not sensitive to HMW HA treatments. Both young and aged cells decreased ERK 1,2 phosphorylation and decreased migration by treating with LMW HA. Interestingly, by using CD44 blocking antibodies, we found a decrease in ERK1,2 phosphorylation and a decreased number of migrating cells. The same results were obtained by treating the cells with U0126, a MAP kinase inhibitor.

These results indicate that hyaluronan could modulate migration during in vitro aging and the regulation of migration could involve CD44 signaling through ERK 1,2 phosphorylation.

Urinary Galactosamine-Containing Acid Oligosaccharides

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Chondroitin sulphate (CS), with dermatan sulphate (DS) galactosamine(GalN)-containing glycosaminoglycan (GAG), is frequently associated, in proteoglycan (PG) form, with the connective tissue (CT) repair progress. In central nervous system (CNS), Rolls et al. (2006) recently suggested that disaccharides derived from CS may positively affect CNS repair. The question rises whether CS degradative fragments may have any role in the CT repair process outside CNS. Acid oligosaccharides with the size of di- and trisaccharides were isolated from urine by Calatroni et al. (1974) and considered blood derived fragments of GAGs, since their urine excretion increases in mucopolysaccharidoses (MPS), inherited lysosomal GAG storage disorders of CT with severe skeletal deformities, and/or mental retardation. In this work the presence of GalN-containing acid oligosaccharides was measured in normal urine and in urine from the different types of MPS and compared with urinary GalN-containing GAGs (CS and DS) levels.

Although CS is not storage material in MPS, urine concentration of GalN-containing acid oligosaccharides was definitely higher than in controls in patients with MPS, especially those with skeletal deformities (and not in the MPS III patients, only showing mental retardation). The expected highest values in MPS I and VI, storing mainly DS. In addition, such concentration turned out to be highly comparable, but in MPS I, with the concentration of urinary GalN-containing GAGs.

Results of the present work suggest that CS metabolism is activated in the presence of deep injured CT to produce and maintain amounts of both CS chains and CS fragments higher than normal. The role of these compounds in repair process will be investigated.

New Capillary Zone Electrophoresis Method for Glycosaminoglycan Analysis

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Glycosaminoglycans (GAGs) are linear polysaccharides whose building blocks consist of an amino sugar and an uronic acid. GAG heterogeneity also depends on sulfation degree and GlcA/IdoA proportion. These posttranscriptional modifications are often responsible for a wide variety of biological functions.

We have evaluated the possibility of using a capillary zone electrophoresis (CZE) analysis protocol for the quantitative and qualitative characterization of the chondroitin sulphate (CS) isomers in human plasma. Plasma samples were freed from protein by papain treatment and GAGs were purified by DEAE-Sephacel chromatography, depolymerized with chondroitinase ABC, and derivatized with 2-aminoacridone.

Analysis was performed in a P/ACE capillary electrophoresis system applying 20 nl of sample under nitrogen pressure in uncoated fused-silica capillary, using 50 mmol/L sodium acetate as electrolyte solution, containing 0.05% methylcellulose, pH 3.8. The separating conditions (28 kV, 175 μA at reversed polarity) were reached in 20 sec and held at a constant voltage for 13 min. Separations were monitored with a laser-induced fluorescence detector at 488 nm excitation and 520 nm emission wavelength. This method allowed us to separate the non- and monosulphated disaccharides obtained from CS isomers in less than 12 min. The proposed method is suitable for measuring total plasma CS isomers content and evaluating the distribution and fine structure of low and high charged forms of plasma GAGs.

This work was supported in part by MIUR funds (PRIN 2005).

Structural Analysis of Glycosamino-Glycans from Fresh Porcine Aortic Valves

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Cardiac valves are specialized forms of cardiovascular connective tissue designed to support high bearing and shearing stresses during their function. Mechanical properties of extracellular matrix are critically important for performance and durability of heart tissue valve substitutes. The valve consists of a semifluid, deformable, avascular matrix that is rich in proteoglycans, glycosaminoglycans (GAGs), and collagen fibres. GAGs play a central role in management of valvular shear stress and cyclic motion of the aortic valve. Our previous studies indicate that localized GAG depletions are produced following cryopreservation protocols (Dainese et al., 2006).

The present investigation was undertaken to study GAG content, distribution, and structure in three different zones (aortic wall, valve commissure and leaflet) from fresh porcine aortic valve. The methodology used for isolation, identification, and structural characterization of GAGs included delipidation, proteolytic treatment, analysis of free GAGs, and of their constituent disaccharides by FACE.

The three selected areas were significantly different in total GAG content. GAG analysis indicated that deep structural dissimilarities in polysaccharide chains exist, depending on their topographic localization. In particular, commissure and leaflet, compared with aortic wall, showed a higher relative percentage of hyaluronan and the presence of a peculiar undersulfated chondroitinfsulfate (slow CS). Structural analysis evidenced differences concerning the sulfation degree of CS. Our findings regarding fresh porcine valve GAG composition provide a basis on which to analyze the integrity of extracellular matrix in both tissue valve substitutes and decellularized scaffolds.

This work was supported by MIUR funds (PRIN 2005).

SESSION 2: ECM METALLOPROTEASES

Glucosamine and its N-Acetylphenyl-Alanine Derivative Down Regulate Metalloprotease Production in Chondrocytes by Affecting MAP Kinase Phosphorylation

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Glucosamine (GlcN), a symptom-control drug used in the treatment of osteoarthritis (OA), has been proved effective in slowing OA progression (Reginster et al., 2001). OA is characterized by a progressive degradation of cartilage due

to an imbalance between synthesis and degradation of the extracellular matrix component. Production of proinflammatory cytokines stimulates chondrocytes to secrete an excess of proteases. We report the effects of GlcN and its N-acetyl phenylalanine derivative (NAPA) on metalloprotease (MMP) production in immortalized chondrocyte cell line, LBPVA55, stimulated with proinflammatory cytokine interleukin (IL)-1β. We analyzed, by quantitative-real time-PCR, MMP-1, -2, -3, -8, -9, 13 mRNA expression level in cells treated with 2.5 and 10 mM GlcN or with 2.5 and 10 mM NAPA, after stimulation with 10 ng/ml IL-1β.

