REVIEW

Cartilage collagens: strategies for the study of their organisation and expression in the extracellular matrix

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The collagens constitute a family of proteins that are assembled into a variety of supra-molecular structures in extracellular matrices. Research conducted over the last 20 years has led to the identification of at least 16 genetically distinct collagen types encoded by over 30 different genes. The structure, function and distribution of these collagens are still the subject of intense investigation, but on the basis of their known gene structure and amino acid sequences it is possible to classify them into different groups. The most studied group is that comprising the collagens capable of forming classical fibrous structures (types I, II, III, V and XI), whose main function is to resist tensile stresses exerted on tissues. Another class of collagenous molecules includes types IX, XII and XIV which are believed to be associated with the fibrous collagens and have been assigned the name of Fibril Associated Collagens with Interrupted Triple-helices (FACIT). Collagen types VIII and X appear to form another class of matrix proteins for they exhibit remarkable homology at the levels of gene organisation and amino acid sequence and have the ability to form regular hexagonal lattice structures. A detailed review of all collagen types is published elsewhere.¹

Individual collagen types are rarely found in isolation in extracellular matrices and generally occur in conjunction with several others. For example, collagen types I, III, V and VI are widely distributed in connective tissues such as skin, tendon, aorta, gut etc. In contrast, collagen IV is localised exclusively to basement membranes and collagen VII has only been described in the anchoring fibrils underlying the lamina densa of many epithelia. During evolution a separate group of collagens has arisen to provide cartilaginous tissues with their own distinct macromolecular organisation. Here we find the cartilage-specific collagens (types II, IX, X and XI) together with the more ubiquitous collagen type VI, and possibly types XII and XIV (fig 1). There is still much to be discovered about the role of these collagens in cartilage and especially about their interactions with the highly hydrated proteoglycans of the cartilage matrix. The collagens must function not only to resist tensile forces and to distribute load on bones articulating in cartilaginous and synovial joints but also to

mediate the growth and development of the skeleton. The critical role of collagen II in maintaining cartilage integrity has been demonstrated by studies on certain heritable chondrodysplasias, whereby aberrant collagen II synthesis gives rise to a spectrum of clinical symptoms ranging from mild dwarfism, myopia and secondary osteoarthritis (OA) in some forms of spondyloepiphyseal dysplasia (SED) to several lethal forms of achondroplasia.² It is clear that the cartilage collagens are crucial determinants of the mechanical properties of all articulating joints and any disturbance of the complex collagenous

Figure 1 The molecular form and chain composition of the cartilage collagens. The molecular forms are represented by schematic scale diagrams with the triple helical domains drawn as thick black lines and the non-triple helical domains (NC) illustrated by filled or shaded circles. The molecules are orientated with their NH_2 -termini to the right.

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architecture is likely to lead to dysfunction and arthritic disease.

In this review we briefly discuss current knowledge of cartilage collagens and highlight how modern technologies are providing greater insight into cartilage collagen structure and function in health and disease.

1) Collagen: a definition

Over the past decade it has become increasingly difficult to define what is a collagen and what is not. Many of the collagens that do not constitute the major fibres of the extracellular matrix are hybrid molecules, where the collagenous domain may only be small in comparison to the non-collagenous part of the molecule. Others, for example type IX collagen, can be considered as protein cores of proteoglycans. However, the triple-helix, in which three left-handed helices (α chains) twist around each other to form a right-handed superhelix, is common to all collagen types. Each α chain must contain glycine (Gly) at every third residue as this is the only amino acid small enough to fit into the centre of the triple-helix. Approximately 10–12% of each of

the remaining X and Y residues of the repeating sequence [Gly-X-Y]_n are proline and hydroxyproline respectively, hydroxyproline being essential for the formation of hydrogen bonds that stabilise the helix.

The fibril-forming collagens are synthesised as large precursor (procollagen) forms with non-collagenous propeptides at the N- and C-termini of an uninterrupted 300 nm triple-helix. The propeptides are removed completely in the case of types I, II and III collagens by specific N- and C-proteinases before/during self-assembly and crosslinking of the triple-helical molecules into stable fibrils. Such fibrils exhibit the classical x ray diffraction patterns of collagen.³ Many of the newly discovered collagens do not fit entirely with the above criteria. For example, some collagens contain interrupted triple-helices and proteolysis leads to their fragmentation into shorter helical domains (types IV, VII, IX, XII and XIV). Others do not undergo the procollagen to collagen processing but retain their terminal non-collagenous domains which influence the supramolecular structures that they assume in the extracellular matrix (types IV, VI, VII, VIII, IX, X, XII and XIV).

