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GENETIC DETERMINANTS OF MATRIX BIOLOGY

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Tissue development is dependent upon the differentiation, function and co-ordinated interaction of various cell types. The synthesis, organisation and turnover of extracellular matrix proteins is important in providing a communication network not only between cells but also during differentiation stages within cell lineages. The identification of genes responsible for connective tissue disorders has highlighted the integral role of matrix proteins, growth, differentiation and transcription factors in tissue growth. A variety of strategies including the candidate gene approach, naturally occurring animal models and transgenic mouse technology have made possible the recent explosion in information concerning gene products involved in disorders.

Defects have been identified arising from the impaired synthesis of most matrix components. Collagens, proteoglycans, hyaluronic acid and elastin have all been implicated in genetic disorders. In most cases this leads to derangement in matrix organisation and in tissue form and function. Degradation of matrix components is also a problem as exemplified by the mucopolysaccharidoses which result from an inability to degrade glycosaminoglycan chains within intracellular organelles. Once again matrix organisation is disrupted due to the accumulation of undegraded matrix components. From the correlation between matrix gene defects and genetic disease we have been able to infer much about the role of matrix components in normal tissue development and growth.

The range of available animal models of genetic disease, both natural and transgenic, will make possible further investigations of the basic mechanism of matrix formation in tissue function, the pathogenesis of dysfunction, and will also permit evaluation of therapies for genetic disorders.

QUANTITATIVE MORPHOMETRIC ANALYSIS OF TRABECULAR BONE ARCHITECTURE IN FELINE MUCOPOLYSACCHARIDOSIS VI.

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Feline mucopolysaccharidosis VI (MPS VI) is a naturally occuring animal model of human MPS VI (Maroteaux-Lamy Syndrome). It is an autosomal recessive glycosaminoglycan storage disorder resulting from a deficiency in the lysosomal enzyme, N-acetylgalactosamine 4-sulfatase. As with human MPS VI the disorder is characterised by a wide range of clinical symptoms including growth retardation and skeletal abnormalities. In this study we compare, quantitatively, measurements of trabecular bone in normal cats, untreated MPS VI cats and MPS VI cats that have undergone various regimes of enzyme replacement therapy (ERT), with an aim to analyse the efficacy of ERT on the skeletal system.

Trabecular bone samples were obtained from the fifth lumbar vertebrae of 6 month old normal (n= 5), MPS VI (n=7) and ERT MPS VI cats (n=6). ERT MPS VI cats received intravenous infusions of varying doses of human recombinant N-acetylgalactosamine 4-sulfatase begun at birth. After sacrifice, the L5 vertebrae was routinely processed, impregnated with silver (von Kossa) and analysed at a magnification of x46 using an automated image analysis system (Quantimet 520 system, version 4.0, Cambridge instruments). Each section was divided into eight equal fields and metaphyseal trabecular bone within the boundary of each field was traced using the Quantimet system, with care taken to avoid cortical bone and growth plate. Sum totals of area, perimeter and frame area values for each field were determined and subsequently summed to obtain total area, perimeter and frame area values for each section. Using these values, parameters commonly used to describe the characteristic features of trabecular bone architecture were calculated.

Data obtained demonstrates that 6 month old MPS VI cats had a decrease in trabecular number and thickness, an increase in trabecular separation and a marked osteopenia (decreased trabecular bone volume), when compared to normal cats of the same age. MPS VI cats that had undergone ERT showed a dose dependent increase of trabecular bone volume, and as such had a lower level of osteopenia than nontreated MPS VI cats. The ERT MPS VI cats also showed a dose dependent increase in trabecular number and thickness, and a dose dependent decrease in trabecular separation. Preliminary data also suggests that frequency of dose is an important factor in determining treatment protocols.

The positive effect of ERT on skeletal development in MPS VI cats demonstrated in this study indicates that ERT may be an effective method of treatment for the devastating skeletal pathology observed in MPS VI children.

IN VITRO EXPRESSION OF MUTATIONS OF TYPE X COLLAGEN

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Type X collagen is a short chain collagen expressed in hypertrophic zone of calcifying cartilage during skeletal development and bone growth. Mutations of the type X collagen NC1 domain in Schmid metaphyseal chondrodysplasia and *in vitro* expression of mutant α1(X) implicated the NC1 domain in chain assembly (1). In these studies we use site-directed mutagenesis to produce mutations to further study the role of α1(X) domains in assembly and helix formation.