We found MMP-1, -3, and -13 upregulated by IL-1β stimulation and brought back by GlcN and NAPA. Same results were obtained when MMP-1, -3, and -13 protein levels were analyzed by ELISA. Mengshol et al. (2000), previously demonstrated that IL-1β induction of MMPs requires MAP kinases activation. To verify if GlcN and NAPA could affect p38, JNK, and ERK MAP kinases activity, we analyzed the whole cell extracts by Western blot analysis, using antiphospho-antibodies. We found phospho-p38, phospho-JNK, and phospho-ERK increased by IL-1β**;** phospho-JNK and phospho p38, but not phospho-ERK, were downregulated by GlcN and NAPA. Finally, we discovered that GlcN and NAPA, by affecting p38 and JNK kinases whose phosphorylation is requested to activate AP1 transcription factor, downregulated MMP-1, -3, and -13 production.

Matrix Metalloprotease Monitoring During Neoadjuvant Breast Cancer Therapy: An Ongoing Study

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Matrix metalloproteinases (MMPs) belong to a large gene family comprising more than 20 genes. Many MMPs have been identified in humans and some of them are involved in cancer, either directly or indirectly. Indeed, recent evidence has shown multiple functions of MMPs, which include the mobilization of growth factors and processing of surface molecules, rather than merely extracellular matrix (ECM) degradation. For these reasons MMPs have received more attention in recent years as putative tumor markers, and also for their easy detection in body fluids. Previous studies by our group have shown that serum levels of MMP-2 and MMP-9 are significantly enhanced in breast and colon cancer patients. In the present study we aimed at verifying if the level of circulating gelatinases was responsive to chemotherapy.

In this study we included 16 breast cancer patients under neoadjuvant treatment, enrolled with consent. For each patient the first assay was done on serum samples taken at the time of first treatment and therefore considered as the reference point. The subsequent assays were done in coincidence with periodical treatments. The activity levels of both MMP-2 and MMP-9 were detected by zymographic assays and quantified by ImageQuant TL. The kinetics of gelatinase activities showed a significant and selective involvement of MMP-9 and allowed us to discriminate subgroups of patients in relation to individual responses to the therapeutic protocols. In 12 of 16 patients the variation of MMP-9 level was positively correlated with the response to therapy, whereas in 4 patients, responsive to the therapy, there was no apparent correlation with the MMP activity levels. This study is in progress to implement the significance of data.

Work supported by Ricerca Scientifica di Ateneo anno 2005 (ex quota 60%).

SESSION 3: EXTRACELLULAR MATRIX STRUCTURE

Is Matrix GLA Protein (MGP) a Key Regulator of Elastic Fiber Calcification?

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It is generally accepted that the high concentration of calcium and phosphate in the extracellular space would readily lead to tissue calcification unless efficiently inhibited. A series of proteins and glycoproteins have been found to operate in soft connective tissues to avoid calcium precipitation. Among these, matrix Gla protein (MGP) is an extracellular protein that inhibits soft connective tissue calcification. Mutations cause ectopic calcification both in humans and in mice. MGP maturation requires gamma-carboxylation of glutamic acid residues by a vitamin K-dependent enzyme and only the carboxylated form of MGP (Gla) exhibits calcium-binding activity and inhibits calcification.

The aim of our study was to investigate whether mesenchymal cells produce the mature form of MGP, and whether MGP may be involved in the mineralization process occurring in Pseudoxanthoma elasticum (PXE). The carboxylated (Gla) and the undercarboxylated (Glu) forms of MGP were assayed by Western blot on human dermal fibroblasts (HDF). Skin biopsies and cell cultures from control and PXE patients were treated by electron microscopy (EM) and sections were immunostained with antibodies specific to Gla or to Glu.

Results indicate were 4-fold: HDFs produce both Glu and Gla, with the amount of Gla significantly higher than that of Glu; by EM, both forms are present within cells, mostly localized on membranes of the endoplasmic reticulum; on sections from skin biopsies, control and normal elastic fibers of PXE patients are slightly positive for both Glu and Gla forms of MGP; on mineralized elastic fibers, Glu and Gla have

distinct localization, with Glu abundantly associated with fine and bulky mineral deposits. However Gla is precisely localized on the calcification front separating normal from mineralized areas within the same fiber. The present data strongly support the hypothesis that Gla to Glu ratio could be crucial for elastic fibers calcification.

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Tropoelastin Deposition in Cultured Human Dermal Fibroblast is Affected by Addition of Heparan Sulphate

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Heparan sulphate (HS) glycosaminoglycan chains are present on numerous proteoglycans of the extracellular matrix as well as on the cellular surface, playing a fundamental role in biological processes as embryonal development, wound healing, inflammation, and cancer progression. Recent data from our laboratory showed that HS is associated with normal elastic fibers in human dermis and that isolated HS chains interact in vitro with recombinant tropoelastin and with peptides encoded by distinct exons of the human tropoelastin gene.

By immunocytochemistry, HS chains were identified as associated with the amorphous elastin component in the human dermis and remained associated with the residual elastin in the partially degenerated fibers of old subjects. This strongly indicated that HS-elastin interactions may play a role in tissue elastin fibrogenesis as well as in modulating elastin stability with time and in diseases.

The aim of our present study was to investigate the role of exogenously added HS to the deposition of tropoelastin by human dermal fibroblast in vitro. HS from pig intestinal mucosa (Opocrin, Modena) was added at concentrations of 0, 10, 50, and 100 μg/ml. Cells were grown for periods up to 20 days after confluence. Tropoelastin secretion was quantified by ELISA, using antielastin polyclonal antibodies (EPC, 1:1000 dilution), whereas tropoelastin aggregation properties were investigated by fluorescent microscopy using antielastin polyclonal antibodies (EPC, 1:200 dilution).

