Mini Review

Chondromodulin-I: A Growth-Modulating Functional Matrix in Cartilage

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Cartilaginous extracellular matrix (ECM) is a unique assembly of macromolecules such as type II collagen and proteoglycans. In association with a number of accessory molecules, it confers special biological functions on cartilage. Among these various accessory molecules, growth-modulating functions have been identified only in several instances, and in many cases functional properties therefore remain to be elucidated. Chondromodulin-I (ChM-I) is a 25-kDa glycoprotein, which was initially identified in our laboratory as a cartilage-specific functional matrix component that stimulates the growth and matrix synthesis of primary chondrocytes *in vitro*. We subsequently purified an endothelial cell growth inhibitor from guanidinium extracts of fetal bovine cartilage, which was surprisingly found to be identical to ChM-I. Purified ChM-I was found to inhibit the proliferation of vascular endothelial cells as well as tube morphogenesis *in vitro*. The generation of a ChM-I null mouse has enabled us to examine the functional roles of this unique ECM component *in vivo*. In this review, we describe the identification and biological characterization of ChM-I as an example of a growth-modulating functional matrix in cartilage.

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The Extracellular Matrix in Cartilage

The mesenchyme supports the animal body by filling the space between the outer and inner surfaces. This space comprises the epithelial and endothelial cell sheets for which cell-cell adhesions are vital for proper functioning. Unlike epithelial and endothelial tissues, however, mesenchymal tissues fulfill their roles mostly through the extracellular matrix (ECM) that they produce. Cartilage is a typical skeletal component of this nature in which the only cell type is the chondrocyte. These cells occupy only about 10% of the tissue volume in articular cartilage, which is comprised largely of the ECM that they synthesize. Hence, chondrocytes are metabolically active in order to fulfill their roles in the synthesis and turnover of ECM components.

Cartilage ECM is hyperhydrated as evidenced by a water content ranging from 60% to almost 80% of the total wet weight. The remaining 20% to 30% of the wet weight of this tissue is

principally composed of two macromolecular materials: type II collagen, which constitutes up to 60% of the dry weight, and a large proteoglycan, aggrecan, which accounts for a large part of the remainder[1]. Minor amounts of other large proteoglycans such as versican, and perlecan are also found in cartilage. Small proteoglycans in cartilage include byglycan, decorin, epiphycan, fibromodulin, and lumican, all of which are classified as a family of small leucine-rich repeat proteoglycans. Type IX, XI, III, V, VI, X, XII and XIV collagens are also present in cartilage as minor components.

The viscoelasticity of cartilage ECM, and its swelling characteristic which is due to hyperhydration, enables articular cartilage to function as a weight-bearing tissue in the synovial joints, where chondrocytes persist to maintain the ECM throughout the lifespan of the animal. Another important function of cartilage is its longitudinal growth capability that facilitates endochondral ossification during which the cartilage preoccupies and rapidly expands its tissue space as a bone primordium during skeletal development. Chondrocytes can achieve tissue growth through proliferation, matrix production, and via an increased cell volume to become terminally differentiated hypertrophic chondrocytes that are eventually replaced by bone and bone marrow. Epiphyseal growth plate chondrocytes survive to adolescence and contribute to the growth of long bone in the same way.

It is therefore of central importance that the cartilage maintains the physicochemical properties of the viscoelastic ECM via the swelling pressure that is due to the hyperhydration of proteoglycans. Hence, cartilage needs to remain avascular and protect itself from vascular invasion as this is accompanied by active proteolysis and degradation of ECM components by vascular endothelial cells to create vascular guidance tunnels (the notable exception to this is the hypertrophic/calcified cartilage that allows vascular invasion to trigger replacement of cartilage by bone). For this reason, tissue-specific anti-angiogenic ECM components have been envisioned in hyaline cartilage and indeed several cartilage-derived angiogenesis inhibitors have been identified to date[2].

