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Review

Noncollagenous, nonproteoglycan macromolecules of cartilage

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Abstract. Extracellular matrix comprises approximately 90% of cartilage, with collagens and proteoglycans making up the bulk of the tissue. In recent years, several abundant cartilage proteins that are neither collagens nor proteoglycans have been characterized in detail. The putative roles of these proteins range from involvement in matrix organization or matrix-cell signaling (PRELP, chondroadherin, cartilage oligomeric protein and cartilage matrix protein) through to molecules that are likely to be involved with modulation of the chondrocyte phenotype (CD-RAP, CDMPs, chondromodulin and pleiotrophin). Other molecules, such as the cartilage-derived C-type lectin and cartilage intermediate layer protein have no role as yet. Due to the difficulties associated with experimentally manipulating a tissue that is 90% extracellular matrix in a manner that can be readily transferred to the whole organism, many of these molecules have been focused on by a surprisingly small number of researchers. This review focuses on newly discovered proteins and glycoproteins in cartilage, with a bias towards those that have structural roles or that are unique to cartilage.

Key words. Cartilage; bone; development; skeleton; protein structure.

Introduction

Cartilage is unusual, in that its cell density is remarkably low. It is a tissue that is 90% extracellular matrix and has therefore been fertile ground for investigators interested in the field of extracellular matrix biology. Cartilage can be considered to be aggregates of the chondroitin sulfate proteoglycan, aggrecan, trapped within a mesh of collagen fibrils that are comprised of type II collagen with lesser amounts of collagens type IX and XI. The proteoglycan is capable of binding large amounts of water to its anionic chondroitin and keratan sulfate glycosaminoglycan chains. As the anionic groups are not saturated, the result is internal hydrostatic pressure that resists compressive forces. The collagen fibrils constrain the proteoglycan aggregates and also provide tensile strength and resistance to shear forces. The result is a tissue that is uniquely suited to being a load-bearing surface in articulating joints. Cartilage is also rigid enough to provide structural support to the developing skeleton in semirigid tissues such as the pinna of the ear, and also in complete skeletal systems, such as those of sharks and rays. Collagen and aggrecan are obviously not the only components of cartilage; other extracellular molecules are involved in organization of the extracellular matrix and organization of chondrocytes with respect to the matrix. For example, leucine-rich proteoglycans such as decorin are

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thought to be involved in the regulation of the diameter of the collagen fibrils and also in the association of collagen fibrils with each other. The leucine-rich proteoglycans have been reviewed recently [1], but other macromolecules involved in cartilage biology are less widely studied. The latter are the primary subject of this review. Those whose location in the human genome is known are outlined in table 1; it is likely that mouse knockouts will produce information on their roles in cases where no human disease is associated with the protein.

A variety of other important macromolecules are found in cartilage, for example members of the transforming growth factor-β (TGF-β) family such as bone morphogenetic proteins [2] and members of the fibroblast growth factor (FGF) family [3]. Mutations in a receptor for FGF, resulting in loss of a suppression effect, have been shown to be responsible for achondroplasia, the most common form of dwarfism [4]. Each of these families of molecules merits a review in their own right and will not be discussed at length here.

Mature cartilage is avascular, and so has been investigated for the presence of compounds that inhibit angiogenesis. As angiogenesis is a primary feature of rapid tumor growth, cartilage, and particularly shark cartilage, has often been used as a source of potential antitumor activity with limited results. Commercially available preparations of crude shark cartilage are likely to be adulterated with fillers, and have not been demonstrated to be efficacious as anticancer agents [5].

In order to understand the role of the various macromolecules found in cartilage, it is necessary to have an acquaintance with cartilage biology and the different types of cartilage found in the body. Articular cartilage is found on the load-bearing surfaces of bones. Fibrocartilage is found in structural tissues that do not bear significant load, such as the trachea, nose and ears and in adults is the cartilage that is usually found in repaired articular cartilage defects. Cartilage is also the progenitor of much of the calcified skeleton.

The skeleton develops from condensations of mesenchymal cells. For the majority of the skeleton, the mesenchymal condensations become chondrogenic and rapidly produce many of the macromolecules found in mature cartilage, such as type II collagen and aggrecan. An exception is the development of membranous bone (such as the skull), where the mesenchymal cells progress directly to osteoblasts.

As bones develop, they acquire a central bony collar, which becomes the diaphysis. Vascular invasion occurs in the diaphysis to form the marrow, and cortical and trabecular bone develops. At the ends of the diaphysis, cartilagenous structures, the epiphyses, continue to add tissue, resulting in bone lengthening. A secondary center of ossification may develop in the center of the epiphysis, and this grows to replace almost all the cartilage with the exception of the articular cartilage at the joint surface. A diagram of the growing end of a long bone, including a secondary center of ossification, is shown in figure 1.

The cartilagenous epiphysis of a developing long bone contains cartilage that is undergoing considerable differentiation. The cartilage at the joint surface is essentially similar to that in mature articular cartilage. Below

Table 1. Gene locations for proteins in cartilage.

