Isolation and Characterization of an Inhibitor of Neovascularization from Scapular Chondrocytes

Marsha A. Moses, Judith Sudhalter, and Robert Langer*

Department of Surgery, The Children's Hospital Medical Center, Boston, Massachusetts 02115; and *Department of Chemical Engineering and Harvard-MIT Division of Health Science and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. An inhibitor of neovascularization from the conditioned media of scapular chondrocytes established and maintained in serum-free culture has been isolated and characterized. To determine whether this chondrocyte-derived inhibitor (ChDI) was capable of inhibiting neovascularization in vivo, this protein was assayed in the chick chorioallantoic membrane assay. ChDI was a potent inhibitor of angiogenesis in vivo (4 μg = 87% avascular zones). This inhibitor is also an inhibitor of fibroblast growth factor-stimulated capillary endothelial cell (EC) proliferation and migration, as well as being an inhibitor of mammalian collagenase. ChDI significantly suppressed capillary EC proliferation in a dose-dependent, reversible manner with an IC_{50} (the inhibitory concentration at which 50% inhibition is achieved) of 2.025 μg/ml. Inhibition by ChDI of growth factor-stimulated capillary EC migration was also observed using a modified Boyden chamber assay (IC_{50} = 255 ng/ml). SDS-PAGE analysis followed by silver staining of ChDI purified to apparent homogeneity revealed a single band having an M_r of 35,550. Gel elution experiments demonstrated that only protein eluting at this molecular weight was anti-angiogenic. These studies are the first demonstration that chondrocytes in culture can produce a highly enriched, potent inhibitor of neovascularization which also inhibits collagenase.

CARTILAGE is a highly specialized connective tissue whose characteristic cell type is the chondrocyte. One major function of these cells is the synthesis of the cartilage extracellular matrix which enables the tissue to withstand compressive stresses (2). An equally important but less understood function of individual chondrocytes is the synthesis of cartilage-specific macromolecules, the physiological significance of many of which still remains poorly understood (2).

Cartilage is an interesting and unique tissue in that it is avascular and relatively tumor-resistant. Early work from several laboratories, including our own, had demonstrated that cartilage contains an inhibitor of neovascularization (4, 9, 21, 26–28), but no single cartilage-derived macromolecule was purified or identified. Despite early suggestions that protease inhibitory activity derived from cartilage might play a role in such processes as the prevention of connective tissue breakdown, inhibition of tumor cell invasion, and inhibition of angiogenesis (21, 22, 25, 27, 37, 39, 41–43, 46, 47, 51), the physiological significance of a cartilage-derived antiprotease activity remained to be elucidated (33). Recently, a cartilage-derived collagenase inhibitor (CDI) has been identified, purified, and shown to be antiangiogenic in vivo and in vitro (34, 36).

A number of groups have recently become interested in the role of the chondrocyte relative to the process of neovascularization. Takigawa et al. (49) have reported that articular chondrocyte conditioned media (CM)[1] contains an inhibitor of angiogenesis, yet this inhibitor has never been purified or identified. Pepper and co-workers have recently demonstrated that chondrocytes, when cocultured with endothelial cell (EC) aggregates in a collagen gel matrix, inhibit capillary EC sprout formation. Addition of anti-TGF-β antibodies to the coculture system only reduced by 50% the inhibitory effect of the chondrocytes on sprout formation. Pepper et al. (40) concluded that these results indicated the presence of an additional antiangiogenic factor.

To identify the antiangiogenic factor produced by cultured chondrocytes, we have established and maintained scapular chondrocytes in serum-free culture and have demonstrated that these cells produce a potent inhibitor of angiogenesis. This chondrocyte-derived angiogenesis inhibitor (ChDI) is also an inhibitor of mammalian collagenase.

1. *Abbreviations used in this paper*: CAM, chorioallantoic membrane; ChDI, chondrocyte-derived inhibitor; CM, conditioned media; EC, endothelial cell; aFGF, acidic FGF; bFGF, basic FGF; IC_{50}, the inhibitory concentration at which 50% inhibition is obtained.

Materials and Methods

Cell Culture

Chondrocytes. Bovine scapular chondrocytes were isolated from the scapular cartilage of newborn calves (1–14 d old) according to the method of Klagsbrun (20) and primary cultures were established and maintained in serum-free culture (see Fig. 1). Veal scapulae were obtained from Arena Co. (Hopkinton, MA) within 24 h of slaughter. Briefly, under sterile conditions the scapular cartilage was excised from the scapular bone and scraped clean of muscle and connective tissue using first a periosteal elevator (Arista, New York, NY) and then a scalpel blade (no. 10; Bard-Parker, Rutherford, NJ). The cleaned cartilage was cut into pieces (0.5 × 0.5 cm) and incubated with Clostridial collagenase (CLS 2; Worthington, Freehold, NJ) (2.0 mg/ml PBS containing Ca^{+2} and Mg^{+2} [Gibco Laboratories, Grand Island, NY]) for 12–24 h. The cell suspension was filtered to remove undigested cartilage and was washed twice with PBS. Cells were then counted using a hemocytometer (Scientific Products, McGaw Park, IL) and 1×10^7 viable cells (as determined by the exclusion of trypan blue) were plated into 75-cm tissue culture flasks (Falcon Labware, Lincoln Park, NJ) containing HAM F-12 (Flow Laboratories, Inc., McLean, VA) supplemented with 50 μg/ml ascorbic acid (Eastman Kodak Co., Rochester, NY). Chondrocyte CM was harvested twice a week and the primary chondrocyte cultures were refed with above media. Upon collection, the media was dialyzed exhaustively against distilled water (mol wt cut off 3,500) (Spectra/Por; Fisher Scientific Co., Pittsburgh, PA) and lyophilized. CM was harvested from several different tissue culture flasks. Scapular cartilage was obtained from at least three calf shoulders per preparation.

Endothelial Cells. Capillary ECs, isolated from bovine adrenal cortex (13) were the generous gift of Katherine Butterfield and Dr. J. Folkman (Children's Hospital, Boston, MA). These cells were maintained in culture in DME (Gibco Laboratories) with 10% calf serum (Hyclone, Logan, Utah) (DME/10), supplemented with retina-derived growth factor (5 μl/ml) (16) in preparation for the assays. These cells were demonstrated to be endothelial by staining with antisera to von Willebrand factor (18) and by their uptake of fluoresceinated, acetylated low density lipoprotein (53).