Unlike many other mesenchymal tissues, in which circulation is vital for the supply of nutrients and endocrine hormones, cartilage has the ability to acquire the necessary nutrients from its surroundings, even in the absence of blood vessels, through a passive flow of solutes in the ECM filled with hyperhydrated proteoglycans. To compensate for the lack of a vasculature, cartilage stores various growth/differentiation factors in its ECM, including fibroblast growth factors and bone morphogenetic proteins, to support of its own rapid growth and facilitate an active metabolic state. However, cartilage contains a large number of other minor ECM proteins that have been characterized only by their physicochemical properties. Only in several instances have the biological functions of these factors been determined and in many cases therefore the functional properties of these components remain to be elucidated.

Identification of Chondromodulin-I

Some of the ECM components in cartilage had been suggested to play a role in the growth and matrix synthesis (expression of the differentiated phenotype) of chondrocytes. We have found in our own experiments that fetal bovine cartilage extracted with 1 M guanidinium chloride stimulates proteoglycan synthesis and DNA synthesis in rabbit growth-plate chondrocytes in culture. The DNA synthesis in these cells was synergistically activated in the presence of an optimal dose of FGF-2 (fibroblast growth factor-2). This activity was found to be heat-stable, but sensitive to reduction by β-mercaptoethanol, and was purified to homogeneity by monitoring the DNA synthesis of cultured chondrocytes in the presence of FGF-2 through heparin affinity and several other chromatographic steps. Figure 1 shows the elution profile of DNA synthesis stimulating activity from a C4 reverse-phase HPLC (RP-HPLC) column. Fraction #4, which gave a single 25-kDa diffuse band on SDS-PAGE analysis, clearly stimulated

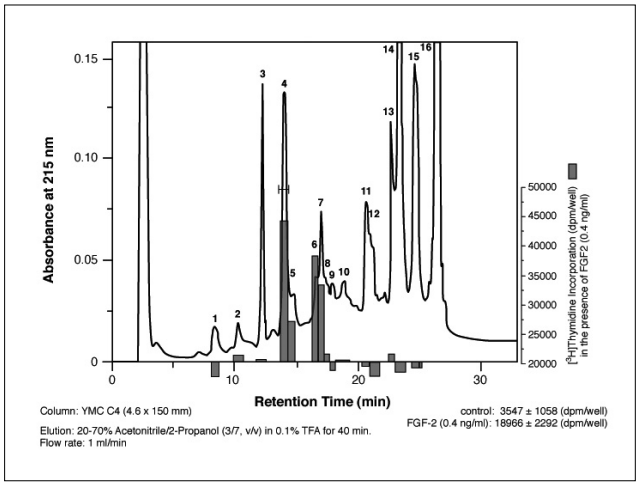

Fig.1 Elution profile of the DNA synthesis stimulating activity from a C4 reverse-phase HPLC column

Shaded bars indicate [^{3}H] thymidine incorporation in rabbit cultured growth-plate chondrocytes in the presence of an optimal dose (0.4 ng/ml) of FGF-2.

the synergistic activation of DNA synthesis in cultured chondrocytes. Amino acid sequencing revealed the N-terminal 27 residues and an internal sequence of 21 residues derived from an endopeptidase digested fragment of a protein denoted Chondromodulin-I (ChM-I)[3]. No amino acids were identified in the 9th and 22nd cycles of sequencing, suggesting that these residues are glycosylated. The shoulder peak (fraction #6) also yielded the same N-terminal amino acid sequence, suggesting a variation due to glycosylation. We subsequently found that purified bovine ChM-I stimulated proteoglycan synthesis in cultured chondrocytes. Chondrocytes are unique among non-transformed cells as they are capable of colony formation (anchorage-independent growth) in soft agar. Purified ChM-I stimulated colony formation of chondrocytes in agarose culture synergistically with FGF-2, which accounts for the chondrocyte colony-stimulating activity that is present in cartilage extracts[4].