Figure 2 Diagrammatic representation of the supramolecular assemblies adopted by the cartilage collagens. Unless otherwise indicated the molecules are orientated with their NH₂-termini to the left. Type II collagen is organised into fibrils illustrated as an array of individual triple helices with a quarter-stagger of 67 nm. Type XI collagen forms heterotypic fibrils with type II collagen (see text), but is distinguished from type II collagen by retaining the N-terminal NC domains as illustrated. Type VI collagen forms beaded microfibrils by antiparallel association of monomers into dimers, lateral association of dimers into tetramers and end-on association of tetramers. Type IX collagen does not form multimeric aggregates on its own, but associates, via cross-links, with the surface of type II collagen fibrils in an antiparallel fashion (see text). The globular NC4 domain and the triple helical COL3 domain project out from the fibril surface. Type X collagen molecules are thought to associate laterally and via their C-terminal NC domains to form a regular hexagonal lattice.

A) THE FIBROUS COLLAGENS: TYPES II AND XI

Collagen type II is the major fibrous collagen found in cartilage as well as in other related tissues such as the vitreous humour of the eye and the intervertebral disc; and normally represents approximately 80–90% of the collagen content of the cartilage matrix. Type II collagen is closely associated with type XI which accounts for less than 5% of the cartilage collagen. As stated above type II and XI collagens are initially synthesised as soluble procollagen precursor molecules with non-collagenous amino- and carboxy-termini. In the case of type II collagen these peptides are removed by N- and C-proteinase, respectively, before fibril formation,⁴ whereas, in type XI collagen all the C-terminal non-collagenous domain, but only a proportion of the N-terminal non-collagenous domain, is removed (fig 2).⁵

Type II collagen is a homotrimer [$\alpha 1(\text{II})$]₃, whereas type XI collagen is a heterotrimer [$\alpha 1(\text{XI})\alpha 2(\text{XI})\alpha 3(\text{XI})$].⁵ The $\alpha 3$ chain of type XI collagen is probably the product of the same gene (COL2A1) as that of $\alpha 1(\text{II})$, although it is more glycosylated. Comparisons of the cDNA and gene sequences of the $\alpha 1(\text{XI})$ ⁶ and $\alpha 2(\text{XI})$ ⁷ chains with those of type V collagen^{8,9} have demonstrated striking similarities. Both type XI and type V collagen have been shown to form heterotypic fibrils with type II and type I collagen respectively^{10–12} and their retention of the N-terminal non-collagenous domains is thought to restrict the overall diameter of these fibrils.

The structural similarities observed for the fibril-forming collagens are reflected at the gene level. They contain many discrete-sized exons of 54 bp or repeats thereof, with each 54 bp encoding 6 Gly-X-Y repeats. Consequently, it has been suggested that the fibrillar collagen

genes evolved from a common 54 bp ancestral coding unit.^{13 14}

Early studies on type II collagen demonstrated it had a higher hydroxylysine:lysine ratio and increased levels of glycosylated hydroxylysine than collagen types I and III.¹⁵ The function of the carbohydrate moieties remains unclear but there appears to be an inverse relationship between glycosylation and fibril diameter.¹⁶ Fibril diameter is clearly under careful control and glycosylation may explain, in part, why type II collagen forms much finer fibrils than type I collagen. However, in mammalian articular cartilage, fine fibrils (10–25 nm) are only found in the pericellular matrix immediately surrounding the chondrocytes, with much thicker fibrils occurring in the territorial and interterritorial matrices.¹⁷

It has recently been discovered that there are two alternatively spliced forms of type II collagen. A 207 bp 5'terminal exon (exon2) coding for a 69 amino acid sequence can either be spliced in (collagen type IIA) or out (collagen type IIB) of the procollagen mRNA in a tissue-specific manner.¹⁸ The domain encoded by the exon is conserved between the fibrillar collagens and may have a functional role in fibrillogenesis. In situ hybridisation studies have revealed that the exon is absent from the pro α 1(II) mRNA species in fully differentiated cartilage and collagen IIA appears to be preferentially expressed by the cells of the perichondrium and in non-chondrogenic embryonic tissues such as nucleus pulposus of the developing intervertebral disc.¹⁸ Studies in the chick system have demonstrated collagen type IIA expression in pre-cartilage limb mesenchyme and also in non-cartilage tissues including embryonic calvaria, skin, heart, skeletal muscle and brain.¹⁹ No studies have yet been carried out to determine whether collagen type IIA is expressed in OA tissue.

