Inhibition of DNA synthesis and tube morphogenesis of cultured vascular endothelial cells by chondromodulin-I

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Abstract Cartilage is an avascular tissue, and exhibits anti-angiogenic properties. Cartilage extracts have been shown to contain an inhibitor for DNA synthesis in vascular endothelial cells in vitro. Here we purified the inhibitory activity in the 10–50 kDa fraction of guanidine extracts from fetal bovine epiphyseal cartilage, and found that the inhibitor was identical with chondromodulin-I (ChM-I). Purified ChM-I inhibited tube morphogenesis of cultured vascular endothelial cells, as well as DNA synthesis. These results indicate that cartilage-specific glycoprotein ChM-I may participate in the maintenance of avascularity and anti-angiogenic properties of cartilage.

Key words: ChM-I; Vascular endothelial cell; Tube morphogenesis; Growth inhibitor

1. Introduction

Angiogenic molecules such as fibroblast growth factor-2 (FGF-2) [1], transforming growth factor-β (TGF-β) [2] and vascular endothelial growth factor (VEGF) [3] are reported to be present in cartilage. Contrary to these observations, cartilage is known to be uniquely avascular among the mesenchymal tissues and exhibits resistance to vascular invasion. The resistance of cartilage to vascular invasion was first studied by Kuettner and his coworkers [4]. They demonstrated that a specific elastase inhibitor can be a potential anti-vascular invasion factor [5]. Since vascular invasion involves local degradation of the basement membrane surrounding the endothelium, the process can be inhibited by inhibition of proteinases [6,7]. Moses and others identified tissue inhibitors of metalloproteinase (TIMP) and its related molecules as an angiogenesis inhibitor [8–10]. However, no cartilage-specific macromolecules capable of inhibiting growth of endothelial cells have been identified.

We previously identified a growth promoting factor chondromodulin-I (ChM-I) in cartilage which stimulated DNA synthesis and proteoglycan synthesis in the cultured growth plate chondrocytes [11]. Chondromodulin-I is a 25 kDa glycoprotein with 121 amino acid residues, and encoded as a C-terminal portion of a larger precursor (335 amino acids) [11]. Mature ChM-I is assumed to be secreted from chondrocytes after proteolytic cleavage by a precursor-endoprotease, furin [12]. During purification, we found that an endothelial cell growth inhibitory activity was closely associated with ChM-I. In the present study, we demonstrated that ChM-I inhibits DNA synthesis and tube morphogenesis of cultured vascular endothelial cells. The inhibitory actions of ChM-I were compared to those of the known endothelial growth inhibitors, platelet factor-4 (PF4) and fumagillin derivatives.

2. Materials and methods

2.1. Materials

Fumagillin and human PF4, fatty acid-free bovine serum albumin were purchased from Sigma Chemicals (St. Louis, MO). Synthetic AGM1470 and synthetic PF4(58-70) were obtained from Mitsubishi Chemical (Tokyo, Japan).

2.2. Purification

Purification of ChM-I was carried out, as previously described [11]. In brief, fetal bovine epiphyseal cartilage (2 kg) was homogenized and extracted with 1 M guanidinium chloride, and then fractionated with 45–60% acetone. The precipitates were subfractionated by successive ultrafiltration into the 10–50 kDa fraction in 4 M guanidinium chloride containing 1 M NaCl and 20 mM Tris-HCl (pH 7.4). The 10–50 kDa fraction (109 mg) was applied to a Sephacryl S-200 column (3.0×90 cm) equilibrated with the same buffer, and the eluate was collected in 8 ml fractions. Bioactive fractions were pooled, and dialyzed. The dialyzed material (13 mg) was applied to a heparin Toyopearl affinity column (0.7×20 cm) equilibrated with 20 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl and 0.03% CHAPS, and then eluted with a linear gradient of 0.15–1.2 M NaCl (2 mg). Finally, ChM-I was purified to homogeneity by HPLC on a reverse-phase column of YMC C_4 (0.46×15 cm) equilibrated with 25% acetonitrile/2-propanol (3/7, v/v) in 0.1% trifluoroacetic acid. The column was developed with a linear gradient of 25–70% of the same solvent. About 60 μg of ChM-I was isolated. The sequence data of ChM-I is available from GenBank under accession number M65081.