Three NC1 mutations comparable to mutations defined in patients were produced by SOEing PCR (Splicing by Overlap Extension); 1952delC; 1963del10 and Y598D. An in-frame helix deletion (amino acid residues 72-354) and a deletion of the NC2 domain (157del258) (amino acid residues 21-54) were also produced. *In vitro* expression of these mutant plasmids using a coupled cell-free transcription and translation system demonstrated that mutations of the NC1 domain all prevented the *in vitro* assembly of type X collagen chains into trimers. In addition, co-translational analysis of mutant and normal type X collagen chains also demonstrated that the mutant chains do not associate with, or interfere with the efficiency of normal chain assembly.

Preliminary studies on transiently transfected cells confirm that the NC1 mutations compromise type X collagen assembly and secretion, but detailed analysis of stably transfected cells is necessary to determine if mutant collagen assembly and secretion is totally prevented *in cellulo*.

In contrast the in-frame helix and the NC2 deletions did not prevent assembly and mutant homotrimers and mutant-normal heterotrimers were formed *in vitro*, and homotrimers in transient transfections, were secreted efficiently.

1. Chan, D., Cole, W.G., Rogers, J.G. and Bateman, J.F. (1995) J. Biol. Chem. **270**, 4558-4562

FACTORS WHICH DETERMINE SKELETAL RESPONSE TO THERAPEUTIC GROWTH HORMONE IN OSTEOGENESIS IMPERFECTA

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Osteogenesis Imperfecta (OI) encompasses a group of connective tissue dysplasias characterized by osseous fragility leading to fractures. OI is heterogeneous and at least 4 phenotypes, OI types IA, IB, IVA and IVB contribute to presentations of non-lethal OI of mild-moderate severity.

Fifteen children have participated in a prospective study of the effect of Genotropin on fracture frequency, bone density as assessed by Dual Energy X-ray Absorbtiometry (DEXA) as well as growth response in various skeletal elements. To better characterize the molecular mechanisms underlying response to synthetic growth hormone, collagen synthesis and secretion by cultured skin fibroblasts from each of these subjects or an affected parent was studied. There were 6 patients with OI type IA, 5 patients with OI type IVA and 4 patients with OI type IVB.

All patients showed reduced type I collagen synthesis. Electrophoretic mobility of pepsin digested type I collagen chains was normal in all but three patients who are being investigated further, one with OI type IA and 2 with OI type IVB. In screening patients in general with OI for collagen protein electrophoretic mutation, the majority of patients with OI type I have shown no abnormality in electrophoretic mobility of pepsin digested collagens. However we have recently observed 2 patients with OI type IB with abnormalities of electrophoretic mobility similar to those observed in the 3 patients in the growth hormone study. These two patients with OI type IB have been demonstrated to have multi-exon deletions in one COL1A2 allele. Differential inclusion of mutant collagens into tissues/cell lines has been demonstrated both *in vitro* and *in vivo* The technique involves the measurement of the proportion of mutant versus normal collagen incorporated (included) into ascorbate induced matrix in long term culture.

A surprising finding from the DEXA studies of the skeleton has been that bone density has been normal for age in some subjects with OI in the Genotropin trial. Thus osteopenia (lit. less bone) may not be a primary consequence of some type I collagen mutations which result in bone fragility but osteopenia may result from a combination of factors including immobilisation after fractures. Further studies of the extent of *in vitro* incorporation *i.e.* inclusion of mutant collagens into bone are needed to explain the findings in OI of mid-moderate severity and the effects of growth factors such as Genotropin on collagenous matrices.

THE COLLAGEN PROTEIN FAMILY: MECHANISMS OF INTRACELLULAR FOLDING, CHAIN ASSOCIATION AND THE GENERATION OF DIVERSE SUPRAMOLECULAR STRUCTURES

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The name "collagen" is used as a generic term to cover a wide range of protein molecules which share the basic structural motif of three polypeptide chains wound in a characteristic triple helical configuration and form highly organised supramolecular assemblies in the extracellular matrix. Some 19 different collagen types comprised of at least 33 individual genetically distinct polypeptide chains have been identified although not all have been well characterised. The most commonly occurring collagens are types I, II, and III which form the long-recognised structural fibrils in which adjacent molecules are staggered with respect to each other and stabilised by covalent intermolecular crosslinks. The other collagen types, including the fibril associated collagens (types IX, XII, XIV and XVI), short chain collagens (types VIII and X), basement membrane collagen (type IV), anchoring fibril collagen (type VII), and microfibrillar collagen (type VI), share the triple helical motif but this may be the only thing these collagens have in common with the fibril-forming collagens. Many have large non-collagenous domains which often remain after deposition of the collagens into the extracellular matrix and direct the formation of diverse supramolecular aggregates. These non-collagenous domains may have important roles in interacting with cells and other matrix components.

