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**LECTURE IN HONOUR OF
ALESSANDRO A. CASTELLANI**

**Human sialidases: molecular biology, biochemistry and
physiopathological implications**

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Sialidases are expressed in viruses, bacteria, protozoa and vertebrates. Viral, bacterial and protozoan sialidases display about 35% homology in their primary structure, and share some specific motifs: six four-stranded antiparallel β -sheets arranged as the blades of a propeller; a F(Y)RIP motif located toward the aminoterminal region; a repetitive motif SXDYGXXT/W in the active site region; some Asp-boxes. In mammalian (including human) organs and tissues, sialidases display different subcellular locations (lysosomes, plasma membrane, cytosol and, possibly, other locations) and substrate specificities. The lysosomal, cytosolic and plasma membrane sialidases, also from the same animal, have different protein structure, antigenic properties and cDNAs.

So far, the following human sialidases have been cloned: the lysosomal sialidase *NEU1* coded by *NEU1* gene mapped in 6p21, the cytosoluble sialidase *NEU2* coded by *NEU2* gene mapped in 2q37, the plasma membrane bound sialidase *NEU3* coded by *NEU3* gene mapped in 11q13, and the sialidase *NEU4* coded by *NEU4* gene mapped in 2q37. The corresponding recombinant sialidases constitute powerful tools for studying the structure-function relationships of these enzymes. As an example, recombinant human cytosolic sialidase (Hs *NEU2*), expressed in *E. Coli*, was purified to homogeneity and its crystal structure defined. The presence of the canonical six blades β -propeller structure, F(Y)RIP motifs and three Asp-boxes, typical of all studied sialidases, was confirmed. In addition, with the use of the inhibitor DANA (2,3-dehydro-3-deoxy-N-acetylneuraminic acid) the dynamic nature of substrate recognition was assessed. Recombinant Hs *NEU2* was proven to differentially recognise: (a) the type of sialosyl linkage; (b) the aglycone part of the substrate (gangliosides act as the best substrates); and (c) the supra-molecular organization (monomer, micelle, vesicle) of the ganglioside substrate. The latter ability might be relevant in sialidase interactions with gangliosides under physiological conditions.

Some plasma membrane bound sialidases appear to be attached to the membrane via a glycan-phosphoinositide (GPI) anchor, possibly indicating their location in specialized plasma membrane "domains" (lipid rafts). Surface sialidase *NEU3* is able to affect sialoglycolipids exposed on the surface of vicinal cells and was shown to be directly involved in neural (with a specific location on the "growth cones") and myoblast differentiation. In the latter case the block of sialidase expression led to inhibition of the differentiation toward myocytes. Sialidases appear also to be involved in human erythrocytes maturation and ageing under physiological and pathological conditions. Very recent results point to the implication of sialidase *NEU3*, and its hydrolysis products gangliosides GM1 and GM2, in the insulin post-receptor signaling events, and of sialidases *NEU2* and *NEU3* in tumor growth. The genetic lack of sialidase *NEU1* is responsible of a very severe lysosomal disease.

**LECTURE IN HONOUR OF
LORENZO GOTTE**

**The supramolecular organization of elastin in the elastic
fiber scaffold of heart valves**

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Elastin is a highly cross-linked polymer that confers elasticity to soft connective tissues in vertebrates. It forms huge branched fibers and lamellae of the order of 0.1 to 10 micron thick and several microns long, according to different organs. The monomeric unit is approximately a 60-kDa protein but still the mechanism and the steps by which the monomer aggregates to give rise to the supramolecular architecture in the tissue is substantially not understood.

The elastic scaffold of pig heart valve was isolated by means of non-degradative procedure and analysed by TEM as well as by OM, SEM and ESEM in controlled conditions in order to evaluate possible distortion and modification of the tridimensional architecture induced by the observation procedures. Eventually the elastic scaffold was fragmented by ultrasonication at different temperature and the resulting fragments observed by SEM and ESEM.

Fiber, lamellar aggregates and thin sheets composed the typology of the morphological elements. Thicker fibers and lamellae apparently resulted from longitudinal and planar net aggregation of thinner fibrillar elements of the order of 100-140 nm exhibiting an elliptical cross-section. However the shape of thicker fibers was not constant and the diameter did not result simply from the addition of smaller discrete elements, branching was frequent and at different angle. The shape of thicker fibers was cylindrical, flat ribbon- and twisted ribbon-like. Very thin sheets were spread between fibers and lamellae and often did roll over becoming integral part of the surrounding fibrous elements. After ultrasonication thicker fibers did not resolve into thinner fibrillar elements but in sheets and cylindrical flakes.

The whole of the experimental evidence does not indicate the presence of a subfibrillar architecture. Apparently fibers may result from the aggregation of laminar components.

CONNECTIVE TISSUES: MORPHOLOGY, MOLECULES AND CELLS

Overexpression of $\alpha 5$ integrin and its role in muscle development

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Integrins are molecules that transmit signals bidirectionally across the plasma membrane. In mammalian skeletal muscle, the $\beta 1$ family of integrins is found at neuromuscular junction (NMJ), myotendinous junction (MTJ) and the sarcolemma, though their expression vary depending on the stage of development. $\alpha 5\beta 1$ and $\alpha 7\beta 1$ are among the major players in muscle differentiation: during embryonic stage $\alpha 5\beta 1$ is found at the MTJ and is substituted by $\alpha 7\beta 1$ in the adult. Both of the subunits have been involved in the development of muscular dystrophy. To understand the crosstalk between $\alpha 5$ and $\alpha 7$, mice overexpressing $\alpha 5$ have been generated: the overexpression was controlled by the human skeletal α -actin promoter whose onset expression is at embryonic day 12 or by the muscle creatine kinase promoter whose onset expression is at birth. Sub-strains overexpressing a mutated form of the $\alpha 5$ subunit have been generated as well. Immunoprecipitation and immunoblotting were used to investigate the formation of $\alpha 5\beta 1$. The co-localization of the subunits was checked by immunostaining of muscles. In the overexpressors, $\alpha 5$ associated with $\beta 1$, with the exception of the mutant. Immunostaining showed a decrease of the two $\alpha 7$ splice variants especially at the MTJ, substituted by an increase of $\alpha 5$. All the mice showed muscular dystrophy. It seems that the two α subunits were competing for association with $\beta 1$, as in the integrin $\alpha 7$ knock out muscle $\alpha 5$ is the only α subunit to co-precipitate with $\beta 1$. This analysis suggests the roles of $\alpha 5$ and $\alpha 7$ in muscle development and integrity and that the correct conformation of integrin $\alpha 5$ is vital to muscle fibre integrity.