B) THE FACIT COLLAGENS: TYPES IX, XII AND XIV

The major role of the collagen fibres of cartilage is to form a stable meshwork to counteract the swelling pressure generated by the hydrated proteoglycan aggregates. The achievement of stability requires the interaction of the collagen fibres with other matrix components and here the first FACIT collagen to be described (type IX collagen) may play a significant role.

Type IX collagen is a heterotrimer composed of three different α chains [α 1(IX) α 2(IX) α 3(IX)] and is found associated with type II collagen. It contains three triple-helical collagenous domains (COL 1, 2 and 3) and four non-collagenous domains (NC1, to 4). Electron microscopical studies have demonstrated the localisation of type IX collagen along the surface of type II collagen fibrils with the COL3 domain and the large (243 amino acids) NC4 domain projecting out from the fibril surface into the perifibrillar matrix.^{10 20} The interaction between types II

and IX collagens has been shown to be stabilised via cross-links²¹ and recent evidence suggests that the type IX and type II collagen molecules are arranged in an anti-parallel fashion²² (fig 2). It has become apparent that the NC domains are of important structural significance. The large NC4 domain on the α 1(IX) chain is very basic, offering the possibility of interaction with acidic proteoglycan molecules. The α 2(IX) chain contains a NC3 domain which is 5 amino acids longer than the other two chains providing a flexible hinge region to the molecule and an attachment site for a glycosaminoglycan (GAG) side chain consisting of either chondroitin sulphate or dermatan sulphate. The GAG chain length is highly variable, is not present on all type IX collagen molecules in cartilage,²³ and its role is presently unknown.

Types XII and XIV collagen are partially homologous to type IX collagen and their proposed function is to associate with type I collagen-containing fibrils in an analogous manner to the association between type IX and type II collagen. However, the precise nature of the interaction remains to be established. Types XII and XIV collagen are homotrimers containing two triple-helical domains (COL1 and COL2) and three non-collagenous domains (NC1 to NC3).^{24 25} The COL1 domain and part of the NC3 domain are homologous to the COL1 and NC4 domains of type IX collagen respectively. However, the NC3 domain of each α chain is very large, being 190 K in non-cartilaginous tissues, giving the molecules their characteristic cross-shape when viewed under the electron microscope by rotary shadowing. In cartilage, the NC3 domain of type XII collagen has recently been shown to be at least 200 K greater.²⁶ There is also evidence indicating the presence of GAG in the NC3 domain of type XII collagen and in one of the other NC domains of type XIV collagen.²⁶ Type XII collagen has been localised to the perichondrium at the articular surface and around cartilage canals whereas type XIV collagen is found throughout the matrix.²⁶ Much is yet to be learned about the functional roles of this unusual family of collagenous extracellular matrix proteins.

C) TYPE X COLLAGEN

Type X collagen is a developmentally regulated short-chain collagen which is transiently expressed by hypertrophic chondrocytes at sites of endochondral bone formation.^{27 28} Such tissue-specific expression makes type X collagen a useful marker for both normal and aberrant bone formation. For example, in addition to its synthesis by developing long bones, type X collagen has also been localised in fracture repair callus²⁹ and osteoarthritic joints.^{30 31} Type X collagen is unusual in that it is encoded by a condensed gene with approximately 95% of the coding sequence being contained in a large single exon.^{32 33} Type X collagen is a homotrimer with α chains of M_r 59 000 and a helical domain of M_r

45 000, approximately half the size of $\alpha 1(\text{II})$ chains. Studies *in vitro* carried out mainly on chick type X collagen have demonstrated that it has a triple-helical domain of approximately 130 nm³⁴ and is capable of forming a hexagonal lattice-like structure³⁵ similar to that observed for the genetically-related type VIII collagen in Descemet's membrane.³⁶ Unlike the fibrillar collagens, type X collagen retains its non-collagenous C- and N-terminal domains and the hexagonal lattice-like structure can be visualised as regularly spaced nodules of aggregated C-termini interconnected with filamentous structures formed via interactions between adjacent triple-helices (fig 2).³⁵ The potential involvement of such structures in the mineralisation of hypertrophic cartilage and the endochondral ossification process remains to be defined.

