Identification of Chondromodulin I as a Novel Endothelial Cell Growth Inhibitor

PURIFICATION AND ITS LOCALIZATION IN THE AVASCULAR ZONE OF EPiphySEAL CARTILAGE

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Cartilage is unique among tissues of mesenchymal origin in that it is resistant to vascular invasion due to an intrinsic angiogenic inhibitor. During endochondral bone formation, however, calcified cartilage formed in the center of the cartilaginous bone rudiment allows vascular invasion, which initiates the replacement of cartilage by bone. The transition of cartilage from the angioreistant to the angiogenic status thus plays a key role in bone formation. However, the molecular basis of this phenotypic transition of cartilage has been obscure. We report here purification of an endothelial cell growth inhibitor from a guanidine extract of bovine epiphyseal cartilage. The N-terminal amino acid sequence indicated that the inhibitor was identical to chondromodulin I (ChM-I), a cartilage-specific growth-modulating factor. Purified ChM-I inhibited DNA synthesis and proliferation of vascular endothelial cells as well as tubule morphogenesis in vitro. Expression of ChM-I cDNA in COS7 cells indicated that mature ChM-I molecules were secreted from the cells after post-translational modifications and cleavage from the transmembrane precursor at the predicted processing signal. Recombinant ChM-I stimulated DNA synthesis and proteoglycan synthesis of cultured growth plate chondrocytes, but inhibited tube morphogenesis of endothelial cells. In situ hybridization and immunohistochemical studies indicated that ChM-I is specifically expressed in the avascular zone of cartilage in developing bone, but not present in calcifying cartilage. These results suggest a regulatory role of ChM-I in vascular invasion during endochondral bone formation.

Cartilage tissue is avascular except during skeletal development, when endochondral bone formation occurs. This process is initiated by the formation of cartilage tissue from aggregated mesenchymal cells, and the cartilage formed serves as a mold for subsequent bone formation. Soon after the cartilaginous mold is formed, chondrocytes in the central part of the mold become markedly hypertrophic and calcified. Capillaries from the surrounding periosteum then invade the calcified zone at the center of the previously avascular cartilage shaft (1). Concomitantly with this neovascularization, osteoclasts and osteoblasts are recruited to gradually replace the calcified cartilage matrix with bone matrix and to excavate the bone marrow cavity (1, 2). The vascular invasion is thus a pivotal event, which coordinates chondrogenesis and subsequent osteogenesis in endochondral bone formation; angiogenesis is switched on at a precise stage during skeletal development. Despite the fundamental importance of this process in endochondral bone formation, our understanding of angiogenic switching in cartilage is limited.

Several angiogenic molecules such as fibroblast growth factor-2 (FGF-2) (3), transforming growth factor β (TGF-β) (4), and vascular endothelial growth factor (VEGF) (5) have been shown to be present in growth-plate cartilage. These molecules could act as potential chemoattractants and mitogens for endothelial cells. These studies suggest that cartilage is potentially “angiogenic,” even though it maintains an “angiostatic” phenotype. However, the regulation of angiogenic switching in cartilage cannot be accounted for only by the expression of these angiogenic molecules, since these are expressed in cartilage and its surrounding tissues even under avascular conditions. There is an equally important component to the angiogenic switch, one governed by angiogenesis inhibitors (6–8). Hanahan and Folkman (9) proposed the hypothesis that angiogenesis is regulated by the balance of both inducers and inhibitors of endothelial cell proliferation and migration. The cumulative levels of inducer and inhibitor signals maintain the endothelial cells in alternative states of quiescence and angiogenesis. The angiogenic switch could be activated either by increasing the levels of the inducers of endothelial cells or by reducing the levels of inhibitors (9).

The resistance of cartilage to vascular invasion was first studied with the use of the chick chorioallantoic membrane by Kuettner and co-workers (10). They demonstrated that the resistance of cartilage was due to a low molecular weight anti-invasion factor extractable by guanidine (11, 12). This anti-
invasion factor turned out to be a specific elastase inhibitor of 15 kDa (13). Vascular invasion can be modulated by the activation or inhibition of proteinases (14–16), since angiogenesis involves a local degradation of the basement membrane surrounding the endothelium (17). Moses previously identified tissue inhibitors of metalloproteinase-1 (TIMP-1) and its related molecules in cartilage, which were associated with the inhibition of angiogenesis (18, 19). TIMP-2 was also isolated as an angiogenesis inhibitor from the culture medium of chondrosarcoma cells (20). However, no cartilage-specific macromolecule capable of inhibiting angiogenesis has been identified.

