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Identification of an Inhibitor of Neovascularization from Cartilage

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Certain tissues such as cartilage are resistant to vascular invasion, yet no single tissue-derived molecule that can inhibit angiogenesis has been reported. A protein derived from cartilage was purified that inhibits angiogenesis in vivo and capillary endothelial cell proliferation and migration in vitro in three separate bioassays. This protein is also an inhibitor of mammalian collagenase. These findings may help elucidate the mechanisms by which neovascularization is controlled in both normal and pathological states.

ANGIOGENESIS, THE PROCESS OF new capillary formation, participates in numerous physiological events, both normal and pathological. Under normal conditions, angiogenesis is associated with wound healing, corpus luteum formation, and embryonic development (*1, 2*). However, a number of serious diseases are dominated by abnormal neovascularization, including solid tumor growth and metastases, diabetic retinopathy, neovascular glaucoma, and rheumatoid arthritis (*3*). The potential therapeutic benefit that a naturally occurring inhibitor of angiogenesis might have in controlling diseases in which neovascularization is involved has prompted a search for angiogenesis inhibitors.

Extracts of cartilage, one of the few avascular tissues in the body, can inhibit angiogenesis (*4, 5*). Although extracts from several different tissue sources have been shown to contain anti-angiogenic activity (*6*), no single tissue-derived macromolecule capable of inhibiting angiogenesis has been identified. Our data from three different bioassays now indicate that a specific protein derived from cartilage inhibited the proliferation and migration of capillary endothelial cells in vitro and angiogenesis in vivo. These three related bioactivities copurified with collagenase inhibitory activity throughout the purification. This molecule was extracted from bovine scapular cartilage with 2 M NaCl and was purified by a series of precipitation and column chromatography steps; NH_2-terminal protein sequence of this cartilage-derived inhibitor (CDI) was determined (Fig. 1).

We measured the effect of CDI on the proliferation of capillary endothelial cells (ECs) in vitro. Capillary ECs proliferate in response to an angiogenic stimulus during neovascularization (*7*). By using the specific cells involved in angiogenesis and stimulating them with a known angiogenesis factor, acidic fibroblast growth factor (aFGF) (*8*),

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1	2	3	4	5	6	7	8	9	10
---	Thr	---	Val	Pro	Pro	His	Pro	Gln	Thr
11	12	13	14	15	16	17	18	19	20
Ala	Phe	---	Asn	Ser	Asp	Val	Val	Ile	Arg
21	22	23	24	25	26	27	28		
Ala	Lys	Phe	[Val]	Gly	Thr	Ala	Glu		

Fig. 1. NH_2-terminal protein sequence of cartilage-derived inhibitor. Automated Edman degradation of CDI was performed on an Applied Biosystems Model 477A protein sequencer with the use of the manufacturer's standard program NORMAL-1. The phenylthiohydantoin–amino acid fractions were identified with an on-line (ABI) Model 120A HPLC. A search of the NBRF-PIR protein sequence database (*25*) revealed that this protein differs from a collagenase inhibitor isolated from human amniotic fluid (which itself is virtually identical to that of a human skin fibroblast inhibitor with the exception of one residue difference) in only two amino acids, at position 17 (Val for Leu) and at position 27 (Ala for Pro) for 28 NH_2-terminal residues (*26*). Samples were not reduced and alkylated, therefore, the dashes (------) represent bona fide blanks that align by similarity with expected cysteine residues of previously reported collagenase inhibitors; all three residues are in agreement (*26, 27*). Sequence differences between this collagenase inhibitor and others (*23, 26, 27*) may be ascribed to species variations, variable forms of this protein in the same tissue (*28*), or both. Brackets indicate determination with less than full confidence.