Intracellular assembly of fibrillar procollagens (types I, II, III, V and XI) is thought to occur via the folding of individual subunit C-propeptide domains and the formation of intrachain disulphide bonds, followed by association and alignment of the chains, stabilisation of the trimeric structure by interchain disulphide links, and finally the zipper-like folding of the triple helix from the C- to N-terminus. The recognition events important for assembly have not been characterised, but are presumed to occur via specific protein-protein interactions between the C-propeptides involving sequence motifs within the propeptide and/or conformational determinants of the folded individual propeptides. Procollagen chains assemble in a highly regulated type-specific manner, despite the sequence homology between the C-propeptide domains and their similar predicted tertiary structure. The goal of our studies is to begin to dissect procollagen folding pathways and the mechanisms directing selective chain association in cells expressing more than one fibrillar collagen type.

To examine the importance of disulphide bond formation in procollagen folding, assembly and secretion we have assessed the ability of proα1(I) chains containing site-directed mutations of cysteine residues to fold and form stable triple-helical molecules. In additional experiments, fibroblasts were incubated with 10mM DTT to completely prevent the formation of disulphide bonds. Together, these data demonstrate that inter- and intrachain disulphide bonds are not required for folding of the triple helix and secretion of proα1(I) homotrimers, however, correct C-propeptide folding and the formation of intrachain disulphide bonds are critical for the heterotrimeric association of proα1(I) and proα2(I) chains.

METABOLISM OF LIVER COLLAGEN IN DIMETHYLNITROSAMINE INDUCED HEPATIC FIBROSIS

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Hepatic fibrosis is characterized by an accumulation of connective tissue components, especially collagen in the liver. In order to obtain more information about the alteration of connective tissue metabolism during the progression of hepatic fibrosis, the biosynthesis and metabolic degradation of liver collagen were investigated in dimethylnitrosamine (DMN) induced liver fibrosis in rats. The metabolism of liver collagen was studied after a single intraperitoneal injection of ^{3}H-proline in a dose of 1110 KBq/100 g body weight. The incorporation of ^{3}H-proline into collagen as ^{3}H-hydroxyproline was measured as an index of collagen biosynthesis. The total and ^{3}H-hydroxyproline were determined after extraction and fractionation of liver collagen into neutral salt soluble, acid soluble and pepsin solubilized fractions. The urinary levels of total and ^{3}H-hydroxyproline were also studied in order to assess the metabolic degradation of liver collagen. A significant increase was noticed in the rate of biosynthesis of liver collagen in all DMN treated animals with a maximum on the 21st day. About 4 fold raise was recorded in the amount of total liver collagen. The urinary excretion of total and labeled hydroxyproline were also increased significantly with a maximum excretion on the 7th day. No correlation was noticed between the increase in liver hydroxyproline content and the increase in urinary excretion of hydroxyproline. The results of the present study indicated a significant increase in the biosynthesis and metabolic degradation of liver collagen during DMN induced hepatic fibrosis. It also revealed that the balance between synthesis and degradation was almost maintained in the early stages of fibrosis as a self defence mechanism, but it was totally impaired in the acute phase of the disease with a net result of accumulation of collagen in the liver.

PROTEOGLYCAN STRUCTURE AND FUNCTION IN NORMAL AND DISEASED CARTILAGE

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Cartilage matrix metabolism undergoes pronounced shifts during the development of OA. These alterations, in interplay with mechanical loading and other exogenous and endogenous factors, over time lead to deterioration of joint function in OA. Experimental evidence points to a marked increase by the chondrocytes of both synthesis and degradation of matrix molecules in the early phases of OA, with little or no net loss of the major matrix molecules. Later, there may still be evidence for an increased synthesis of matrix components, but due to defects in the structure of new molecules or their extracellular assembly, or shifts in degradative activity, net loss of matrix occurs. In the end, the compensatory efforts mounted by the chondrocytes collapse while degradation continues and the matrix and joint fail. The 'point of no return' on this pathway has not been defined and may be different in different joints, individuals and experimental models of OA. Aging may cause a shift of the 'point of no return' so that a lesser joint insult may initiate the OA process in the aged cartilage than in the young cartilage.

During cartilage matrix degradation molecular fragments and other products of tissue metabolism are released into the synovial fluid and subsequently other body fluid compartments. These 'markers' may be used to monitor the dynamics of matrix metabolism in vivo in the human and in animal models. Analysis of the structure of the released molecular fragments may help to elucidate the disease mechanisms in OA.

We have shown the increased release of fragments of aggrecan and COMP and of MMP-1 and MMP-3 to human joint fluid after knee injury and in OA. MMP-3 levels are 15-70 fold that of MMP-1. MMP concentration in synovial fluid is a sensitive indicator of joint pathology, especially when combined with other markers. Serum concentrations of MMP-3 increase after joint injury. Concurrent with these indications of increased matrix degradation, we find increases in other synovial fluid markers which suggest an increased synthesis of both aggrecan and collagen II.