2.3. Cell culture and thymidine incorporation

Bovine carotid artery endothelial (BCAE) cells were isolated from adult bovine carotid arteries and cultured as reported [13,14]. In brief, carotid arteries were opened lengthwise. The endothelial cell layer was obtained by gently scraping the intimal surface with a scalpel. The cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 in air. After primary culture, the cells were subcultured for making a stock of frozen cells. Cells at passages 10–20 were used. For determination of DNA synthesis in the cells, BCAE cells (4×10^3 cells/well) were inoculated onto 96-multiwell plated (Corning) and cultured in 0.1 ml of MEM containing 10% FBS. When the culture became subconfluent approximately two days before the cells stop growing at confluency, the medium was replaced by MEM containing 10% FBS and a test sample. The cells were incubated for 16 h and labeled with [^{3}H]thymidine (5 μCi/ml, NEN) for the last 4 h. Radioactivity incorporated into DNA was determined with a scintillation spectrometer.

2.4. Tube morphogenesis

Experiment on tube morphogenesis of BCAE cells were carried out in duplicate by the method described previously [14]. A mixture of 0.3% type I collagen solution (0.6 ml; Koken, Tokyo, Japan), 0.1 M NaOH (75 μl) and 10-fold concentrated MEM (75 μl) were poured into 12-multiwell plates and allowed to form a lower gel at 37°C. Then, 1×10^5 cells in 2 ml of MEM containing 10% FBS were inoculated 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 layer. Lastly, MEM containing 10% FBS

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was added on the collagen gel. On the third day of culture, morphological changes of the cells were observed under a phase-contrast microscope. In the absence of an inhibitor, 70–90% of the cells formed tube-like cellular networks. For semiquantitative analysis of tube morphogenesis [15], cells forming tube-like 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 (MTT) [16]. By serial dilutions of test samples, we determined a dose range of a test sample to cause 50% inhibition of tube formation over the control.

3. Results and discussion

Previously we reported that the 100–300 kDa fraction of cartilage extracts contained an endothelial cell growth inhibitor [17]. The fractionated cartilage extracts inhibited growth of melanoma due to its anti-angiogenic action. Unlike other angiogenesis inhibitors [8–10], our preparation of cartilage extracts did not associate with the inhibitory activity on collagenase. During purification of cartilage-specific growth promoting factor ChM-I [11], we found that the 10–50 kDa fraction also exhibited an inhibitory action on DNA synthesis in subconfluent BCAE cells growing in MEM containing 10% FBS. The concentration required for 50% inhibition (IC_{50}) on DNA synthesis was about 30 μg/ml (Fig. 1). As shown in Fig. 2A, the DNA synthesis inhibitory activity was coeluted with materials having a molecular mass of 10–30 kDa. The active fractions (fractions 25 to 31) were pooled and dialyzed against 20 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl. Then the materials were subjected to heparin Toyopearl affinity chromatography (Fig. 2B). Materials not bound to the column (Hep.-pass.) exhibited no inhibitory action on [^{3}H]thymidine incorporation by BCAE cells in culture (Fig. 1). The bound materials were eluted with a linear gradient from 0.15 to 1.2 M NaCl (Fig. 2B). Bioactivity was recovered in the fraction eluted with 0.5 M NaCl. The active fractions were pooled (Hep. 0.5–1.2 M). The IC_{50} of the Hep. 0.5–1.2 M fraction was approximately 3 μg/ml (Fig. 1).