Cell Proliferation Assays

Capillary ECs were plated in DME/5 onto gelatin-coated (1.5% gelatin, wt/vol [Difco, Detroit, MI]) 24-well tissue culture dishes (Nunc, Roskilde, Denmark). At the indicated times, inhibitor samples and acidic FGF (aFGF) (F.G.F. Co., La Jolla, CA) were added to some of the wells. Wells containing PBS alone and PBS and aFGF were included as controls. These substances were added simultaneously in volumes that did not exceed 10% of the final volume. After incubation with the test substances for the designated times, medium was aspirated, cells were washed with 0.5 ml of PBS, removed by trypsinization (0.5 ml 0.05% trypsin-0.02% EDTA; Gibco Laboratories), and counted electronically with a cell counter (Coulter Electronics, Hialeah, FL).

This cell counting assay was supported by an additional proliferation assay which measures the number of endothelial cells in culture on the basis of the colorimetric measurement of cellular acid phosphatase (7).

Reversibility of ChDI Inhibition of Capillary EC Growth

To determine whether the inhibition of capillary EC proliferation was due in part to cytotoxicity, reversibility studies were conducted as described previously for capillary EC (3, 8, 36). The proliferation assay described above and in Fig. 5 was repeated with ChDI (IC_{50} concentration), with the following modification: on day 5, cells from representative wells of three conditions—cells unexposed to either growth factor or ChDI, cells exposed to growth factor alone, and cells exposed to both growth factor and ChDI—were counted as usual. Duplicate wells of each condition were also washed twice with 0.5 ml PBS and either refed with DME/5 alone (controls) or with DME/5 and growth factor. These wells were then counted 3 d later. Cells were examined morphologically under light microscopy.

Migration Assays

Capillary EC migration was measured using a modification of the Boyden chamber assay (10). Blind well chambers (no. 025-187; Neuroprobe, Pleasanton, CA) were utilized. Polycarbonate membranes with 8-μm pores (Nucleopore, Pleasanton, CA) were precoated with human fibronectin (6.67 μg/ml in PBS; Cooper, Freehold, NJ). Basic FGF (bFGF) (Takeda Chemical Co., Osaka, JP) was added to the lower wells. The upper wells received capillary EC and increasing concentrations of ChDI. Control wells received DME/1, either with or without FGF. After incubation, the cells on the upper surface of the membrane were wiped off by drawing the membrane over a wiper blade (Neuroprobe, Freehold, NJ) as described previously (36). The cells which had migrated through the membrane onto the lower surface were fixed in 2% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) followed by MeOH (4°C) and stained with hematoxylin. Migration was quantified by counting the number of cells on the lower surface in 16 oil immersion fields per well.

Angiogenesis Assays

To determine whether or not ChDI could inhibit angiogenesis in vivo, the chorioallantoic membrane (CAM) assay was utilized. Briefly, on day 3 of development, fertilized chick embryos were removed from their shells and placed in plastic petri dishes (1005; Falcon Labware). They were maintained in humidified 5% CO_2 at 37°C. On day 6 of development, samples of ChDI were mixed in methylcellulose disks and applied to the surfaces of the growing CAMs above the dense subectodermal plexus. After 48 h exposure of the CAMs to ChDI, CAMs were observed using a binocular dissecting microscope at × 7–10 magnification. The CAMs were injected intravascularly with India-ink/Liposyn as described previously (50). Other CAMs were prepared for histological analysis by first being fixed in formalin (Fisher Scientific Co., Fairlawn, NJ) at room temperature and rinsed in 0.1 M cacodylate buffer, pH 7.4. The specimens were embedded in JB-4 plastic (Polysciences, Warrington, PA) at 4°C and 3-μm sections were cut using a Reichert 2050 microtome. Sections were stained with toluidine blue and micrographs were taken on a Zeiss photomicroscope using Kodak TM × 100 and a green filter.

Protein Assays

Protein concentration of chondrocyte CM and ChDI was determined by the Lowry method (29) using BSA as the standard.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (24); 0.75-mm 15% gels were used. Samples were reduced with mercaptoethanol (Sigma Chemical Co.) before application. Silver staining was carried out according to a modification of the method of Wray et al. (54). The gel was fixed for 30 min in 50% methanol (Pierce Chemical Co., Rockford, IL), 10% acetic acid followed by an overnight incubation in 5% methanol, 7% acetic acid. The next morning, gels were placed in 10% glutaraldehyde (Sigma Chemical Co.) for 30 min and then washed with four changes of water over a period of 2 h. Gels were then silver stained for 15 min and developed as described by Wray et al. (54). Unless otherwise indicated, reagents were obtained from Fisher Scientific Co. (Fairlawn, NJ).

Chromatography

To isolate ChDI from the chondrocyte CM, media were exhaustively dialyzed against distilled water before chromatography. The dialyzed samples were lyophilized and diluted in 0.1 M Tris-HCl (Schwarz Mann, Biotec, Cleveland, OH) pH 7.6, containing 4 M guanidine-HCl (ICN Biomedicals, Cleveland, OH), 0.001 M $CaCl_2$ and 0.02% sodium azide. Alternatively, samples were diluted and chromatographed in buffer containing 0.05 M Tris-HCl, pH 7.6 containing 0.2 M NaCl and 0.001 M $CaCl_2$ (collagenase assay buffer [CAB]). Samples of chondrocyte CM (2–4 mg protein) were then applied to a Sephadex G-75 (Superfine) (Pharmacia, Uppsala, Sweden) column (1.6 × 50 cm) equilibrated with either of the appropriate buffers at a flow rate of 10 ml/h. 1-ml fractions were collected. Molecular weight markers used were blue dextran (M_r 2×20^6), ovalbumin (M_r 43,000), chymotrypsinogen (M_r 26,000), and ribonuclease A (M_r 13,000). Unless otherwise indicated, all reagents were obtained from Sigma Chemical Co. Fractions enriched in collagenase inhibitory activity (1 inhibitory unit, i.e., IC_{50} or greater) were pooled for further study.

Collagenase Inhibitor Assays

Collagenase inhibitory activity was determined using a modification (38) of a radiometric collagen film assay (19) as described by us previously.