D) TYPE VI COLLAGEN

Type VI collagen forms a distinct sub-class within the collagen family and assembles into

beaded microfibrils which occur in cartilage and in a number of other tissues including aorta, placenta, uterus, skin, tendon and cornea. It has a relatively short triple-helical domain (105 nm) flanked by exceptionally large N- and C-terminal globular domains that account for more than two thirds of the mass of the molecule (fig 1). Three distinct α chains have been attributed to type VI collagen although there is evidence that all three are not coordinately expressed and there may be a family of collagen VI isoforms.³⁷ The most common form is the heterotrimer [$\alpha 1(\text{VI})\alpha 2(\text{VI})\alpha 3(\text{VI})$], where the $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ chains have M_r 140 K and share some sequence homology, and the unrelated $\alpha 3(\text{VI})$ chain has M_r 240–280 K. The larger size of the $\alpha 3(\text{VI})$ chain is accounted for by a much larger N-terminal non-collagenous domain which is subject to variation as a result of alternative splicing of the $\alpha 3(\text{VI})$ mRNA.^{38 39}

The structure adopted by type VI collagen is very different from other collagen types. Following its synthesis, monomers assemble intracellularly into antiparallel staggered dimers, two of which in turn align in register to form a tetramer. Following secretion, end to end polymerisation of the tetrameric units results in the formation of typical microfibrillar networks (fig 2).

The function of type VI collagen is ill-defined, but it possesses a number of interactive properties which suggest it may have a bridging role between cells and their extracellular matrix. The triple-helical domain of type VI collagen contains a number of sequences capable of supporting cell binding (Arg-Gly-Asp or RGD) but although it has been shown to bind to cells, at present it is not known which of the RGD sequences are involved. The type VI collagen microfibrils have been shown to be stabilised by specific non-covalent interactions with hyaluronan⁴⁰ and type VI collagen has also been shown to interact with collagen types I and II, and the small proteoglycan decorin.⁴¹ The globular domains of type VI collagen contain repetitive 200 amino acid motifs [three in $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ and 11 in $\alpha 3(\text{VI})$] which share a high degree of homology to the fibrillar collagen-binding A domains in von Willebrand factor.⁴²

E) NON-COLLAGENOUS PROTEINS

Collagens, virtually by definition, are assigned a structural role in cartilaginous tissues but in addition to the proteoglycans there also exists a number of other proteins which may play key structural roles. Some of the proteins have defined functions but, for many, their function remains unknown. It is beyond the scope of the present review to give a detailed account of all the proteins isolated to date but some brief information is included in the table. The defined ultrastructure of the cartilage matrix therefore is undoubtedly complex and there is still much work to be done to determine the specific mechanisms by which matrix assembly takes place.

