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A Metalloproteinase Inhibitor as an Inhibitor of Neovascularization

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Abstract Metalloproteinases and their endogenous inhibitors are key components of an enzyme system which is important in a number of fundamental biochemical and cellular processes. Our recent work has focused on the role of a particular metalloproteinase, collagenase, and the role of an endogenous inhibitor of this enzyme in the control of neovascularization. The proteolytic degradation of extracellular matrix components by capillary endothelial cells (EC) has been shown to be one of the key prerequisites of the angiogenic process. As part of a study of the effect(s) of the inhibition of collagenase on neovascularization, we have recently reported the purification, characterization and partial NH_2-terminal sequence of a cartilage-derived inhibitor (CDI) of angiogenesis in vivo and in vitro. Evidence is presented which suggests that one means of controlling deregulated vascular growth characteristic of a number of "angiogenic diseases" may be at the level of the control of metalloproteinase activity.

Key words: collagenase, collagenase inhibitor, endothelial cell, angiogenesis, vascular growth control, cell proliferation, cell migration, tumor growth

Proteolysis is a key component of many important biochemical and physiological events, such as embryonic morphogenesis, trophoblast implantation, tissue remodelling, angiogenesis, and bacterial and parasitic invasion [for further review see 1]. Like neovascularization, proteolysis has been described as being limited in duration and strictly regulated under normal conditions [1]. Under pathological conditions, however, these regulatory controls fail. For example, deregulated proteolytic degradation has now been causally related to malignant conversion, that stage in cancer progression when tumorigenic cells acquire the ability to invade and metastasize [1,2].

Increased attention has been focused on the matrix metalloproteinase family of proteolytic enzymes. These enzymes have been broadly subdivided into three categories on the basis of substrate specificity: the interstitial collagenases, the Type IV collagenases (gelatinases), and the stromelysins [for further review see 3]. The degradation of extracellular matrix barriers which is a prerequisite of those physiological events mentioned above involves the proteolytic activity of a number of enzymes, the most important of which is the collagenase family of metalloproteinases [4].

Like other enzyme families, the metalloproteinases are a key component of a system of "balanced proteolysis" wherein an equilibrium exists between the amount of active enzyme and its endogenous proteinase inhibitor(s). These natural metalloproteinase inhibitors comprise a family of proteins which are in many cases structurally similar and in most cases, functionally similar. This family of inhibitors can be subdivided into two groups based on significant sequence similarities (Table I). One group, the first member of which is TIMP-1 (tissue inhibitor of metalloproteinase-1), whose purification was reported in 1983 from the conditioned media of human skin fibroblasts [5], also includes CDI (cartilage-derived inhibitor), a cartilage-derived collagenase inhibitor [6], reported in 1990 to share significant sequence similarity with TIMP-1 [7,8], and the 21-kDa protein, an avian metalloproteinase inhibitor whose NH_2-terminal amino acid sequence was reported in

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TABLE I. NH_2-Terminal Sequence of Metalloproteinase Inhibitors*

	5 10 15
CDI[a]	——-Thr——-Val-Pro-Pro-His-Pro-Gln-Thr-Ala-Phe——Asn-Ser
Human TIMP[b]	Cys-Thr-Cys-Val-Pro-Pro-His-Pro-Gln-Thr-Ala-Phe-Cys-Asn-Ser
Murine TIMP[c]	Cys-Ser-Cys-Ala-Pro-Pro-His-Pro-Gln-Thr-Ala-Phe-Cys-Asn-Ser
Bovine TIMP[d]	ND-Thr-ND-Val-Pro-Pro-His-Pro-Gln-Thr-Ala-Phe-ND-Asn-Ser
21-kDa protein[e]	ND-Thr-Cys-Val-Pro-Ile-His-Pro-Gln-Asp-Ala-Phe-Cys-Asn-Ser
BCDI[f]	Cys-Ser-Cys-Ser-Pro-Val-His-Pro-Gln-Gln-Ala-Phe-Cys-Asn-Ala
Human TIMP-2[g]	Cys-Ser-Cys-Ser-Pro-Val-His-Pro-Gln-Gln-Ala-Phe-Cys-Asn-Ala
Bovine MI[h]	ND-Ser-ND-Ser-Pro-Val-His-Pro-Gln-Gln-Ala-Phe-ND-Asn-Ala
	20 25
CDI	Asp-Val-Val-Ile-Arg-Ala-Lys-Phe-*Val*-Gly-Thr-Ala-Glu
Human TIMP	Asp-Leu-Val-Ile-Arg-Ala-Lys-Phe-Val-Gly-Thr-Pro-Glu-Val
Murine TIMP	Asp-Leu-Val-Ile-Arg-Ala-Lys-Phe-Met-Gly-Ser-Pro-Glu-Ile
Bovine TIMP	Asp-Val-Val-Ile-Arg-Ala-Lys-Phe-Val-Gly-Thr-Ala-Glu-Val
21-kDa protein	Asp-Ile-Val-Ile-Arg-*Ala*-Lys-Val-*Val-Gly-Lys-Lys-Ile-Met*
BCDI	Cys-Ile-Val-Ile-Arg-Ala-Lys-Ala-Val-Asn-Lys-Lys-Glu-Val
Human TIMP-2	Asp-Val-Val-Ile-Arg-Ala-Lys-Ala-Val-Ser-Glu-Lys-Glu-Val
Bovine MI	Asp-Ile-Val-Ile-Arg-Ala-Lys-Ala-Val-Asn-Lys-Lys-Glu-Val