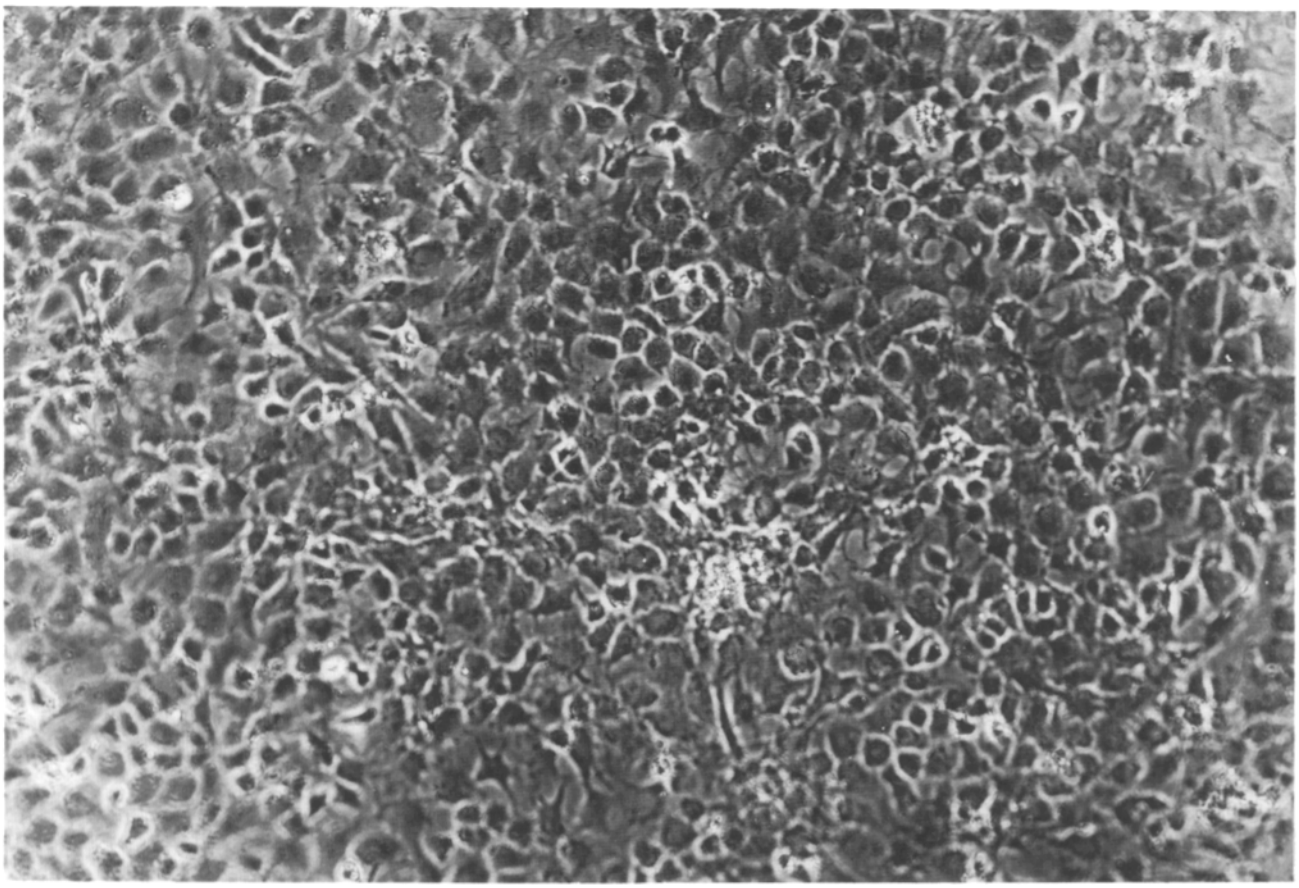

Figure 1. Phase-contrast micrograph depicting confluent monolayer of scapular chondrocytes. The cells are refed twice a week with HAM F-12 supplemented with 50 μg/ml ascorbic acid. The chondrocytes are polyhedral and demonstrate their characteristic cobblestone morphology.

Elution of Antiangiogenic Activity from SDS-Polyacrylamide Gels

After chromatography on Sephadex G-75, ChDI was subjected to electrophoresis on SDS-PAGE slab gels as described above with the following exceptions. Samples were dissolved in buffer that did not contain mercaptoethanol. Samples were incubated at 37°C for 1 h before application to gels rather than being boiled. Electrophoresis was carried out at 250 V for 1 h as above. After electrophoresis, lanes containing protein markers together with a lane containing ChDI were cut out of the slab and silver stained according to a shortened protocol (38). Gels were fixed for 30 min in 50% methanol, 10% acetic acid. The water washes after glutaraldehyde fixation were shortened to 20 min each. While gels were being fixed and stained, the unstained portion was stored at 4°C in the refrigerator. The stained gels were placed in 50% methanol until they were the same size as the unstained gels. Gels were then lined up and regions corresponding to these approximate M_r were excised: 65–67, 31–40, 21.5–31, 15–21.5, and 13–15 kD. Although no other protein bands were observed after electrophoresis on the silver-stained SDS-PAGE gel (see Fig. 3), we chose to include these other regions of the gel as controls in our elution studies because cartilage has been shown to contain other collagenase inhibitors (15, 36, 38). Some areas from lanes in which only sample buffer had been electrophoresed were excised to serve as controls. Gel slices were minced finely and incubated for 24 h in 1–2 ml of CAB containing 0.5% Brij 35 (38). The eluate was dialyzed overnight at 4°C against 1,000 vol of CAB containing 0.05% Brij 35. Protein concentrations of ChDI were estimated by comparing the intensities of silver-stained ChDI bands on SDS-PAGE gels to those of the protein standards as previously described (45). Samples were assayed both for their ability to inhibit capillary EC proliferation and collagenase as described above. To test these eluted samples for their ability to inhibit capillary EC proliferation, Brij 35 was removed from each eluted sample using prepacked columns of Extracti-Gel D (Pierce Chemical Co.) according to manufacturer's specifications.

Results

Primary cultures of chondrocytes isolated from the scapular cartilage of newborn calves were established and maintained in HAM F-12 supplemented with ascorbic acid (Fig. 1). The serum-free nature of these primary chondrocyte cultures eliminated the need to remove any contaminating serum-derived proteins. To further purify and characterize the ChDI from the serum-free chondrocyte CM, the media was chromatographed on a Sephadex G-75 (superfine) column eluted in buffer containing 0.01 M Tris-HCl, pH 7.6, containing 4 M guanidine-HCl and 0.001 M $CaCl_2$. Fractions were then screened for collagenase inhibitory activity as described previously (36, 38); inhibitory activity was recovered in a molecular weight region centered at ~32,000 (Fig. 2). Fractions enriched in collagenase inhibitory activity from this step were pooled and protein concentrations determined. SDS-PAGE analysis of ~5 μg of ChDI followed by silver staining revealed a band at an M_r of 35,550 (Fig. 3). This same result was observed even when 10 and 15 μg of ChDI were electrophoresed.

To determine whether ChDI was capable of inhibiting angiogenesis in vivo, the chick CAM assay (50) was used. Fig.