Non-collagenous proteins isolated from cartilage

Protein	Localisation	Function	Reference
Anchorin CII (M_r 34 000)	Chick chondrocyte cell surface	Binds type II collagen	43, 44
Cartilage matrix protein CMP (M_r 148 000 homotrimer subunit M_r 54 000)	Bovine tracheal and epiphyseal cartilage, nasal septum, not articular cartilage	Interacts with proteoglycans and binds type II collagen	45, 46
Cartilage matrix protein (M_r 36 000 monomeric)	Articular cartilage	Not known	45
Chondrocalcin (C-propeptide of type II collagen) (M_r 105 000 homotrimer, subunits M_r 35 000)	Hypertrophic cartilage	Binds hydroxyapatite. Possible role in cartilage mineralisation	47
Collagen-binding protein CBP (M_r 54 000)	Swarm chondrosarcoma Cartilage	Binds type II collagen and interacts with proteoglycans. Possible role in regulating collagen fibril diameter	48
Cartilage matrix glycoprotein (CMGP) (M_r 550 000 tetramer, subunits M_r 116 000–130 000)	Articular cartilage Fibrocartilage Vitreous	Not known	49
Cartilage oligomeric matrix protein (COMP) Similar/identical to CMGP (M_r 524 000 pentamer, subunits M_r 100 000)	Swarm chondrosarcoma Cartilage	Not known	50
Ch21 protein (M_r 21 000) Identical to p20K protein	Chondrocytes and granulocytes undergoing terminal differentiation	Not known (binding and transport of small hydrophobic molecules?)	51
Chondronectin (M_r 180 000, subunits M_r 56 000–77 000)	Cartilage and serum	Mediates attachment of chondrocytes to type II collagen	52
Fibromodulin (M_r 59 000) Structurally related to decorin and PG-S1	Cartilage and other connective tissues	Collagen binding regulation of fibrillogenesis	53, 54
Fibronectin (M_r 440 000) M_r 58 000	Most tissues	Binds to chondrocytes and type II collagen	55, 56
M_r 55 000 (monomer) Similar to M_r 58 000	Bovine articular cartilage Other tissues?	Not known	53
	Adult human articular cartilage	Not known (Immunoglobulin binding?)	57
PARP (Proline/Arginine Rich Protein) M_r 24 000 monomer 49% identical to N-term. end of $\alpha 1(\text{XI})$	Cartilage	Not known	58
Tenascin (M_r >10 ⁶ daltons)		Binds to aggrecan	59

2) Current strategies in the study of the collagens in normal and diseased cartilage

To enhance our understanding of cartilage structure and the complex interplay between cells and matrix that occur in this avascular tissue, a variety of techniques have been developed. A brief synopsis of the basic strategies currently in use in the study of cartilage collagens is outlined below.

A) BIOCHEMICAL EXTRACTION

Although the existence of 'collagen' in connective tissues has been known since the nineteenth century it was not until the 1950s that the structure of type I collagen was defined,⁶⁰ followed by type II collagen in the late 1960s.⁶¹ The early studies on chick sternal cartilage demonstrated that type II collagen, which is not readily solubilised in neutral salt solutions, can be extracted following limited pepsin digestion.⁶² Such a relatively crude procedure relies on the fact that collagens comprise three polypeptides in a triple-helical conformation which is resistant to proteolytic cleavage. The other cartilage collagens have since been successfully extracted using similar, but more elaborate adaptations. Extraction of collagens from mature mammalian cartilages requires preliminary extraction of the proteoglycans with dissociative agents such as 4 M guanidinium hydrochloride to ensure a more efficient solubilisation of the collagens by pepsinisation. Differential salt fractionation of the pepsin digests at acid pH can readily separate out the different collagen types. A detailed breakdown of the fractionation of pepsin-solubilised collagens from cartilage has been given elsewhere.⁶³ Analyses of the major fibrillar cartilage collagen types are particularly amenable to such extraction procedures. However, the helical domain of collagen VI represents less than 30% of the native molecule and fragmentation of collagen IX arises due to proteolytic cleavage at the non-collagenous (NC) domains separating the helical segments. Consequently, alternative methods for the extraction of the intact forms of these cartilage collagens have had to be developed.^{64 65}

Biochemical extraction has allowed much information to be gained into the macromolecular composition and gross quantities of particular collagen types in normal and diseased cartilage, but the approach does have its limitations. The procedures are lengthy and require relatively large amounts of tissue which restricts the application of studies on small animal model systems of cartilage disease where early phases of damage and repair can be observed. In contrast, human material is normally available only from severely affected joints and therefore it is only the resultant end-stage of the disease that is being studied.

B) IMMUNOLocalISATION

Immunocytochemical studies commonly use indirect immunofluorescence in which the antigenic epitopes are visualised by an

enzymic system coupled to a development of a coloured end product which is recognised by light microscopy. To achieve greater definition, antigenic epitopes can be recognised by electron microscopy by using a second antibody coupled to colloidal gold.