The role of the hypoxic condition on the protein profile of human fibroblasts

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Tumor progression is influenced by extracellular matrices and by soluble factors or cytokines that are locally produced by host tissue cells and that may sustain one of the most insidious aspect of tumors, namely their propensity to invade normal tissues and to form metastases in organs at distant sites from the primary tumor. It is generally accepted that growth of solid tumors is dependent on the formation of new blood vessels. The angiogenic response of the host is mediated by angiogenic molecules that are released from cancer and normal stroma cells, especially fibroblasts. Moreover, under hypoxic conditions such as those found during tumor growth, a variety of angiogenic growth factors are upregulated in normal and transformed cells, suggesting that an interrelationship between hypoxia and angiogenesis may exist. Most studies on oxygen-dependent regulation of gene expression have been performed with tumor cells; however, besides tumor cells, normal stroma cells, eg, endothelial cell, immune competent cells and fibroblasts, may change their protein profile and phenotype, thus influencing neighbouring cells. Human dermal fibroblasts were grown for

96h in 5% CO₂ and 21% O₂ (pO₂=140mmHg) or 2% O₂ (pO₂=14mmHg). The hypoxic condition does not influence the proliferation rate as well as the protein content of fibroblasts. By proteome analysis we demonstrated that 27 protein were differentially expressed at least three times in the two experimental conditions, in particular 30% appeared upregulated whereas 70% were downregulated in the hypoxic conditions. By mass spectrometry we identified elongation factor 1- $\alpha 1$, α -enolase, protein disulfide isomerase, pyruvate kinase, NDRG1 protein, 40S ribosomal protein SA, calmodulin, fetuin-A and parathymosin among the proteins whose expression was influenced by changes in the pO₂. In conclusion, data indicate that fibroblast respond to the hypoxic condition by activating stress response pathways and possibly altering post-translational protein maturation.

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Cytokines-induced increase of cell hyaluronan synthase activity: studies after oxidative injury in fibroblast cultures

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It was previously reported that high levels of glycosaminoglycans (GAGs) are found after free radical injury (Campo GM *et al.*, Chem Biol Interact, 148: 125, 2004). The addition of hyaluronic acid (HA) was able to protect cells from oxidative stress. These outcomes were positive by using both commercial HA and purified plasma GAGs of human origin (Campo GM *et al.*, Free Radic Res, 38: 601, 2004; Campo GM *et al.*, Toxicol In Vitro, 19: 561, 2005). HA synthesis is regulated by three hyaluronan synthases (HAS1, HAS2 and HAS3). Recently it has been reported that several cytokines such as interferon- γ (IFN- γ), tumor necrosis factor- α , (TNF- α), etc. are able to modulate HASs transcription in cell cultures (Stuhlmeier KM and Pollaschek C, J Biol Chem, 279: 8753, 2004). The net consequence of this modulation is an increase in HA levels. The aim of this research was to evaluate the protective effects of the increased endogenous HA concentration, obtained by using cytokines stimulation, in fibroblast cultures exposed to oxidative stress. IFN- γ and TNF- α were added to the fibroblast cultures before oxidative stress induction by using the oxidizing system employing Fe²⁺ plus ascorbate. We assessed HAS1, HAS2 and HAS3 mRNA content, HA levels, cell death, lactate dehydrogenase activity, membrane lipid peroxidation, and endogenous antioxidant depletion. The treatment of fibroblasts with cytokines up-regulated HASs gene expression and increased HA production, while the exposure of the cultures to Fe²⁺ produced cell death, enhanced lipid peroxidation and lactate dehydrogenase activity and decreased antioxidant defences. The effect of the endogenous increase of HA, induced by the cytokines, to protect cell from oxidative injury is especially discussed.

The effect of glycosaminoglycan and proteoglycan addition to murine chondrocytes in culture on gene expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs)

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The functional integrity of articular cartilage is determined by a balance between chondrocyte biosynthesis of extracellular matrix and its degradation. The matrix metalloproteinases (MMPs) constitute a family of secreted/cell-surface-anchored zinc endopeptidases, which are essential mediators of normal tissue remodelling as well as of tissue destruction in both inflammatory and degenerative joint diseases. The proteolytic activity of the MMPs is normally regulated by endogenous specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). In this study we examined the effect of murine chondrocytes culture treatment with commercial Glycosaminoglycans (GAGs: Hyaluronan, HA, Chondroitin-4-sulphate, C-4-S, Chondroitin-6-sulphate, C-6-S, Dermatan sulphate, DS) and purified human plasma Proteoglycan (PG) preparation (mainly composed of C-4-S PGs) on the gene expression of MMPs (MMP1a, MMP1b, MMP2, MMP3, MMP8, MMP9, MMP13, MMP14, MMP16), TIMPs (TIMP1, TIMP2, TIMP3 and TIMP4), IL1 β and TGF β . GAGs are expected to affect matrix biosynthesis; plasma PGs are considered breakdown product of cartilage metabolism. A dose dependent effect was studied by using three different amounts for each additive product. Furthermore, the mRNAs transcription was evaluated at 24 and 48 hours. The addition, to the chondrocytes cultures, of commercial HA and plasma PGs preparation showed a more evident effect with respect to the other molecules. The HA handling, in a dose dependent way, increased MMP8 and MMP9 gene expression, while purified plasma PGs treatment increased both MMP3 and MMP9 mRNA levels with no apparent dose effect.

Integrin and laminin chain expression in alpha 7 deficient smooth muscular tissue

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In muscular tissues, alpha7beta1 is the major integrin receptor for laminins, which are represented by isoforms laminin 2 and 4 (basal laminae of skeletal and cardiac muscle cells) 8 and 10 (smooth muscle cell basal laminae). The absence of alpha7 integrin chain leads to the onset of skeletal muscular dystrophies (Mayer U *et al.*, Nature Genet 1997, 17, 318), and cardiac hypertrophy (Ortolani F *et al.*, *in press*). In alpha 7 integrin chain deficient mice, we previously observed different degrees of ultrastructural alteration between *viscera* wall smooth muscle cells of those in vascular walls. Since moderate up-regulation of alpha 5 integrin chain was described for alpha 7 deficient skeletal muscle (Nawrotzki R *et al.*, Hum Mol Genet 2003, 12, 483), here compensatory overexpression was investigated of other alpha integrin chains and laminins in alpha 7 deficient mice, concerning smooth muscle cells of *muscularis externa* of oesophagus, stomach, *ileus*, colon, bladder, *uterus* as well as those in *tunica media* of vessels of the heart,

different skeletal muscles, and *viscera* walls as above. No differences appeared for alpha 5 and alpha 6 integrin chain distribution pattern in both control and K.O. mice, whereas remarkable increase in alpha 3 integrin chain was apparent in K.O. smooth muscle cells *versus* control ones, more evident in *viscera* walls than in vascular ones, consistently with the weaker damage observed. Concerning the distribution of laminin chains, no differences was detected by comparing w/t and K.O. mice.