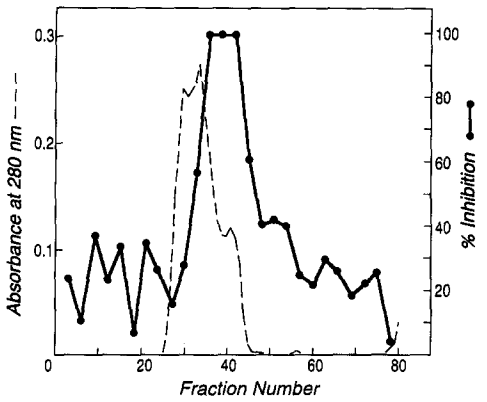

Figure 2. Sephadex G-75 chromatography. Chondrocyte CM was exhaustively dialyzed against distilled water and concentrated by lyophilization. CM samples (2–4 mg) were diluted in 0.1 M Tris-HCl, pH 7.6, containing 4 M guanidine-HCl, and 0.001 M $CaCl_2$ and applied to a Sephadex G-75 (superfine) column (1.6 × 50 cm) equilibrated with the buffer described above at a flow rate of 10 ml/h; 0.8-ml fractions were collected and 75-ul aliquots were assayed for collagenase inhibitory activity. ---, A280; ●, percentage of inhibition.

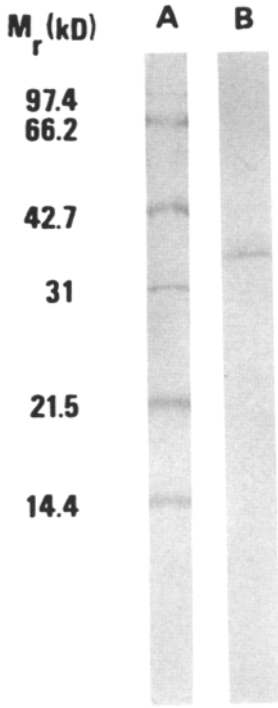

Figure 3. Silver-stained SDS-PAGE of ChDI. SDS-PAGE was performed on ChDI (5 ug) according to the method of Laemmli (24); samples were reduced with mercaptoethanol before application. Silver staining was carried out by a modification of the method of Wray et al. (54). To determine molecular weight, the following reduced standard proteins were used: phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad Laboratories, Richmond, CA).

4 *a* shows the significant inhibition of embryonic neovascularization as evidenced by the large avascular zone, caused by 4-μg samples of ChDI placed in methylcellulose discs, after 48 h of exposure to ChDI. This effect was observed in 87% of the eggs tested. This observation was reproduced in four separate sets of CAM assays using different ChDI preparations. Inhibitor eluted from G-75 columns in the absence of 4 M guanidine-HCl also demonstrated the same amount of inhibition. In contrast, control CAMs implanted with empty methylcellulose discs did not develop avascular zones (Fig. 4 *b*). Histological studies of ChDI-treated CAMs reveal an absence of normal capillaries (Fig. 4 *c*) relative to controls which show normal vascular development (Fig. 4 *d*).

To understand the mechanism(s) by which ChDI inhibits angiogenesis we studied the effect of this inhibitor on two key components of the angiogenic process, capillary EC proliferation and migration (1). By utilizing the specific cells involved in angiogenesis and by stimulating them with known angiogenesis factors, we could mimic the angiogenic process in vitro. Unfractionated serum-free media conditioned by these primary chondrocyte cultures inhibited aFGF-stimulated capillary EC proliferation in a dose-dependent manner, with an IC_{50} of 200 μg/ml (Fig. 5 *a*). The ChDI obtained from the G-75 chromatography step inhibited capillary EC proliferation in a dose-dependent manner with an IC_{50} of 2.025 μg/ml (Fig. 5 *b*). An additional bioassay which determines the number of endothelial cells in culture on the basis of colorimetric measurement of cellular acid phosphatase (7) was also utilized to determine the effect of ChDI on capillary EC proliferation. Results from this assay verified the cell counting assay results (data not shown).

To demonstrate that the protein migrating at ~35.5 kD was responsible for the antiangiogenic activity, we conducted the gel elution experiments described in Materials and Methods. Material eluted from each of the zones as described above was tested for its ability to inhibit FGF-stimulated capillary EC proliferation as well as for its ability to inhibit mammalian collagenase. Only protein corresponding to the band at M_r 35,500 inhibited capillary EC proliferation, doing so in a dose-responsive manner (Fig. 5 *c*). This material also inhibited collagenase in the radiometric enzyme assay (IC_{50} = 51.15 ng/ml).

To determine whether the inhibition of capillary EC proliferation was due to cytotoxicity, reversibility studies were conducted as described previously (3, 8, 36). These studies showed that not only did the endothelial cells grow normally after removal of the inhibitor, but these cells were also capable of responding to mitogen to the same degree as did control cells never exposed to ChDI.

To examine the specificity of the inhibitor's antiproliferative effect, we tested other substances for their ability to inhibit capillary EC proliferation. Other enzyme inhibitors such as trypsin ovoinhibitor, α-2 macroglobulin (a collagenase inhibitor), and chondroitin sulfate (a glycosaminoglycan found in cartilage) did not have a significant effect (<20% inhibition or stimulation, at all doses tested) on growth factor–stimulated capillary EC proliferation even when tested at concentrations of 50 μg/ml. These factors also do not inhibit angiogenesis in vivo (36).

Given that capillary EC migration in response to a stimulus is another key component of the angiogenic process (1), we tested the chondrocyte CM and ChDI derived from G-75 columns eluted in the absence of 4 M guanidine-HCl for their ability to inhibit bFGF-stimulated EC migration using a modification (10) of the Boyden assay. Chondrocyte CM inhibited growth factor–stimulated capillary EC migration at an IC_{50} of 28 μg/ml and ChDI inhibited at an IC_{50} of 255 ng/ml (Fig. 6).