*Italicized residues are those determined at less than full confidence. Dashes represent bona fide blanks (samples not reduced or alkylated before sequencing) which align by similarity with expected cysteine residues of previously reported metalloproteinase inhibitors. ND, not determined.

[a]Ref. 6
[b]Ref. 7, 8.
[c]Ref. 41.
[d]Ref. 12.
[e]Ref. 9.
[f]Ref. 10.
[g]Ref. 11.
[h]Ref. 12.

1991 to be > 60% identical to a consensus sequence of TIMP [9]. Another group, the first member being BCDI (bovine cartilage-derived inhibitor), reported in 1986 as a specific mammalian collagenase inhibitor purified from bovine scapular cartilage [10] and found to share 65% amino terminal sequence similarity with TIMP-1, also includes TIMP-2, purified and characterized from the conditioned media of human melanoma cells in 1989 [11]. TIMP-2 was reported to be highly homologous to BCDI [11] (approximately 86%) over the first 45 amino acids and is now considered to be the human homolog of BCDI. Another member of this group is MI (metalloproteinase inhibitor), purified from the conditioned media of bovine aortic endothelial cells and whose sequence was reported in 1990 to be nearly identical to BCDI and TIMP-2 [12]. Table I compares the NH_2-terminal sequences of the best characterized metalloproteinase inhibitors in these two groups. There are also a number of less well-characterized, lower molecular weight metalloproteinase inhibitors which await complete purification and amino acid sequence analysis. Statistical analysis of amino acid composition data by Staskus et al. [9], using the Cornish-Bowden index, SΔn [13], also defines two distinct groups of tissue inhibitors of metalloproteinases, one representing the TIMP-1 group and the second representing the BCDI/TIMP-2 group. As first noted by Murray and coworkers [10] in their comparison of BCDI and TIMP and extended by Staskus and coworkers to include a larger number of metalloproteinase inhibitors, (CDI was not analyzed in this study), these two groups of inhibitors do not appear to be related to each other as judged by SΔn as a measure of protein similarity despite the similarities in NH_2-terminal sequences [9].

As mentioned above, proteolysis is an important prerequisite for a number of important physiological events, one of which is angiogenesis, the process of new capillary formation. With this in mind, we became interested in studying the effect(s) of metalloproteinase inhibition on the "process" of angiogenesis. During angiogen-

esis, capillary endothelial cells (EC) comprising the parent venule are stimulated by an angiogenic stimulus to produce proteolytic enzymes, such as collagenase and plasminogen activator, which facilitate EC release via the degradation of the venule's basement membrane. Proteolytic activity is also required for the migration of EC into the perivascular stroma. These events are followed by EC sprout extension and subsequent lumen formation [14]. As is EC "escape" from the parent venule, capillary sprout elongation, lumen formation, and EC migration are all events which are dependent on a shift in the proteolytic balance in favor of enzymatic activity [15–17]. Vascular morphogenesis and invasion are also regulated by shifts in the balance of proteases and their inhibitors [18,19].