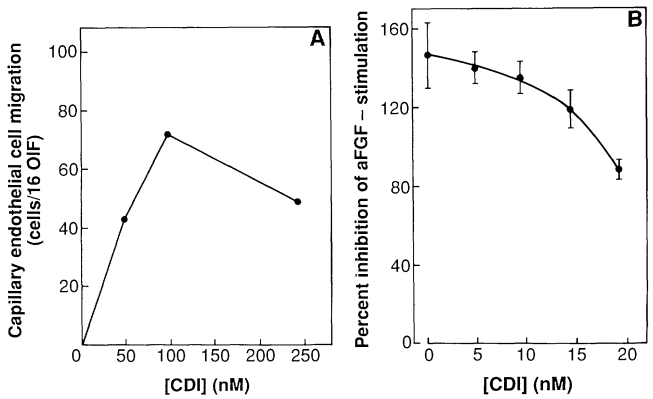


Fig. 2. (**A**) Inhibition of capillary EC proliferation by CDI. Capillary endothelial cells (2×10^3) in 0.2 ml were plated onto gelatin-coated 96-well tissue culture dishes (Nunc) on day 1. On day 2, cells were fed with Dulbecco's modified Eagle's medium (Gibco) with 5% calf serum (Hyclone) (DMEM/5) and aFGF (10 ng/ml) (FGF Company), and increasing concentrations of freshly purified CDI. Wells containing phosphate-buffered saline (PBS) (Gibco) alone and PBS + aFGF were included as controls. On day 5, media were removed and cells were washed with PBS and assayed for acid phosphatase activity (*12*). This assay exhibited a linearity between acid phosphatase activity and endothelial cell number up to 10,000 cells per well (*12*). We verified this linearity in the presence of the cartilage inhibitor and other inhibitors of capillary EC proliferation such as vitreous and platelet factor–4. Percent inhibition was determined by comparing wells exposed to stimulus with those exposed to stimulus and inhibitor. Each point represents the mean of quadruplicate control wells and triplicate inhibitor wells, and the SEM is less than 7% of the mean. (**B**) Effect of cartilage-derived angiogenesis inhibitor on capillary endothelial cell migration. Migration was measured with the use of blind well chambers (Neuroprobe, no. 025–187) and polycarbonate membranes with 8-μm pores (Nucleopore) coated with human fibronectin (6.67 μg/ml in PBS) (Cooper). Basic FGF (bFGF; 10 ng/ml; Takeda Company) diluted in DMEM/1 was added to the lower well. The upper wells received 2.5×10^4 capillary ECs and increasing concentrations of purified CDI (used within 24 hours of purification). Control wells receive DMEM/1, either with or without bFGF. The cells that had migrated through the membrane onto the lower surface were fixed, stained, and quantified by counting the number of cells on the lower surface in 16 oil immersion fields (OIF per well). Each point represents the mean ± SEM of four wells. In control wells without bFGF, the number of migrated capillary ECs was 61 ± 7 cells. IC_{50} values were 143, 38, and 8 μg/ml for CDI obtained at the A-1.5m, Bio-Rex 70, and Sephadex G-75 chromatography steps, respectively.

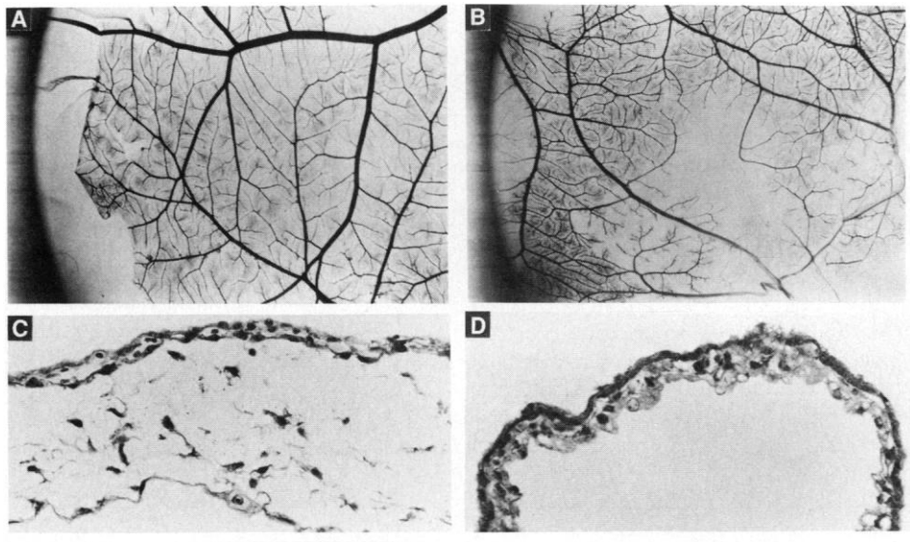
we could mimic the angiogenesis process in vitro. This assay can be used to demonstrate the stimulation of capillary EC proliferation by various angiogenic factors (*9*). Fractions enriched in collagenase inhibitory activity from column steps throughout the purification (Table 1) were monitored for their ability to inhibit aFGF-stimulated bovine adrenal cortex capillary endothelial cell proliferation. Collagenase has been shown to have a key role in the angiogenic process (*5, 10, 11*). We determined the number of capillary ECs in culture on the basis of the colorimetric measurement of cellular acid phosphatase (*12*). The collagenase inhibitory activity and the anti-proliferative activity copurified throughout the purification of CDI. Purified CDI was a potent inhibitor of aFGF-stimulated capillary EC proliferation; 96 nM caused 72% inhibition (Fig. 2A). Electronic cell counting supported these results.