Hyperforin inhibits PMN migration, inflammation-triggered angiogenesis and pulmonary fibrosis

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Hyperforin (Hyp), a polyphenol-derivative of St. John's Wort, has emerged as key player in the anti-depressant activity of the plant, but also as inhibitor of bacteria, lymphocyte and tumour-cell proliferation, and matrix proteinases. We tested whether, besides inhibition of LE activity, Hyp might efficaciously contain both polymorphonuclear (PMN) leukocyte recruitment and unfavorable eventual tissue responses. The study analyzed human PMN-conditioned media by gelatin zymography, PMN chemotaxis and chemoinvasion through gelatin and Matrigel, expression of PMN chemokine-receptors and integrins by cytofluorimetry, inhibition of PMN-triggered angiogenesis in an IL-8-induced murine model, restraint of inflammation-triggered lung fibrosis in a bleomycin-induced murine model. Without affecting PMN viability, chemokine receptor or CD11b/CD18 integrin array, the compound exerts efficient dose-dependent restraint of their chemotaxis and chemoinvasion (IC50 1 μ M, for both), blocking the LE-triggered activation of the extravasation-instrumental gelatinase MMP-9, preserving its zymogen form from spontaneous rapid turnover, and up-modulating ERK1/2 activation. PMN-mediated angiogenesis *in vivo* is blocked by both local injection, and daily i.p. administration of Hyp; the latter protocol enhances resolution in a pulmonary inflammation model, significantly reducing consequent fibrosis. These results indicate that Hyp is a potent anti-inflammatory compound with therapeutic potential, and shed light on mechanistic keys.

The 3D structure of crimps in stretched and relaxed collagen fibers of rat gastrocnemius tendon

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Stretching training enhances joint range of motion and is recommended to improve performance during human movements and prevent tendon and muscle injuries.¹ No *in vivo* morphological study has been carried out on tendon stretching both in humans or animals. In this study we reproduced the *in vivo* mechanical conditions of a static stretching single training applied to gastrocnemius muscle and Achilles tendon of six rats (Sprague Dawley) 3 months old. A resin adapted tutor was applied to a posterior leg of each animal kept under anaesthe-

sia dorsally flexing the foot of 55° for 10 minutes. Achilles tendon of the controlateral leg was kept relaxed as control. Tendons of both legs were fixed in Karnovsky solution and then excised to be processed for direct light microscopy (DLM), polarized light microscopy (PLM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Control tendons showed at DLM, PLM, SEM and TEM parallel wavy collagen fibres corresponding to collagen crimps described in literature as helical structures, flattened helices, spirals or smooth waves. Control crimps (angle of 7°) were morphologically studied.² In stretched tendons crimps (angle of 7°) almost disappeared. The mechanism of elongation of tendon during stretching consists in an almost complete disappearance or evident flattening of crimp structures. In fact, when present, crimps of stretched tendon showed a reduction of the angle of about 40% vs. control crimps.

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Hyaluronan metabolism in *in vitro* culture of endothelial cells

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Hyaluronan (HA) is a non sulphated glycosaminoglycan present in extracellular matrix and it is involved in a variety of physiological processes, such as cellular growth, migration and tumor progression. The mechanisms underlying the overproduction of HA by stroma cells surrounding tumors have not been completely elucidated. The biosynthesis of HA is regulated by three mammalian HA synthases, HAS1, HAS2 and HAS3, that differ from each other in the expression profile in embryonic development and in their ability to form HA with different length. As eucariotic cells show different efficiency in HA production, we have established a model to compare two vein endothelial cells cultures *in vitro* (ECV, immortalized cell from endothelial vein and HUVEC, human umbilical vein endothelial cells). ECV and HUVEC (Cambrex) have been cultured in complete EGM2 medium according to protocols. To measure the HA production, conditioned media were collected and analysed by electrophoresis technique (FACE). From each cultures we extracted the HA cytoplasmatic precursors (UDP-sugars) using Triton X-100 and acetonitrile and we separated UDP sugars by capillary electrophoresis. Gene expression studies were carried out by Taqman RealTime RT-PCR on total RNA extracted from ECV and HUVEC in order to study the gene expression of HA synthases, hyaluronidases (Hyal) and HA membrane receptor CD44. Using membrane impedance quantification we evaluated cell migration ability.

Culture medium of ECV has been found to have higher HA content than HUVEC whereas UDP-sugars showed similar concentration. Gene expression experiments have shown a substantial induction of the mRNA coding for only HAS3 in ECV. As the induction of HA degrading enzymes in ECV have been slighter than HAS3 increase, the HA accumulation in these cells could mainly depends on HAS3 up regulation rather than Hyal down regulation in ECV. Moreover, the higher level of HA in ECV correlated with an elevated migratory capacity of this cells.

β2-microglobulin isoforms display an heterogeneous affinity for type I collagen

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Amyloidosis associated with long term haemodialysis (DRA) is caused by fibrillar deposition of β2-microglobulin (β2-m) and it is localized in muscle skeletal system. It has been claimed that β2-m interacts with type I and type II collagen and this property has been linked to deposits tissue specificity. The binding parameters of the interaction between collagen and β2-m were determined by band shift electrophoresis and surface plasma resonance by using bovine collagen of type I and various isoforms of β2-m. Wild type β2-m binds collagen type I with a K_d of 4.1×10^{-4} M and type II with 2.3×10^{-3} M. By the BIAcore™ system we monitored the binding properties of the conformers of the slow phase of folding of β2-m (Chiti *et al.* 2001 *J. Mol. Biol.* 307:379-391). The folding intermediates during the slow phase of folding don't display any significant difference with respect to the binding properties of the fully folded molecule. Increased affinity for collagen type I is found in the case of N-terminal truncated species lacking of six residues. The K_d of this species is 3.4×10^{-5} M at pH 7.4 and its affinity increases to 4.9×10^{-6} M at pH 6.4 (Giorgetti *et al.* *Protein Sci.* 2005 14:696-702). Fluctuations of the affinity caused by β2-m truncation and pH change can cause modifications of protein concentration in the solvent that surrounds the collagen and could contribute to generate locally a critical protein concentration able to prime the protein aggregation.

Human recombinant prolidase: synthesis, purification and characterization

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Prolidase is a dipeptidase involved in the latter stage of proteins catabolism. It hydrolyses imidodipeptides containing proline or hydroxyproline at the C-terminal position. Mutations in the prolidase gene (PEPD) cause Prolidase Deficiency (PD), an autosomal recessive connective tissue disorder. The enzyme structure, catalytic site and kinetic are still mainly unknown as well as the genotype-phenotype relationship in PD.

We generated a recombinant human prolidase in eukaryotic (CHO) and prokaryotic (E.Coli) systems. Total RNA from human fibroblasts was extracted, retrotranscribed, PCR amplified with specific prolidase primers and subcloned respectively in pcDNA4HisMax and pET16b+ vectors, both containing an N-terminal poliHis Tag. The enzyme was purified by affinity chromatography using the Ni-NTA Agarose resin and a step imidazole gradient from 50 to 500 mM. Enzyme activity and Western Blotting with Anti His antibody were used to follow the purification. Both recombinant enzymes had temperature and pH range of activity similar to human fibroblasts prolidase, with a maximum at 50°C and pH ≈ 8. They showed also similar inhibition susceptibility to Cbz-Pro and similar long term stability at 37°C. Enzymatic digestion of the His Tag results in an increase of activity, but did not alter the other parameters. Western blotting on cytosolic and nuclear fractions of CHO transfected cells and confocal microscopy demonstrated a cytosolic localization of the prolidase enzyme.