Immunolocalisation can provide valuable information on the temporal and spatial deposition of various matrix proteins. The extraction and subsequent purification of particular collagen types has permitted collagen type-specific antibody production for use in studies such as immunolocalisation. Although antibodies to type I and II collagens are now commercially available, the production of antibodies which specifically recognise the other minor cartilage collagens has proved to be a more difficult task. The helical domains of collagen molecules usually give only a weak immunological response when injected into rabbits or other species. However, the non-collagenous extension peptides have proved to be much more immunogenic. Polyclonal antibodies can be readily raised to purified collagens but the structural similarity between the triple helical domains requires that they be cross-absorbed against other known collagen types. This approach is becoming increasingly difficult as more and more collagens are being discovered. Monoclonal antibodies, demonstrating high specificity and affinity for the initial immunogen, represent an alternative strategy in antibody production technology. Unfortunately, most monoclonal antibodies raised to the various collagen types also show a high degree of species specificity so that antibodies raised against a collagen type from one species may not cross-react/recognise its human counterpart. Caution must always be exercised when using monoclonal antibodies in immunolocalisation studies as it is possible that the single epitope recognised may be masked for example, by interactions between other matrix macromolecules or by mineral. It is often a prerequisite to de-calcify tissue sections before subsequent immunolocalisation. Due to epitopes being conformationally determinant, it is essential that the antibody is known to recognise the native protein. This will depend largely on the biochemical extraction procedure adopted during the initial purification of the immunogen.

Biochemical and immunolocalisation analyses of surgically removed joints from patients with later stages of OA give some insight into the resultant effect of cumulative changes within the joint over a number of years. Although this information is useful, in order to identify the root cause of the disease it is necessary to obtain tissue at a much earlier stage. Animal models can be used, but it is very often difficult to extrapolate the results back to humans. The removal of small cartilage samples by arthroscopy from patients with early OA should allow subsequent analyses by *in situ* hybridisation and immunolocalisation.

C) MEASUREMENT OF COLLAGEN BREAKDOWN

There are comprehensive data documenting proteoglycan degradation in OA but there is little understanding of what happens to the collagens. The distinctive features of collagenous proteins such as their relatively high content of hydroxyproline, hydroxylysine and pyridinium cross-links have provided useful urinary and/or serum markers for detecting abnormal cartilage/bone degradation. Hydroxyproline, the most common marker studied,⁶⁶ is extremely non-specific due to the possibility of it arising in body fluids from several sources including the C1q complement component and from the high proportions of newly synthesised collagen which are degraded intracellularly.⁶⁷ Urinary levels of pyridinium cross-links, which are present in mature collagen fibrils, have been shown to correlate with severity of OA⁶⁸ and may provide a means of monitoring disease progression and therapy. Analysis of synovial fluid for such markers can indicate collagen breakdown in specific joints since the fragments formed following degradation are not interacting with other matrix macromolecules and are free to diffuse out into the synovial fluid. Assays have not yet been developed to detect degradation products of collagen types VI, IX, X, XI, XII or XIV which may appear in synovial fluid or serum following the breakdown of the fibrillar collagen meshwork.

A major limitation in detecting and studying collagen degradation in normal and OA joints has been the lack of a technique for identifying collagen breakdown *in situ*. Recently, antibodies have been raised which specifically recognise only fragmented and denatured type II collagen α chains.⁶⁹ Immunolocalisation studies have demonstrated that staining for denatured type II collagen is much greater in arthritic cartilage and has a different distribution pattern to that in normal tissue. It is conceivable that the levels of denatured fragments of type II collagen in the synovial fluid of affected joints may correlate well with the extent of disease. If so, then the ability to detect them specifically would provide a sensitive marker system.

D) IN VITRO SYSTEMS

In vitro culture experiments on chondrocytes or cartilage explants accompanied by SDS-PAGE analysis of [³H]proline-labelled biosynthetic products have been extremely valuable in providing an insight into the nature of collagen precursors, their processing and deposition in the extracellular matrix.⁶³ The effects of various growth factors and cytokines on collagen gene expression are most readily investigated using *in vitro* systems but are subject to the criticism that chondrocytes *in vivo* do not exist in monolayers on plastic bathed in serum. Consequently attempts have been made to develop culture systems having somewhat more biologically-relevant environments. Chondrocytes cultured within 3-D collagen gels⁷⁰ or over agarose⁷¹ have been

