Chondromodulin-I expression in rat articular cartilage

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Summary. The localization and expression of chondromodulin-I (ChM-I), an angiogenesis inhibitor, in the rat articular cartilage during maturation from 2 to 10 weeks of age were examined by immunohistochemistry, Western blot analysis and ribonuclease protection assay, and the results were compared with those in the epiphyseal cartilage. ChM-I was found to be diffusely immunostained in the inter-territorial space of the cartilage matrix from the intermediate to the deep layers at the immature stage. As the articular cartilage matured, the immunoreactivity was localized around the hypertrophic chondrocytes in the deep layer and the immunoreactivity became weak after maturation. In contrast, the ChM-I immunoreactivity was intense in the epiphyseal cartilage at all ages examined. ChM-I was detected by Western blotting as a broad band or occasionally as a cluster of multiple bands (~25 kDa) in both the articular and the epiphyseal cartilage. The intensity of the bands decreased gradually with age in the articular cartilage, but was unchanged in the epiphyseal cartilage at all ages. Ribonuclease protection assay revealed that ChM-I mRNA also decreased gradually with age in the articular cartilage in parallel with the maturation of the articular cartilage, while no decrease in ChM-I mRNA was found in the epiphyseal cartilage. The expression of ChM-I mRNA in the articular cartilage was less than that in the epiphyseal cartilage at all ages. The decrease in amount of ChM-I in the mature articular cartilage suggests that ChM-I plays a more important role in the maintenance of vascularity in the immature articular cartilage than in the mature one. The avascular condition may be preserved by angiogenic inhibitors or mechanisms other than ChM-I in the mature articular cartilage.

Introduction

The avascular nature of cartilage has led to suggestions of intrinsic inhibitors of angiogenesis in it (Kuettnner and Pauli, 1983). During the development of embryonic skeletons, mesenchymal cells aggregate and a cartilaginous bone primordium appears as a mold for bone formation. The chondrocytes in the central part of the mold then become hypertrophic and calcified. The calcified cartilage permits vascular invasion into the cartilaginous bone while allowing capillaries from surrounding peristeam recruit osteoblasts and osteoclasts to replace the cartilage matrix with the bone matrix, the so-called endochondral bone formation. This endochondral bone formation in the epiphyseal cartilage is fundamental for embryonic skeletal development and longitudinal bone growth after birth. Hiraki and his colleagues isolated a cartilage-derived molecule, chondromodulin-I (ChM-I) (Hiraki et al., 1991) as a chondrocyte-growth and anti-angiogenic factor.

ChM-I is a 25kDa glycoprotein purified from the fetal bovine epiphyseal cartilage on the basis of its growth stimulating activity for chondrocytes (Hiraki et al., 1991). This glycoprotein stimulates the proliferation, proteoglycan synthesis, and colony formation of cultured growth plate chondrocytes in vitro (Inoue et al., 1997). Interestingly, ChM-I was found to inhibit the DNA synthesis and tube morphogenesis of cultured vascular endothelial cells (Hiraki et al., 1997b), and tumor growth in vivo (Hayami et al., 1999). Thus, ChM-I has various functions for different cell types. In the epiphyseal cartilage, ChM-I mRNA and protein was
strongly detected in the avascular zones and its expression was significantly reduced in the lower hypertrophic and calcified zones where vascular invasion occurs (Hiraki et al., 1997a, 1999). On the other hand, vascular endothelial growth factor (VEGF), a potent vascular endothelial cell specific mitogen, was absent or only weakly present in the avascular zones but abundant in the lower hypertrophic and calcified chondrocyte zones (Gerber et al., 1999; Horner et al., 1999; Carlevaro et al., 2000). These findings suggest that the decrease in ChM-I protein and the increase in VEGF protein permit vascular invasion and subsequent endochondral bone formation in the epiphyseal cartilage. Thus, ChM-I is presumed to play an important role in the regulation of vascular invasion during endochondral ossification in the epiphyseal cartilage.

While many studies on ChM-I in cartilage have been performed in the epiphyseal cartilage, no study has reported on the expression and localization of this molecule in the articular cartilage. The articular cartilage is also avascular and is resistant to vascular invasion in physiological conditions throughout life. However, the mechanism to maintain the avascularity in this cartilage is still unclear. The aim of
Materials and Methods

Animals and tissues
Animals used in all experiments were inbred male Wistar-Kyoto (WKY) rats (Charles River Japan Inc., Kanagawa) at 2, 4, 6, 8 and 10 weeks of age. Their knee joints were decalcified in a 10% ethylene diamine tetraacetic acid (EDTA) solution (pH 7.4) at 4°C with gentle shaking for 7 days, dehydrated in graded ethanol, and embedded in paraffin. The tissues were sectioned at a 4 μm thickness for immunohistochemistry. For protein and RNA extraction, the articular and the epiphysial cartilage were separated from the proximal and distal femur and proximal tibia.