Cell proliferation was unaffected by addition of HS at the concentrations used. During the first days of culture, HS-treated cells, differently from controls, showed close to the cells surface small globules of tropoelastin that, with time, progressively aggregated into elongated fibers. These data confirm the existence of close interactions between HS and tropoelastin, further suggesting the hypothesis that HS may interfere with elastin deposition and assembly.

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Type I Collagen Has Multiple Binding Sites for Biglycan

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Biglycan (BGN) and decorin (DCN) are small leucine-rich proteoglycans (SLRPs) interacting with collagen I, regulating fibril formation and morphology. BGN and DCN have chondroitin/dermatan sulfate chains (CS/DS) and localize at bands d/e of the type I collagen fibril banding pattern (Iozzo 1999; Scott, 1998). Localization and specific sequences in type I/II collagen for the binding to SLRPs are unknown however. We used collagen CNBr peptides to narrow the search of binding sites for BGN.

Recombinant BGN was purified from culture media of a clone of CHO cells stable transfected with an expression vector containing the human sequence of BGN. The interaction of BGN with collagenous samples was studied by a solid-phase assay, as performed with DCN (Tenni et al., 2002).

BGN was found to bind collagen I ($K_d \sim 22$ nM) and collagen II ($K_d \sim 36$ nM). Only a few CNBr peptides of collagen I were active: $K_d \sim 20$ nM, ~ 9 nM, and ~ 41 nM have been determined for $\alpha 1$(I) CB6, $\alpha 1$(I) CB7, and $\alpha 2$(I) CB4, respectively. Only 1 CNBr peptide from collagen II ($\alpha 1$(II) CB11) has some binding ability to BGN. The triple helical conformation is essential for the interaction. In addition, binding of collagens/peptides to BGN has an ionic character and is not influenced by 1% Triton X-100. Collagen Lys/Hyl residues are essential since their N-acetylation inhibited binding to BGN. All peptides interacting with BGN contain sequences falling in bands d/e; their binding is specific since other peptides with sequences in bands d/e do not interact with BGN.

In conclusion, BGN is similar to DCN (Tenni et al., see above) for its ability to interact with multiple binding sites in collagen I and the involvement of collagen Lys/Hyl residues.

Fibrillar Crimp as a Function of Collagen Microfibrils in Connective Tissues

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Crimps have been described in connective tissues bearing unidirectional forces, whose collagen fibrils run straight and parallel showing only occasionally a steep change in direction. The crimp apex reveals a localized deformation of the collagen fibrils (flattening, buckling, or torsion patterns), that lose their distinctive D-banding and reveal their microfibrillar structure.

We named these original aspects of collagen fibrils in Achilles' tendon crimps "*fibrillar crimps*" (Franchi et al. in press).

Polarized light microscopy shows crimp-like structures in almost all connective tissues, including unrelated tissues whose collagen fibrils have a different structure and a dissimilar functional significance. Ottani et al. (2001) have shown that tissues bearing unidirectional forces are mainly composed of large heterogeneous parallel fibrils (T-type fibrils) exhibiting a period of 67 nm. Tissues withstanding multidirectional forces are formed by thinner, uniform helical collagen fibrils (C-type fibrils) exhibiting a period of 64 nm.

Our present study aims to compare the structure and ultrastructure of crimps in different tendons (Achilles', flexor digitorum profundus, patellar tendons) with ligaments (collateral and cruciate ligaments of the knee) and with a wide range of other tissues (tendon sheaths, nerve sheaths, aortic wall, fascia communis) in rats. Angled fibrillar crimps, always associated with T-type fibrils, are present in all tendons and also in some ligaments where it is possible to find also C-type fibrils lacking the distinctive structure of fibrillar crimps. Fibrillar crimps were never observed in the remaining connective tissues Our data suggest that fibrillar crimps are only associated with T-type fibrils and are strictly related to the microfibril arrangement.

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SESSION 4: BIOMATERIALS

Decellularization and *In Vivo* and *In Vitro* Repopulation with Endothelial Cells of Porcine Heart Valve Leaflets

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Bioprostheses are widely used to replace cardiac valves. Besides favorable hemodynamic properties, however, these prostheses suffer from the major disadvantage of limited duration linked to sclerosis and dystrophic calcification events. These are linked, at least in part, to defective extracellular matrix (ECM) preservation and incomplete removal of native cells in currently glutaraldehyde-treated scaffolds. In addition, the absence of the endothelial coat may represent a relevant pathogenetic factor of the graft sclerotic process. As previously reported in Spina et al. (2003), we achieved complete cell extraction from porcine valve leaflets with concurrent preservation of their extracellular matrix. Moreover, as reported by Bertipaglia et al. (2003), these acellular scaffolds allowed in vitro repopulation with homologous valve interstitial cells, which also redifferentiated into all four cell phenotypes existing in heart valves.

Porcine pulmonary valvulated segments (PVCs) were decellularized using combined nondenaturating neutral detergents Triton X-100 and cholate, followed by Benzonase® digestion. Acellular PVCs were orthotopically implanted in recipient pigs for 1–2 months, or in vitro seeded with endothelial cells derived from human umbilical cord (HUVEC), and incubated for 2 weeks. Histological and TEM-SEM ultrastructural analysis was performed, also after histochemical reactions for glycosaminoglycan (GAG) localization, laminin immunolocalization, immune reactions for endothelial cell inflammatory or thrombotic phenotype.

The treated PVCs exhibited complete cell remotion, good ECM preservation, and surface reactivity for laminin. After 2-month implantation, in vivo cell colonization spontaneously occurred by two distinct cell populations: endothelial-like cells, adhering to PVC luminal areas, and mesenchymal-like cells, migrating through PVC interstitium. After cell seeding and 2-week incubation, monolayers of antiinflammatory and antithrombogenic human endothelial cells completely covered PVC luminal surfaces. Cell adhesion to the retained basal lamina and cell junction formation also were observed. In addition, valve interstitium was enriched by newly secreted GAGs. After cell seeding and 2-week incubation, micropinocytotic activity by endothelium and increased GAG-reactivity were observed.