We previously reported that extracts of cartilage contain an endothelial cell growth inhibitor (21, 22), while it showed no inhibitory activity for collagenase. Taking advantage of its affinity to heparin, we purified the endothelial cell growth inhibitor to homogeneity from cartilage. Here we report for the first time the amino acid sequence of the inhibitor and establish that the inhibitor is identical to a cartilage-specific growth-modulating component, chondromodulin I (ChM-I) (23). The expression pattern of ChM-I in a developing bone strongly implies its physiological significance as a key component in the phenotypic transition from angiostatic to angiogenic cartilage during endochondral bone development.

MATERIALS AND METHODS

Cell Culture and Bioassy—Bovine cartilaginous endothelial (BCE) cells were isolated from adult bovine cartilaginous arteries and cultured by the reported method (24, 25). Briefly, fresh bovine cartilaginous arteries were opened lengthwise with a scalpel. The endothelial cell layer was obtained by gently scraping the intimal surface with a scalpel. These BCE cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37 °C under 5% CO2 in air. After the primary culture, the cells were subcultured at a rate of one generation per passage to make a stock of frozen cells. For the determination of the DNA synthesis in the cells, BCE cells (4 × 105 cells/well) were inoculated onto 96-multiwell plates (Corning Glass Works, Corning, NY) and cultured by the reported method (24, 25). BCE cells were cultured in RMPI 1640 medium containing 0.1% fatty acid-free bovine serum albumin (BSA; Pharmacia, Uppsala, Sweden) equilibrated with 20 mM sodium phosphate (pH 7.4) containing 5 mM guanidinium chloride and 0.3 mM NaCl, and the eluate was collected in 8-m fractions. Bioactivity was recovered in fractions 25–31, which were dialyzed against 20 mM sodium phosphate (pH 7.4) containing 0.15 mM NaCl. The material (110 mg) was applied to a heparin-Toyopearl affinity column (0.7 × 20 cm; Tosoh, Tokyo, Japan) equilibrated with 20 mM sodium phosphate (pH 7.4) containing 0.15 mM NaCl and 0.03% CHAPS. Bound materials were eluted with a linear gradient of 0.15–1.2 mM NaCl. The cartilage-derived endothelial cell growth inhibitor was purified to homogeneity from the fraction eluted with 0.5–1.2 mM NaCl (Hep, 0.5–1.2 mM fraction) by high performance liquid chromatography (HPLC) on a reverse-phase column (YMC C-4, 0.46 × 30 cm; YMC, Kyoto, Japan) and identified by SDS-PAGE (32).

Protein Purification—Fetal bovine epiphyseal cartilage (2 kg of cartilage from 3–6-month-old embryos) was homogenized and extracted with 1 M guanidinium chloride, and then fractionated with 45–60% acetone at 4 °C, as described (30). The resultant precipitates (12) were dissolved in 4 M guanidinium chloride containing 50 mM Tris-HCl, 0.5 mM caproic acid, 20 mM Tris- HCl, and 1 mM NaCl (pH 8.0). Subfractions of the cartilage extracts (CE) were prepared by successive ultrafiltration through Amicon (Beverly, MA) XM300 and Amicon YM100 filters (CE<sub>100</sub>–300-kDa, and finally concentrated on Amicon YM10 filters (CE<sub>10</sub>–50-kDa). The CE<sub>100</sub>–300-kDa fraction was dialyzed to a Sephadex G-50 (Pharmacia, Piscataway, NJ) eluted with 20 mM sodium phosphate (pH 7.4) containing 5 mM guanidinium chloride and 0.3 mM NaCl, and the eluate was collected in 8-m fractions. Bioactivity was recovered in fractions 25–31, which were dialyzed against 20 mM sodium phosphate (pH 7.4) containing 0.15 mM NaCl. The material (110 mg) was applied to a heparin-Toyopearl affinity column (0.7 × 20 cm; Tosoh, Tokyo, Japan) equilibrated with 20 mM sodium phosphate (pH 7.4) containing 0.15 mM NaCl and 0.03% CHAPS. Bound materials were eluted with a linear gradient of 0.15–1.2 mM NaCl. The cartilage-derived endothelial cell growth inhibitor was purified to homogeneity from the fraction eluted with 0.5–1.2 mM NaCl (Hep, 0.5–1.2 mM fraction) by high performance liquid chromatography (HPLC) on a reverse-phase column (YMC C-4, 0.46 × 30 cm; YMC, Kyoto, Japan) and identified by SDS-PAGE (32). The purified inhibitor was further purified to homogeneity from cartilage. Here we report for the first time the amino acid sequence of the inhibitor and establish that the inhibitor is identical to a cartilage-specific growth-modulating component, chondromodulin I (ChM-I) (23). The expression pattern of ChM-I in a developing bone strongly implies its physiological significance as a key component in the phenotypic transition from angiostatic to angiogenic cartilage during endochondral bone development.