Demineralized bone matrix (DBM), which contains bone morphogenetic protein (BMP), induces cartilage that is subsequently replaced by bone via the endochondral ossification pathway. Experimentally, this has been found to occur within three weeks of DBM implantation into the fascia of mice[5]. In contrast, we found in our previous study that when the DBM had been mixed with the guanidinium extracts of bovine cartilage, the induced cartilage persisted without capillary ingrowth and failed to form bone, implying the presence of cartilage-derived angiogenesis inhibitor(s) in the cartilage extract preparation[5]. Thus, by monitoring the inhibition of DNA synthesis of cultured aortic endothelial cells, we were able to successfully purify the endothelial cell growth inhibitor from cartilage extracts[6]. The inhibitor that was finally purified by RP-HPLC showed an identical SDS-PAGE profile, and was identified by its N-terminal amino acid sequence to be ChM-I. We confirmed later that mature ChM-I inhibits the *in vitro* tube morphogenesis of human umbilical vein endothelial cells, retinal endothelial cells and bovine aortic endothelial cells[7,8].

Figure 2A shows the primary amino acid sequences of human and mouse ChM-I (both of 120 residues) predicted from the nucleotide sequences of ChM-I precursor cDNA. Mature ChM-I was found to be encoded as the C-terminal portion of a larger type II transmembrane precursor of 334 amino acids, from which the mature protein is cleaved by a furin-like endopeptidase and secreted from the cell. The human ChM-I precursor gene, mapped to chromosome 13 (13q14.3), comprises seven exons, the last two of which encode the mature 120 amino acid portion of the precursor. Exon 6 encodes a processing signal sequence and the N-terminal hydrophilic domain (domain 1) that contains glycosylation sites. Exon 7 encodes the cysteine-rich region and the subsequent C-terminal hydrophobic domain (domain 2; 71 amino acids), which is essential for the growth-modulating functions of ChM-I. The amino acid sequence of the human ChM-I precursor was found to be well conserved across species including fish (Fig.2B)[9]. Domain 2 in particular is highly conserved across species at the amino acid level and only a single amino acid difference exists between the mouse and the human sequences in this domain (Val^{320} to Ile^{320}). Thus far, no invertebrate ortholog of ChM-I has been found. ChM-I precursor and tenomodulin (TeM) constitute a family of type II transmembrane proteins harboring a homologous cysteine-rich anti-angiogenic domain at the C-terminus. TeM is not expressed in cartilage but found to be specifically expressed in hypovascular dense connective tissues such as tendons and ligaments[10].

Localization of ChM-I Expression and Abnormalities in ChM-I Deficient Mice

Cartilage is the most prominent site of ChM-I expression in the mammalian body[6,11]. During limb development, the immunoreactivity for mature ChM-I has been shown to specifically localize in cartilaginous bone primordia, where it is maintained at a high level in the avascular zone of cartilage, i.e. in the resting, proliferating, and early hypertrophic zones (green signals in Fig.3). The overlapping expression pattern of ChM-I transcripts was confirmed by *in situ* hybridization[5,6,11] and expression was found to be completely abolished in the late hypertrophic/calcified zone of cartilage. Taking advantage of ATDC5 mouse chondroprogenitor cells *in vitro*, ChM-I transcripts were found to be induced in tandem with the overt chondrogenic differentiation that occurs after mesenchymal condensation of chondroprogenitor cells[5]. Following the induction of type II collagen mRNA, the level of ChM-I transcripts increases as the chondrocytes mature, and declines as the cells progress to the hypertrophic and calcified stages.

In contrast, as visualized by an anti-PECAM-1 antibody (Fig.3; red signal), although the vascular networks are well developed to nourish all of the limb structures, they are clearly segregated from cartilage by the perichondrium. Cartilage switches its phenotype during endochondral bone formation from anti-angiogenic to pro-angiogenic at the center of cartilaginous bone primordia where the hypertrophic and calcified cartilage zone is formed. Concomitant with the disappearance of ChM-I, calcified cartilage begins to express vascular endothelial growth factor-A (VEGF-A) and matrix metalloproteinase 13 (MMP13), which initiates the remodeling of calcified cartilage matrix (Fig.3).