The decellularized PVCs are propensive for both in vivo homologous cell repopulation and in vitro heterologous endothelization with HUVEC. In addition, PVC stroma acquired more and more hybrid character because human-endothelium-generated GAGs were added to the native ECM macromolecules retained within the treated porcine PVCs. Thus, these engineered PVCs appear as promising autologous-like, glutaraldehyde-free, and antithrombogenic bioprostheses.

Differentiation Patterns of Human Mesenchymal Cells after Seeding on Homograft-Derived Acellular Pulmonary Valve Scaffolds

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Calcification risk and immune response are reduced after cardiac valve decellulariztion and subsequent repopulation with recipient cells can provide durable, autogenic-like implants. Previously, we showed that porcine aortic valve leaflets subjected to decellularization (Spina et al., 2003) allowed proper in vitro colonization by valve-derived porcine fibroblasts/myofibroblasts with additional re-expression of endothelial cells and smooth muscle cells (Bertipaglia et al., 2003).

Here, we used human mesenchymal stem cells (hBM-MSC) to repopulate human valve homografts that had undergone decellularization with Triton-X-100/sodium-cholate and

aspecific benzonase treatments and incubation in fetal bovine serum (FBS) and bovine fibronectin (50 μg/ml in PBS). hBM-MSC were isolated from bone marrow by Ficoll density-gradient centrifugation, selected for plastic adhesion, expanded in MEMα plus FBS, and characterized by FACS-analysis and cytocentrifugation. Seeding with 2×10^6 cell/cm^2 was performed on ventricularis aspect of leaflets under incubation in modified DMEM Hepes plus FBS for 30 days.

Immunohistochemical analysis revealed the reconstitution of endothelial cells (von Willebrand factor), myofibroblasts (smooth muscle actin, platelet myosin, vimentin and SM22), and matrix synthesis (collagen I, III and elastin), as well as for native valves. A minor percentage of the seeded cells maintained stem cell markers (OCT4, SSEA4). The differentiated cells showed no osteogenic phenotype. Proper cell differentiation was also revealed by TEM analysis.

These data show that our decellularization procedure allows suitable in vitro cell repopulation by human mesenchymal stem cells and suggests the feasibility of a novel, autogenic-like valve bioprosthesis.

Primary Stability and Biological Fixation in Endosseous Dental Implants

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Long-term success of an endosseous implant is directly related to the amount of newly formed bone strictly adhering to its surface. Neo-osteogenesis is quicker and more extensive when the neo-osteogenetic tissue is in an initial condition of greater stability. This is why the implant, soon after its insertion, should always be firmly anchored to the bone. This clinical condition is called primary stability.

A screw-shaped implant well meets these requirements of quick neo-osteogenesis, because it alternates areas where the implant is strictly anchored to the bone (thread area) with areas where the bone-implant gap is filled with a blood clot (between two adjacent threads). In this interthreads area a very quick osteointegration process takes place, playing an important role during the first month after the surgical insertion (Franchi et al., 2004).

In the first week after surgery a bow-shaped blood vessel scaffold fills the implant-bone gap and is soon surrounded by the newly differentiating bone. During the second and third week bow-shaped trabeculae of woven bone are present. They initially follow the vascular network orientation and then turn into a lamellar shape. Then 4 weeks after surgery the lamellar-shaped trabeculae become thicker and completely fill the bone-implant gap; the newly formed bone is strictly adherent to the implant surface and is comparable to the neo-osteogenetic tissue observed 3 months after implant insertion.

Biomechanical tests and histomorphometric parameters of this research suggest that, from the second week after surgery, the peri-implant bone gives the implant a gradual "biological fixation" (Franchi et al., 2005). This biological fixation replaces the primary stability that disappears because of the bone degeneration in the thread area. The sooner the biological fixation takes place the faster the implant can be subjected to mechanical loading.

Immunohistochemical Localization of Osteopontin and In-Situ Detection of Interstitial Cell Apoptosis in Aortic Valve Leaflets Subjected to In Vivo Experimental Calcification

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A distinct pattern of cell degeneration has been described for porcine calcifying aortic valve leaflets (AVLs) after xenogenic subdermal implantation (Ortolani et al., 2002; *Histol Histopathol* 2003; *Histol Histopathol*, in press). It this situation degraded acidic lipids cluster at cell surfaces in association with calcium-binding protein Annexin-V, acting as primary nucleators of apatite. As reported, ectopic mineralization could depend on the expression of proteins involved in regulating normal osteogenesis instead of a mechanistic one. Of these, osteopontin (OPN) is an acidic phosphoprotein also present at high levels in calcified vascular tissues and plays an inhibiting role on mineralization process. In the present investigation, immunohistochemical reactions and immunogold labelling (mouse antimouse OPN monoclonal Ab, Santa Cruz) showed that actually OPN is present in AVLs after the usual preimplantation treatment with 0.6% glutaraldehyde, and subdermal implantations for 2 days, 2 weeks, and 6 weeks. Additionally, TUNEL assay (ApopTag-Peroxidase—Chemicon Int.) and ultrastructural evidences suggested that incomplete apoptosis takes place, anticipating the degradation cascade as previously reported to occur.

These data support the concept that subdermal model dependent hypoxic/anoxic conditions affect AVL cells inducing both active reactions; the transient expression of osteogenesis-involved proteins, and cell death. The latter primes the onset of the mechanistic phenomena culminating in apatite salt crystallization.

Extracellular Matrix Composition and General Conformation of Porcine Aortic and Pulmonary Root Before and After Decellularization Process

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Aortic (AR) and pulmonary (PR) root are widely used for preparation of heart valve bioprostheses. Few studies have been carried out on the extracellular matrix (ECM) organization of their components, particularly in case of decellularized scaffold preparation. Here we report quantitative data concerning both distribution of collagen and elastin in different sectors of AR and PR, as well as hydration, morphology, and dimensional variations of AR and PR leaflets before and after removing collagen and other ECM components.