Protein	Human gene location	Human mutation phenotype
CDMP-1	20q11.2	acromesomelic chondrodysplasia (Hunter-Thompson type) chondrodysplasia (Grebe type) brachydactyly
CD-RAP	19q13.1 (based on location in mouse)	
Chondroadherin	17q21.33	
Chondrocalcin (collagen II C-propeptide)	12q13.11–13.2	no defects specific to the C-propeptide, but loss of C-terminal region by a frameshift mutation results in arthro-opthalmopathy (Stickler syndrome)
Chondromodulin-1	13q21.1 (based on EST mapping)	
Chondromodulin-II	5q31.1–32	
CILP	15q22	
CLECSF1	16q23	
CMP/matrilin-1	1p35	
COMP	19p13.1	pseudoachondroplasia multiple epiphyseal dysplasia
Pleiotrophin	7q33	
PRELP	1q23	
PARP (collagen XIa2 propeptide)	6p21	no defects observed in the N-propeptide region of *COL11A2*
Matrix Gla protein	12p12.2	

Proteins and the chromosomal locations of their corresponding gene. The phenotypes exhibited as a result of mutations in human genes are described, where known.

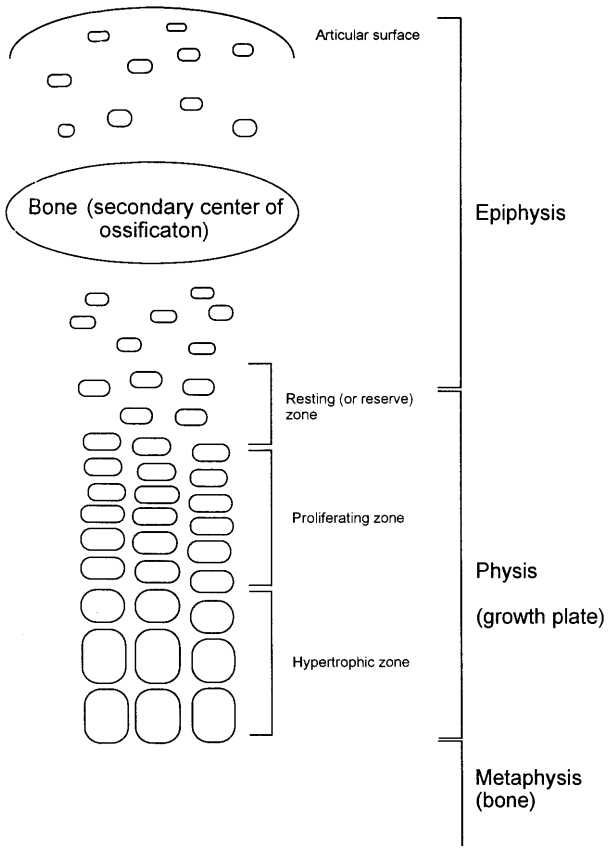

Figure 1. An overview of the structural features of the epiphysis of a developing bone. At the top of the diagram is the articular surface. Below this is the secondary center of ossification, a structure which develops in the epiphyseal cartilage at different times in some, but not all bones. The growth plate is below the resting zone, starting with a region in which chondrocytes are proliferating prior to entering into hypertrophy. Hypertrophy is characterized by cell expansion followed by apoptosis. In the proliferating zone, the chondrocytes begin to form columnar arrays, and in the hypertrophic zone these columnar arrays are separated by regions in which active calcification occurs. In larger animals, vessels, the cartilage canals, penetrate from the metaphysis to the cartilage below the secondary center of ossification. A recent publication by the American Academy of Orthopedic Surgeons [139] gives considerable detail on the topic of growth plate [7] and skeletal growth.

this, are dispersed chondrocytes in what is termed the 'resting zone'. Further down, closer to the bone are proliferating chondrocytes which form columnar arrays and transition into hypertrophic chondrocytes. The hypertrophic chondrocytes closest to the bone are surrounded by calcified cartilage (fig. 1). This region contains a number of specialized molecules, including type X collagen, osteopontin and bone sialoprotein [6].

Depending on the specific bone, vascular invasion of the epiphysis can occur, resulting in a secondary center of ossification. This results in a cartilaginous region, epiphyseal cartilage, between the secondary center of ossification and the articular surface. A second cartilagenous region that continues into the growth plate is found between the secondary center and the bone. In large animals, this region is vascularized [7]. Eventually, the secondary center absorbs the growth plate, and growth ceases.

A fundamental issue in cartilage biology is why articular cartilage in the mature individual does not usually repair effectively. What are the factors or matrix components that enabled articular cartilage to form at one point but which are absent in the mature skeleton? Why, in the adult, does newly synthesized cartilage not integrate well into existing cartilage? What is the interplay between the mechanical forces that are essential for skeletal development [8], and what are the proteinaceous signals or products of cellular differentiation?

During skeletal maturation, the cartilagenous extracellular matrix is degraded and replaced by bone in an orchestrated fashion. The role of specific proteases and glycosidases in this process has recently received considerable attention, particularly in degradative diseases of cartilage such as rheumatoid arthritis and osteoarthritis.

There are a variety of factors involved in the control of chondrocyte maturation, as well as a variety of factors controlling the type of extracellular matrix that is laid down. It is important, in analysis of any cartilage-derived molecule, to have a clear picture of where the molecule is from, both physically and temporally. The extremely polyanionic matrix makes histological techniques difficult, and it is quite possible that molecules that are present, and critical for cartilage function, are not being made locally by chondrocytes except in disease.

Due to its acellular nature, cartilage is a 'difficult' tissue to analyze by modern molecular techniques. Thus, many of the newly discovered macromolecules in the tissue have not been exploited as they might have been in a more 'popular' tissue. Basic information for these molecules, such as developmental location, gene structure and biosynthetic regulation, has not been determined, and there are many areas that are ripe for analysis.