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, pancreatic trypsin inhibitor, α_2-macroglobulin (a collagenase inhibitor), and chondroitin sulfate A (a glycosaminoglycan found in cartilage) did not have a significant effect (less than 20% inhibition or stimulation, at all doses tested) on growth factor–stimulated capillary EC proliferation, even when tested at concentrations of 50 μg/ml (*13*). These factors also do not inhibit angiogenesis in vivo. The inhibitor had no detectable inhibitory effect on the growth of nonendothelial cells, such as bovine aortic smooth muscle cells and Balb/c 3T3 cells. Preliminary experiments, conducted to determine the effect of CDI on capillary EC synthesis of DNA, indicated that the cartilage-derived angiogenesis inhibitor suppressed the incorporation of radioactive thymidine by capillary ECs in response to aFGF in a concentration-dependent manner with an IC_{50} (the concentration of inhibitor at which 50% inhibition is obtained) of approximately 300 nM (*14*).

Another component of the angiogenic process is the migration of capillary ECs in response to angiogenic stimuli (*7*). We studied the effect of CDI on capillary EC migration with a modification of the Boyden chamber technique (*15*). A blind-well Boyden chamber (*16*) consists of two wells (upper and lower) separated by a porous membrane. The lower wells received a known concentration of growth factor and the upper wells received a predetermined number of cells and CDI. Cells attach to the upper surface of the membrane, migrate through and attach to the lower membrane surface. The membrane was then fixed and stained for counting (*17*). An inhibitor of growth factor–stimulated capillary EC migration copurified with the collagenase in-

Table 1. Purification of CDI. CDI appeared as a single band of apparent relative molecular mass (M_r) of 27,650 as determined by SDS–polyacrylamide gel electrophoresis (PAGE) analysis followed by silver staining. This protein was purified approximately 1000-fold with the use of a modification of a previously reported method (*23*), and collagenase inhibitory activity was determined according to Johnson-Wint (*24*). Veal scapulae were obtained from Berliner and Marx, Inc., within 24 hours of slaughter and immediately frozen until use. They were then scraped free of muscle and connective tissue. CDI was purified with a combination of extraction in 2 M NaCl, precipitated with HCl and ammonium sulfate (25 to 60%), and analyzed by a series of chromatography steps: (i) gel filtration on A-1.5m Sepharose (Bio-Rad) in the presence of 4 M guanidine-HCl; (ii) ion exchange on a Bio-Rex 70 (Bio-Rad) cation exchange column; (iii) gel filtration on a Sephadex G-75 (superfine) (Pharmacia) column, and (iv) reversed-phase high-performance liquid chromatography (HPLC) on a Hi-Pore 304 column (Bio-Rad). A unit is defined as the amount of protein required to inhibit 1 unit of corneal collagenase by 50%. One unit of collagenase produced 10% cleavage of collagen in 2.5 hours at 37°C as described previously (*23*).

Step	Specific activity (units/mg)	Purification (-fold)
Concentrated extract	15.4	
Acid and $(NH_4)_2SO_4$ precipitation	55.6	3.6
A-1.5m	222.2	14.4
Bio-Rex 70	877.2	57.0
Sephadex G-75	2,824.9	183.4
Reversed-phase HPLC	14,084.5	914.6



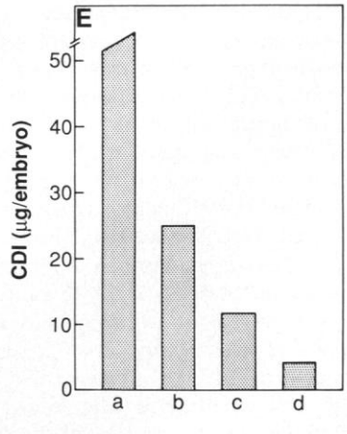


Fig. 3. Inhibition of angiogenesis by CDI. On day 3 of development, fertilized chick embryos were removed from their shells and placed in plastic petri dishes. On day 6, purified CDI (4 μg) was mixed in methylcellulose disks and applied to the surfaces of the growing CAMs above the dense subectodermal plexus. After a 48-hour exposure of the CAMs to CDI, India ink–Liposyn was injected intravascularly as described (*18*). (**A**) Normal CAM containing an empty methylcellulose disk, (**B**) CAM with a CDI-containing disk; 33% of the eggs tested in at least two separate sets of CAM assays of several different batches of cartilage starting material had avascular zones. (**C**) Histological sections of day 8 normal CAMs (×800) or (**D**) CDI-treated CAMs. Tissue specimens were fixed in formalin, rinsed in 0.1 M cacodylate buffer, pH 7.4, and embedded in JB-4 plastic (Polysciences) at 4°C. Three-micrometer sections were cut with a Reichert 2050 microtome, stained with toluidine blue, and micrographs were taken on a Zeiss photomicroscope with Kodak TM ×100 and a green filter. Fractions enriched in collagenase inhibitory activity from each successive purification step (Table 1) were also tested for anti-angiogenic activity in the CAM. These two activities copurified. (**E**) Bars represent the dose per embryo of CDI from each step required to obtain the same degree of inhibition as purified CDI. The bars on the graph indicate the following: (a) CDI from A-1.5m step, (b) CDI from Bio-Rex 70 step, (c) CDI from Sephadex G-75 step, and (d) purified CDI. The slanted line in (a) indicates that even at doses of 50 μg and higher, the percent inhibition equivalent to that of purified CDI was not achieved. Each of these bars (a to d) represents at least two separate sets of CAM assays conducted on separate weeks with CDI preparations from several different batches of starting material. A total of 108 embryos were tested. All tests were conducted double-blind.