Immunohistochemistry
The paraffin-embedded tissue sections were dewaxed with
xylene and hydrated through graded ethanol and distilled water. The sections were treated with testicular hyaluronidase (500 U/ml, type IV; Sigma-Aldrich, Tokyo) for 60 min at 37°C and rinsed in 0.01M phosphate buffered saline (PBS). They were incubated with 5% normal goat serum for 30 min at room temperature to reduce non-specific reaction, and then incubated with the affinity-purified rabbit anti-rat ChM-I antibody (Funaki et al., 2001) diluted at 1:2000 for 16 h at 4°C. After being rinsed in 0.1M PBS, they were reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5 dilution, EnVision; DAKO, Kyoto) for 30 min at room temperature and the peroxidase reaction products were visualized with 3′-diaminobenzidine tetrahydrochloride-0.01% hydrogen peroxide (Kamite et al., 2002). The sections were counterstained with hematoxylin.

Western blot analysis

The articular and the epiphyseal cartilage were homogenized in a lysis buffer (8M Urea, 0.2% sodium dodecyl sulfate, 0.8% Triton X-100, 3% 2-mercaptoethanol) and stored at 4°C overnight. The homogenates were centrifuged at 20,000×g for 20 min at 4°C to remove insoluble debris and supernatants were collected. The protein concentrations of the supernatant samples were assayed by Lowry’s method (Paterson, 1977). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the supernatant was mixed with an equal volume of a Laemmli sample buffer solution (0.5M Tris-HCl pH6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue) and boiled for 5 min. Proteins (2.5 μg/lane) were run on 15% polyacrylamide slab gels and transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% skimmed milk in 0.1% PBS with 0.05% Tween 20 for 1 h at room temperature, and incubated with the affinity-purified rabbit anti-rat ChM-I antibody (Funaki et al., 2001) diluted at 1:5000 for 16 h at 4°C. After washing in 0.1M PBS with 0.05% Tween 20, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000 dilution, EnVision; DAKO) at room temperature for 40 min. The membranes were washed in 0.1M PBS with 0.05% Tween 20 and the immunoreactivity was visualized using the ECL plus western blotting detection system (Amersham Pharmacia Biotech, Tokyo).

Ribonuclease protection assay

The expression of ChM-I mRNA in the articular and the epiphyseal cartilage was examined by ribonuclease protection assay as reported previously (Sambrook et al., 1989, Yamamoto et al., 1994). Total cellular RNA was isolated from the cartilage samples by an acid guanidium thiocyanate phenol chloroform extraction method (Trizol; Life Oriental Technologies Inc., Tokyo). 32P-labeled antisense cRNA probes for ChM-I mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized using ChM-I cDNA (392 bp corresponding to nucleotides 868-1259 of the full-length cDNA) and GAPDH cDNA (114 bp corresponding to nucleotides 674-787 of the full-length cDNA) as templates, respectively. The specific radioactivity of 32P-labeled cRNA probes was adjusted to 1×107 cpm/μl each in a hybridization buffer (80% formamide, 40 mM 1,4-piperazinediethanesulfonic acid, 0.4 M NaCl, 1 mM EDTA). Five μg of total RNA extracted from the articular and the epiphyseal cartilage were hybridized with the cRNA probes (1×106 cpm each) at 45°C for 16 h in the hybridization buffer. Unhybridized probes were digested with ribonuclease A (4.0 μg/ml) and ribonuclease T1 (120 U/ml) mixtures at 30°C for 1 h, and then the ribonucleases were digested with protease K (0.5 mg/ml) at 37°C for 30 min. After phenol chloroform extraction, the hybridized probes were precipitated with ethanol and heat-denatured at 93°C for 5 min, and electrophoresed on 6% polyacrylamide gels. The dried gels were exposed to X-ray films for 16 h at -80°C. Detection and analysis of bands were performed by phosphor imaging techniques using Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA). The results were represented as the ratio of integrated density for ChM-I mRNA to that for GAPDH mRNA.

Results

Immunohistochemistry of ChM-I in the articular and the epiphyseal cartilage

The localization of ChM-I protein in rat articular and epiphyseal cartilage was studied by immunohistochemistry. Immunoreactive ChM-I was found only at the articular and the epiphyseal cartilage in longitudinal sections of the knee joints (Fig. 1, 2). No immunoreactivity was demonstrated in the soft tissue surrounding the cartilage, bone tissues, or bone marrow cells.