Sinotubular junction and sinuses of PR exhibited higher hydration (88 $\pm$ 5 and 88 $\pm$ 5%) with respect to AR (79 $\pm$ 3 and 80 $\pm$ 3%). On the contrary hydration of AR leaflets (94 $\pm$ 1%) was similar to that of 2 PR leaflets (95 $\pm$ 1%) while hydration of a third one was significantly different (89 $\pm$ 1%). In general hydration properties did not change significantly after decellularization with detergents (TRICOL) with the exception of aforementioned third PR leaflet that then conformed to other ones.

With reference to dry defatted weight, decellularization removed about 3–6% and 3–4% of material from sinotubular junction (ST) and sinuses (SS) of AR and PR, respectively. However in leaflets the percent loss of material was significantly higher in PR (23.1 $\pm$ 12) with respect to AR (4 $\pm$ 2), particularly in the third leaflet (26 $\pm$ 4).

In leaflet concentration of elastin (12%) and collagen (51%), respectively, was similar for both vessels, whereas in ST and SS collagen resulted significantly higher in PR with respect to AR (25–26 versus 19–21%). After decellularization, concentration of collagen and elastin appeared to be increased in all AR and PR sectors. This indicated preservation of their original content by balancing loss of other components in ST and SS and AR leaflets but otherwise accounted for about 11% loss of collagen in PR leaflets. Moreover different loss of such components and hydration variation revealed existence of likely structural difference even between PR leaflets.

Isolation of elastin component in AR leaflet, resulting in loss of $\sim$88% by weight, revealed that elastic fiber scaffold was organized in a way to reproduce exactly the morphology of whole leaflet. Surface area of such scaffold accounted for $\sim$40% of that of corresponding decellularized leaflet, thus indicating pretensioning of elastic fibers in presence of collagen and other ECM components. In addition, to known thickness difference between AR and PR our present investigation revealed also differences in hydration, stability of ECM components, as well as internal variations between sectors of the same vessel.

SESSION 5: PROTEOMIC APPLICATIONS

Proteomic Modulation of Neoplastic Cells by Extracellular Matrix Components

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The mutual interactions between epithelium and mesenchyme play a pivotal role during morphogenesis, tissue homeostasis, renewal, and repair. The oncogenic transformation progressively modifies the normal tissue environment, often inducing a form of transdifferentiation epithelium/mesenchyme (loss of cell polarity and stationary state, gain of cell motility) that has been correlated with high levels of malignancy. This suggests that the metastatic propensity, rather than being associated to specific genetic alteration, is the result of epigenetic influences exerted by microenvironment conditions.

One of the major aims of our research group in the last years has been the study of neoplastic cell modulation under the influence of various microenvironment components. Along this line, several biological assays demonstrated that OF/LB collagen, a tumor derived collagen (Pucci-Minafra et al., 1993) is able to induce an increase of both cell proliferation and motility of breast cancer cells. More recently we have demonstrated that cells grown on plastic, OF/LB, and type V collagen, respectively, display different expression of a representative group of proteins (Fontana et al., 2004).

As a model for the present studies we have used the 8701-BC cell line, derived from a primary ductal infiltrating breast cancer and extensively characterized (Minafra et al., 1992). At first, we constructed a reference proteomic map, where more than 160 protein spots were identified by N-terminal or internal sequencing (Pucci-Minafra et al., 2006). To verify the effects of representative extracellular matrix molecules (collagens and SLRPs) on protein expression, we compared the proteomic profile of cells grown on plastic with that of cells alternatively grown in the presence of different ECM molecules.

Present data have shown that the proteomic profile is modulated at varied extent by individual ECM components. Protein categories that are more responsive to external stimuli are metabolic enzymes and chaperonines. This is of great interest since metabolism and protein homeostasis play key roles on tumor progression. Collectively, our present data suggest that the microenvironment may emanate influences of opposing signal, resulting from local composition of ECM, that may contribute in directing the neoplastic cells toward a more or less aggressive phenotype.

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Response of Confluent Human Dermal Fibroblasts to Serum Deprivation Investigated by Proteome Analysis

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The importance of serum factors for cell growth and cell maintenance in vitro is well known as well as the influence of serum withdrawal on cell cycle. By contrast, few data are available on the effect of serum deprivation on quiescent cells in primary cell culture. Fibroblasts may frequently experience changes in growth factors availability, as during wounding, aging, or as a result of fibrotic proceses.

The aim of our present study was to investigate the response of human dermal fibroblasts in primary cell culture to serum withdrawal applied for 48 hr after cell confluence. To avoid the effect of serum deprivation on the cell cycle and because quiescent cells are in a condition more similar to the in vivo situation, normal human dermal fibroblasts were grown for 48 hr after confluence, in 5% CO_2 and 21% O_2 in the presence or absence of 10% FBS. Cell viability, cell morphology, and reactive oxygen species (ROS) production were evaluated by light microscopy and FACS analysis. Moreover, the protein profile was investigated by two dimensional gel electrophoresis and differentially expressed proteins were identified by mass spectrometry.

Serum withdrawal caused cell shrinkage, without significantly modifying the total cell number. ROS production, as evaluated by the DH2 probe, was markedly increased after serum deprivation. By proteome analysis, 41 proteins appeared to change their expression. In particular, 11 proteins appeared significantly more expressed upon serum deprivation, whereas 30 proteins were significantly decreased. The majority of differentially expressed proteins belongs to cell metabolism, cellular cytoskeleton, and protein synthesis and folding. Interestingly, in our experimental conditions, annexin 2 exhibited the most dramatic changes, being downregulated more than 10-fold in the absence of serum.