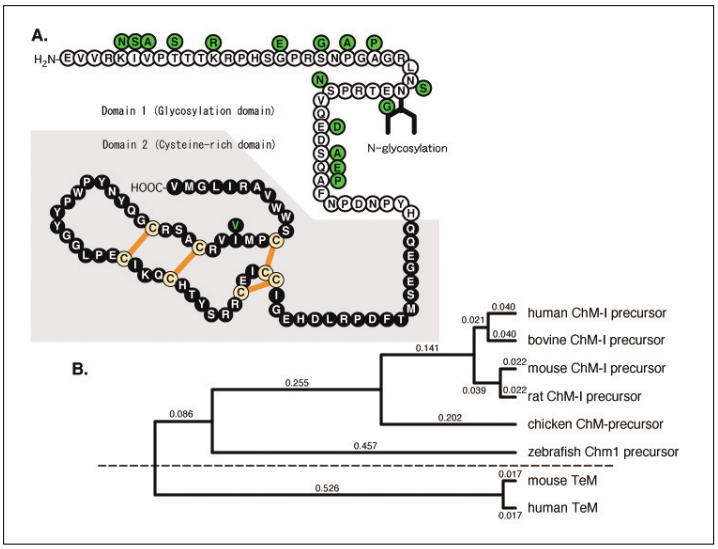

Fig.2 Molecular structure of ChM-I

(A) The amino acid sequence of human mature ChM-I. The 17 amino acid residues that are not conserved in the mouse counterpart are indicated in green. A conserved N-glycosylation site is found in domain 1. Domain 2 contains four disulfide bonds that are indicated by orange bars. (B) The phylogenetic tree of the chondromodulin-I precursor and a ChM-I related protein, TeM.

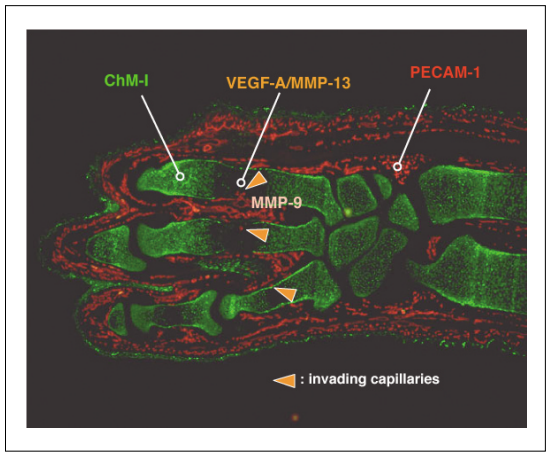

Fig.3 Immunolocalization of ChM-I in the avascular mesenchyme of the developing mouse forelimb at E16.5[10]

A frozen section of mouse forelimb was double-stained with anti-rat PECAM-1 antibody (*red*) and anti-ChM-I antibody (*green*). Positive staining for ChM-I is observed in the avascular cartilage ECM zones of the phalanges, carpal bones and ulna and radius in the PECAM-1-negative domains. Orange arrowheads indicate the sites of vascular invasion into hypertrophic/calcified cartilage of cartilaginous bone primordia.

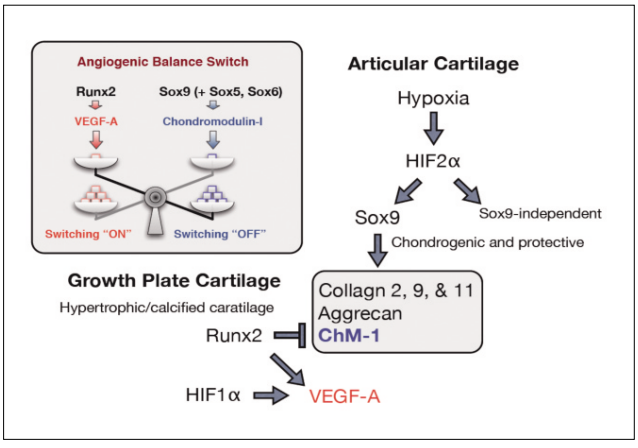

Fig.4 A schematic representation of the regulation of ChM-I expression and a putative angiogenic switch balanced by VEGF-A and ChM-I

Concurrent VEGF-A-induced MMP9 expression and activation facilitates vascular invasion by creating vascular guidance tunnels within the three-dimensional matrix environment at the center of bony collar surrounding the late hypertrophic and calcified zone of cartilage.