In conclusion the recombinant enzyme reproduces the biochemical characteristic of the human enzyme representing a valid tool to better investigate and characterize this enzyme and to develop an enzyme replacement therapy for human PD.

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Characterization of ECM scaffolds obtained by decellularization of bovine nasal cartilage

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The object of this study is the characterization of scaffolds obtained by decellularization of bovine nasal cartilage. The aim is to prepare intact structured ECM scaffolds with increasing porosity which in turn could act as substrate for repopulation with chondroblasts.

Such scaffolds have been prepared removing cell components by combining ipotonic conditions in presence of protease inhibitors followed by detergent like Tris and Deoxycholate (TRIDOC). By an alternative procedure samples were previously treated with 0.9 M Urea (U-TRIDOC). The porosity of the system was tentatively increased by subsequent partial removal of GAG with testicular hyaluronidase. Residual scaffolds were analysed for collagen, uronate and water content and finally observed by TEM.

Hyaluronidase removes 33% of total dry weight from original untreated samples (NT), while TRIDOC and U-TRIDOC 32 and 23%, respectively, when followed by hyaluronidase. Thus pretreatment with urea apparently stabilizes the matrix by reducing the amount of material solubilized by hyaluronidase itself. This material is composed by collagen and accounts to about 10% of the initial dry weight. Hyaluronidase solubilizes 25% of total uronate from NT sample and 35 and 34% when in combination with TRIDOC and U-TRIDOC, taking into account, however, that 33 and 32% of total uronate had already been removed by detergent treatment, respectively. When examined by TEM cells appear to have been removed from ECM lacunae.

In addition to cells, detergents remove significant amount of collagen and GAG by themselves. Pretreatment with urea prevents the specific removal of collagen following hyaluronidase treatment but does not influence its action on GAG.

Physiological and pathological aspects of the shape modular structure of cartilage, involving decorans. The aetiology of osteoarthritis

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The extracellular matrix (ECM) shape module concept has evolved through successive stages; Morphological demonstration of regular interfibrillar bridges of lengths characteristic of the ECM, present in all non-mineralised ECMs; Biochemical identification of decorans non-covalently attached at specific binding sites to collagen fibrils via decoron proteins with antiparallel dermocondan sulphate (DS) aggregates bridging the fibrils; Physiological, contributing vital elasticity to the ECMs.¹ What pathologies are there?

Cartilages are built of shape modules, explaining for the first time anisotropic responses of articular cartilage to compressive and tensile stresses.² Degradation of shape modules in

osteoarthritis, the major crippling disease, reduces these responses. Loss of shape modular function in binding together collagen fibrils via decoran bridges, results in sucking-in of water as fibrils drift apart under the swelling pressure of the aggrecan, etc. Elastic decoran helps to define and maintain cartilage shape whereas expansile aggrecan has less influence on morphology. DS contains L-iduronate, a sugar that switches elastically between conformers of different lengths under tension.³ Stanesco claimed loss of a decoran-like PG in osteoarthrotic cartilage. Decoran may be a primary target of the OA disease process.

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Molecular effects of glucosamine and its derivative on cartilage homeostasis

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Introduction. Glucosamine (GS), a symptom-control drug, was found to entail also joint structural changes in Osteoarthritis. We report the effects of GS and its derivative N-Acetyl Phenylalanine Glucosamine (NAPA) on an immortalized chondrocyte cell line LBPVA 55 (Osteoarthritis and Cartilage, 2002, 10:879) to explain the *in vitro* results previously obtained (Eur. J. Med. Chem. 1991, 26:753). Methods. LBPVA 55 cells were stimulated with TNF α (10 ng/mL) and treated with GS and NAPA at 0.25 mM and 10 mM respectively, the latter mimicking therapeutic concentration. mRNA expression was analysed by cDNA microarray and by Quantitative Real Time PCR (Q-RT-PCR). Results and discussion. A number of genes, analyzed by microarray, were up-regulated by TNF α and modulated by GS and NAPA. To confirm the microarray results, Q-RT-PCR analysis was performed. Only 15% of genes detected by microarray showed the same trend with Q-RT-PCR, probably due to difference in sensitivity of the two methods. TNFR1, a receptor for TNF α and TRAF6, a molecule involved in TNF α pathway, resulted up-regulated by TNF α and modulated by GS and NAPA. Cullin2, rho6, a small GTPase, IL-1 β and IL-6 mRNA expression was modulated as well. At NAPA and GS 10mM concentration, IL1 β resulted modulated by GS, but not by NAPA, whilst NAPA gave greater modulation of TNFR1, TRAF6 and rho6. We previously demonstrated that both drugs successfully re-organize articular cartilage *in vivo* (XIX FECTS, 2004, PM11), and now our findings assess that they affect gene expression of proteins involved in inflammatory cascade and cartilage homeostasis.

Identification of free decorin core protein in tendon

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Decorin is a multifunctional proteoglycan expressed in the extracellular matrix of any connective tissue and its functions are correlated to the biological activity of both the core protein and the glycosaminoglycan moiety. It is known that decorin core protein is able to bind several molecules such as fibronectin, thrombospondin and TGF β , so it is of relevant interest to try to identify the eventual presence of the free core in the tissues, besides the mature form, to define the correct biological potentiality of the proteoglycan. Decorin was purified from bovine tendon; the whole proteoglycan and the GAG-free decorin core were obtained by 4 M guanidine hydrochloride extraction of tendons, ultracentrifugation in CsCl gradient and DEAE Sephacel chromatography. The identity of the GAG-free core was determined by western blotting stained with polyclonal anti decorin antibody and its migration pattern was similar to the one obtained after ABCase treatment of the mature form of the proteoglycan. Roughly, the weight ratio between GAG-free core and the whole decorin was 1:10. We are now performing preliminary binding studies of the native core protein with acid-soluble type I collagen and pepsinized type II collagen, in order to compare the preparation with the core protein obtained by enzymatic digestion of the glycosylated proteoglycan. Moreover, it will be interesting to determine the variation of the free core protein content in different physiological and pathological conditions.