The immunoreactivity was intense in the inter-territorial space of the cartilage matrix in the intermediate and deep layers of the articular cartilage obtained from 2- and 4-week-old rats. The intermediate layer consisted of fifteen to twenty cell layers in these rats (Fig. 1A, B). At 6 and 8 weeks, intense immunoreactivity was limited in the articular cartilage matrix around the hypertrophic chondrocytes.
Fig. 3. Western blot analysis of ChM-I in the articular and the epiphyseal cartilage. A: A broad band or a cluster of multiple bands corresponding to about 25KDa is detected in both the articular and the epiphyseal cartilage samples. A: Density of the band in the articular cartilage decreases gradually with age. Lane 1: 2 weeks, Lane 2: 4 weeks, Lane 3: 6 weeks, Lane 4: 8 weeks, Lane 5: 10 weeks. B: There is no tendency for a decrease in the band intensity in the epiphyseal cartilage samples. Lane 1: 2 weeks, Lane 2: 4 weeks, Lane 3: 6 weeks, Lane 4: 8 weeks, Lane 5: 10 weeks.

Fig. 4. Ribonuclease protection assay of ChM-I in the articular and the epiphyseal cartilage. The expression of ChM-I mRNA is detected in all the articular and epiphyseal cartilage samples. A: ChM-I mRNA expression is the most intense in the 2 week sample, while its expression decreases gradually with age in the articular cartilage samples. Lane 1: 2 weeks, Lane 2: 4 weeks, Lane 3: 6 weeks, Lane 4: 8 weeks, Lane 5: 10 weeks. B: No clear-cut decrease in the expression of ChM-I mRNA in epiphyseal cartilage samples occurs with age. Lane 1: 2 weeks, Lane 2: 4 weeks, Lane 3: 6 weeks, Lane 4: 8 weeks, Lane 5: 10 weeks.
in the deep layer (Fig. 1C, D). In 10-week-old rats, the immunoreactive ChM-I was almost negligible in the articular cartilage where no hypertrophic chondrocytes were found, and the morphological alignment of the articular cartilage was as mature as that of the adult one (Fig. 1E).

In contrast, the ChM-I immunoreactivity was intensely demonstrated in the inter-territorial space of the cartilage matrix in the resting, proliferating, and early hypertrophic cartilage zones in the epiphyseal cartilage at all ages examined. The immunoreactivity was faint in lower hypertrophic cartilage zones, and no immunoreactivity for ChM-I was detected in the calcified cartilage zones in the epiphyseal cartilage (Fig. 2). Here the immunoreactivity remained unchanged from 2 to 10 weeks of age.

**Western blot analysis**

The presence of ChM-I in the articular and epiphyseal cartilage was semi-quantified by Western blot analysis (Fig. 3). The bands of ~25kDa were detected in both the articular (Fig. 3A) and the epiphyseal cartilage (Fig. 3B) obtained from rats at all ages examined and appeared broad or as a cluster of multiple ones, corresponding to various glycosylated forms of ChM-I.

The ~25kDa bands were intensely immunoblotted in the articular cartilage samples of 2- and 4-week-old rats. The intensity of the band in the articular cartilage samples decreased gradually with age and became faint in 10-week-old rats (Fig. 3A). On the other hand, the intensity of the band in the epiphyseal cartilage was not reduced with age (Fig. 3B). These results accorded with those obtained by immunohistochemistry.

**Ribonuclease protection assay**

The expression of ChM-I mRNA was examined in the articular and the epiphyseal cartilages by ribonuclease protection assay (Fig. 4) and was quantified by phosphor imaging techniques (Molecular Imager FX: Bio-Rad Laboratories, Hercules, CA, USA) (Fig. 5).

In the articular cartilage, the ChM-I mRNA expression was most intense in 2-week-old rats, and the intensity of the band decreased gradually with age (Fig. 4A). The ratios of ChM-I to GAPDH mRNA expression were 2.52, 1.72, 0.93, 0.32 and 0.34 at 2, 4, 6, 8 and 10 weeks in the articular cartilage, respectively (Fig. 5).

In the epiphyseal cartilage, there was no tendency for the ChM-I mRNA expression to decrease with age (Fig. 4B). The ratios of ChM-I to GAPDH mRNA at 2, 4, 6, 8 and 10 weeks in the epiphyseal cartilage were 3.78, 2.95, 3.38, 3.61, and 2.69, respectively (Fig. 5).