In this review, we focus on macromolecules in which cartilage is their primary or only location. The roles of many of these macromolecules are unknown; many will be likely to have multiple functions. Conceptually they can be divided into two types of molecules, structural and regulatory, although there is considerable overlap between the categories. 'Structural' matrix components are likely to become 'regulatory' when they bind to components in the pericellular matrix, as they will in part be responsible for transmitting mechanical forces from the extracellular matrix to the cell.

The smaller structural molecules can be conceived of as a molecular 'glue', helping to orient the large structural components, proteoglycan aggregates and collagen fibrils, with respect to each other and with respect to the chondrocytes. The regulatory molecules, such as growth factors, maintain the specific chondrocyte phenotype and help to control maturation to hypertrophic chondrocytes. Vascular invasion into the epiphysis is predicted to be controlled in this fashion.

Isolation

Cartilage components have classically been isolated by extraction with guanidine hydrochloride [9]. This procedure, originally designed to dissociate aggregates of high molecular weight components, has proved to be useful for isolation of other components that appear to be bound tightly to either the territorial matrix, the pericellular matrix or the cell surface. Briefly, the tissue is extracted with 4 M guanidine HCl and the extract is dialyzed to bring the chaotrope concentration down to 0.4 M. This enables proteoglycan aggregates to re-associate. The aggregates are then isolated by centrifugation in an isopycnic cesium chloride gradient. Non-proteoglycan components of the extract will be in the low density, upper portion of the gradient.

Molecules less than 50 kDa are then readily purified by sequential gel permeation chromatography and reversed-phase high performance liquid chromatography [10, 11]. Higher molecular weight proteins and glycoproteins are generally isolated after one or more ion-exchange steps. Frequently, a DEAE-cellulose ion-exchange step at pH 7.5 is used to remove proteoglycans. For example, chondroadherin and PRELP, while they can be isolated by physical methods as described above, were originally isolated from fractions that did not bind to DEAE-cellulose by sequential CM-cellulose steps at pH 7.0 and 5.0 [12].

The isolation of macromolecules from extracellular matrix-rich tissues generally requires chaotropic agents (6–8 M urea or 4 M guanidine hydrochloride). While some proteins appear to be relatively unperturbed by this treatment (for example, link protein and aggrecan, which still associate with hyaluronic acid and with each other after removal of chaotropic agents), others do not refold so readily. For example, the recent preparation of the leucine-rich proteoglycans decorin and biglycan from eukaryotic cells infected with modified vaccinia virus has enabled circular dichroism analysis of the secondary structure of the native proteoglycans to be performed. These studies show that there is a strong β-sheet component that is lost and not regained after denaturation in chaotropes [13].

Enzymes

There are a number of enzymes found in the cartilage extracellular matrix. In particular, a variety of enzymes are associated with matrix vesicles, which bud off the chondrocyte plasma membrane [14]. For example, alkaline phosphatase, required for bone formation, is abundant. Phospholipase A2 is not found in newborn cartilage, but is quite prominent in salt extracts of adult articular cartilage [15]. Matrix vesicles are considered to be part of chondrocytes and so have been excluded from this review.

We have also chosen to exclude the family of matrix metalloproteinases (MMPs) and their inhibitors [tissue inhibitors of MMPs (TIMPs)] from this review. These enzymes and inhibitors are extremely important in tissue modeling, but as they have relevance to almost all tissues, they are not cartilage-specific.

Chitinase family

Two related proteins that are in the chitinase family have been isolated from chondrocyte-conditioned medium. Human cartilage gp-39, also known as YKL-40, is a 39-kDa heparin-binding glycoprotein [16]. A closely related protein, YKL-39, copurifies with YKL-40 on gel filtration chromatography of conditioned medium [17]. Both these proteins are very abundant in chondrocyte-conditioned medium; YKL-39 accounts for 4% of the protein, whereas YKL-40 accounts for 33% of the protein but is not found until cells have been cultured for 2 days. The closest relative of these proteins is chitotriosidase, an enzyme that cleaves glycosidic bonds. Neither YKL-39 or YKL-40 has been shown to have enzyme activity [16]. It has been speculated that these proteins are involved in tissue remodeling [17]. YKL-40 is not detectable in newborn or adult articular cartilage, but can be found in the cartilage of patients with rheumatoid arthritis [16, 18].

Lysozyme

Lysozyme, a 14.5-kDa protein, is present in cartilage, although its role is unknown [19, 20]. Lysozyme increases in human intervertebral disks with age, increasing eight-fold between less than 20 years to ages greater than 65, where the level can be as high as 0.35% wet weight of tissue [21]. Lysozyme does not appear to be the culprit for age-related degradation of proteoglycans, as it has no effect on them [22].

Structural macromolecules

One of the advantages of working with cartilage is the relatively large amount of extracellular matrix and

therefore the ease with which structural matrix macromolecules can be isolated and characterized. This review will focus on the matrix macromolecules that are unique or especially prevalent in cartilage and not those which are ubiquitous and also found in cartilage, such as fibronectin.