Formation of Tubulike Cellular Networks by Vascular Endothelial Cells in Vitro—Formation of tubulike cellular networks of BCEA cells was assayed in duplicate by the method of Kanyasuyu et al. (31) with slight modifications. A mixture of 0.3% type I collagen solution (0.6 ml; Koken, Tokyo, Japan), 0.1% NaOH (75 μl), and 10% concentrated MEM (75 μl) was poured into 12-multicell plates and allowed to form a lower gel at 37 °C. Then, 1 × 10<sup>5</sup> BCE cells in 2 ml of MEM containing 10% FBS were introduced onto the gel and incubated for 24 h. Subsequently, the medium was aspirated and an aqueous solution (40 μl) containing a test sample was added. The mixed collagen solution (0.75 ml) was overlaid on the cells to form an upper gel at 37 °C. Finally, MEM was changed to the condition on the third day of culture, phase-contrast microscopic pictures were taken. For semiquantitative analysis of tube morphogenesis (32), cells forming tubulike cellular networks (>100 μm in length) were counted after viable staining of the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (33). By serial dilution of ChM-I, a dose range to cause 50% inhibition of tube formation over the control was determined. ChM-I was first dissolved in 0.1% fatty acid-free BSA, and used at 100-fold dilution for the bioassy.

Expression of Bovine ChM-I Precursor cDNA in COS Cells—For the expression studies, the plasmid pUChM261 was constructed by ligation of the EcoRI linker into the ScoI site of a plasmid harboring bovine ChM-I precursor cDNA, pUChM261 (23). The 1.25-kilobase pair EcoRI fragment released by the EcoRI digestion of pUChM261 was then cloned into the EcoRI site of the pcDL-SRoz296 vector (34). The result-ant plasmid, containing the bovine ChM-I precursor in the sense direction, was named pDLChM1-I. A plasmid containing the ChM-I precursor sequence in the opposite direction (pDLCChM1-I-3) was used as a negative control vector. Monkey COS cells were transfected with pcDLChM1-I or pcDLChM1-I-3 by the application of calcium phosphate-DNA coprecipitates to cells grown in 10-cm diameter culture dishes. After 4 h of culture in DMEM containing 5% FBS, the culture medium was replaced and the culture was conditioned for another 60 h. The medium (23 ml) was then recovered and applied to a heparin-Toyopearl affinity column. Bound materials were eluted stepwise with buffer containing 0.5 M NaCl and buffer containing 1.2 M NaCl. The fraction eluted with 1.2 M NaCl was concentrated 30-fold with a Centricon (Amicon) and recombinant bovine ChM-I was further purified to homogeneity by HPLC on a reverse-phase column, and its N-terminal amino acid sequence was determined.

Anti-ChM-I Antibody and Immunoblotting—The concentrated hepa- rin-bound fraction of the conditioned medium (0.9 ml) was separated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane BA-85 (Schleicher & Schuell). The membrane was immersed in TBST buffer
The probe was labeled with [35S]thymidine to chemistry or in 4% paraformaldehyde in 0.01M PBS (pH 7.4) for at 4 °C in periodate-lysine-paraformaldehyde solution for immunohistochemistry or in 4% paraformaldehyde in 0.01M PBS (pH 7.4) for in situ hybridization. The ChM-I antiserum was purified using Staphylococcus protein A (Pharmacia Biotech Inc.) according to standard protocols, and the antibody was stored at approximately 2 mg/ml.