These data suggest that membrane stability, endo- and exocityc transport mechanisms, and/or cytosketal interactions could be affected by serum withdrawal. This effect may represent a stress condition capable of influencing cell proliferation as well as cell metabolism, depending on such cell culture conditions as cell cycle and cell density. We could suggest that dermal fibroblasts can adapt themselves to environmental changes and that these cells represent a good experimental model to investigate the response of quiescent cells to serum deprivation. The model could also highlight pathways that may be a prelude to irreversible changes and to cell death after a prolonged serum withdrawal.

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Two Dimensional Gel Electrophoresis of Murine Articular Cartilage

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Many diseases of an aging population and many heritable skeletal disorders include conditions leading to cartilage degeneration causing chronic pain and loss of mobility. Despite the advances in our understanding of the molecular basis of diseases, substantial gaps remain either in our understanding of disease pathogenesis or in the development of effective strategies for early diagnosis and treatment. The current interest in disease proteomics is due in part to the prospects that a proteomic approach will overcome some limitations of other approaches.

Proteomic analysis of cartilage tissue presents several problems such as difficulties in obtaining homogeneous tissue biopsies from human patients and controls and the high amount of proteoglycans that, due to their high anionic charge, interfere with focusing, resolution, and reproducibility of two dimensional electrophoresis (2-DE). These problems might be overcome using the several animal models of cartilage disorders available to date. In this work we have tested different protocols for 2-DE analysis of murine articular cartilage. Some of which already have been applied to proteomic analysis of chondrocyte or cartilage organ cultures including proteoglycan removal before 2-DE by cetyl pyridinium chloride precipitation, ultrafiltration, or enzyme digestion.

The different strategies for 2-DE analysis have been compared, allowing a method that requires very low amount of cartilage, is consistent and reproducible, and allows the identification of both intracellular and extracellular proteins.

This method was applied to cartilage analyses of a mouse model of dyastrophic dysplasia (dtd mouse). Four spots were detected only in dtd samples and differences of expression between dtd and wild-type mice were observed in 13 spots suggesting that this method would allow protein expression studies.

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Differential Proteomic Analysis of Human Atherosclerotic Plaque Extracts

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Atherosclerosis is a form of chronic inflammation characterized by the accumulation of lipids and fibrous elements in medium and large arteries. The leading event in the atheromatous plaque development is the subendothelial retention of apoB-100 containing lipoproteins (Williams and Tabas, 1995). Subsequently, oxidative and proteolytic events, ox-lipoprotein accumulation, foam cells formation, inflammation, and cell death lead to the formation of a necrotic core surrounded by a cap of new extracellular matrix (Libby, 2002). The mechanisms underlying plaque stability are still largely unknown (Lutgens et al., 2003).

The aim of our work is the identification, by means of a proteomic approach, of intraplaque markers of both presence and stability of atherosclerotic lesions. We analyzed 48 protein extracts from segments obtained by carotid endarterectomy (19 stable and 29 unstable plaques). Proteins extracted were first separated by isoelectric focusing (pH 4–8) and subsequently by SDS-PAGE on 10% polyacrilamide gels. After blue Coomassie staining, gels were analyzed for differences by means of PDQuest v8.0 software. Peptide mass fingerprintings obtained by MALDI-TOF MS were used to identify the spots of interest.

Plaque extracts contained both plasma (∼70%) and constitutive (∼27%) proteins. We found some proteins that were either increased or decreased in abundance in relation to plaque stability ($p < 0.05$), many of which are known to possess important functions in tissue inflammation and oxidative stress.

SESSION 6: TUMOR INVASION AND ANGIOGENESIS

Effects of Endothelial Cells on Breast Cancer Cells in a Coculture System

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Endothelial cells play a pivotal role during invasion and metastasis of malignant carcinomas. Cancer cells must cross the endothelial barriers at least twice: first to gain access to the circulation and second to exit and form metastases. Therefore, reciprocal cross-talking between endothelial and cancer cells is an expected event, still not fully elucidated. The aim of our present study was to contribute to the knowledge of the biological effects exerted by human endothelial cells on 8701-BC breast cancer cells, derived from a primary breast cancer.

The cancer cells were cultured with microvascular endothelial cells (MVEC) in mixed culture conditions. The same number of endothelial cells and cancer cells were initially seeded. Coculture conditions were maintained for 48 hr. Cells were then detached by EDTA and separated by cell sorting, based on the differential expression of CD31 antigen, which was highly expressed in MVEC and almost absent in 8701 BC cells. Control and cocultured neoplastic cells, after harvesting, were divided into aliquots and separately used to extract RNA for expression analyses and proteins for proteomic assays respectively. For transcription assays, an oligo GEarrays (SuperArray), which include 288 genes involved in breast cancer and relevant candidate biomarkers, was used. Normalized data, obtained after hybridization with dUTP-biotin labelled cDNAs, were compared for signal intensity with the GEarray analyzer program. To validate the microarrays results, selected genes were analyzed by semiquantitative RT-PCR. The proteomic analyses, performed in the parallel samples, were carried out as previously described (Pucci-Minafra et al., 2006) in a Immobilin/2D IPG system.

Date collected so far demonstrated that endothelial cells exert significant influences on breast cancer cells, both at protein and transcripts levels. However, for some genes it was not possible to correlate the level of transcription with that of related proteins, suggesting that some effects of endothelium on cancer cells may act at posttranscriptional level. This study is in progress to implement the significance of data.

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Hypoxia Influence on Differential Gene Expression of 8701-BC Cancer Cells

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Hypoxia (oxygen tension [pO2] < 7 mmHg) is a frequent condition of locally advanced solid tumors associated with malignant progression and with diminished therapeutic responses. In normal cells, hypoxia can induce growth arrest or differentiation, apoptosis, and necrosis (Vaupel, 2004). Conversely, in tumor cells hypoxia can stimulate a set of genes that allow cells to fruitfully adapt to low oxygen tension and nutrient-deprivation and to survive and spread in their hostile environment. This is mainly accomplished since hypoxia blocks HIF-1α hydroxylation and thus activates HIF-1α-mediated gene expression.