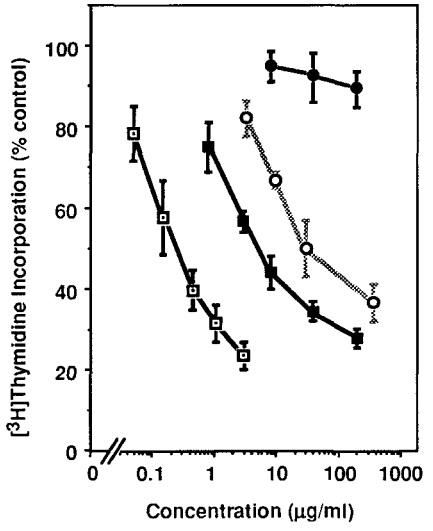

Fig. 1. Dose-response curves of purified ChM-I. Subconfluent BCAE cells were treated for 16 h with various concentrations of the 10–50 kDa fraction (○), the Hep.-pass. fraction (●), the Hep. 0.5–1.2 M fraction (■) and purified ChM-I (□). The [^{3}H]thymidine incorporation into control wells treated with 0.1% BSA in phosphate-buffered saline was 112682 ± 6032 dpm/well (100 ± 5%). Points represent means ± standard deviations in triplicate assays.

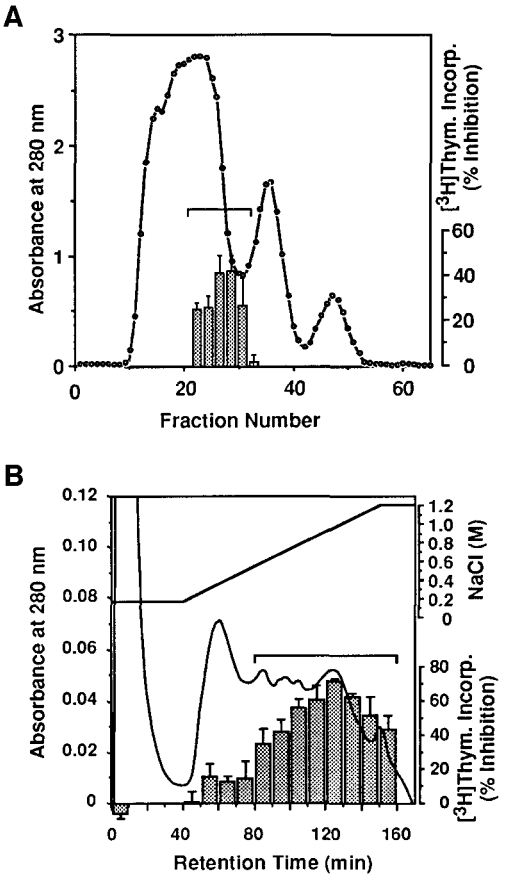

Fig. 2. Chromatographic separation of an DNA synthesis inhibitory activity in cartilage extracts. Subconfluent BCAE cells were incubated with MEM containing 10% FBS and a test sample for 16 h, and labeled with [^{3}H]thymidine for the last 4 h. Aliquots from each chromatographic fraction were assayed. In A, elution profile of the 10–50 kDa fraction on Sephacryl S-200 chromatography is shown. Bioactive fractions were pooled, and then separated by a heparin affinity column (B). The shaded bars represent the inhibitory activity on [^{3}H]thymidine incorporation into BCAE cells in triplicate assays.

The DNA synthesis inhibitory activity was finally purified to homogeneity by YMC C_4 reverse-phase HPLC. Bioactivity was recovered only in the fraction corresponding to ChM-I. SDS-PAGE of the active fraction gave a single diffuse band of 25 kDa (data not shown). Purified ChM-I inhibited DNA synthesis in BCAE cells in a dose-dependent manner (Fig. 1). The IC_{50} of ChM-I was approximately 200 ng/ml.