**Immunohistochemistry and in Situ Hybridization**—Developing bovine tails were collected from 5-month-old fetuses, and fixed overnight at 4 °C in periodate-lysine-paraformaldehyde solution for immunohistochemistry or in 4% paraformaldehyde in 0.01M PBS (pH 7.4) for in situ hybridization. The caudal vertebrae were dissected out, dehydrated in a graded series of ethanol, and embedded in paraffin. Longitudinal serial sections were cut at 6 μm and processed for histological examinations. Deparaffinized sections were treated with 1% H2O2 in methanol for 30 min to reduce endogenous peroxidase activity, and washed in PBS. Sections were treated with 500 units/ml testicular hyaluronidase (type V, Sigma) in PBS for 20 min at 37 °C and rinsed in PBS. The slides were covered with 5% normal goat serum in PBS for 30 min and then with anti-ChM-I polyclonal antibody, and further incubated overnight at 4 °C. Preimmune rabbit IgG was used as a negative control. Anti-ChM-I antibody was used at the dilution of 1:300. Immunoreactions were performed using a Vectorstain peroxidase rabbit ABC kit (Vector Laboratories, Burlingam, CA). Sections were washed with PBS, and the antigenic sites were demonstrated by treating the sections with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Dojin Chemicals, Tokyo, Japan) in 0.05 M Tris-HCl buffer (pH 7.6), and 0.01% H2O2 for 7 min. Nuclei were stained with methyl green. The sections were then dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific, Pittsburgh, PA). Consecutive sections were stained with hematoxylin-eosin for histological characterization using von Kossa’s technique for the identification of calcium phosphate.

The in situ hybridization procedures were essentially as described by Hayashi et al. (35) and Iyama et al. (36). The Chm-I cDNA probe used for in situ hybridization was a 900-base pair EcoRI-BamHI fragment. The probe was labeled with [32P]thymidine 5'-γ-thiotriphosphate (DuPont) by nick translation to the specific activities of 3.1 × 108 cpmpg DNA and 1.1 × 108 cpmpg DNA, respectively. After hybridization, the slides were washed under high stringency conditions as described previously (35). The dried tissue sections were dipped into Kodak NTB2 emulsion and exposed for 3–7 days at 4 °C. After autoradiography, the sections were counterstained with hematoxylin. For the semiquantitative analysis of labeling intensity, the number of autoradiographic silver grains per cell was counted in 100 cells in five zones of the epiphyseal cartilage at magnification of ×250 under oil immersion. The specificity of the hybridization signals was confirmed by RNase treatment of the sections (2 mg/ml, 1 h at room temperature) before hybridization with the DNA probe as a negative control. Only a few autoradiographic silver grains were found on the sections digested with RNase, implying the specificity of the hybridization with the Chm-I probe in a manner dependent on the presence of the RNA in the tissue sections.

**RESULTS**

**Purification of an Endothelial Cell Growth Inhibitor from Cartilage**—Acetone precipitates of cartilage extracts were subfractionated by ultrafiltration into three portions: the first fraction containing materials with apparent molecular masses of 100–300 kDa (CE100–300 kDa), the second with materials of 50–100 kDa (CE50–100 kDa), and the third containing materials of 10–50 kDa (CE10–50 kDa). The test samples were incubated with confluent monolayers of BCAE cells for 16 h, and their inhibitory activity on DNA synthesis was assayed as described under “Materials and Methods.” In agreement with our previous observations (22), CE100–300 kDa inhibited the [3H]thymidine incorporation of the cells in a dose-dependent manner, whereas CE50–100 kDa had only a marginal effect (Fig. 1A).