shown to exhibit a rounded cell morphology, elaborate an extracellular matrix containing cartilage collagens and maintain their phenotype for longer periods than those cultured on plastic. Manipulation of the surrounding matrix by incorporation of other components into the collagen gel system such as collagen II, proteoglycan aggregates and calcium salts have been shown to have profound effects on embryonic chondrocyte collagen gene expression⁷²⁻⁷⁴ It remains to be established whether cells from mature cartilage can respond to external factors in a similar manner. However, studies on OA cartilage have demonstrated the presence of at least three different chondrocyte phenotypes expressing different patterns of collagenous polypeptides.³² There is undoubtedly a need to develop *in vitro* systems whereby the effects of factors, particularly those known to be elevated in OA tissue, can be examined on immortalised chondrocytes exhibiting a stable phenotype. The availability of stable chondrocyte cell lines would also benefit molecular biological approaches such as the study of DNA-protein interactions controlling and modulating collagen gene expression. The isolation of sufficient nuclear proteins for performing DNA footprinting and gel retardation assays requires large numbers of cells with a defined phenotype. Cell lines have been produced by infection of fetal rat costal chondrocytes with viruses carrying the *myc* and *raf* oncogenes⁷⁵ or by transfection of rabbit articular chondrocytes with a plasmid containing the SV40 early function segment.⁷⁶ However, the immortalised cells produced only maintain some of the properties of differentiated chondrocytes. The main problem with current methods of cell immortalisation is that they rely on the random integration of the responsible gene into the host genome leading to the possibility of it having adverse effects on the expression of certain host cell genes. Studies on the molecular mechanisms involved in cellular immortalisation will hopefully provide a means of producing more reliable cell lines in the future. Alternatively, cells from chondrosarcomas may be of value to *in vitro* analyses provided they are able to respond to external stimuli in a manner similar to normal chondrocytes. Karyotyping of such cells is essential due to a number of chondrosarcomas having been shown to contain chromosomal translocations.⁷⁷

The elucidation of the mechanisms regulating the complex interactions between different collagen types and between collagens and other extracellular matrix components *in vivo* is still in its infancy. For example, although the longitudinal packing of fibrillar collagen molecules into a quarter-stagger array has been known for a long time, much less is known about the lateral packing of collagen molecules to form circular cross-sectioned fibrils of uniform diameter. However, advances have been made with the development of an *in vitro* system for generating collagen fibrils by enzymic cleavage of type I procollagen with purified procollagen

C-terminal proteinase.^{78 79} The technique has provided an insight into fundamental aspects of collagen fibril formation and has been successfully applied to the study of abnormal type I collagen fibrils produced by patients with osteogenesis imperfecta.⁸⁰ It is conceivable that similar studies could be carried out on type II collagen from patients with genetically inherited OA showing linkage to COL2A1. Whether such systems can be exploited to gain a better understanding of overall matrix assembly involving multiple macromolecular components remains to be established.

E) MOLECULAR BIOLOGICAL APPROACHES

Reports of the first cloned collagen DNA sequences appeared over a decade ago⁸¹ and since then rapid progress has been made in the isolation, characterisation and chromosomal localisation of all the genetically distinct collagens discovered to date.⁸² Complementary DNA (cDNA) probes have proved to be an extremely useful and sensitive tool for determining the levels of collagen gene expression (as measured by the level of specific mRNA) and the sites of collagen synthesis (by *in situ* hybridisation).

Steady-state mRNA levels for a given gene can be estimated by transferring known amounts of either total RNA or polyA RNA extracted from a tissue sample onto nylon or nitrocellulose membranes (Northern or slot blot) followed by hybridisation to a labelled probe. The technique is limited by its requirement of microgram quantities of RNA which may not be readily available from a clinically removed sample. However, the development of the polymerase chain reaction (PCR) has provided a method for the detection of mRNA species from small amounts of tissue.⁸³ Essentially, RNA extracted from the tissue is reverse transcribed into cDNA and amplified by consecutive rounds of annealing of specific oligonucleotide primers, elongation of the target sequence using Taq DNA polymerase and denaturation of the double-stranded product. Due to the primer extension products from one cycle acting as templates in the next, the copy number approximately doubles at every cycle so that 20 cycles of PCR yields about a million-fold amplification. Unfortunately, the sensitivity and simplicity of the technique is not matched by quantitative accuracy. Anything capable of interfering with the exponential amplification effectively nullifies any quantitative assessment of the end products. However, PCR can be a quantitative procedure if appropriate internal controls are used and precautions are taken to minimise the amount of interference with the doubling of the target sequence.⁸⁴ Quantitative PCR therefore, could potentially be of great use in measuring the gene expression of a variety of collagenous and non-collagenous proteins from limited amounts of OA tissue.