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Molecular cloning and characterization of UDP-glucose dehydrogenase from the amphibian *Xenopus laevis* and its involvement in hyaluronan synthesis

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UDP-glucose dehydrogenase (UGDH) supplies the cell with UDP-glucuronic acid, a precursor of glycosaminoglycan and proteoglycan synthesis. In our study, we cloned and characterized the UGDH from the amphibian *Xenopus laevis* that is one of the model organisms for developmental biology. We found that *Xenopus* UGDH (xUGDH) maintained a very high degree of similarity with other known UGDH sequences both at the genomic and the protein levels. Also its kinetic parameters are similar to those of UGDH from other species. During *X. laevis* development, UGDH is always expressed, but clearly increases its mRNA levels at the tailbud stage (i.e., 30 hours post fertilization). This result fits well with our previous observation that hyaluronan, a glycosaminoglycan that is synthesized using UDP-glucuronic acid and UDP-N-acetyl glucosamine, is abundantly detected at this developmental stage. The expression of UGDH was found to be related to hyaluronan (HA) synthesis, in fact in human smooth muscle cells the overexpression of xUGDH or endogenous abrogation of UGDH altered UDP sugar concentration in cells and modulated specifically HA synthesis. Our findings were confirmed by *in vivo* experiments where the silencing of xUGDH in *Xenopus* embryos determined a HA decrease and severe malformations at neural tube during early development.

Effects of EPO on the structural recovery of the nervous tissue in a rat model of traumatic spinal cord injury

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Recent studies have shown that the acute administration of recombinant human erythropoietin (rhEPO) attenuates the effects of traumatic spinal cord injury and accelerates functional recovery in a rat experimental model (Gorio *et al.*, PNAS 99:9450-9455, 2002). Our investigation aimed at elucidating the mechanisms, at present largely unknown, through which EPO exerts its beneficial actions. Since the formation of a glial scar enriched in chondroitin sulfate proteoglycans (CSPGs) has a repulsive effect on axonal regrowth and compromises functional outcome, we investigated whether rhEPO reduces the extension of the glial scar and influences the expression of CSPGs. To this end, we applied immunocytochemical methods at light and confocal microscopy to study the distribution of neurocan, aggrecan and versican in the spinal cord of rats at 30 days after contusive spinal cord injury, with or without rhEPO administration. Our results showed that, in comparison to controls, in the injured spinal cords 1) all the molecules considered were upregulated throughout the whole grey and white matter, and not only at the lesion site; 2) the administration of rhEPO did not significantly modify their levels, although it significantly reduced the size of the glial scar, the extent of the gliosis and the amount of macrophages and microglia. The complex of the results indicates that rhEPO does not influence the expression of CSPGs considered in the present study, but exerts an anti-inflammatory effect that improves the structure of the nervous tissue by reducing the extent of the secondary damage.

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CONNECTIVE TISSUE DISEASES AND CANCER

Calcification in human stenotic aortic valves: involvement of acidic lipids and annexin-V

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Acidic lipid clustering at the surface of cells and matrix vesicles and associated calcium-binding protein Annexin-V (Anx-V) have been found to act as major apatite nucleators in aortic valves subjected to experimental calcification (Ortolani *F et al.*, *Connect Tissue Res* 43: 44-55, 2002; *Histochem J* 34: 41-50, 2002; *Histol Histopathol* 18: 1131-40, 2003). Here, possibility was investigated that these degenerative processes also occur in pathological valve calcification.

After explantation, human calcified valves affected by aortic stenosis were subjected to reaction with 0.05% Cuprolinic Blue + 2.5% glutaraldehyde + 0.05 M MgCl₂ in phosphate solutions, pH 4,8 (GA-CB). Semithin sections of GA-CB-reacted samples underwent von-Kossa-silver-staining and re-embedded to achieve reacted thin sections (GA-CB-S). Histological sections underwent von-Kossa-silver-staining (S). Cryosections underwent immunohistochemical reactions for Anx-V. LR-White-thin sections underwent immunogold reactions for Anx-V. Polyclonal AB anti-Anx-V R88 was used (courtesy of Klaus von der Mark).

As for experimental valve calcification, it was observed clustering of acid lipids around cell debris, matrix vesicles and elastic fibers, colocalization between GA-CB and GA-CB-S reactivity, and immunogold labelling for Anx-V which was closely associated with the accumulating lipidic material.

These results suggest common mechanisms to be shared by this pathological calcification and experimental one, including initial hypoxia-induced up-regulation of Anx-V (Denko *N et al.*, *Clin Cancer Res* 6: 480-7, 2000) and subsequent translocation due to protein avidity for the exuding lipids.

On the pathogenesis of PXE-like clinical manifestations in β -thalassemia patients

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A number of β -thalassemia patients, with no mutations in the ABCC6 gene, have clinical and histological connective tissue alterations almost identical to those in genetic PXE. In vitro dermal fibroblasts from β -thalassemia patients with (PXE+) and without (PXE-) PXE-like alterations were compared with those from genetic PXE and from controls as to their redox balance and ability to accumulate and to extrude calcein, a marker used to evaluate the efficiency of MDR/MDR membrane transporters. Cells from β -thal-(PXE+) behave as those from genetic PXE, since they have a higher level of vitamin E-sensitive ROS compared to controls and to β -thal-(PXE-) cells. Moreover, β -thal-(PXE+) cells accumulate significantly more and release significantly less calcein compared to controls and to β -thal-(PXE-) cells, revealing a behaviour almost identical to cells from PXE. These findings indicate that β -thal-(PXE+) cells suffer from a mild oxidative stress and

have an impaired function of MRP/MDR transporters similar to that in PXE. However, the effect of inhibitors (indomethacin, benzbromarone, MK571) of calcein release is different in PXE and in β -thal-(PXE+) cells, suggesting that the MRP/MDR deficiency in β -thal-(PXE+) cells is due to different mechanisms compared to that in PXE. This agrees with the datum that ABCC6 gene and expression are normal in β -thal-(PXE+) cells and indicate that the impaired transport activities may depend on other genes and/or epigenetic factors. In conclusion, data suggest that PXE-like clinical manifestations in β -thalassemic patients could depend on an oxidative stress associated with or causing a deficient activity of membrane transporters, leading to metabolic alterations similar to those in PXE.

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Cross-talk between breast cancer cells and fibroblasts in coculture system

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Among solid tumors, breast cancer is one of the most frequent and potentially aggressive forms. The transition from a benign to a malignant tumor is correlated to cell invasion through the basal lamina. In this phase, several cascade processes start to be activated, involving the progressive transition of the epithelial phenotype (stationary and polarised) into a mesenchymal motile phenotype. In the mean time, the host stroma undergoes severe changes, including ECM modifications and host cell's recruitment. Therefore, the extracellular environment of primary tumors may be depicted as a complex microecosystem, in which the neoplastic cells, the extracellular matrix, mesenchymal cells, blood and lymphatic vessel, and a variety of active factors play a dynamic role in the progression of the cancer.

Between host cells of the tumor microenvironment, fibroblasts are likely to be the most representative ones. To investigate the possible interactions between neoplastic cells and fibroblasts we have performed proteomic analyses of breast cancer cells (8701-BC) cocultured with normal fibroblasts.

In the coculture experiments the two cell types were grown separated by a microporous membrane. The protein profiles were obtained by 2D-IPG and identified by Nt-microsequencing, Maldi-Tof and Western Blot. The spots, with assigned identity, were grouped into functional categories, as previously described (Pucci Minafra *et al.* *Annals NY Acad. Sci.* 963: 122-139, 2002). The comparative analysis between control and cocultured cells revealed significant variations of the expression levels especially with regards to cytoskeletal proteins. In particular an increased expression level of vimentin and a decrease of cytokeratins were observed. These data might be related to a more marked epithelium-mesenchyme transition and/or an increase in the motility of 8701/BC cells when cocultured with fibroblasts.