COMP

Cartilage oligomeric protein (COMP), a member of the thrombospondin family (thrombospondin-5), is a pentameric molecule, forming a structure similar to a bouquet of flowers. Each monomer has a calculated molecular weight of 82.7 kDa. COMP can be isolated from cartilage in EDTA-containing buffer [23]. COMP has a high content of acidic residues, and the five monomers are held together by disulfide bonds [24]. The 84 amino acids at the N-terminal are unique to COMP and not found in other members of the thrombospondin family [25]. Each monomer consists of an N-terminal domain, four epidermal growth factor (EGF)-type repeats, followed by eight calmodulin-like repeats and a globular C-terminal domain. Immunohistochemistry reveals that COMP is primarily found in the interterritorial matrix in adult cartilage, whereas in fetal cartilage it is primarily in the territorial matrix, adjacent to chondrocytes [26]. Developmentally in the rat, COMP is expressed at highest levels in the center of the growth plate and in articular cartilage is expressed at relatively low levels [27]. The globular, C-terminal domain of COMP binds four separate sites in collagen in a zinc-dependent manner and with a dissociation constant of 1.5 nM [28].

The gene for COMP in humans has been located on chromosome 19p13.1 [29]. Pseudoachondroplasia and some forms of multiple epiphyseal dysplasia have been shown to be caused by mutations in COMP [30, 31]. Pseudoachondroplasia has a cellular phenotype characterized by accumulation of material in the endoplasmic reticulum [32]; this accumulated material appears to be COMP. Interestingly, COMP only accumulates in the endoplasmic reticulum in vivo, not in long-term cultured chondrocytes. This suggests that a cartilage-specific matrix component may be responsible for transport of COMP out of the cell [33]. Thus, some matrix assembly may occur intracellularly.

COMP is not the only member of the thrombospondin family in cartilage. During development, thrombospondin-3 (which binds heparin) has also been shown to be present in cartilage, as well as other tissues [34].

COMP is a useful marker for the characterization of erosive cartilage disorders, as fragments of COMP can be found in the synovial fluid of patients with erosive cartilage damage [35]. Serum levels of COMP are associated with progression of osteoarthritis [36].

CMP/matrilin I

Cartilage matrix protein (CMP) is a trimeric 148-kDa molecule with the monomers disulfide-bonded together at the C-terminal [37]. Human (chromosome 1p35) [38], chick [39] and mouse [40] forms have been characterized. The monomer contains 12 cysteines distributed over four domains. Two matrilin-type domains, which are in the same family as von Willebrand factor and complement components B and C2, are separated by an EGF superfamily domain. At the C-terminal is a heptad repeat consisting of α helices which enables monomers to associate by coiled-coil interactions [41], even in reductive conditions [42]. CMP is in the same family as matrilin 2 (which is localized to tissues other than cartilage [43]) and matrilin 3, with which it can form heterocomplexes in the epiphysis (see below).

Developmentally, CMP messenger RNA (mRNA) is present early in skeletal development. In later life, however, it is absent from the articular surface and is also absent from intervertebral disks [40]. However, it is present in other cartilages, notably tracheal, nasal and auricular. CMP is found in both the territorial and the interterritorial matrix [42]. The location of CMP mRNA in the developing joint is highly localized to the region between hypertrophic chondrocytes and proliferating chondrocytes [44] and appears to be associated with the cessation of mitosis [45]. Interestingly, and perhaps surprisingly, mice containing the chicken gene for CMP express the chick CMP in the same locations as they express their own CMP gene, suggesting that the promoter elements are retained even across this quite large evolutionary distance [46].

CMP can be isolated from cartilage using relatively gentle conditions; EDTA-containing buffers release the molecule in what is presumably its native structure [42]. Some CMP becomes cross-linked to aggrecan. Of the pool that is isolated associated with aggrecan, approximately one-third is covalently attached to the chondroitin sulfate region. Increasing amounts are bound with increased age, and there appear to be five discrete sites of attachment [40], correlating with the observation that CMP is more difficult to extract with increasing age of an animal [47].

CMP has been produced in the laboratory by retrovirally transfected cells. In vitro, it is associated with two types of filament in the extracellular matrix. One type is composed of type II collagen. CMP has been shown to self-associate [48], and when type II collagen is trapped intracellularly by removal of ascorbate, a second type of filament, presumably comprised of CMP, can be seen [49].

CMP is an example of a pluripotent matrix molecule that is capable of mediating interactions between a variety of matrix components. It is clearly tightly, even covalently, linked to the aggrecan chondroitin sulfate

domain. As it can also bind to collagen [50], it is reasonable to assume that it could immobilize chondroitin sulfate-containing proteoglycan fragments in the tissue after their release by aggrecanase or matrix metalloproteinases. The role of a filamentous form is unclear, but this might form an alternative to a collagen stabilized matrix in, for example, the pericellular matrix or the territorial matrix.

Cartilage-derived, C-type lectin (CLECSF1)

An 18-kDa member of the C-type lectin family whose closest relative was tetranectin was found to be abundant in shark cartilage and represented a major low-molecular weight component of the tissue [51]. Isolation of matrix components from cartilage in the spinal nucleus pulposus, from articular cartilage and from epiphyseal cartilage in the region of the proliferating zone yielded the bovine equivalent of this protein [52]. Subsequently, the sequence of the human gene (*CLECSF1*) has been determined and the gene located on chromosome 16q23 [P. J. Neame and H. Tapp, unpublished]. Based on the overall similarity of the cartilage-derived lectin to tetranectin, which forms a trimer, the quaternary structure of the cartilage lectin is likely to also be a trimer. The residues that form the contact sites in tetranectin trimers [53] are highly conserved in the cartilage lectin. Neither the role of tetranectin nor the role of the cartilage lectin are known. However, tetranectin binds to plasminogen via its globular domain [54], and it is possible that the cartilage lectin has a similar role as a binding protein.