In situ hybridisation is also a highly sensitive technique, allowing the detection of gene expression by individual cells by hybridisation of cDNA probes to suitably prepared tissue

sections. Since the functional half-life of many mRNAs is relatively short (minutes or hours) their detection *in situ* can allow for an accurate assessment of the temporal and spatial expression of a given gene. It should be emphasised, however, that the transcription of a gene and its subsequent translation are two independently controlled events and consequently any data obtained by *in situ* hybridisation can only be substantiated by concomitant immunolocalisation of the gene product. This should also be taken into account with results from quantitative PCR and Northern and slot blot analyses. To ascertain whether the level of gene transcription is equivalent to its translation *in situ* hybridisation and immunolocalisations should be performed on serial sections^{31 32} and ideally, the techniques should be combined to allow for simultaneous localisation of the mRNAs and their respective gene products. It is not yet possible to carry out such a study on mineralised cartilage tissue, although a method has been described for a cell culture system.⁸⁵

The application of recombinant DNA technology to the study of cartilage collagen gene expression has so far led to only a relatively basic understanding of the mechanisms involved in collagen gene regulation and many areas remain to be investigated. For example, little is known of how multiple DNA sequences and transcription factors interact to modulate the level of gene transcription. An understanding of such regulatory mechanisms is essential for both elucidating normal patterns of collagen gene expression in endochondral bone formation and aberrant gene expression in OA. Ultimately, it may be possible to alter the controlling mechanisms of and stimulate the formation/regeneration of cartilage in diseased tissue.

F) TRANSGENIC ANIMALS

The latest and perhaps most far reaching technology which will benefit the study of OA is that of transgenics. Any gene which has been cloned and sequenced can potentially be manipulated in the germ line, usually of mice, enabling more precise information to be gained about its function. Homologous recombination of the gene of interest into embryonic stem cells is currently the most effective method for ensuring that the gene is expressed at the correct genomic locus.⁸⁶ The mice which subsequently develop from this procedure are chimeric heterozygotes; mating of siblings allows the creation of a homozygous founder line carrying the mutant gene.

The mechanical properties of bone and cartilage are functions of the molecular organisation of their extracellular matrices, and OA involves a malfunction of that structure. Consequently, the creation of transgenic mice harbouring defects in specific structural components may predispose them to early onset of OA. However, the use of transgenics in the study of cartilage matrix function is still in its infancy. To date transgenic mice have

been produced harbouring minigene constructs of $\alpha 1$ (II),⁸⁷ $\alpha 1$ (IX)⁸⁸ and $\alpha 1$ (X).⁸⁹ Each study has employed the method of micro-injection into pronuclei of one cell mouse embryos for the introduction of the construct into the mouse genome. This method which relies on the random integration of the minigene into a site favourable for its transcription is highly inefficient. Consequently caution should be taken in drawing conclusions from the phenotypes obtained as it is possible that the integrated construct may have disrupted the expression of another gene. A prerequisite therefore when studying mice harbouring cartilage-specific transgenes is that the expression of the construct is restricted to cartilaginous tissues. When the minigene pro α chains are co-expressed in the presence of normal pro α chains they associate together. However, the mutant collagen produced is rapidly degraded intracellularly due to incorrect folding and the inability to form a stable triple helix, a process referred to as 'procollagen suicide'.⁹⁰ Transgenic mice harbouring the type II and type X minigenes showed varying forms of chondrodysplasia⁸⁷⁻⁸⁹ whereas histological analyses of those harbouring the type IX collagen minigene showed the presence of early onset of OA in the knee joint and corneal disorders.⁸⁸

Although the results obtained from the limited number of studies described above are preliminary, there is clearly a significant contribution to be made from transgenics to the study of cartilage matrix. In addition to being able to examine the functional roles of the non-fibrillar collagens it will also be possible to use cartilage collagen-specific promoters to drive the expression of other factors much implicated in controlling matrix synthesis and degradation. Such factors could include the cartilage-degrading enzymes, which are known to be elevated in OA tissue,⁹¹⁻⁹² and their inhibitors, as well as various cytokines and growth factors. As transgenic technology improves, it will be of great interest to discover the extent to which transgenic animals can contribute towards the understanding of the pathological processes involved in OA.

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