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Homozygosity modulates OI phenotype in BrlIIV mouse

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BrlI mouse is a murine model for Osteogenesis Imperfecta generated by knocking a G349C substitution into one col1a1 allele. BrlI/+ pups have 30% perinatal lethality. Surviving BrlI mice are small, with weaker and more brittle bones than WT.

By mating the BrlI/+ mice we made the surprising observation that the OI phenotype was milder in BrlI/BrlI homozygous mice. BrlI/BrlI have normal perinatal survival rate and their weight and size is intermediate between WT and BrlI/+. They lack the rib fractures and flared thorax, as well as the vertebral and calvarial demineralisation seen in BrlI/+. BrlI/BrlI 2 month femurs have normal BMD and intermediate CSA, BV/TV and TbTh, withstand normal loading to fracture and are less brittle than BrlI/+. Cell numbers, MAR and BFR/BS were unchanged at 2 and 6 months, compared to WT.

Matrix insufficiency and collagen chain composition may contribute to the difference in phenotype between animals with different genotype. In BrlI/+, type I collagen with one mutant chain is selectively retained by the cells, whereas the collagen with two mutant chains is better secreted. Since BrlI/BrlI mice make exclusively collagen with two mutant $\alpha 1(I)$ chains, this results in a 33% matrix insufficiency in homozygotes vs the 40% insufficiency in heterozygous. Additionally, the reactive -SH group in BrlI/+ type I collagen might form illegitimate cross-links with intracellular or matrix components. We are investigating the relative importance of matrix insufficiency and collagen composition to phenotype modulation.

ABCC6 gene analysis in Italian subjects affected by

Pseudoxanthoma elasticum

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Pseudoxanthoma elasticum (PXE) is a genetic disorder due to mutations in the ABCC6 gene, a member of the ABC sub-family of membrane transporters, characterized by high genetic heterogeneity. More than 80 different mutations have been identified; a few of them are recurrent mutations, the majority are sporadic mutations. The analysis of the 31 exons and of the flanking intron sequences of the gene is often required for mutational screening.

In order to identify mutations in the Italian PXE families, to characterize Italian genotypes and to expand the mutational spectrum of the gene, we developed an efficient strategy for DNA diagnosis of PXE by combining different methodological approaches.

A cohort of 83 Italian PXE families was examined. The two most frequent mutations, p.R1141X and ABCC6 E23_29del, as well as deletion ABCC6 E15del, were identified by restriction enzyme digestion (p.R1141X) or selective amplification with specific primers (ABCC6 E23_29del, ABCC6 E15del) and size separation on agarose gels. In a number of patient, where only one or no mutant allele was found, the direct sequencing of the gene was performed. In addition, dHPLC was applied to the study of the ABCC6 gene in 7 families. The results show that mutant alleles are present mostly in com-

found heterozygous state. The great majority of mutations are located in the carboxy-terminal of the protein and almost all mutations involve aminoacidic residues located in the intracellular site. Several new variants, confirmed as causative mutations by excluding their presence in 100 control alleles, were found. In conclusion, our study confirms the genetic heterogeneity of PXE and underlines the usefulness of combining different techniques for the screening of mutations in the ABCC6 gene in families with PXE.

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Hyaluronan content and gene expression for hyaluronan synthases and hyaluronidases in the umbilical cord of healthy and Down syndrome fetuses

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The mechanisms by which the excess genetic material of chromosome 21 results in the dysmorphic features of Down syndrome (DS) are largely unknown. Since HA plays a central role in many morphogenetic processes during embryogenesis, an alteration in its metabolism could be involved in the pathogenesis of several structural defects of DS. The extracellular matrix of umbilical cord (UC) is the mammalian tissue with one of the highest content of HA. Therefore we sought to explore the quantitative HA modifications during gestation, tissue distribution and HA metabolism in euploid and DS UCs. Quantitative and molecular size analyses were performed using HPLC and FPLC. Tissue distribution was visualized by immunohistochemistry. Gene expression for HA synthases (HAS) and hyaluronidases (HYAL) were quantified by real-time PCR techniques and HYAL activity was detected by zymography. Immunohistochemistry showed a larger amount of Wharton's jelly HA in DS UCs than in euploid UC. Real-time PCR analysis showed a higher expression of HAS 2 and a lower expression of HYAL 2 in the Wharton's jelly of DS fetuses compared to that of euploid fetuses at 14 weeks of gestation. On the contrary, at term HYAL 2 expression was higher in DS specimens. Zymographic studies showed a similar behaviour with a lower HYAL activity at early gestation and a higher HYAL activity at term gestation in DS UCs compared to euploid specimens. We can conclude that HA is more represented in DS UCs than in euploid. A complex alteration of the HA metabolism characterized by an increased synthesis of lower weight HA molecules is a peculiarity of DS UCs.

New insight on the pathogenesis of clinical manifestations in *Pseudoxanthoma Elasticum*

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Pseudoxanthoma elasticum (PXE) is a genetic disease inducing calcification and fragmentation of elastic fibers in skin, vessels and eye. It is due to mutations of ABCC6 gene encoding for the membrane transporter MRP6. The physiological role of MRP6 and the pathogenesis of lesions are unknown. Clinical and histological damages similar to those in

PXE are present in genetic hemolytic disorders. The hypothesis is that an imbalanced redox status, already observed in hemolytic disorders, may be present in PXE. Dermal fibroblasts from PXE patients have been assayed for ROS, malondialdehyde (MDA), and glutathione (GSH) contents, and for the activities of enzymes involved in the cell redox balance. Mitochondrial activities have been investigated as possible source of oxidant species.

Results. PXE fibroblasts suffer from a vitamin E-sensitive mild chronic oxidative stress due to imbalance between production and degradation of oxidant species. Compared to controls, PXE cells have significantly higher content of ROS and MDA, higher ratio of oxidised to reduced GSH, a higher activity of superoxide dismutase (SOD) and of glutathione transferase (GT). By contrast, catalase (CAT) and glutathione reductase (GR) activities are similar to controls. The significantly higher levels of Mn-SOD and of mitochondrial membrane potential in PXE cells compared to controls indicate mitochondria as source of superoxide anion and of ROS. Therefore, the imbalance between production and degradation of oxidant species in PXE cells may also depend on loss of mitochondrial $\Delta\psi_m$ control with overproduction of H_2O_2 . Whatever induced, PXE cells suffer from a mild chronic oxidative stress and this can explain the great majority of structural and biochemical alterations already reported in PXE.