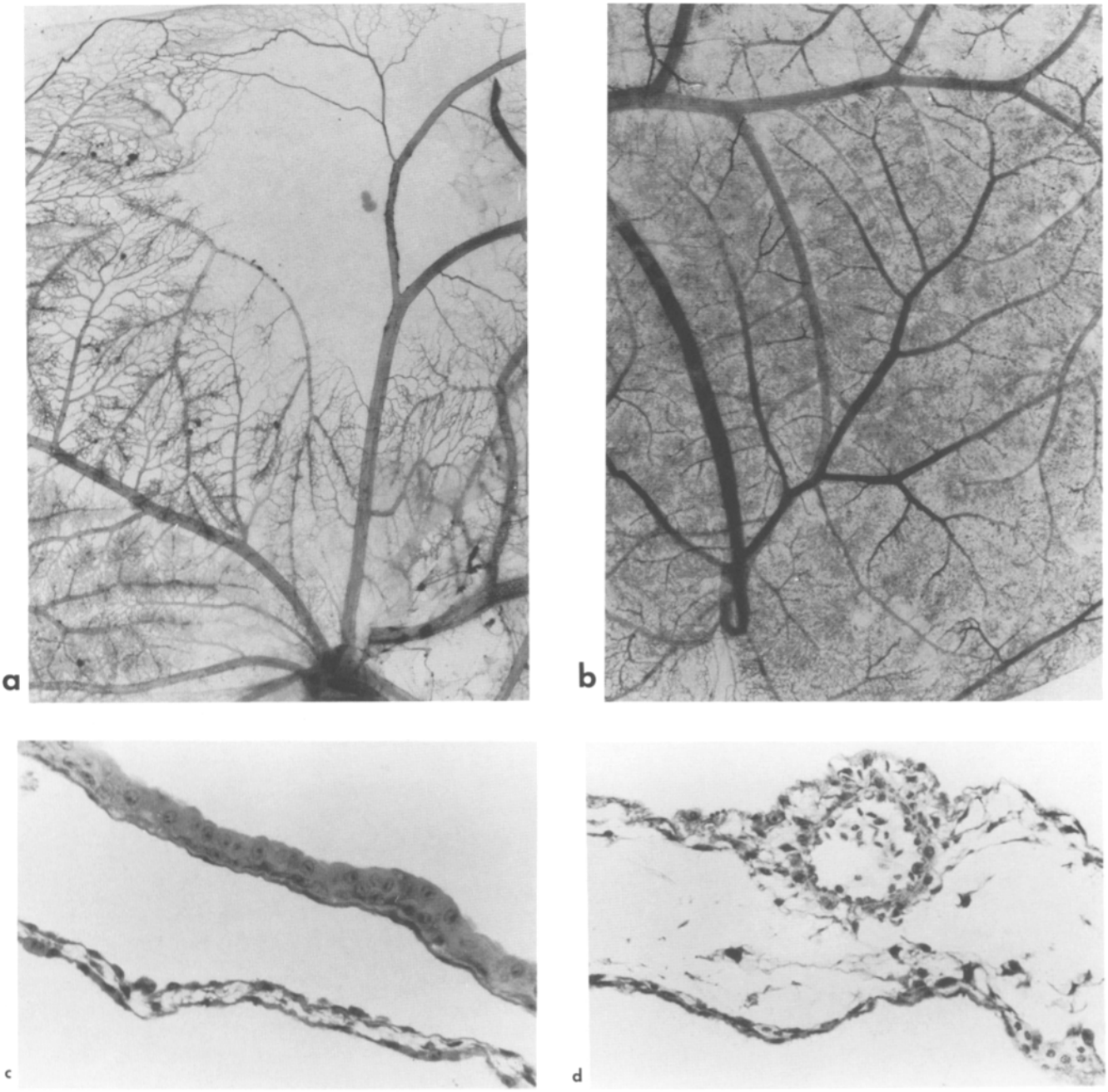

Figure 4. Inhibition of angiogenesis by ChDI. On day 3 of development, fertilized chick embryos were removed from their shells and placed in plastic petri dishes. They were maintained in humidified 5% CO_2 at 37°C. On day 6 of development, 4-μg samples of ChDI were mixed in methylcellulose disks and applied to the surfaces of the growing CAMs. After 48 h exposure of the CAMs to ChDI, avascular zones, free of capillaries and small vessels were observed using a binocular dissecting microscope at ×7–10 magnification. The CAMs were injected intravascularly with India ink/Liposyn as described previously (50). The centers of the avascular zones are free of India ink–filled capillaries and contain the methylcellulose disks (*a*). A normal CAM containing an empty methylcellulose disk is shown in *b*. Histological sections of day 8 CAMs reveal normal capillary development in untreated controls (*d*) in comparison to ChDI-treated CAMs (*c*). 22 embryos were used. All tests were conducted double-blind.

Discussion

Neovascularization is a complex process characterized by penetration of basement membrane by capillary EC, migration of cells through matrix towards a stimulus, and subsequent proliferation (1, 11). Although the role of various growth factors in the process of vascular proliferation has been well characterized (12), relatively little is known about the identity or role of naturally occurring inhibitors of neovascularization (31). Due to previously reported difficulties in obtaining or purifying biologically active inhibitory mate-