To the extent that proteolysis is an important component of the angiogenic process, it can be argued that inhibitors of proteolytic activity should inhibit neovascularization. Indeed, early studies of cartilage, an avascular, tumor-resistant tissue, strongly suggested that protease inhibitors might be responsible for its avascularity.

These studies began with the work of Eisenstein and coworkers, who reported that of a larger number of tissue samples implanted on the CAM (chick chorioallantoic membrane), only cartilage failed to become vascularized [20]. This group further showed that extracts of cartilage inhibited the proliferation of cultured aortic endothelial cells [21]. Sorgente and coworkers demonstrated that cartilage lost its resistance to vascular invasion when extracted with 1 M guanidine and that these guanidine extracts contained protease inhibitory activity [22]. Brem and Folkman showed in 1975 that pieces of native cartilage could inhibit tumor-induced neovascularization, in contrast to denatured cartilage samples, which did not [23]. Langer and coworkers next reported that cartilage extracts containing a collagenase inhibitor inhibited tumor-induced neovascularization when administered both locally and systemically [24,25]. This group went on to suggest that this anti-angiogenic activity was responsible for the resistance of cartilage to vascular invasion. Takigawa, Shirai, and coworkers reported that extracts of cartilage and chondrocyte conditioned media contain an inhibitor of angiogenesis [26,27]; however, it is not known whether or not these extracts contained protease inhibitory activity.

Work began to focus on the purification of protease inhibitors from cartilage. Kuettner and coworkers showed that bovine nasal cartilage contained inhibitory activities against trypsin and mammalian collagenase [28]. Roughley et al. [29] recovered a collagenase inhibitory activity in a 22,000 Mr fraction purified from the same source in 1978. Later, Pauli et al. reported that cultured osteosarcoma cells could invade cartilage only after it had been guanidine-extracted and that this invasion could be prevented by the re-addition of this "anti-invasion" extract. They proposed that the resistance of cartilage to invasion was regulated, at least in part, by tissue-derived proteinase inhibitors [30]. The first purification and characterization of a collagenase inhibitory activity from cartilage was in 1986 by Murray and coworkers, who purified and obtained amino terminal sequence of a bovine scapular cartilage-derived collagenase inhibitor (BCDI) which shares 65% sequence similarity with a TIMP-1 over the first 45 amino acids [10]. Gabrielides and Barreau purified another collagenase inhibitor (Mr 19000) from the same source in 1987 [31]. Collagenase inhibitors have also been isolated from the culture medium of cartilage tissue and chondrocytes [32–35].

Taken together, these studies provided strong evidence that cartilage contained powerful proteinase inhibitors which could be responsible for its avascularity and which would support the choice of cartilage as a potential source of angiogenesis inhibitors. Despite these important bioactivities found in cartilage, no single tissue-derived macromolecule was shown to be an active anti-angiogenic factor.

As mentioned earlier, it had been reported in 1980 that cartilage extracts containing a collagenase inhibitor could inhibit tumor growth and angiogenesis in rabbits bearing corneal implants of V2 carcinoma and in mice which carried subconjunctival B16 melanoma implants. Most interesting was the observation that the cartilage factor(s) utilized in these experiments did not interfere with the growth of the tumor cell populations directly. The inhibitor did not retard the growth of either tumor cell type in culture, even at concentrations as high as 1 mg/ml [25]. Before vascularization, both treated and untreated tumors grew slowly in two dimensions, suggesting that the tumor cells themselves were not affected by the inhibitor. In addition, histological sections showed non-necrotic tumor cells present in the treated mice and rabbits. These

data suggested that the inhibitor acted to prevent tumor growth by inhibiting angiogenesis, rather than directly on the tumor itself [25].

With this hypothesis in mind, and in light of the critical importance of regulated proteolysis in the process of neovascularization, we chose to directly study the role of a cartilage-derived collagenase inhibitor (CDI) in the regulation of angiogenesis. Our laboratory reported the purification, characterization, and partial amino terminal sequence of this protein in 1990 [6]. CDI was purified to homogeneity by a series of extraction and precipitation steps followed by a series of chromatography steps, including gel filtration in the presence of 4M guanidine-HCl, ion-exchange, a second gel filtration step, and finally reverse phase HPLC. NH_2-terminal sequence data was obtained for the first 28 amino acids and is shown in Table I.