Cartilage intermediate layer protein (CILP)

CILP is a 91.5-kDa glycoprotein that is found in the interterritorial matrix of the deeper layers of cartilage and is more prevalent in older tissue [55]. CILP is largely absent from the superficial layer and the cartilage nearest to the bone in the joint. It is also particularly prevalent in rib cartilage. CILP is unique in that it derives from a proform, possibly by intracellular, furin-type cleavage. The C-terminal of the CILP precursor encodes for an enzyme, nucleotide pyrophosphorylase, which is a 51.8-kDa protein [56].

Chondrocalcin—the C-propeptide of type II collagen

Chondrocalcin is a 35-kDa protein that is abundant in fetal cartilage [57]. The name derives from its affinity for calcium and its primary location in regions where the growth plate is beginning to calcify [58]. Sequence analysis showed that chondrocalcin was the C-propeptide of type II collagen [59]. Like PARP (below), it is likely a direct indicator of de novo collagen biosynthesis. The C-propeptide of type I collagen exerts a negative feedback regulatory influence on the biosynthesis of type I collagen precursor by binding to integrin $\alpha 2\beta 1$ [60], and it is tempting to suggest that chondrocalcin may play a similar role. The collagen type II C-propeptide has been shown to bind to the enhancer region of the type II collagen gene [61]. Chondrocalcin binds to anchorin CII (annexin V), a protein associated with the plasma membrane of chondrocytes and with matrix vesicles [62] that has been shown to play a major role in the process of calcification [63].

Histones

Histone H2b was first identified as a cartilage component by Marshall Urist using hydroxyapatite purification of potential bone-inducing factors [64]. Histone H2b, H3 and H4 are present in significant amounts in dissociative extracts of both fetal and adult cartilage [P. J. Neame, unpublished]. The N-terminal of histone H4 could not be determined by Edman degradation, suggesting that it is alkylated. While detailed structural analysis of guanidine-extractable histones in cartilage has not been performed, lysine residues within the first 10 amino acids of histone H2b and H3 did not appear to be modified. Whether histones have a role in the cartilage matrix is unclear; it is possible to speculate that they have a role in binding glycosaminoglycan chains

Lamprin

A protein most closely related to elastin has been isolated from lamprey cartilage [65]. It has a GGLGV repeat sequence and an unknown function. Monoclonal antibodies to elastin also cross-react to lamprin.

Link protein and aggrecan G1

Link protein (LP) is a 45–50-kDa glycoprotein that binds to aggrecan and hyaluronic acid and stabilizes the cartilage aggrecan complex with hyaluronic acid. LP has a structural similarity to the G1 domain of aggrecan, which also binds to hyaluronic acid. The G1 domain of aggrecan can be found as an isolated molecular species in cartilage as a result of aggrecanase- or matrix metalloproteinase-mediated cleavage of the aggrecan core protein [66, 67]. Accumulation of G1 increases with the age of tissue [68]. The G1 domain has a tissue half-life of 25 years, approximately seven times that of the parent proteoglycan, as measured by racemization of aspartic acid [69].

The structural features of LP and its role have been reviewed [70]. Briefly, LP consists of an N-terminal domain which is likely to form an immunoglobulin-type

fold and two closely related C-terminal domains that are similar to domains in the hyaluronate-binding proteins CD44 and TSG-6. Recently, the NMR-derived structure of the hyaluronate-binding region of TSG-6 was described and found to belong to the family of C-type lectin folds [71]. Residues that are important for HA binding have been mapped on CD44 [72]. These residues differ from those in LP, G1 and TSG-6, but define a similar surface to those used for carbohydrate binding by other members of the C-type lectin family.

Leucine-rich proteins

Leucine-rich proteins and proteoglycans form an extensive and varied family of molecules that are likely to share a common protein fold but which have diverse functions [73]. The leucine-rich proteoglycans (decorin, biglycan, fibromodulin, lumican and epiphycan) are well-known components of the cartilage extracellular matrix. Reviews on these proteoglycans have been published recently [1, 74]. All these macromolecules share a similar sequence motif. RNAse inhibitor shares a similar motif and folds into a horseshoe-shaped molecule constructed from a coil of repeating β-sheet-turn-α-helix motifs. It is unclear whether the leucine-rich proteins and proteoglycans of the extracellular matrix form a similar structure to the pronounced, horseshoe-like form of RNAse inhibitor. Evidence from circular dichroism spectroscopy suggests that they possess considerably less α-helix than RNAse inhibitor, and so are likely to curve in a less pronounced fashion [75]. Nevertheless, the residues that contribute to the β-sheet-turn-α-helix structure are highly conserved.

Small, leucine-rich proteoglycans (SLRPs). These, of course, do not fall into the molecular niche that this review covers, as they are proteoglycans. However, some of these molecules are 'part-time' proteoglycans and so might be found in protein fractions. Fibromodulin and lumican can be found as glycoproteins with N-linked oligosaccharides that have not been modified by conversion into keratan sulfate chains. In the mature adult, fibromodulin is an N-linked oligosaccharide-containing leucine-rich glycoprotein, with little evidence of either polylactosamine chains, or the sulfated product, keratan sulfate [76]. Similarly, lumican exists as a proteoglycan in younger cartilage but is expressed at high levels as a glycoprotein in adult cartilage [77]. Biglycan that has lost its N-terminal, glycosaminoglycan-containing domain can be found in the nucleus pulposus of the spine [78]. Nonglycanated forms of biglycan increase significantly with age in human cartilage, in contrast to decorin, which is primarily found as a proteoglycan at all ages [79].