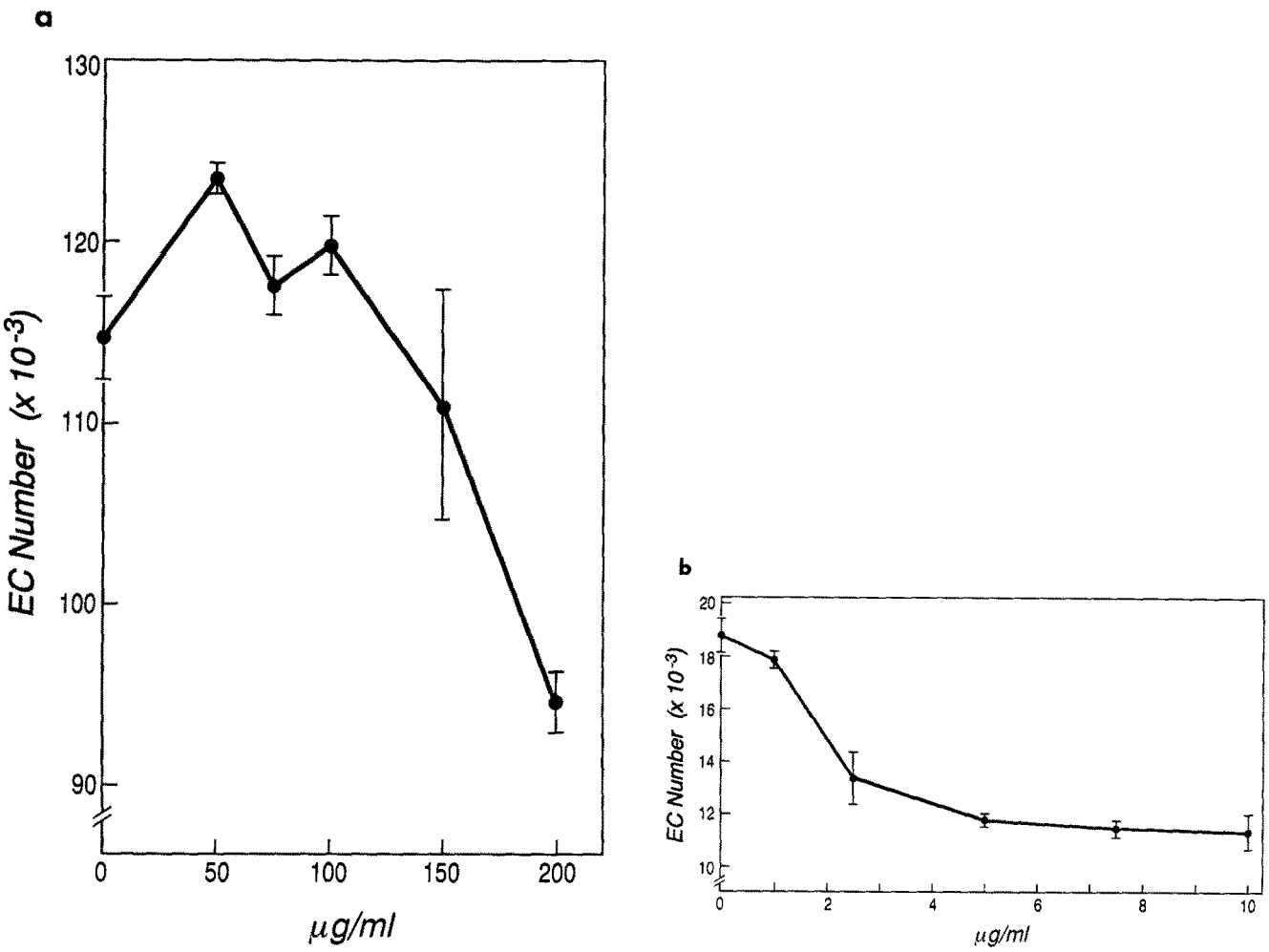

Figure 5. Effect of chondrocyte CM and ChDI on aFGF-stimulated capillary EC proliferation. Capillary ECs (10^4 cells/0.5 ml DME/5) were plated onto gelatin-coated 24-well tissue culture dishes and allowed to attach overnight. On day 2, unattached cells were removed and attached cells were refed with DME/5. Inhibitor and aFGF (12 ng/ml) were added. Wells containing PBS alone and PBS + aFGF were included as controls. On day 5, medium was aspirated, cells were washed with 0.5 ml of PBS, removed by trypsinization, and counted. *a* and *b* demonstrate the antiproliferative effect of increasing concentrations of chrondrocyte CM and ChDI, respectively, on capillary EC proliferation. Control wells containing PBS alone contained 75,630 ± 129.45 cells (*a*) 10,807 ± 911.67 cells (*b*) and 18,907 ± 129.45 cells (*c*). *a* and *b* are representative of three separate experiments (*b* is representative of three separate Sephadex G-75 column runs); *c* is representative of two separate experiments and demonstrates the antiproliferative activity of increasing amounts of chrondrocyte-derived angiogenesis inhibitor corresponding to an M_r of 35,500 obtained from the gel elution experiments. Percent inhibition was determined by comparing the cell number of wells exposed to stimulus with those exposed to stimulus and inhibitor. Each data point of each experiment represents the mean of triplicate wells; SEM was <3% of the mean.

rial from human and animal tissue extracts, alternate sources of antiangiogenic factors have recently begun to be explored (31). The studies reported here demonstrate that scapular chondrocytes established and maintained in serum-free culture produce a potent inhibitor of angiogenesis in vivo. The potency of this inhibitor can be appreciated when compared to that of other inhibitors of neovascularization previously reported. Other angiogenesis inhibitors (e.g., vitreous, protamine, platelet factor 4, AGM-1470, and others (14, 23, 30, 50) required doses of 10–100 μg of material to obtain ~50%

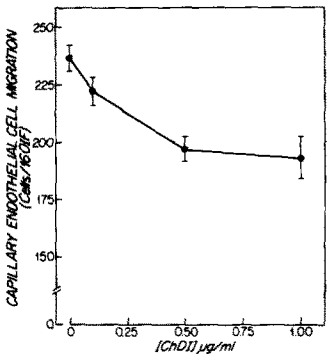

Figure 6. Inhibition of capillary EC migration by ChDI. Migration of capillary EC was measured using a modification of the Boyden chamber assay as described by us previously (36). FGF (Takeda Co., Osaka, Japan) diluted in DME/1 (29 μl) was added to the lower wells at a concentration of 10 ng/ml. The upper wells received 50 μl of a cell suspension containing 5 × 10^5 capillary EC/ml and increasing concentrations of ChDI. Control wells received DME/1, either with or without bFGF. The migration chambers were incubated at 37°C in 10% CO_2 for 4 h. Each point represents the mean ± SEM of four wells. In control wells without bFGF, the number of migrated capillary ECs was 186 ± 4 SEM.

avascular zones, whereas just 4 μg of ChDI caused 87% avascular zones.

It is interesting to note that this chondrocyte-derived antiangiogenic protein (ChDI) is also an inhibitor of mammalian collagenase. Although it has been known that chondrocytes synthesize proteases and some of their modulators (activators and inhibitors), it has been assumed that these macromolecules are produced solely for the purpose of synthesizing and remodelling the cartilage extracellular matrix (52). This work demonstrates an additional function for a negative modulator of collagenase activity.

This report represents the first demonstration that a chondrocyte-derived collagenase inhibitor can inhibit angiogenesis in vivo. In 1984, Bunning and co-workers reported that a collagenase inhibitor produced by bovine nasal cartilage and articular chondrocytes did not inhibit angiogenesis on the chick CAM (5). Takigawa, Shirai, and co-workers subsequently reported that undefined extracts of articular cartilage and articular chondrocyte CM contain an inhibitor of angiogenesis (49); whether these extracts contain collagenase inhibitory activity is still not known.

This study also confirms earlier reports which noted the production of collagenase inhibitors by articular chondrocytes. A number of groups have shown that metalloproteinase inhibitors are produced by chondrocytes in culture, yet until now, the physiological significance of such an inhibitor remained unknown. These inhibitors include a partially purified collagenase inhibitor having an M_r of 32,000 isolated from the conditioned media of bovine articular chondrocytes as reported by Bunning and co-workers (5) and Murphy et al. (37), a partially purified collagenase inhibitor having an M_r of 35,000 from the same source as described by Morales and co-workers (32) and a higher molecular weight collagenase inhibitor made by rabbit articular chondrocytes in culture (M_r 66,000) described by Morris (33).

Whether ChDI is identical to the tissue-derived inhibitor of angiogenesis (CDI) or to other chondrocyte-derived collagenase inhibitors remains to be seen since lack of sequence information of these chondrocyte factors precludes direct comparison. We are currently conducting studies to obtain sequence information for ChDI. We note here that in terms of its capillary EC antiproliferative activity, the specific activity of ChDI is virtually the same as that reported for CDI, the cartilage-derived angiogenesis inhibitor (IC_{50} of 62.5 nM) (36). ChDI is a slightly more potent (IC_{50} of 7.18 nM) inhibitor of capillary EC migration than is CDI (IC_{50} of 16 nM). In terms of its antiangiogenic activity in vivo ChDI is at least as potent an inhibitor of neovascularization as CDI (36). ChDI differs from CDI in its apparent M_r (35,500 and 27,650 respectively) as determined by both gel filtration and SDS-PAGE analysis.