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Contribution of amino acid sulfur to proteoglycan sulfation in a mouse model of diastrophic dysplasia

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Sulfate transporter chondrodysplasias are caused by mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene that lead to impaired uptake and intracellular depletion of sulfate with ensuing undersulfation of newly synthesized cartilage proteoglycans (PGs). We have used our mouse model of diastrophic dysplasia to estimate the contribution of cysteine-derived sulfur to cartilage PG sulfation. Cartilage fragments from newborn wild-type and mutant animals were double labeled with [³H]glucosamine and [³⁵S]cysteine and the ³⁵S/³H ratio in glycosaminoglycans was determined; the ³⁵S/³H ratio was 4 fold higher in mutant animals compared to the controls. Subsequently, wild-type and mutant animals at 1 day of age were injected with [³⁵S]cysteine and after 24 h, the specific activity (dpm/nmol) of the 4-sulfated disaccharide was measured in skin and cartilage. In both tissues the specific activity was increased in mutant compared to wild-type littermates. On the basis of these data, newborn mutant and wild-type mice were injected daily with N-acetylcysteine; after 1 week cartilage PG sulfation was measured by HPLC disaccharide analysis. In mutant mice a moderate increase in PG sulfation was observed in the animals treated with the drug compared to the ones treated with a placebo. These data confirm that the pathway by which sulfate is recruited from the intracellular oxidation of thiol compounds for PG sulfation is active in cartilage in vivo and suggest potential therapies of diastrophic dysplasia with thiol compounds.

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Angioprevention: angiogenesis in a chemopreventive setting

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Acquisition of an angiogenic potential, the angiogenic switch, is a key step in progression to tumor malignancy. Chemoprevention focuses on cancer prevention using agents devoid of collateral effects. We note that angiogenesis is a common key target of most chemopreventive molecules, a concept termed *Angioprevention*. We are investigating angioprevention drugs to reveal mechanisms and identify novel candidates. Common mechanisms of action involve inhibition of protease activity or of leukocyte infiltration, whose contribution to cancer through MMP-9 production is documented.

N-acetyl-cysteine (NAC) prevents angiogenesis in the Matrigel sponge assay and reduced growth of xenografts of the highly angiogenic Kaposi's sarcoma *in vivo*. NAC inhibits MMP-2 and MMP-9 activity via the free sulfhydryl group that mimics the inhibition conferred by the free cysteine in the propeptide, thus is a broad MMP inhibitor. The green tea flavonoid, epigallocatechin-3-gallate (EGCG), linked to reduced tumor incidence by epidemiological evidence, shows strong inhibition of MMP-2 and to a slightly lesser extent MMP-9. The green tea flavonoid EGCG, also represses neutrophil infiltration and neutrophil-mediated inflammation-associated angiogenesis, thus EGCG also indirectly targets MMP-9. EGCG has also been observed to potently inhibit neutrophil elastase as well.

We analyzed the regulation of gene expression they exert in primary human umbilical endothelial cells (HUVEC) in culture with functional genomics. Expression profiles obtained through Affymetrix GeneChip arrays identified overlapping sets of genes regulated by the anti-oxidants and possible mediators potentially representing a *fingerprint* of the anti-angiogenic switch.

Ectopic decorin induces proteomic and cytoskeleton modulation of breast cancer cells

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During the invasive growth neoplastic cells enter in dynamic contact with several components of the extracellular matrix that may influence gene expression and induce phenotypic modulation of neoplastic cells. Among these environmental factors, decorin, a representative member of the small leucine-rich proteoglycan family, occupies a central role because of its ability to interact with collagens and cellular receptors and to modulate biological activities. To test the effects of ectopic decorin expression in neoplastic cells we performed a comparative study of decorin-transfected 8701-BC clones vs the parental cell line, applying 2D-IPG, western blot and RT-PCR methodologies. Our preliminary results showed that the protein complement expressed by cells following transfection undergoes significant modifications. Protein modulation involves some cytoskeletal proteins, metabolic enzymes and chaperonins. Cell morphology assays show remarkable cell surface modifications of transfected clones. Since cytoskeleton, besides its role in maintaining cell polarity, is also involved in signal transduction, its modification in the transfected clones is probably associated with complex responses induced by ectopic decorin. In addition, transfected clones display a significant

reduction of the levels of matrix proteases released into the media culture, which correlates with a downregulation of the transcription of the corresponding genes. These results provide additional insights into the reported effect of decorin in neoplastic cell behaviour.

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Mutations in collagen I affect the binding to small leucine-rich proteoglycans

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Mutations in collagen I cause Osteogenesis Imperfecta (OI). We studied the effect of mutations in the $\alpha 2(I)$ chain on the interaction with decorin (DCN), biglycan (BGN) and fibromodulin (FM), all small leucine-rich proteoglycans (SLRPs). Collagens were purified from culture media of skin fibroblasts from skin biopsies of three lethal and four non-lethal OI patients. DCN and FM were purified from bovine tendon, recombinant BGN from a clone of stable transfected CHO cells. The interaction was determined by ELISA using biotinylated SLRPs (1. Tenni *et al.*, Eur J Biochem 269, 1428, 2002). Our results show that mutations affect type I collagen interactions with the three SLRPs, the affinity apparently decreasing as the mutation is more C-terminal. At least for DCN and FM, this can be explained by the presence in collagen of multiple binding sites for these two SLRPs and the fact that collagen Lys/Hyl are essential for the binding to DCN (see 1.) and FM (unpublished results); in particular, post-translational Hyl overglycosylation in the portion of the triple helix N-terminal with respect to the mutation probably causes steric hindrance for SLRP binding. Superimposed on these findings, we have found a very low binding to DCN and FM and a low binding to BGN by lethal mutations G421D and G622D, both in the binding region for CS/DS sulfate PGs (2. Di Lullo *et al.*, J Biol Chem 277, 4223, 2002). Different affinities for the three SLRPs, in particular a very low binding for FM, were obtained for two more C-terminal mutations (non-lethal G688S and lethal G706S), both in the binding region for KS PGs.

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Hyaluronan metabolism in human breast cancer cell line 8701BC.

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Qualitative and quantitative changes are frequently observed in the extracellular matrix stroma surrounding cancer. High level of hyaluronan (HA) is often associated with malignant progression in many cancers, such as breast cancer, colorectal cancer, and glioma.¹ Hyaluronan-rich matrices around tumors favor the cancer cells migration and infiltration of newly formed blood vessels.² Changes in hyaluronan content within primary tumors result from complex interactions between the cancer cells and associated stroma: growth factors and cytokines produced by the carcinoma may stimulate fibroblasts embedded in the stroma to increase the production of hyaluronan and associated hyaluronan-binding matrix components. In a recent paper carcinoma-associated fibroblasts are reported to be responsible for recruiting endothelial cells into tumor thereby boosting tumor angiogenesis.³ Human breast cancer cell line 8701BC, expresses the enzymes involved in HA metabolism, the synthases HAS2 and 3 and the hyaluronidases; nevertheless cells do not produce HA in ECM but only few molecules of HA are evident within the cells. In co-culture in Transwell system with fibroblasts and tumor cells it was evident that 8701BC cells and fibroblasts increased the expression of the HA synthases, demonstrating a cross-talk between these cells; further analysis are in progress to elucidate the molecules involved in this interaction.