The roles of these molecules as glycoproteins, rather than proteoglycans, are not clear. Lack of glycosaminoglycan chains will obviously reduce the ability of these molecules to bind water. Decorin, fibromodulin and lumican have all been shown to bind collagen fibrils, whereas biglycan is found primarily in the pericellular matrix and is thought to bind to collagen type VI in the cornea [80]. Decorin and fibromodulin bind to different sites on collagens types I and II [81]. The lack of glycosaminoglycan chains in the adult would result in a collagen fibril that is less hydrated on its surface. Biglycan, decorin and fibromodulin have all been shown to bind TGF-β and may act as a reservoir, or as an inhibitor of this important growth factor [82]. Lack of glycosaminoglycan chains is unlikely to make a difference to growth factor binding.

PRELP. PRELP is a 55–58-kDa, cartilage-specific glycoprotein containing four N-linked oligosaccharides [83]. PRELP is not abundant in fetal or neonatal human cartilage and accumulates with age [84]. The human gene for PRELP is located on chromosome 1q23 [85]. In spite of the overall similarity of PRELP to keratan sulfate proteoglycans, particularly fibromodulin and lumican, no evidence has been found for glycosaminoglycan chains [D. Heinegård, personal communication]. The N-terminal of PRELP is particularly proline- and arginine-rich (hence the name: proline and arginine-ended leucine-rich protein).

In view of its tissue-specific and temporally restricted distribution, the promoter of PRELP is likely to generate new insights into cartilage-specific gene regulation. A protein that is likely to be a repressor member of the Ets family of gene regulatory molecules has been found by gel retardation analysis in nuclear protein extracts from fibroblasts and neonatal chondrocytes; this protein is absent in adult chondrocytes and so is a potential candidate for cartilage-specific gene regulation [86].

Chondroadherin. Chondroadherin is a 36-kDa, leucine-rich protein that has been shown to promote chondrocyte attachment to plastic [87]. It is thought that this is achieved through binding to the integrin $\alpha 2\beta 1$ [88]. The developmental location of chondroadherin is well defined. It is associated with the interterritorial matrix, initially in the growth plate. As the epiphysis develops, expression continues in the proliferating zone and also extends to articular cartilage [89].

The most obvious difference between chondroadherin (CHAD) and other leucine-rich proteins and proteoglycans is that the signal peptide is immediately followed by the first cysteine of the molecule. Thus there is no domain that might be an acceptor for glycosaminoglycan addition (as in decorin or biglycan) or sulfation (as in PRELP, fibromodulin or lumican). CHAD has no N-linked oligosaccharides, and thus cannot be converted into a keratan sulfate proteoglycan in the same manner as lumican or fibromodulin, although it does

have an O-linked oligosaccharide [12]. Chondroadherin has a somewhat different C-terminal from the other members of this family that are found in extracellular matrix and contains four, instead of two, cysteines that are disulfide-bonded [90]. The human gene is found on chromosome 17q21.33 [91], and the mouse gene is found on chromosome 11 (D11Mit14) [92].

N-propeptide of *COL11A2* (PARP)

PARP (proline-arginine rich protein) is the 24-kDa N-terminal globular domain of collagen α2(XI) [93]. It is not to be confused with poly(adenosine diphosphate-ribose) polymerase, an enzyme involved in DNA repair. PARP is very abundant in epiphyseal cartilage and less abundant in adult cartilage [11]. It is probable that PARP accumulates in the matrix as a result of removal of the N-propeptide of collagen α2(XI) and its considerably basic character. Whether PARP plays a role in the structure of cartilage or whether it provides feedback to chondrocytes has not been determined. It is likely that the N-propeptides of collagens XI, IX, V, XII, XIV and XVI form a similar structure, probably primarily β-sheet [94].

Epiphyseal and regulatory cartilage proteins

The epiphysis and the growth plate are the center of growth of the majority of the skeleton. As such, they are very dynamic structures. They are the last regions in the body to be replaced by adult tissue. The epiphysis is a rich source of molecules that may be growth factors, or at least that may be involved in modulation of cellular phenotype. In particular, chondromodulins I and II and pleiotrophin have been isolated from dissociative extracts of fetal bovine nasal and epiphyseal cartilage with yields of at least 15 μg/g wet weight of cartilage [10, 95].

Angiogenin

Angiogenin has been identified in dissociative extracts of bovine tibial epiphysis and fetal nasal cartilage, and, while only present at molar levels of about one-tenth that of pleiotrophin or chondromodulin, is present in significant amounts. It is not present in articular cartilage [P. J. Neame, unpublished]. The role of angiogenin may be to encourage the ingrowth of a vascular system prior to ossification. In this role, it would be antagonized by chondromodulin I.

Cartilage-derived morphogenetic proteins (CDMPs)

Cartilage-derived morphogenetic proteins 1 and 2 are members of the TGF-β superfamily and were isolated after extraction of articular cartilage with 0.15 M sodium chloride. Their closest relatives are bone morphogenetic proteins 5 and 6 [96]. CDMP-1 (in mouse, growth/differentiation factor 5, or Gdf5) is primarily found in the mesenchymal condensations that precede the development of bone. It is upregulated in tissue culture. A mutation in CDMP-1 is associated with acromesomelic chondrodysplasia, Hunter-Thompson type [97]. A cysteine-tyrosine change that results in defective secretion is associated with chondrodysplasia Grebe type [98], and an arginine change to a stop codon is linked to brachydactyly type C [99]. In contrast to CDMP-1, CDMP-2 is found in hypertrophic cartilage postnatally and is downregulated in cultured chondrocytes. In this regard it is more typical of other markers of the cartilage phenotype, such as collagen type II. Both CDMPs stimulate production of proteoglycans [100], and both bind to the bone morphogenetic protein receptor-IB and II [101].