We began our studies of the effect of CDI on neovascularization by first studying its effect on endothelial cell (EC) proliferation. In order to best mimic the process of neovascularization in vitro, capillary EC were utilized as the cells of choice and these cells were stimulated with known angiogenesis factors, in this case either acidic or basic fibroblast growth factor (aFGF or bFGF). When the effect of CDI on capillary EC proliferation was analyzed using an assay which determines the number of endothelial cells in culture on the basis of the colorimetric measurement of acid phosphatase [36], we found that CDI was a potent inhibitor of EC growth, suppressing growth factor-stimulated EC proliferation by 72% at a concentration of 96 nM. This assay also provided the unique opportunity to screen large numbers of column fractions from each purification step for anti-proliferative activity in a reliable and sensitive manner. Importantly, we found that the antiproliferative activity of CDI copurified with the anti-collagenase activity of this protein throughout the purification of CDI. These results were supported by electronic cell counting assays. We also found that CDI suppressed the incorporation of tritiated thymidine by capillary EC in response to aFGF in a concentration-dependent manner with an IC_{50} of 300 nM.

To study the specificity of CDI's antiproliferative effect, a number of other substances were tested for their ability to inhibit EC proliferation. Enzyme inhibitors, such as trypsin ovoinhibitor, pancreatic trypsin inhibitor, alpha 2-macroglobulin, and chondroitin sulfate A (a cartilage glycosaminoglycan) did not have a significant effect on FGF-stimulated EC growth. Even when tested at concentrations of 50 μg/ml, these factors are not angiogenesis inhibitors in vivo. Preliminary studies also suggest that CDI is an endothelial cell specific inhibitor having no detectable inhibitory effect on aortic smooth muscle cells or Balb/c 3T3 cells.

We next examined the effect of CDI on growth factor-stimulated capillary EC migration, another key component of the angiogenic process. Utilizing a modification of the Boyden chamber technique, we screened each of the purification steps of CDI for an anti-migration activity. We found that the anti-collagenase activity of CDI copurified with a potent inhibitor of FGF-stimulated capillary EC migration throughout the purification of CDI. Purified CDI inhibited EC migration with an IC_{50} of 16 nM.

To study the effect of CDI on neovascularization in vivo, it was first tested in the CAM assay. Picomolar amounts of purified CDI implanted in methylcellulose discs caused large avascular zones (Fig. 1a) on the CAM surface in contrast to the control CAMs which never developed avascular zones (Fig. 1b). Dose response curves generated using CDI obtained from each step of the purification protocol demonstrated that CDI's anti-collagenase and anti-angiogenic activities copurified. Histological studies of CDI-treated CAMs revealed a mesoderm that was thinner than usual and avascular relative to controls. CDI is a very powerful inhibitor of angiogenesis when compared with the lowest reported doses of other described angiogenesis inhibitors, both those tested alone as well as those tested in conjunction with other factors [6]. These results were particularly striking because they represented, to our knowledge, the first demonstration that a collagenase inhibitor, with no other known biological activity, could inhibit neovascularization in vivo.

In light of the observation that CDI could inhibit embryonic neovascularization, this protein was next tested for its ability to inhibit tumor-induced neovascularization in vivo. This was accomplished by using the rabbit corneal pocket model, in which V2 carcinoma was used as the angiogenic stimulus. Capillary growth towards the tumor was significantly inhibited by 40 μg of highly purified CDI in this in vivo model [37].

The results of these studies on the anti-angiogenic effect of CDI make it tempting to