Angiogenesis is a complex process which includes local degradation of the basement membrane, migration and proliferation of endothelial cells, and formation of new capillaries [18]. In this study, we examined the effect of ChM-I on tube morphogenesis in vitro by monitoring cell-shape changes of BCAE cells cultured in collagen gel [14,19]. We plated BCAE cells onto type I collagen gel and covered them with an upper layer of collagen gel. In the presence of 10% FBS, the cells elongated and reorganized into a network of capillary-like structures within three days (Fig. 3). In contrast, tube morphogenesis was markedly inhibited by 1 μg/ml purified ChM-I (Fig. 3). The Hep. 0.5–1.2 M fraction (10 μg/ml) similarly inhibited tube morphogenesis (data not shown). These results suggest that ChM-I also affects the last step of angiogenesis, tube morphogenesis, as well as growth of endothelial cells.

Platelet factor-4 (PF4) is a prototype of endogenous angio-

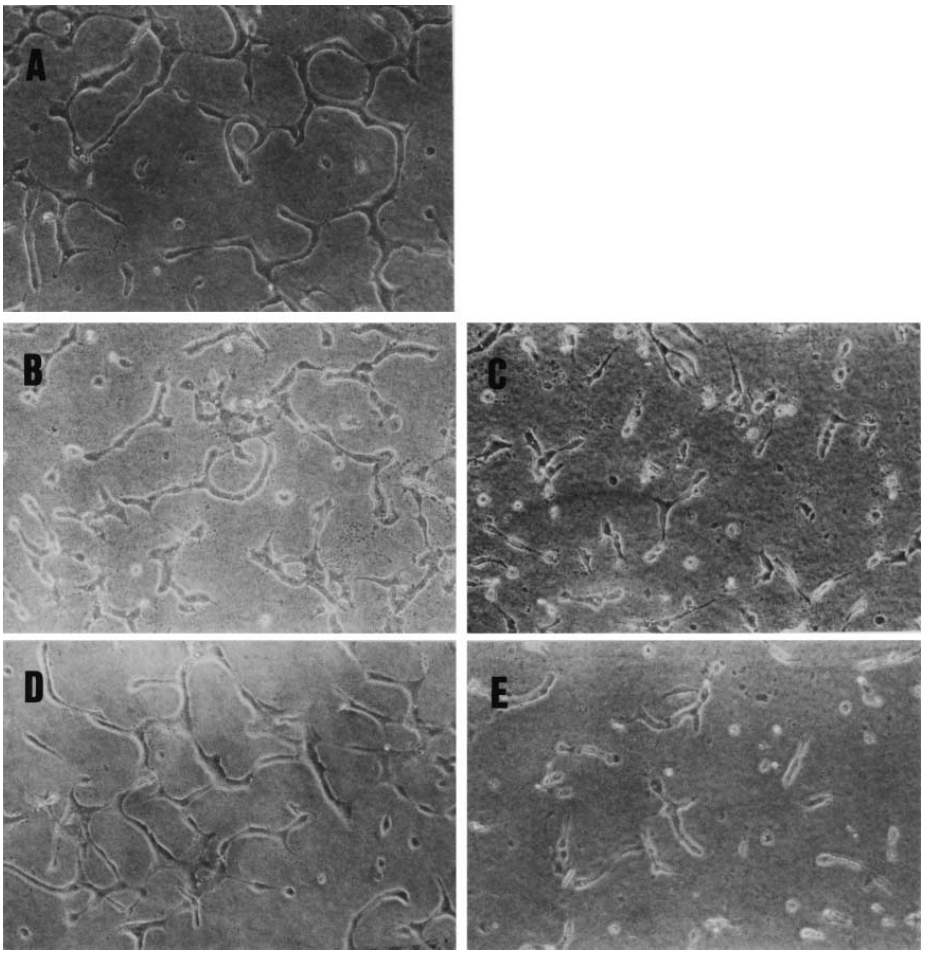

Fig. 3. Effect of ChM-I on tube morphogenesis of BCAE cells in a collagen gel. BCAE cells were inoculated onto a collagen gel in 12-multiwell plates in the presence of 0.1% BSA alone (A), 0.1 μg/well purified ChM-I (B), 1 μg/well purified ChM-I (C), 0.01 μg/well PF4 (D), or 0.1 μg/well PF4 (E). Bar represents 200 μm.