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Microdetermination of chondroitin sulfate in normal and PXE-affected human plasma by HPLC and fluorophore-assisted carbohydrate electrophoresis

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An inexpensive, simple, sensitive and reproducible analytical method for the quantitative and qualitative evaluation of chondroitin sulfate (CS) from human blood plasma samples by using fluorophore-assisted carbohydrate electrophoresis (FACE) has been developed. After treatment with a nonspecific protease to convert proteins into small peptides, CS from 100 μ l of normal human plasma was extracted by using a filter membrane (molecular mass cut-off of 3,000 Da) or purification by using an anion-exchange resin. The recovered CS was converted into unsaturated disaccharides through the action of chondroitin ABC lyase, derivatized with 2-aminocridone by reductive amination in the presence of cyanoborohydride and separated by FACE. The linearity, sensitivity and reproducibility of the method were determined in comparison with HPLC equipped with postcolumn derivatization and fluorescence detection using 2-cyanoacetamide as a fluorogenic reagent. This method was applied to the characterization of CS in healthy human subjects, PXE-affected and carriers ageing from 13 to 74 years old.

BIOMATERIALS

Electromagnetic bioreactor effects on the growth of SAOS-2 cells seeded onto a titanium fiber mesh scaffold

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Several porous biomaterials are used as 3D scaffolds for bone tissue engineering. Moreover, increasing evidence suggests that physical stimuli, such as shear stress and electromagnetic field, can modulate bone histogenesis and calcified matrix production *in vitro* and *in vivo*.^{1,2} Our aim was to investigate the effects of an electromagnetic wave on human osteosarcoma SAOS-2 cells in terms of proliferation and calcified matrix production. SAOS-2 cells were seeded onto 3D titanium fiber mesh scaffolds, and electromagnetically stimulated (electromagnetic culture) or not (control culture). Immunofluorescence, Scanning Electron Microscopy (SEM), ELISA and RT-PCR analysis were performed on both scaffolds. Immunofluorescence and SEM analysis revealed that the electromagnetic stimulation improved the cell distribution on the scaffold surface in the electromagnetic culture compared to control culture. Furthermore, the electromagnetic stimulation significantly increased the scaffold coating with calcified matrix, and with bone proteins such as type I collagen, decorin, and osteopontin. RT-PCR analysis showed an up-regulation of the bone matrix protein transcription, suggesting that the electromagnetic stimulation could be used to improve osteoblast growth and calcified matrix development *in vitro*.

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Heart valve *Elastin Leaflet* as starting material for tissue engineering

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Objectives: The elastic fiber scaffold is known to play a major role in the biomechanics of heart valve leaflets. It is also known that elastic fibers are not normally turned over in adult valves. The elastin scaffold could thus be used as a novel substrate for a tissue engineered aortic valve. Although the elastin scaffold has been studied mechanically, detailed investigations on its ultrastructure have been lacking. Our objective therefore are to more completely understand heart valve elastic fiber ultrastructure.

Methods: Elastic fiber scaffolds were obtained from pig aortic and pulmonary heart valve leaflets. Initially a detergent based decellularizing procedure was performed, followed by a collagenase (purified on an elastin column) treatment. Third passage smooth muscle cells (SMC), from rat neonatal ascending aorta and human fibroblasts (HF) from skin, were seeded on the scaffold. Decellularized and repopulated scaffolds were examined with transmission and scanning electron microscopy and by immunohistochemistry.

Results: No collagen was observed after treatment with collagenase, in either the aortic or in the pulmonary valve. Elastin microfibrils were not affected by this procedure. Histologic analyses demonstrated the absence of cell nuclei in the digested scaffolds. Preliminary results show that SMC and fibroblast are attached to both aortic and ventricularis surface of the leaflets after one week of culture. Conclusions: SMC and HF seeding on valve elastin scaffolds has the potential to rebuild a viable aortic and pulmonary valve.

Histologic, histomorphometric and ultrastructural evaluation of titanium particles detached from titanium plasma sprayed endosseous dental implants

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The implant surface topography is one of the factors enhancing the osseointegration of endosseous dental implants. Rough implant surfaces favour early adhesion of blood clot and cells to implant, shortening the implant biological fixation, increasing the device area which is in contact with host tissues and favouring implant primary stability and peri-implant osteogenesis, but can also improve the detachment of titanium particles that limit the osteoblast activity and the peri-implant osteogenesis (Urban RM *et al.*, 2000). Our previous research (Franchi M. *et al.*, 2004) demonstrated that titanium granules detach from TPS implant surface during the implant insertion, and are still detectable in peri-implant tissues 14 days after surgery.

24 TPS screws were inserted in sheep tibia. The implants were removed 14 and 90 days after insertion. Implants with surrounding peri-implant tissues were analyzed by LM, SEM, EDAX. Ti granules of 3-60 µm were detectable in peri-implant tissues of 14 and 90 days samples. The Ti particles were observed in marrow spaces of peri-implant bone, in medullary canals of tibia, but also englobed in the newly formed bone. Morphometric analysis investigated the number, the distance from the implant surface, the tissue site (medullary space or bone) and the area of the Ti particles observed in peri-implant tissue. The decreasing of the particles number was higher (66.55%) than the reduction of their area (6%) from 14 to

90 days after surgery. These data suggest that the small Ti particles detached from the TPS during implant insertion may be degraded or attached by host cells from 14 to 90 days after surgery.

***In vitro* and *in vivo* studies of titanium implants surface microtopography**

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Titanium implant osteointegration is strictly connected to the extension of the implant surface which comes into direct contact with the host bone tissue. During implant insertion direct contact to bone is limited to the thread surface, whereas the space between threads is filled with the blood clot. It has been shown that the surface in contact with the clot needs to be roughened to speed up osteointegration (Martini D. *et*

al., 2003; Franchi M. *et al.*, 2004).

This study aims to demonstrate that the rough surface which better favours new bone formation is that most closely simulating the natural bone architecture.

Smooth surface titanium plates, plates with surfaces roughened by sandblasting with Al₂O₃ and ZrO₂ powders and screw implants with the same surfaces were comparatively analyzed at LM, SEM and TEM.

Human osteoblasts were seeded on the different plates and cell proliferation and differentiation were more pronounced on plates with a surface porosity reproducing the natural bone situation. In particular osteoblasts showed more affinity for U-shaped microconcavities similar in size to those of the bone lacunae (Guizzardi S. *et al.*, 2004).

Implants were inserted in sheep femur and tibia. Screws analysis showed a better outcome of those with U-shaped microconcavities in terms of affinity index, bone ingrowth and bone maturation index from the early weeks after surgery.

Our findings show that rough surfaces with U-shaped microconcavities enhance retention of the blood clot and osteoblasts adhesion, affecting both qualitatively and quantitatively implant osteointegration.

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