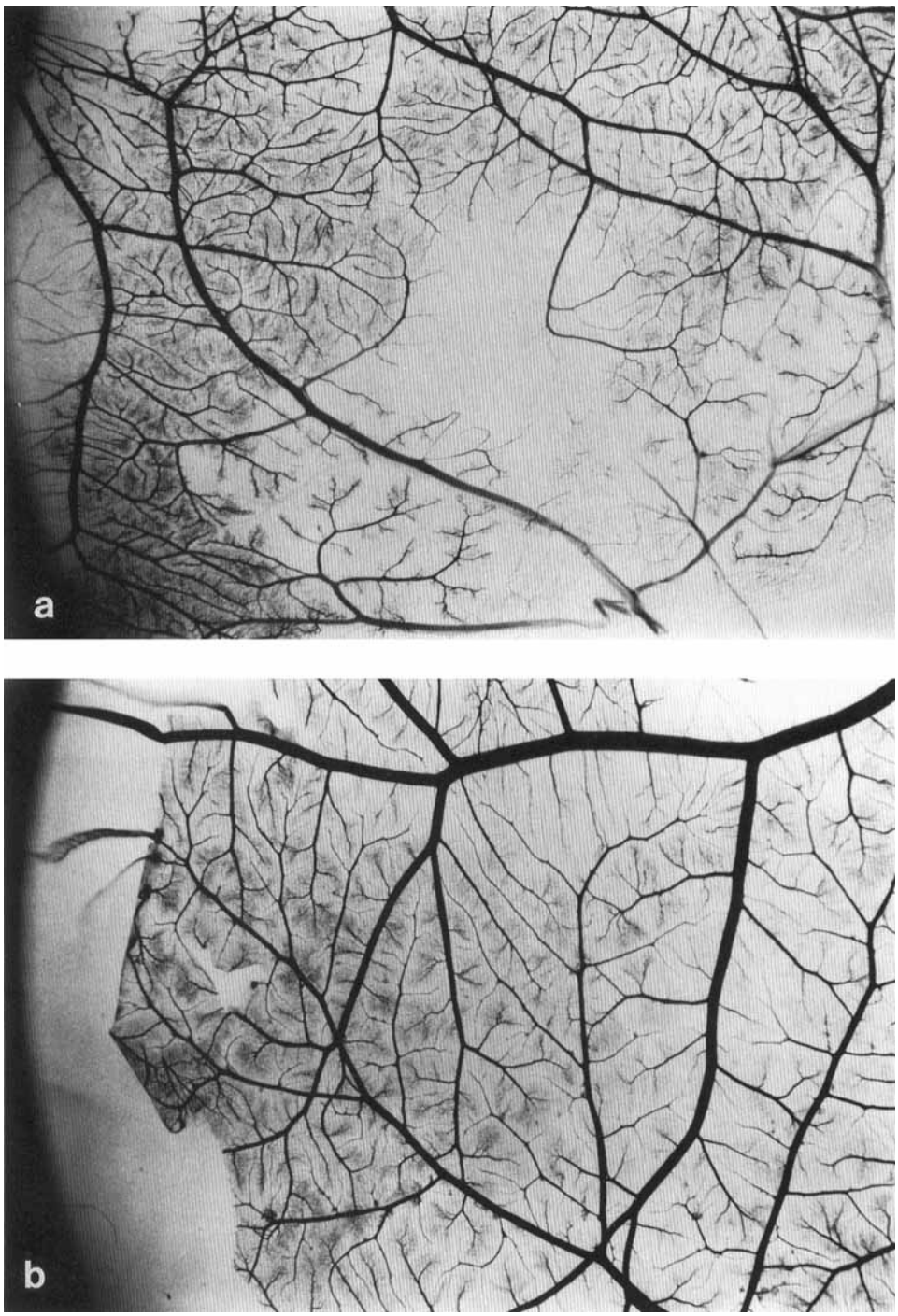

Fig. 1. Inhibition of angiogenesis by CDI. Avascular zone caused by picomolar concentrations of CDI implanted in methylcellulose disc (**a**) and normal control (**b**) implanted with blank methylcellulose disc. CAMs were injected intravascularly with India ink. Reprinted from Moses et al. [6] with permission of Cell Press. Copyright 1990 by AAAS.

speculate that CDI is the inhibitory factor responsible for the inhibition of neovascularization by cartilage first observed nearly twenty years ago, as well as for maintaining the distinctive avascularity of cartilage and its relative tumor resistance. Cartilage has, in fact, been shown to contain a number of key molecules important in the regulation of neovascularization, including at least two related, but distinct, metalloproteinase inhibitors, CDI and BCDI, as

well as CDGF, the cartilage-derived growth factor since shown to be basic FGF [38] and TGF-β [39]. Future studies with these purified factors should elucidate the relationship between these and other modulators of angiogenesis and their respective roles in maintaining the balance which results in the distinct avascular state of cartilage under normal conditions. Furthermore, these results demonstrate an important role for metalloproteinases in the modulation of neovascularization and suggest that one means of controlling the deregulated vascular growth characteristic of a number of "angiogenic diseases" [40] may be at the level of the control of metalloproteinase activity.

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