genesis inhibitors [20,21]. The C-terminal 13 amino acids of PF4(58-70) have been suggested to participate in the inhibitory activity of PF4 [22]. The fumagillin derivative AGM1470 (or otherwise called TNP-470) was also shown to inhibit growth of cultured endothelial cells and tumor induced angiogenesis [23,24]. In this study, ChM-I was compared to PF4 and AGM1470 in terms of their inhibitory activities on DNA synthesis and tube morphogenesis of BCAE cells in vitro. The results were evaluated as the concentrations required for 50% inhibition of [^{3}H]thymidine incorporation and tube morphogenesis of the cells in the presence of 10% FBS (Table 1). Tube morphogenesis was semiquantitatively assessed by counting cells forming tube-like cellular networks after staining of the viable cells with MTT [16,25]. Fumagillin and AGM1470 inhibited DNA synthesis at concentrations of 5 pmol/ml and 0.5 pmol/ml, respectively. These were about 100-fold lower than those required for inhibition of tube morphogenesis. On the other hand, PF4 inhibited formation of tube-like cellular networks as characterized by its IC_{50} (1.2–12 pmol/well; Fig. 3), whereas a much higher dose was required for inhibition of DNA synthesis. Similarly PF4(58-70) inhibited formation of cellular networks, but it was almost inactive

Table 1
Effects of various inhibitors on DNA synthesis and tube morphogenesis

Addition	Inhibition of DNA synthesis (IC_{50})[a]	Inhibition of tube morphogenesis (IC_{50})[b]
Fumagillin	5 pmol/ml	> 2500 pmol/well
AGM1470 (TNP-470)	0.25–0.5 pmol/ml	25–250 pmol/well
PF4(58-70)	> 11000 pmol/ml	> 770 pmol/well
PF4	> 2000 pmol/ml	1.2–12 pmol/well
ChM-I	8–23 pmol/ml	11–38 pmol/well

[a]The relative potencies of various inhibitors were compared in terms of their concentrations required for the 50% inhibition of [^{3}H]thymidine incorporation (IC_{50}) into BCAE cells cultured in the presence of 10% FBS, determined from the dose-response profiles. Two independent experiments were performed.

[b]BCAE cells were cultured on a collagen gel containing 10% FBS in the presence or absence of the inhibitory agents for three days. Cells forming tube-like cellular networks (> 100 μm in length) were counted. The relative potencies of the agents were compared in terms of their concentrations required for the 50% inhibition of tube formation stimulated by 10% FBS (IC_{50}). Two independent experiments were performed.

on DNA synthesis of the cells. Thus, AGM1470 inhibits angiogenesis due to its inhibitory action on growth of endothelial cells, whereas PF4 acts on tube morphogenesis. In contrast, ChM-I inhibited both DNA synthesis and tube morphogenesis at a similar dose (Table 1).

ChM-I was first discovered as a functional matrix component in cartilage which stimulates growth and phenotypic expression of chondrocytes [11]. The ChM-I transcripts were specifically expressed in cartilage. Recently, we demonstrated by using a chondrogenic cell line ATDC5 that ChM-I transcripts are expressed in chondrocytes in a differentiation-dependent manner [26]. Although ChM-I stimulated growth of osteoblasts as well as chondrocytes, it exerted no growth stimulation in cultured fibroblasts [26]. Members of thrombospondin (TSP) family are other extracellular matrix components with anti-angiogenic properties [27,28]. These molecules are also found in cartilage, but have a broader distribution in the body [29–31]. It is not clear at present whether these molecules also function as cartilage-derived anti-angiogenic factor. Recently we described isolation of chondromodulin-II (ChM-II) which stimulates growth of osteoblasts, but has no structural similarity to ChM-I [32]. ChM-II did not affect growth of cultured vascular endothelial cells (data not shown). These results indicate a possibility that ChM-I may confer anti-angiogenic properties on cartilage as a cartilage-specific matrix component. The in vivo studies of ChM-I to show an anti-angiogenic action in vivo are now underway.

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