Differences in molecular weight between ChDI and other chondrocyte-derived collagenase inhibitors may be ascribed to differences in the types of cartilage (articular or scapular) used as a source of chondrocytes, species variations (bovine or rabbit), the conditions under which these cultures were established and maintained (with or without serum), differences in purification methods, and the potential existence of different members of a family of related metalloproteinase inhibitors (35, 48).

Difficulties in obtaining and purifying biologically active inhibitory material from human and animal tissue extracts have greatly limited the identification and availability of angiogenesis inhibitors. The fact that these primary chondrocyte cultures were established and maintained in serum-free culture greatly facilitated purification of ChDI by eliminating the need to remove any contaminating serum-derived proteins as well as by eliminating any effect that these serum-derived factors might have on normal chondrocyte secretion (33).

These studies suggest that ChDI may be at least one of the molecules responsible for the maintenance of cartilage's avascularity. A number of serious connective tissue pathologies including rheumatoid arthritis can be characterized by persistent and deregulated angiogenesis (12, 17, 44) as well as by uncontrolled extracellular matrix degradation (6). The further study of ChDI, as a chondrocyte-derived antiangiogenic factor, may lead to potential therapeutic agents for the treatment of the large number of serious diseases, connective tissue and otherwise, which might be controlled via the use of an antiangiogenic strategy.

We thank Dr. J. Folkman for his help with the bioassays and for the capillary endothelial cells, and Dr. Yuen Shing for his advice and assistance. We also thank Dr. B. Zetter for his critical review of the manuscript; Dr. D. Ausprunk, S. Dethlefsen, and G. Jackson for their help with the in vivo studies; and J. Behm for her excellent technical assistance. Supported by National Institutes of Health (NIH) grant EY05333 to R. Langer and NIH postdoctoral fellowship EY06047 to M. A. Moses.

Received for publication 6 April 1992 and in revised form 16 July 1992.

References

1. Ausprunk, D., and J. Folkman. 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* 14:53–65.
2. Block, J. A., S. E. Inerot, S. Gitelis, and J. H. Kimura. 1991. Synthesis of chondrocytic keratan sulphate-containing proteoglycans by human chondrosarcoma cells in long-term culture. *J. Bone and Joint Surg.* 73: 647–658.
3. Braunhut, S. J., and M. Palomares. 1991. Modulation of endothelial cell shape and growth by retinoids. *Microvasc. Res.* 41:47–62.
4. Brem, H., and J. Folkman. 1975. Inhibition of tumor angiogenesis mediated by cartilage. *J. Exp. Med.* 141:427–439.
5. Bunning, R. A. D., G. Murphy, S. Kumar, P. Phillips, and J. J. Reynolds. 1984. Metalloproteinase inhibitors from bovine cartilage and body fluids. *Eur. J. Biochem.* 139:75–80.
6. Cambray, G. J., G. Murphy, D. P. Page-Thomas, and J. J. Reynolds. 1981. The production in culture of metalloproteinases and of inhibitor by joint tissues from normal rabbits and from rabbits with a model arthritis. *Synov. Rheumatol.* 1:11–16.