Cartilage-derived, retinoic acid responsive protein (CD-RAP)

The complementary DNA (cDNA) for CD-RAP was isolated by differential display analysis of mRNA from chondrocytes that had been treated with retinoic acid. Similarly to collagen type II, transcription of CD-RAP mRNA was downregulated by retinoic acid [102]. The approximately 12-kDa mature protein is the same as melanoma inhibitory protein, a protein that inhibits growth of melanoma cells in an autocrine fashion [103]. The biological role of CD-RAP is unknown, although in cultured chondrocytes, the protein downregulates DNA synthesis. Based on the location of the mouse gene on chromosome 7 [104], the human gene is likely to be on chromosome 19q13.1.

Chondromodulin I

Chondromodulin is an 18–24-kDa glycoprotein that is abundant in fetal cartilage. It was originally characterized as a small, cartilage-derived, glycoprotein (SCGP, small cartilage-derived glycoprotein) with a glycosylated N-terminal and a disulfide-bonded C-terminal. It could also be isolated in a truncated 9.6-kDa form that was missing the glycosylated N-terminal [10]. Subsequently, SCGP was found to be identical to a protein, chondromodulin, that enhanced the activity of FGF on chondrocytes [105] and that had been purified based on its affinity for heparin-Sepharose [106]. Chondromodulin derives from a larger precursor that has a membrane insertion domain 40 residues from the signal peptide [105].

Chondromodulin has two roles: First, it potentiates the effect of FGF-2 on chondrocytes, increasing the rate of cell division as measured by thymidine incorporation in cultured chondrocytes and increasing the rate of proteoglycan synthesis [105]. Second, chondromodulin I acts as an inhibitor of proliferation of vascular endothelial cells [107]. Chondromodulin I also promotes proliferation of osteoblasts. Chondromodulin I is downregulated by FGF-2, TGF-β and parathyroid hormone [108].

LECT2/chondromodulin II

Chondromodulin II is a 16-kDa protein that is quite abundant in fetal cartilage [109]. It bears no similarity to chondromodulin I, except in its name, and in its location in the epiphysis. Like chondromodulin I, chondromodulin II enhances chondrocyte and osteoblast proliferation [110] and proteoglycan biosynthesis, but unlike chondromodulin I, it does not inhibit vascular endothelial cell growth. Chondromodulin II also promotes osteoclast differentiation [111]. The human gene is found on chromosome 5q31.1–32 [112]. Chondromodulin II is identical to LECT2 (leukocyte cell-derived chemotaxin 2), was isolated from liver and has been shown to be chemotactic for neutrophils in vitro [113].

Matrilin 3

Matrilin 3, 48.9 kDa, is a member of the same family of proteins as cartilage matrix protein (matrilin 1) and is cartilage-specific. Unlike CMP, matrilin 3 only has a single von Willebrand factor domain. This is followed by four EGF-type modules and a likely coiled-coil oligomerization domain at the C-terminal [114]. In the epiphysis, matrilin-3 forms trimeric heterocomplexes with CMP in an overall ratio of 1:1 [115].

Matrix Gla protein

Matrix Gla protein is a small, vitamin K-dependant, 8.5-kDa protein found primarily associated with cartilage formation and calcification but also synthesized by vascular smooth muscle cells. It is upregulated by vitamin D [116]. It contains four to six γ-carboxy-glutamate (Gla) residues and is closely related to the bone Gla protein [117]. It is initially found at the interface of mesenchymal condensations and epithelium and then is found associated with resting, proliferative and late, but not early, hypertrophic chondrocytes [118].

Matrix Gla protein appears to be associated with the maintenance of an uncalcified matrix. Absence of Matrix Gla protein in transgenic mice results in calcified arteries which contain cells that are characteristic of chondrocytes. A deficiency, rather than an absence of Matrix Gla protein, results in poor skeletal growth [119].

Pleiotrophin / HB-GAM

Pleiotrophin (PTN) is an 18-kDa secreted heparin-binding, developmentally regulated protein that is abundant in fetal, but not mature, cartilage [95]. While PTN is by no means restricted to cartilage, it is so abundant in fetal cartilage that we have included it here. Immunohistochemical analysis of its distribution in fetal epiphysis indicated that it is associated with the cell surface of chondrocytes [95], whereas reverse-transcription followed by polymerase chain reaction or Northern analysis show PTN message levels are highest in the resting zone of the cartilage growth plate [A. Azizan and P. J. Neame, unpublished].

PTN, together with midkine and chicken retinoic acid-induced heparin-binding protein, are members of a family of heparin-binding cytokines that share sequence, structural and functional similarity [120–122]. PTN has been shown to enhance neural outgrowth in developing neurons [123] and stimulate angiogenesis in tumors [124]. In the developing chick limb, PTN appears shortly after mesenchymal condensation (stages 27–28) and continues to be present until stages 42–44. The protein persists for some time after the mRNA has disappeared [125].