![Cartilage-derived Endothelial Cell Growth Inhibitor](image)

**Fig. 1.** Purification of endothelial cell growth inhibitor from cartilage extracts. A, inhibition of thymidine incorporation in BCAE cells. Subconfluent BCAE cells were incubated with MEM containing 10% FBS in a 96-multiwell plate and a test sample for 16 h, and were labeled with [3H]thymidine for the last 4 h. The inhibitory activities of CE100–300 kDa ( ), CE50–100 kDa ( ), CE10–50 kDa ( ), the Hep.-1.2M fraction ( ● ), and purified endothelial cell growth inhibitor ( ○ ) are shown. The [3H]thymidine incorporation into control wells incubated with PBS and 0.1% BSA was 97642 ± 1748 dpm/well (100 ± 2%). Points represent means ± standard deviations of the results of triplicate assays. B, SDS-PAGE of endothelial growth inhibitor purified from CE100–300 kDa. The purified inhibitor (10 μg) was subjected to SDS-PAGE analysis in 15% gel and stained with Coomassie Brilliant Blue (lane 1). The electrophoretic mobility of marker proteins is shown in lane 2. C, the N-terminal amino acid sequence of the purified inhibitor.

The concentration of CE100–300 kDa required for 50% inhibition (IC50) was approximately 50 μg/ml. It was also noted that the inhibitory effect on DNA synthesis of CE10–50 kDa was as potent as that of CE100–300 kDa (Fig. 1A).

The inhibitory activity in CE100–300 kDa was further sepa-
Cartilage-derived Endothelial Cell Growth Inhibitor

The protein content of the bioactive fraction at each purification step was determined by the Bio-Rad protein microassay, except for the final HPLC step. Amount of purified ChM-I after HPLC was estimated by UV absorption at 280 nm and amino acid analysis of the protein. The bioactivity was evaluated by the concentration required for 50% inhibition (IC₅₀) of DNA synthesis in cultured BCAE cells.

| Purification step                  | Total protein | Yield | IC₅₀ (µg/ml) |
|----------------------------------|---------------|-------|-------------|
| Fetal epiphyseal cartilage       | 2 kg (wt)     |       |             |
| Acetone fraction                 | 12 g (wt)     |       |             |
| Ultrafiltration (CP₁₀₀–₃₀₀ kDa)  | 512 mg        | 100   |             |
| Sephacryl S-200                  | 110 mg        | 21.5  |             |
| Chromatography                   |               | 20    |             |
| Heparin-Sepharose chromatography | 7.8 mg        | 1.5   | 3           |
| Reverse-phase HPLC               | 0.2 mg        | 0.04  | 0.2         |

Rated by Sephacryl S-200 chromatography in 5 M guanidinium chloride and 0.3 M NaCl. Bioactivity was coeluted with materials having an apparent molecular mass of 10–30 kDa. There was no inhibitory activity in the materials of a higher molecular mass under this highly dissociative condition, suggesting the possibility that the inhibitor was partitioned in CP₁₀₀–₃₀₀ kDa and CE₁₀–₅₀ kDa by forming aggregates or binding to a certain component in cartilage. The active fractions were pooled and dialyzed. The materials were then subjected to heparin-Toyopearl affinity chromatography. The bound materials were eluted with a linear gradient from 0.15 to 1.2 M NaCl. Bioactivity was recovered in the fractions eluted with 0.5 M NaCl. The active fractions were pooled (Hep. 0.5–1.2 M). The IC₅₀ of the Hep. 0.5–1.2 M fraction was approximately 3 µg/ml (Fig. 1A). The materials not bound to the column (Hep.-pass) exhibited no inhibitory activity. The endothelial cell growth inhibitor was finally purified to homogeneity by YMC C₄ reverse-phase HPLC. Bioactivity was coeluted with a single protein peak. The SDS-PAGE analysis showed a single diffuse band of 25 kDa (Fig. 1B). The N-terminal amino acid sequence of the inhibitor was then determined (Fig. 1C) and found to be identical to that of ChM-I, which was previously found to have a growth-promoting activity on cultured chondrocytes (23). The purified ChM-I from the CE₁₀₀–₃₀₀ kDa fraction inhibited the DNA synthesis by BCAE cells in a dose-dependent manner (Fig. 1A). The IC₅₀ was approximately 200 ng/ml. The protein recovery and relative inhibitory activity at each purification step are summarized in Table I. We recently isolated a novel growth-promoting factor chondromodulin II (ChM-II) from cartilage extracts (37). ChM-II is distinct from ChM-I, having no sequence similarity with each other. However, similar to ChM-I, it stimulated DNA synthesis and proteoglycan synthesis in cultured chondrocytes. On the other hand, unlike ChM-I, ChM-II exhibited no inhibitory action on DNA synthesis in BCAE cells (Fig. 2A).