hibitor throughout the purification. Purified CDI inhibits capillary EC migration with an IC_{50} of 16 nM (Fig. 2B).

To determine whether CDI could inhibit neovascularization in vivo, we tested it in the chick chorioallantoic membrane assay (CAM) (*18*) as a third bioassay (Fig. 3). Over 100 CAMs were used. Purified CDI (4-μg samples) in methylcellulose discs caused significant inhibition of embryonic capillaries in the yolk sacs, resulting in large avascular zones. In contrast, control CAMs implanted with empty methylcellulose discs never developed avascular zones. Histological studies of CDI-treated CAMs revealed a mesoderm that was thinner than normal and avascular (Fig. 3D) relative to controls. We monitored the anti-angiogenic activity of CDI throughout its purification using this CAM assay, and dose dilution curves were generated for the inhibitor from each successive purification step through homogeneity. The increasing potency of the angiogenesis inhibitor with its increasing purification is shown in Fig. 3E. The cartilage-derived inhibitor is a potent inhibitor of angiogenesis, since inhibition was observed with as little as 4 μg. The lowest reported doses of described angiogenesis inhibitors tested alone in the CAM assay are 50 μg of protamine (*18*), 200 μg of bovine vitreous extract (*19*), and 10 μg of platelet factor–4 (*18, 20*). The lowest reported doses of angiogenesis inhibitors effective as combinations include heparin (50 μg) and hydrocortisone (60 μg) (*2*), and β-cyclodextrin tetradecasulfate (14 μg) and hydrocortisone (60 μg) (*2*).

Our data identify a single, tissue-derived macromolecule, CDI, as a potent inhibitor of angiogenesis. In vitro studies with this inhibitor indicate that it also negatively modulates two key components of the angiogenic process, the proliferation and migration of capillary endothelial cells. Because this angiogenesis inhibitor is also a collagenase inhibitor, additional interpretations of earlier studies may now be possible. For example, in 1978, platelet factor–4 was described as a collagenase inhibitor (*21*). Subsequently, several research groups have tested this factor for its ability to inhibit angiogenesis because of its high affinity for heparin and have found that it was anti-angiogenic (*18, 20*) and inhibited capillary EC proliferation (*13*). Our studies suggest that the collagenase inhibitory activity of platelet factor–4 may have a role in inhibiting neovascularization.

This study supports earlier work in which the importance of collagenase in the invasiveness of capillary endothelial cells during angiogenesis was demonstrated (*11, 22*). It may also help to explain why tissues such as cartilage are resistant to invasion and may contribute to a broader understanding of the control of vascular proliferation.

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Fibroblast Growth Factor Receptor Is a Portal of Cellular Entry for Herpes Simplex Virus Type 1

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Herpes simplex virus type 1 (HSV-1) is a ubiquitous pathogen responsible for considerable morbidity in the general population. The results presented herein establish the basic fibroblast growth factor (FGF) receptor as a means of entry of HSV-1 into vertebrate cells. Inhibitors of basic FGF binding to its receptor and competitive polypeptide antagonists of basic FGF prevented HSV-1 uptake. Chinese hamster ovary (CHO) cells that do not express FGF receptors are resistant to HSV-1 entry; however, HSV-1 uptake is dramatically increased in CHO cells transfected with a complementary DNA encoding a basic FGF receptor. The distribution of this integral membrane protein in vivo may explain the tissue and cell tropism of HSV-1.

THE PATHWAY FOR HERPES SIMPLEX virus type 1 (HSV-1) entry into vertebrate cells remains undefined. The putative sequence of events in herpes infection involves initial virion attachment to the cell surface through an interaction with heparin-like cell-associated glycosaminoglycans (GAGs) (*1*), fusion of the viral envelope with the plasma membrane (*2, 3*), removal of the envelope (*4*), and release of the viral nucleocapsid into the cytoplasm of the cell (*5*). The process of viral entry involves specific viral-associated glycoproteins as well as target cell binding sites and receptors (*3*); however, no specific receptor has been described for HSV-1. In contrast, several other viruses use binding sites for known physiological ligands. For example, rhinovirus, an