The ratios of ChM-I to GAPDH mRNA in the articular

![Fig. 5. Detection and analysis of bands performed by phosphor imaging techniques. The results are represented as ratios of the integrated density for ChM-I mRNA to that for GAPDH mRNA. The ratios at 2, 4, 6, 8 and 10 weeks in the articular cartilage (closed bar) are 2.52, 1.72, 0.93, 0.32, and 0.34, respectively, while in the epiphyseal cartilage (open bar), they are 3.78, 2.95, 3.38, 3.61, and 2.69, respectively. At 2 weeks, the ratio in the articular cartilage is roughly two-third less than that in the epiphyseal cartilage, and it decreases gradually to one-tenth of that at 10 weeks.](image-url)
cartilage were always less than those in the epiphyseal cartilage at all ages (Fig. 5). At 2 weeks, the ratio in the articular cartilage was roughly two-thirds less than that in the epiphyseal cartilage. Following this, the ratio of the articular cartilage to the epiphyseal cartilage decreased with age to one-tenth at 10 weeks.

Discussion

The presence of ChM-I and its physiological roles in angiogenesis inhibition have been shown in the mature epiphyseal cartilage (Inoue et al., 1997; Hiraki et al., 1997a, 1999; Shukunami and Hiraki, 1998; Hiraki and Shukunami, 2000). However, the role of ChM-I in the regulatory mechanism of avascularity in the articular cartilage has not been previously investigated.

The present study examined the expression and the localization of ChM-I in the articular cartilage in the developing rat and found that the expression of ChM-I at both protein and mRNA levels decreased gradually with age in the articular cartilage. This decrease occurred in parallel to the morphological maturation of the articular cartilage: the loss of hypertrophic chondrocytes and increase of extracellular cartilage matrix. However, no decrease in ChM-I protein and mRNA expression was observed in the epiphyseal cartilage. These results suggest that the role of ChM-I as an angiogenesis inhibitor in the immature articular cartilage may weaken as the articular cartilage matures. During the immature stage of the articular cartilage at 2 to 8 weeks of age, the bone formation continues at the secondary ossification center as endochondral bone formation (Moss and Moss-Salentijn, 1983). Then the articular cartilage contacts the bone at the intermediary site of the hypertrophic chondrocyte zone where the endochondral bone formation and active vascular invasion occur. This knowledge leads us to consider that a potential anti-angiogenic mechanism might be strongest in the immature articular cartilage, especially around the hypertrophic chondrocytes at the bone-articular cartilage interface. The intense expression of ChM-I in the immature articular cartilage at the hypertrophic chondrocyte zone may indicate that ChM-I acts as a molecule to inhibit vascular invasion in the immature state of the articular cartilage.

On the other hand, the expression of ChM-I in both protein and mRNA was weak in the articular cartilage at 10 weeks of age. The articular cartilage at this time consisted of larger amounts of extracellular cartilage matrix and smaller number of chondrocytes that were not hypertrophied as compared with these at 2-8 weeks, indicating that the articular cartilage had matured at 10 weeks. At this stage, endochondral bone formation at the bone-articular cartilage interface subsides and vascular invasion from the bone to the cartilage also terminates, suggesting that any anti-angiogenic protection might be unnecessary in the mature articular cartilage. Thus, the weakened expression of ChM-I in the mature articular cartilage may imply that the antiangiogenic property of ChM-I is no more important there than in the immature one. On the other hand, substantial amounts of endogenous angiogenic molecules such as bFGF and VEGF have been detected in the articular cartilage (Weseman and Bollnow, 1997; Tajima et al., 1998; Ichigatani et al., 2001; Pfander et al., 2001). These facts may indicate that a certain anti-angiogenic mechanism other than the expression of ChM-I is involved in maintaining its avascularity in the mature articular cartilage.

Beside its antiangiogenic property, ChM-I has a growth-stimulating activity for chondrocytes (Hiraki et al., 1991). A difference in mitotic activity of chondrocytes has been demonstrated between the immature articular cartilage and the mature one (Hinchcliffe and Johnson, 1983). This mitotic activity is higher in the immature articular cartilage than the mature one. The abundance of ChM-I expression in the immature articular cartilage and the decrease in the mature one may correlate with the difference in proliferative activity between the immature and mature articular cartilage. The high proliferative activity in the immature articular cartilage may also account for the higher regenerative when the immature articular cartilage is injured (Namba et al., 1998).

In conclusion, our study clarified that the expression of ChM-I in the articular cartilage decreases gradually with age, though that in the epiphyseal cartilage does not. It suggests that ChM-I, as an angiogenesis inhibitor and a chondrocyte growth stimulating factor in the articular cartilage, is more important in the immature articular cartilage than in the mature one, and that the mechanism to preserve avascularity may differ between the mature articular cartilage and the epiphyseal one.

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References