Finally, we examined the effect of ChM-I on the proliferation of BCE cells isolated from bovine brain cortex. BCE cells (2 x 10⁴ cells) were plated in 60-mm diameter culture dishes. The cell numbers in the cultures increased exponentially in RPMI 1640 medium containing 10% FBS and 2 ng/ml FGF-2 (Fig. 2B). The proliferation of the cells was markedly inhibited by ChM-I (1 µg/ml). There was no substantial loss of cells due to detachment from the culture plate during incubation.

Effects on the Formation of Tubelike Cellular Networks by Endothelial Cells—The effect of ChM-I on tube formation was examined by analyzing the cell-shape changes of endothelial cells (31). BCAE cells (1 x 10⁵ cells/well) were plated onto type I collagen gel in 12-multiwell plates and covered with an upper layer of collagen gel. In the presence of 10% FBS, the cells changed their morphology and reorganized into a network structure within 3 days (Fig. 3A). The Hep. 0.5–1.2 M fraction (10 µg/well) derived from CE₁₀₀–₃₀₀ kDa markedly inhibited this process (Fig. 3B). Similarly, purified ChM-I (1 µg/well) inhibited the tube formation of the cells (Fig. 3C). In either case, no network structure of the cells was formed during a 7-day culture, suggesting that ChM-I acts on the tube morphogenesis of vascular endothelial cells as well as their growth. The dose range of ChM-I required for 50% inhibition of tube formation was determined to be 0.1–1 µg/well.

Next, we attempted to express the recombinant molecule of ChM-I in COS cells for confirmation of its bifunctional activities on endothelial cells and chondrocytes. As reported previously (23), ChM-I is encoded as a C-terminal portion of a larger precursor, which contains a putative transmembrane domain near the N-terminal part. The mature ChM-I sequence is preceded by a precursor cleavage site (Arg-Glu-Arg-Arg) for endoprotease furin (38). In the present study, the 1.25-kilobase pair EcoRI fragment of bovine ChM-I cDNA harboring the entire coding region of the ChM-I precursor was cloned into the pcDL-SRko296 expression vector (34). The constructed vectors were introduced into monkey COS7 cells by calcium phosphate transfection. After incubation for 5 days, the conditioned medium was collected and concentrated with a heparin affinity column. Presence of mature ChM-I secreted in the conditioned medium was analyzed by immunoblotting with anti-ChM-I antibody (Fig. 4). In the medium recovered from cultures transfected with a vector with ChM-I cDNA in the sense direction, immunoblotting revealed a single diffuse band corresponding to an average molecular mass of 25 kDa, which was indistinguishable from that of naturally occurring ChM-I. No immunoreactive material was found in the medium from cultures transfected with a negative vector with the cDNA in the opposite direction.

Then, recombinant ChM-I expressed in the medium was further purified to homogeneity by reverse-phase HPLC. The N-terminal amino acid sequence of the recombinant molecule was identical to that of naturally occurring ChM-I, indicating that ChM-I is secreted from cells after the cleavage of the
Purified recombinant ChM-I (0.5 mg/well) (lane 2), ChM-I purified from CE 100–300 kDa (1 mg/well) (C), or recombinant ChM-I (0.5 mg/well) purified from the conditioned medium of COS7-cell culture (D). After 3 days of culture, the changes in cell shape were examined under a phase-contrast microscope. The micrographs represent typical results of two independent experiments. Bars represent 200 μm.

Localization of ChM-I in a Developing Bone—Since the expression of ChM-I mRNA was specifically detected in cartilage by the previous Northern blot analysis (23), the localization of mature ChM-I protein was studied here by immunohistochemistry. Longitudinal sections of bovine developing caudal vertebrae were treated with the anti-ChM-I antibody (Fig. 6A). Antigenic sites were found only in cartilage in the developing bone. There was no immunoreactive material in the surrounding soft tissues, including non-cartilaginous cells filling the future joint space (top of figure), perichondrium and periosseous tissue (right and left of figure), and muscle. No immunoreactivity was found in the bone or marrow cells at the primary ossification center (bottom of figure). At higher magnification, intense immunoreactivity was found in the inter-territorial space of the cartilage matrix in the resting and proliferating cartilage zones (Fig. 6B), as well as in the cytosol of chondrocytes. The treatment with preimmune rabbit IgG of semiserial sections showed no positive staining (Fig. 6B'). Specific immunoreactivity against ChM-I was also found in the upper hypertrophic zone, but no immunoreactivity was seen in the lower hypertrophic and calcified cartilage (Fig. 6C).