The aim of our present research was to study in a broad proteomic context, the effects exerted by hypoxic conditions on the 8701-BC breast cancer cells. This cell line derives from a ductal infiltrating carcinoma of the breast, and it has been extensively characterized also for its proteomic profile (Pucci-Minafra et al., 2006). For this purpose, cells were grown at low oxygen content (pO2 2%) and examined in parallel with normoxic cells (pO2 20%).

A preliminary set of experiments were performed to establish the expression levels of a set of key-genes linked to aggressiveness of breast cancers, among which are ErbB2 and c-Myc. The results obtained by semiquantitative RT-PCR showed that, while ErbB2 expression levels underwent low modifications during hypoxia, Myc was increased several fold. To determine global protein pattern changes during hypoxia, we used the Immobilin/2DIPG electrophoresis system. Using the silver stain method, we were able to resolve about 1000 proteins from both normoxic and hypoxic samples. Presently 160 of them were identified by direct methods. Proteins to which an identity was assigned were grouped into functional

categories and the quantitative variations were calculated by the ImageMaster algorithms, using the percentage of relative volumes to normalize data.

Surprisingly, total protein pool detectable in the maps was not altered significantly by the hypoxia, suggesting that this cell line, isolated from an advanced malignant breast cancer, was already basically adapted to anaerobic metabolism.

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Renaissance of Hypericum Perforatum: A Fresh Look at its Actions Against Inflammation, Angiogenesis, and Tumor Invasion

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The use of *Hypericum perforatum* (St. John's wort) in Western medicine was well known even before the 1600 s for wound and burns healing, for diuretic activity, and to relieve various types of nervous disease. Today, controlled trials confirm the efficacy of this plant extract over placebo in the treatment of mild to moderately severe depression.

Of the different classes of *H. perforatum* secondary metabolites, the prenylated acylphloroglucinol hyperforin (Hyp) has emerged as key player for antidepressant activity. But Hyp also exerts remarkable antibiotic activity against several Gram–positive bacteria and has antiproliferative effect *in vitro* in PHA–stimulated blood lymphocytes as well as in several tumor cell lines, where it also inhibits proliferation *in vivo*.

We have demonstrated that Hyp is a good inhibitor of leukocyte elastase, exerts strong inhibition of *in vitro* tumor cell chemoinvasion, and *in vivo* produces remarkable reduction of neovascularization, size of experimental colon carcinoma metastases, and inflammatory tumor infiltration. The latter finding, the recently reported modulation of immune-inflammatory responses in intestine and liver, as well as of microtubule formation by endothelial cells, led us to investigate in more detail the effects of Hyp on polymorphonuclear (PMN) leukocytes, on their lytic capabilities, and on new vessel growth.

The results show that without affecting *in vitro* human PMN viability and chemokine-receptor expression, micromolar Hyp was able to inhibit in a dose-dependent manner their chemotaxis and chemoinvasion. PMN-triggered angiogenesis in a murine model also is blocked by both local injection and daily *i.p.* administration of the Hyp; the latter protocol enhances resolution in a pulmonary bleomycin-induced inflammation model, significantly reducing consequent fibrosis. These results indicate that Hyp is a powerful antiangiogenic and anti-inflammatory compound with therapeutic potential.

Involvement of Hyaluronan During *In Vitro* Angiogenesis of Endothelial Cells

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Hyaluronan (HA) is a large nonsulphated glycosaminoglycan of extracellular matrix and an important regulator of angiogenesis—in particular, the growth and migration of vascular endothelial cells (EC). Angiogenesis, the formation of new blood vessels, is essential for the growth and repair of tissues and is prevalent in a variety of physiological conditions. Excessive vascularization occurs in rheumatoid arthritis, diabetic retinopathy, psoriasis, and neoplasia.

To investigate the HA mechanism that regulates angiogenesis, we used culture of human vein endothelial cells (HUVEC). By polyacrilammide gel electrophoresis of fluorophore-labeled saccharides (PAGEFS) and immunohistochemistry (IHC) we showed that HUVEC did not produce HA in culture medium. Real-Time RT-PCR studies showed that HUVEC have mRNA coding for HA synthases (HAS) 2 and 3. Moreover, we showed that HUVEC-isolated membranes could be able to synthetize HA when incubated with UDP-sugar precursors.

On this basis we cloned human HAS2 and HAS3 and transfected HUVEC with these constructs. Whereas transfected HUVEC with HAS2 construct did not change in HA synthesis, HUVEC transfected with HAS3 started to produce a considerable amount of HA, evident both in medium by PAGEFS and on cell membranes by IHC. This increment in HA synthesis was coupled with a marked modification of cell migration; in particular HAS3-5 transfected HUVEC reduced dramatically their capacity to migrate in a transwell apparatus. Moreover, the same effect was observed adding HA to medium culture of no transfected HUVEC. Interestingly, the HA oligos showed a more marked effect. The decreasing capacity of migration was related to a significant vascularization of Matrigel support by HUVEC after HAS3 transfection and suggested the importance of HAS3 in HA synthesis and angiogenesis. Moreover, treatement with HA oligos added to culture medium increased HUVEC angiogenesis suggested a pivotal role of HA in the formation of new blood vessels.

SESSION 7: CONNECTIVE TISSUE DISEASES

Structural Analisys of Plasma Glycosaminoglycans in Patients with Atheromatous Lesions

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Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. Numerous risk factors such as hypertension, diabetes, and hyperlipidemia are involved in the development and progression of this pathology (Lusis, 2000). However, plasmatic markers for early diagnosis and prognosis of atheromatous lesions have not yet been identified. Some studies have evidenced variations in plasma glycosaminoglycans content associated with conditions, such as mucopolysaccharidosis and diabetes (Tomatsu et al., 2005).