7. Connolly, D. J., M. B. Knight, N. K. Harakas, A. J. Wittwer, and J. Feder. 1986. Determination of the number of endothelial cells in culture using an acid phosphatase assay. *Anal. Biochem.* 152:136–140.
8. Doctrow, S. R., and J. Folkman. 1987. Protein kinase C activators suppress stimulation of capillary endothelial cell growth by angiogenic endothelial mitogens. *J. Cell Biol.* 104:679–687.
9. Eisenstein, R., K. E. Kuettner, C. Neopolitan, L. W. Sobel, and N. Sorgente. 1975. The resistance of certain tissues to invasion. III. Cartilage extracts inhibit the growth of fibroblasts and endothelial cells in culture. *Am. J. Pathol.* 871:337–348.
10. Falk, W., R. H. Goodwin, Jr., and E. J. Leonard. 1980. A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods.* 33:239–247.
11. Folkman, J. 1987. Angiogenesis. *In* Thrombosis and Haemostasis. M. Verstrate, J. Vermylen, R. Lijnan, J. Arnont, editors. Leuven Univ. Press., Leuven, Belgium, 583–596.
12. Folkman, J., and M. Klagsbrun. 1987. Angiogenic factors. *Science (Wash. DC).* 235:442–447.
13. Folkman, J., C. Haudenschild, and B. R. Zetter. 1979. Long-term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. USA.* 76:5217–5221.
14. Folkman, J. 1985. Angiogenesis and its inhibitors. *In* Important Advances in Oncology. V. T. DeVita Jr., S. Hellman, S. A. Rosenberg, editors. Lippincott, Philadelphia, PA: 42–62.
15. Gabrielides, C., and F. Barreau. 1987. A vertebrate interstitial collagenase inhibitor from bovine scapular cartilage: purification and characterization. *Biochim. Biophys. Acta.* 924:238–247.
16. Gitlin, J. D., and P. A. D'Amore. 1983. Culture of retinal capillary cells using selective growth media. *Microvasc. Res.* 26:74–80.
17. Harris, E. D., Jr. 1976. Recent insights into the pathogenesis of the proliferative lesion in rheumatoid arthritis. *Arthritis Rheum.* 19:68–72.
18. Hoyer, L. W., R. P. de Los Anstos, and J. R. Hoyer. 1973. Antihemophilic factor antigen. Localization in endothelial cells by immunofluorescent microscopy. *J. Clin. Invest.* 5:2737–2744.
19. Johnson-Wint, B. 1980. A quantitative collagen film collagenase assay for large numbers of samples. *Anal. Biochem.* 104:175–181.
20. Klagsbrun, M. 1979. Large-scale preparation of chondrocytes. *Methods Enzymol.* 58:560–564.
21. Kuettner, K. E., J. Hiti, R. Eisenstein, and E. Harper. 1976. Collagenase inhibition by cationic proteins derived from cartilage and aorta. *Biochem. Biophys. Res. Commun.* 72:40–46.
22. Kuettner, K. E., B. U. Pauli, and L. Sobel. 1978. Morphological studies on the resistance of cartilage to invasion by osteosarcoma cells in vitro and in vivo. *Cancer Res.* 38:277–287.
23. Kusaka, M., K. Sudo, T. Fujita, S. Marui, F. Itoh, D. Ingber, and J. Folkman. 1991. Potent anti-angiogenic action of AGM-1470: comparison to the fumagillin parent. *Biochem. Biophys. Res. Commun.* 3:1070–1076.
24. Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage-T4. *Nature (Lond.).* 227:680–685.
25. Langer, R., and J. Murray. 1983. Angiogenesis inhibitors and their delivery systems. *Appl. Biochem. Biotech.* 8:9–24.
26. Langer, R., H. Brem, K. Falterman, M. Klein, and J. Folkman. 1976. Isolation of a cartilage factor that inhibits tumor neovascularization. *Science (Wash. DC).* 193:70–72.
27. Langer, R., H. Conn, J. Vacanti, C. Haudenschild, and J. Folkman. 1980. Control of tumor growth in animals by infusion of an angiogenesis inhibitor. *Proc. Natl. Acad. Sci. USA.* 77:4331–4335.
28. Lee, A., and R. Langer. 1983. Shark cartilage contains inhibitors of tumor angiogenesis. *Science (Wash. DC).* 221:1185–1189.
29. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. R. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:2265–2275.
30. Lutty, G. A., D. C. Thompson, J. Y. Gallop, R. J. Mello, A. Patz, and A. Fenselau. 1983. Vitreous: an inhibitor of retinal extract-induced neovascularization. *Invest. Ophthalmol. Vis. Sci.* 24:52–56.
31. Maione, I. E., G. S. Gray, J. Petro, A. J. Hunt, A. L. Donner, S. I. Bauer, H. F. Carson, and R. J. Sharpe. 1990. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science (Wash. DC).* 247:77–79.
32. Morales, T. I., K. E. Kuettner, D. S. Howell, and J. T. Woessner. 1983. Characterization of the metalloproteinase inhibitor produced by bovine articular chondrocyte cultures. *Biochem. Biophys. Acta.* 760:221–229.
33. Morris, G. M. 1989. A high molecular weight collagenase inhibitor made by rabbit chondrocytes in cell culture. *Matrix.* 9:127–134.
34. Moses, M. A., and R. Langer. 1991. Inhibitors of angiogenesis. *Bio/Tech.* 9:630–634.
35. Moses, M. A., and R. Langer. 1991. A metalloproteinase inhibitor as an inhibitor of neovascularization. *J. Cell Biochem.* 47:230–235.
36. Moses, M. A., J. Sudhalter, and R. Langer. 1990. Identification of an inhibitor of neovascularization from cartilage. *Science (Wash. DC).* 248: 1408–1410.
37. Murphy, G., M. B. McGuire, R. G. Russell, and J. J. Reynolds. 1981. Characterization of collagenase, other metalloproteinases and an inhibitor (TIMP) produced by human and cartilage in culture. *J. Clin. Sci.* 61:711–716.
38. Murray, J. B., K. Allison, J. Sudhalter, and R. Langer. 1986. Purification and partial amino acid sequence of a bovine cartilage-derived collagenase inhibitor. *J. Biol. Chem.* 261:4154–4159.
39. Pauli, B., V. Memoli, and K. Kuettner. 1981. Regulation of tumor invasion by cartilage-derived anti-invasion factor in vitro. *J. Natl. Cancer Inst.* 67:65–73.
40. Pepper, M. S., R. Montesano, J. D. Vassalli, and L. Orci. 1991. Chondrocytes inhibit endothelial sprout formation in vitro: evidence for involvement of a transforming growth factor-beta. *J. Cell. Phys.* 146:170–179.
41. Presta, M., and D. B. Rifkin. 1988. New aspects of blood vessel growth: tumor and tissue-derived angiogenesis factors. *Haemostasis.* 18:6–17.
42. Rifkin, D. B., J. L. Gross, D. Moscatelli, and E. Jaffe. 1982. Proteases and angiogenesis: Production of plasminogen activator and collagenase by endothelial cells. *In* Pathobiology of the Endothelial Cell. H. L. Nossel and H. J. Nogel, editors. Academic Press, New York. 191–197.
43. Roughley, P. J., G. Murphy, and A. J. Barrett. 1978. Proteinase inhibitors of bovine nasal cartilage. *Biochem. J.* 169:721–724.
44. Sano, H., R. Forough, J. A. M. Maier, J. P. Case, A. Jackson, K. Engleka, T. Maciag, and R. L. Wilder. 1990. Detection of high levels of heparin binding growth factor-1 (acidic fibroblast growth factor) in inflammatory arthritic joints. *J. Cell Biol.* 110:1417–1426.
45. Shing, Y. 1988. Heparin-copper biaffinity chromatography of fibroblast growth factors. *J. Biol. Chem.* 263:9059–9062.
46. Shirai, E. 1987. Production of an anti-angiogenesis factor by cultured chondrocytes and establishment of a cell line which produces the factor. *J. Osaka Univ. Dent. Soc.* 32:163–180.
47. Sorgente, N., K. E. Kuettner, L. W. Sobel, and R. Eisenstein. 1975. The resistance of certain tissues to invasion. II. Evidence for extractable factors in cartilage which inhibit invasion by vascularized mesenchyme. *Lab. Invest.* 32:217–222.
48. Staskus, P. W., F. Masiarz, L. J. Pallanck, and S. Hawkes. 1991. The 21-kDa protein is a transformation-sensitive metalloproteinase inhibitor of chicken fibroblasts. *J. Biol. Chem.* 266:449–454.
49. Takigawa, M., E. Shirai, M. Enomoto, Y. Hiraki, M. Fukuya, F. Suzuki, T. Shiio, and Y. Yugari. Cartilage-derived anti-tumor factor (CATF) inhibits the proliferation of endothelial cells in culture. *Cell Biol. Int. Rep.* 9:619–625.
50. Taylor, S., and J. Folkman. 1982. Protamine is an inhibitor of angiogenesis. *Nature (Lond.).* 297:307–312.
51. Thorgeirsson, U. P., L. A. Liotta, T. Kalebic, I. M. Margulies, K. Thomas, M. Rios-Candelore, and R. J. Russo. 1982. Effect of natural protease inhibitors and a chemoattractant in tumor cell invasion in vitro. *J. Natl. Cancer Inst.* 60:1049–1054.
52. Treadwell, B. V., M. Pavia, C. A. Towle, V. J. Cooley, and H. J. Mankin. 1991. Cartilage synthesizes the serine-protease inhibitor PAI-1: support for the involvement of serine proteases in cartilage remodeling. *J. Orth. Res.* 9:309–316.
53. Voyta, J., D. Via, C. Butterfield, and B. Zetter. 1984. Identification and isolation of endothelial cells based on their increased uptake of acetylated low density lipoprotein. *J. Cell Biol.* 99:2034–2040.
54. Wray, W., T. Boulikas, V. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197–203.