The localization of ChM-I transcripts in bovine developing caudal vertebrae was further studied by in situ hybridization (35, 36). The 900-base-pair EcoRI-BamHI fragment of bovine ChM-I cDNA was labeled with [35S]thymidine and hybridized to semiserial sections. Hybridization signals were found only in the cartilaginous tissue (Fig. 6D). The localization of the gene transcripts was observed to coincide with the ChM-I immuno-
reactive areas of the sections. The ChM-I cDNA fragment labeled with \(^{3}H\)thymidine was also used to obtain a higher resolution of hybridization signals. Abundant hybridization signals were found in the area overlapping the locations of the proliferating cartilage zone (Fig. 6). High power views showing strongly stained ChM-I in the inter-territorial matrix in the proliferating cartilage zone (B), as well as the resting cartilage zone and the upper hypertrophic cartilage zone. The absence of ChM-I staining became evident in the lower hypertrophic and calcified cartilage zones (C). Preimmune rabbit IgG exhibited no positive staining as shown by the high power view of the proliferating cartilage zone (B'). D, low power view indicating the cartilage-specific expression of ChM-I mRNA from the resting cartilage zone to the upper hypertrophic cartilage zone using \(^{32}P\)-labeled cDNA probe. High resolution autoradiographs using the \(^{3}H\)-labeled cDNA probe showing intense signals of ChM-I mRNA in the proliferating cartilage zone (E), but very few signals in calcified chondrocytes (F, arrows). The specificity of the hybridization signals was confirmed by RNase treatment of the sections before hybridization with the DNA probe. E', only a background level of signals was found over the cells in the proliferating cartilage zone. Bars, 500 \(\mu m\) in A and D and 30 \(\mu m\) in B, B', C, E, E', and F.

Taking advantage of the high resolution of signals with the \(^{3}H\)-labeled probe, the relative levels of gene expression were estimated by counting the numbers of autoradiographic silver grains per cell in each cartilage zone. Since the size and packing density of cells significantly changes in each zone, the relative numbers of silver grains per unit area were compared (Fig. 7). Small chondrocytes in the resting cartilage zone (Zone II) expressed a high level of ChM-I mRNA, whereas non-cartilaginous cells filling the future joint space (Zone I) showed only the background level of hybridization signals. The proliferating cartilage zone (Zone III) expressed the highest level of ChM-I transcripts. The level in the lower hypertrophic and calcified zone (Zone V) became as low as that in Zone I.

**DISCUSSION**

**Purification and Properties of Cartilage-derived Endothelial Cell Growth Inhibitor—**Angiogenesis proceeds through multiple steps: 1) the disruption of the basement membrane surrounding the endothelial cells prior to invasion into the surrounding stroma, 2) the proliferation of the endothelial cells, and 3) the changes of cell shape and the formation of new capillary tubes behind the advancing front of the vasculature (9, 17). Therefore, an agent that interferes with any one of the above steps can be an angiogenesis inhibitor. Since the first step is the regional degradation of the basement membrane by collagenase or other proteases (39), it is reasonable to speculate that the collagenase inhibitors TIMPs might act as angiogenesis inhibitors (20, 40). A specific elastase inhibitor was also isolated from cartilage as an anti-invasion factor (13). In the present study, we attempted to purify an endothelial cell growth inhibitor from bovine fetal epiphyseal cartilage, which acts on the second step of angiogenesis.

We suspected in the early stage that CE\(_{100–300\, kDa}\) contained an active component distinct from the inhibitor present in CE\(_{10–50\, kDa}\) since CE\(_{50–100\, kDa}\) had only a weak inhibitory activity (Fig. 1A). However, the apparent molecular size of the inhibitor was 10–30 kDa under highly dissociative conditions. The N-terminal amino acid sequence of the purified inhibitor was identical to that of ChM-I (Fig. 1C) (23, 41), suggesting that the inhibitory activity of both CE\(_{100–300\, kDa}\) and CE\(_{10–50\, kDa}\) was derived from the same molecule (Figs. 1A and 2A). ChM-I probably formed self-aggregates or bound to another matrix component(s) in CE\(_{100–300\, kDa}\). ChM-I inhibited the proliferation of BCE cells (Fig. 2B) and the formation of tubulike cellular networks of BCAE cells in collagen gel at comparative doses (Fig. 3).