The Runx2 transcription factor participates both in the anti-angiogenic to pro-angiogenic phenotypic switching of cartilage at the center of cartilaginous bone primordia and in osteoblastic differentiation[12]. In this regard, the targeted disruption of Runx2 results in a loss of endochondral bone formation due to the lack of vascular invasion of the cartilaginous bone primordia, although ChM-I expression was persistent in the bone primordia of Runx2 deficient mice[12]. The phenotype of these animals was rescued by the forced expression of Runx2 in cartilage under the control of the type II collagen promoter. Moreover, in rescued Runx2 null mice in which ChM-I expression was suppressed, VEGF-A was found to be induced. Following the restoration of maturation so that hypertrophic chondrocytes develop, vascular invasion occurs even in the absence of osteoblastic differentiation[12]. Thus, Runx2 is likely to participate in the genetic switch of an-

giogenesis that confers a coordinated change in the induction of VEGF-A and downregulation of ChM-I in hypertrophic chondrocytes. This generates the mutually exclusive expression pattern of ChM-I and VEGF-A (Fig.4).

Based on the preferential expression of ChM-I in the avascular region, it was reasonable to assume that ChM-I null mice would show some angiogenic phenotypes upon vascular invasion into the cartilage during endochondral bone formation. However, any apparent vascular abnormalities were not observed during endochondral bone formation in these mice[13]. This is subsequently explained by the presence of a robust anti-angiogenic barrier in the perichondrial tissue surrounding the cartilaginous bone primordia which blocked vascular invasion in the early stages of endochondral bone formation[10].

Instead of the angiogenesis-related phenotype, we found in our analysis that ChM-I null adult mice displayed a marked reduction in bone remodeling. A significant increase in the bone mineral density was observed in ChM-I null mice due to an imbalance between osteoclastic bone resorption and osteoblastic bone formation[13]. Recently, we have reported that adult ChM-I null mice exhibit aberrant cartilage formation during fracture repair[14]. Radiological examination of these knockout animals revealed a delayed union although the fracture site was covered with both external and internal calluses. Interestingly, the majority of the chondrocytes in the periosteal callus in ChM-I null mice failed to differentiate into mature chondrocytes, resulting in a marked reduction of the external cartilaginous callus. Hence, in the absence of ChM-1, predominant primary bone healing occurs due to an indirect effect induced by this reduction of cartilaginous callus rather than by a direct effect on osteogenesis, thus leading to a delayed union[14].

Sox9, a transcription factor of the Sox family, is a potent transactivator of the chondrocyte-specific enhancer of the type II collagen gene and has an essential role in chondrogenesis. The adenoviral co-expression of *Sox9*, *Sox5*, and *Sox6* induces chondrogenic differentiation in mouse embryonic stem cells (Fig.4)[15]. Following the induction of the characteristic chondrocyte marker genes, i.e. the *type II*, *type IX*, and *type XI* collagens and aggrecan, *ChM-I* transcripts are also induced. Similarly, the expression of these Sox family members successfully induces *ChM-I* mRNA, even in adult dermal fibroblasts, which are non-chondrogenic. Therefore, Sox9 is considered to be a part of genetic switch that regulates *ChM-I* expression[16]. *Sox9* is also activated when endocardial endothelial cells undergo a mesenchymal transition and migrate to form endocardial cushions. This induces the cardiac valve precursor cells to develop cardiac valves which are maintained as avascular tissue in the heart[17]. ChM-I was detected throughout the valvular interstitial cells of all four cardiac valves and their extracellular matrix, but not in the outer endothelial cell layer. The non-overlapping localization of ChM-I and VEGF-A was apparent at all development stages of the heart under normal conditions[18].