The aim of our work is the quantitative and qualitative characterization of plasma chondroitin sulfate (CS) isomers to evaluate their eventual changes related to atherosclerosis presence and progression. Plasma samples obtained from patients with atheromatous lesions classified in stable ($n = 12$) and unstable ($n = 10$) plaques were freed from protein by papain treatment, and GAGs were purified by DEAE-Sephacel chromatography, depolymerized with chondroitinase ABC and derivatized with 2-aminoacridone. Analysis of derivatization products was performed in a P/ACE capillary electrophoresis system.

The quantitative analysis showed significantly increased levels of CS isomers in pathological samples compared with controls. Moreover, differences in plasma CS content between the two subgroups of pathological samples exist. This preliminary study suggests that the content of CS isomers could be a plasmatic marker both of presence and stability of atherosclerotic plaques.

New Insights on the Pathogenesis of Pseudoxanthoma Elasticum by Proteome Analysis

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Pseudoxanthoma elasticum (PXE) is a genetic disorder characterized by cutaneous, retinal, and cardiovascular lesions, basically due to progressive calcification and fragmentation of elastic fibers. The PXE gene belongs to the ABC-binding cassette family (ABCC6) and encodes for the transmembrane transporter MRP6 (multidrug resistance protein 6), whose biological function is unknown. The pathogenesis of PXE is still unclear, albeit it has been suggested recently that oxidative stress may be regarded as the pathogenetic link between ABCC6 mutations and elastic fiber calcification (Pasquali-Ronchetti et al., 2006).

The aim of our present study was to elucidate at least some of the molecular mechanisms and/or the metabolic pathways responsible for connective tissue abnormalities in PXE. The cell monolayer from in vitro cultured normal and PXE fibroblasts underwent two-dimensional gel electrophoresis using the Immobiline/polyacrylamide system. Quantitative and qualitative evaluations were performed with the Melanie 3 software. Differentially expressed proteins were analyzed by MS/MS sequencing.

Results indicate that 40 proteins were significantly differentially expressed in PXE fibroblasts compared with normal cells. The great majority of changes involved proteins of the endoplasmic reticulum, consistently with the possible derangement of these organelles in PXE. Furthermore, in PXE cells, there was a significant upregulation of calumenin, an endoplasmic reticulum chaperone that plays a central role in the regulation of gamma-carboxylation of vitamin K dependent proteins, such as matrix Gla-proteins (MGP). Interestingly, the recent literature indicates MGP acting as a local calcification inhibitor in soft connective tissues, especially in elastic fibers (Schurgers et al., 2005). Increased expression of calumenin may inhibit gamma-carboxylase activity, causing a possible decrease of MGPs. These data bring new light to the increased susceptibility of elastic fibers to calcify, as in PXE.

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Fibroblasts from Patients with β-Thalassemia and Pseudoxanthoma Elasticum-Like Clinical Manifestations

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Several reports have pointed to the frequency of skin, eye, and vessel clinical manifestations in β-thalassemia patients resembling those typical of inherited pseudoxanthoma elasticum (PXE). Moreover, ultrastructural studies have shown that in both disorders the dermis exhibits calcification of elastic fibers, deposition of abnormal matrix constituents in the extracellular space, and abnormal collagen fibrillogenesis. Beta-thalassemia patients with PXE-like clinical manifestations do not exhibit mutations in the ABCC6 gene.

In the present study, in vitro dermal fibroblasts from β-thalassemia patients with (PXE+) and without (PXE−) PXE-like clinical manifestations were compared with those from PXE and from controls as far as their redox balance and ability to

accumulate and to extrude calcein, a marker used to evaluate the efficiency of MRP/MDR membrane transporters. Fibroblasts from β-thal-(PXE+) behave similarly to those from PXE having a significantly higher level of reactive oxygen species (ROS) compared with controls and to β-thal-(PXE-) cells ($p < 0.05$). In addition, β-thal-(PXE+) cells accumulate significantly more and release significantly less calcein compared with controls and to β-thal-(PXE−) cells, revealing a behavior almost identical to cells from inherited PXE. However, the effect of inhibitors (indomethacin, benzbromarone, MK571) of calcein release differs between PXE and β-thal-(PXE+) cells, indicating that the MDR/MRP deficiency in β-thal-(PXE+) cells is due to a mechanism different from that in PXE.

Data suggest that PXE-like clinical manifestations in a number of β-thalassemia patients may depend on mild chronic oxidative stress associated with or causing deficient activity of membrane transporters and leading to metabolic alterations similar to those in PXE.

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Bone Phenotype in a Mouse Model of Diastrophic Dysplasia

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Mutations in the *diastrophic dysplasia sulfate transporter (DTDST* or *SLC26A2)* gene cause a family of recessively inherited chondrodysplasias that includes diastrophic dysplasia (DTD). This gene encodes for a sulfate/chloride antiporter expressed on the surface of many cell types (including osteoblasts), whose function is crucial for the uptake of inorganic sulfate needed for proteoglycan sulfation.

We recently have generated a mouse model for diastrophic dysplasia (dtd mouse); dtd mice show a partial loss of function of the DTDST, causing a chondrodysplastic phenotype that recapitulates essential aspects of DTD phenotype in humans.

To better characterize the bone phenotype, which has never been studied in DTD patients, we performed static and dynamic histomorphometry in long bones from 1- and 2- months-old-mutant and wild-type mice. For evaluation of bone dynamic histomorphometry, prior to sacrifice, the animals received by intraperitoneal injection two fluorochromes at set intervals of time. After dissection long bones were fixed in 70% ethanol. Tibiae were used for X-ray and DXA analyses. Femurs were dehydrated, embedded without demineralization in methyl methacrylate, cut with a microtome, and stained for microscopic viewing. Consecutive sections of the same sample were used to evaluate static and dynamic parameters. The measurements were performed in compliance with the recommendations of the American Society for Bone and Mineral Research Histomorphometry Committee.

At all the age points considered, alterations in the bone phenotype of dtd mice were observed, even though neither severe osteoblastic defects nor mineralization defects were detected.

Furthermore, these data suggest that the bone phenotype in dtd mice should be due to defects in bone resorption or to systemic factors but not to a primary osteoblastic defect.

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