The mode of action of PTN is unclear at present. PTN binds to heparan sulfate and is found associated with chondrocytes when fetal epiphyseal cartilage sections are stained with anti-PTN antibody. Syndecan III [126] and phosphacan [127] have both been shown to be high-affinity acceptors for PTN. Given the amounts of PTN that are found in fetal cartilage, and the fact that it seems to be primarily associated with the cell surface, it is possible that the mode of action may be to block heparan sulfate binding sites which could be used by other cytokines such as FGF2. PTN has been shown to inhibit the proliferation of mesenchymal and epithelial cells in cultured limb buds [128]. The increased thymidine uptake observed on addition of FGF2 was reversed by PTN addition, suggesting that PTN may block part of the FGF receptor. When recombinant PTN was added to chondrocytes, they stopped dividing, they exhibited increased metabolic activity and synthesis of aggrecan and biglycan, and they reduced decorin expression [H. Tapp and P. J. Neame, unpublished].

Regulation

Mechanical forces play a prominent role in the regulation of limb development. As soon as the musculoskeletal system, including the nervous system, has developed to the point where movement is possible, mechanical loads form across bones. These forces play an important role in limb development and have been demonstrated to have profound effects on the type of

extracellular matrix that is synthesized. For example, decorin synthesis is higher in cartilage loaded with 5-s pulses [129]. Pulsatile compression increases matrix biosynthesis by 20–40%, as measured by incorporation of proline and sulfate into macromolecules [130].

The mode whereby mechanical forces can transmit signals to chondrocytes varies. As cartilage is compressed, the effective concentrations of solutes will change to a much larger extent than would be expected by the distortion of the tissue. This is because the majority of the water is immobilized by the anionic GAG chains of proteoglycans. Indeed, pH has been shown to have a dramatic effect on chondrocytes [131].

Compressive and shear forces that the cells are exposed to also have a direct effect on their phenotype via interactions between cell surface macromolecules and the surrounding extracellular matrix. Thus a mechanical force can be transmitted to a chondrocyte via an integrin that is indirectly (or directly) bound to an extracellular macromolecule [132].

Recently, it has become possible to readily analyze the regulatory (promoter) regions of genes and deduce which regions are important or which promoters and enhancers are required for transcriptional regulation. While the precise chondrocyte type that should be used for these studies is not always clear, useful information has been obtained for a number of genes including the collagens and some proteoglycans. Of the molecules described here, CMP [133], CD-RAP [134] and PRELP [86] have had their promoters characterized in detail. Upstream gene sequences are available for chondroadherin (130 bp) [91] and CLECSF1 (12 kbp) [P. J. Neame and H. Tapp, GenBank accession no. AF077344].

The role of regulatory molecules such as parathyroid hormone-related peptide (PTHrP) and the hedgehog family of genes in limb development has received considerable attention recently. PTHrP is a regulatory factor in organogenesis of a variety of tissues [135], and in the growth plate appears to prevent entry of prehypertrophic chondrocytes into the hypertrophic pathway. In chick limbs, a mitogenic response to PTHrP is only observed in growth plate chondrocytes and not articular chondrocytes [136]. The importance of PTHrP in development of the growth plate is illustrated by a mutation of the receptor that causes Blomstrand chondrodysplasia [137]. The hedgehog family of genes (for example indian hedgehog and sonic hedgehog) regulate both chondrocyte progression and PTHrP expression [138]. For thorough descriptions of current knowledge in this area, a review volume is recommended [139].

Conclusion

Advances in protein isolation techniques combined with the power of modern molecular biology have taken cartilage from being thought of as a relatively simple example of extracellular matrix to its present status of a complex, tightly regulated tissue in which cells are exposed to gradients of a variety of components that have an effect on their phenotype.

Chondrocytes synthesize extracellular matrices that are uniquely suited to their environment. Unlike most tissue types, where tissue strength derives at least in part from cell-cell interactions, tissue strength in cartilage derives from the interactions of the extracellular matrix. Thus the nature of the matrix macromolecules and their interactions with each other are of considerable importance.

Furthermore, during development, chondrocytes are exposed to a milieu of regulatory molecules. Some of these, such as PTHrP and chondromodulins I and II, appear to stimulate proliferation of chondrocytes, whereas others, such as pleiotrophin and CD-RAP, appear to inhibit cell proliferation. It is likely that these regulatory molecules also have different effects on matrix macromolecules. In order to investigate the events that control matrix production and chondrocyte differentiation, suitable experimental models have to be used.

In spite of the enormous gains in knowledge that have been made in recent years, there are still no universally accepted 'good' experimental models of cartilage that are applicable to most experimental techniques. While it is generally accepted that monolayer culture is not an ideal model, it is the only model that enables chondrocytes to be studied over the short term without concern over the effects of the extracellular matrix. Culture of chondrocytes in alginate beads mimics the environment in the cartilage matrix, where chondrocytes do not come in contact with each other, or at least with only a few other cells [140]. It is particularly appropriate for long-term culture. It does not approach the mechanical stresses faced by chondrocytes in cartilage. Mechanical loading is a regulatory force on chondrocytes as soon as the musculoskeletal system is capable of generating force. Mechanically loaded explants [130] are a good model of cartilage, but make it difficult to determine whether a given molecule, applied to determine its effect on chondrocytes, has actually reached the majority of cells unless sophisticated histological techniques are used. Culturing cells in monolayers on flexible sheets attempts to mimic some of the mechanical forces [141], but does not mimic longer-range forces derived through the extracellular matrix.

Developing cartilage also possesses a variety of cell types in the growth plate. Some of these cells are not particularly abundant, or are hard to culture, or are hard to isolate, and so will continue to provide interesting problems for some time to come. The human distress caused by skeletal problems, whether induced by trauma or induced by developmental disorders, contin-

ues to make cartilage biology an important and dynamic research area.

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