Cardiac valves have the same level of avascularity as cartilage under normal conditions, but under pathological conditions, such as aortic stenosis, these valves express angiogenic factors that cause neovascularization[19]. A deficiency in ChM-I caused no apparent abnormalities in young mice (8 week old), but resulted in increased angiogenesis in the cardiac valves of 90-week-old mice. This age-dependent neovascularization was associated with valve thickening, calcification and turbulent blood flow. This pathology is similar to the clinical findings in patients with aortic stenosis, which poses an 80% 5-year risk of progression to heart failure, valve replacement or death[19]. In a mouse model of atherosclerosis (the apolipoprotein E null mice) that develops abnormal lipid deposits and calcification with age, ChM-I was found to be absent in calcified regions of the cardiac valves and this was associated with increased microvessel density and VEGF-A upregulation[18]. Similarly, numerous vessels have been observed in the cardiac valves of patients with valvular heart disease. ChM-I was shown to be markedly downregulated in the regions of neovascularization that strongly expressed VEGF-A. In normal valves, ChM-I was detectable in the laminae fibrosa, spongiosa and ventricularis layers, whereas VEGF-A was absent in all cell layers. Cardiac valve disease has therefore been linked for the first time to a deficiency in an endogenous angiogenesis inhibitor.

Therapeutic Implications

Angiogenic disease has been extensively studied with a focus on angiogenesis stimulators such as VEGF, FGF, and platelet-derived growth factor. As the list of endogenous angiogenesis inhibitors has been added to, the further elucidation of the angiogenic balance has become increasingly important. The cardiac phenotype of the ChM-I null mouse indicates that a lack of an endogenous angiogenesis inhibitor underlies some significant heart pathologies[18]. Transcripts for ChM-I are substantially reduced in many cases of chondrosarcoma, compared with normal articular cartilage and other benign cartilage tumors[11]. The susceptibility of chondrosarcoma to tumor angiogenesis may therefore be accounted for by a loss of ChM-I expression. When injected into nude mice, human chondrosarcoma cells produce tumors with a cartilaginous ECM that abundantly expresses type

II collagen and aggrecan. In addition, The local administration of recombinant human ChM-I (rhChM-I) in these mice completely blocked vascular invasion and tumor growth[11]. Furthermore, the adenoviral transduction of human ChM-I in mouse melanoma cells results in the suppression of tumor growth in association with decreased tumor angiogenesis, when injected into syngenic mice[8].

ChM-I has also been found to be expressed in the cortex of the thymus in mice[5, 20], suggesting that it may participate in T cell development and function. Indeed, rhChM-I has been found to suppress the proliferative response of both mouse splenic T cells and human peritoneal blood T cells that had been stimulated with anti-CD3/CD28 antibodies[20]. Production of interleukin-2 is decreased in rhChM-I-treated mouse CD4 T cells. Moreover, rhChM-I suppresses the proliferation of synovial cells prepared from the joints of patients with rheumatoid arthritis[20]. Hence, in addition to angiogenesis inhibition, the suppression of T cell responses and synovial cell proliferation indicates a unique therapeutic potential for ChM-I in rheumatoid arthritis[20].

Articular cartilage expresses ChM-I at a lower level than growth plate cartilage and intense immunoreactivity for ChM-I has been detected in the intermediate and deep layers of articular cartilage in rats[21]. Upon maturation of the rat, this immunoreactivity is localized around the hypertrophic chondrocytes in the deep layer[21]. Osteoarthritis (OA) is a chronic degenerative joint disorder characterized by articular cartilage destruction and osteophyte formation, and is a major cause of disability in the elderly. Although the etiology of OA is not yet fully understood, the cumulative mechanical loading and instability of the joints may play a role in the pathogenesis of this disease to result in a gradual loss of proteoglycans. In the early phase of OA, ChM-I immunoreactivity is lost from the articular surface in rats[21]. The loss of proteoglycans may reduce the availability of oxygen and the supply of other nutrients to induce early protective responses. This includes the production of hypoxia-inducible factors (HIF) in articular chondrocytes in a manner both dependent and independent of Sox9. Recently, ChM-I has been characterized as a new hypoxia-inducible and Sox9-regulated gene in human articular chondrocytes (Fig.4)[16]. In contrast to cartilaginous bone primordia or ephiphyseal cartilage, which allows vascular invasion prior to replacement by bone, normal articular cartilage remains avascular throughout life. In OA, articular cartilage appears to shift to an ephiphyseal-like phenotype and eventually allows blood vessels to penetrate its calcified zone to facilitate the further progression of cartilage degradation. The expression of the Runx2 transcription factor is suggested to participate in this phenotypic shift that upregulates VEGF-A and MMP13 and downregulates ChM-I and other cartilage-protective agents[22].

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