Bovine ChM-I is a glycosylated protein with 121 amino acid residues and an apparent molecular mass of 25 kDa on SDS-PAGE (Fig. 1B). The nucleotide sequence of ChM-I cDNA suggests that ChM-I is encoded as a C-terminal portion of a larger protein.
transmembrane precursor (335 amino acids) (23). The mature ChM-I sequence is preceded by a cleavage site for the precursor-processing endoprotease, furin (38, 42). Consistent with our prediction, the expression of ChM-I precursor cDNA in COS cells resulted in the recovery of the mature cleaved form of ChM-I in the conditioned medium (Fig. 4). Therefore, Ch-M-I is secreted from cells as a glycosylated form after cleavage with the endoprotease from the precursor membrane protein. The bioactivities of the recombinant molecule unequivocally established that the cartilage-derived endothelial cell growth inhibitor was identical to Chm-I, and revealed its bifunctional nature depending on the target cell type (Figs. 3D and 5).

Angiogenic Switching of Cartilage by Chm-I During Endochondral Bone Formation—Angiogenesis plays an important role in endochondral bone formation. Vascular invasion ensures the linkage of cartilage formation with subsequent osteogenesis (2, 10). Despite the apparent avascularity of cartilage, a number of angiogenic molecules have been found in cartilage, including FGF (3, 43), VEGF (5), and a 120-kDa angiogenic molecule (44). Cartilage has also been described as one of the major sources of TGF-β (4), which stimulates angiogenesis in vivo (45). FGF-2 is found in virtually all tissue types including bone and cartilage (3, 43). Therefore, the anti-angiogenic properties of cartilage cannot be accounted for by angiogenic molecules alone.

In situ hybridization defined the specific localization of Chm-I mRNA within the cartilaginous tissue in developing bone (Fig. 6D). No expression of the transcript was detected in bony tissues or the surrounding soft tissues. The level of Chm-I mRNA was apparently up-regulated in the proliferating and upper hypertrophic zones of cartilage (Figs. 6E and 7). However, the expression of the gene was reduced in the lower hypertrophic and calcified zone, allowing vascular invasion (Figs. 6F and 7). This pattern of expression is compatible with the finding that capillaries invade the lower hypertrophic and calcified cartilage at the site of contact with the bony collar and periosteum during endochondral bone development (2). Our immunohistochemical study clearly indicated that the localization of Chm-I protein completely overlapped the area of its gene expression (Fig. 6, A and B). Chm-I was accumulated in the inter-territorial space of cartilage matrix (Fig. 6B). In contrast, our immunohistochemical study revealed that FGF-2 protein was confined to the cell surface or pericellular space with scarcely any in the inter-territorial space. Thus, the distribution of Chm-I protein in cartilage was clearly distinct from that of FGF-2, suggesting that the FGF-containing pericellular space of chondrocytes was wrapped within a barrier of Chm-I. This unique localization pattern may explain how Chm-I confers an anti-angiogenic property on cartilage. The anchoring mechanism is not known yet, but the requirement of an anchor seems critical for inhibitors that act locally to confer anti-angiogenic properties on a specific type of tissue. Folkman and colleagues defined a class of cryptic angiogenesis inhibitors (8, 9, 46). These inhibitors are generated from larger precursors that are not themselves inhibitors of angiogenesis. Their activation scheme seems relevant for inhibitors present in the circulation, since they must be conveyed to remote sites in the body for expedient use. In contrast, a short range action must be important for local inhibitors that act in restricted areas in the body. Therefore, we speculate that Chm-I is placed in the extracellular matrix by a specific anchoring mechanism after cleavage from a larger transmembrane precursor within cells. The identification of the anchoring component is a key issue for the molecular analysis of the angiogenic switch in cartilage, and for the future application of Chm-I as a therapeutic agent.

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