

Stimulation of angiogenesis through cathepsin B inactivation of the tissue inhibitors of matrix metalloproteinases

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Abstract The tissue inhibitors of matrix metalloproteinases (MMPs), TIMP-1 and TIMP-2, are also angiogenesis inhibitors. Cathepsin B and MMPs are found at sites of neovascularization in pathologies such as cancer and osteoarthritis. Treatment of TIMP-1, TIMP-2, and of a mixture of both inhibitors from human articular chondrocytes with cathepsin B resulted in their fragmentation, whereby they lost their MMP-inhibitory and anti-angiogenic activities. Our data suggest that, besides directly participating in tissue destruction, cathepsin B can be harmful for two further reasons: it raises the activity of the MMPs also in the absence of mechanisms up-regulating these enzymes, and it stimulates angiogenesis. This is a prerequisite for blood vessel invasion in a variety of pathological situations of which cancer and osteoarthritis are prominent examples.

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1. Introduction

Angiogenesis is a pivotal step in tumor growth and metastasis, and in non-neoplastic situations such as ocular, myocardial, intestinal, reproductive and rheumatic diseases [1]. Neovascularization depends on angiogenic factors and extracellular proteolysis by matrix metalloproteinases (MMPs), which are regulated by the tissue inhibitors of matrix metalloproteinases (TIMPs), a family of proteins consisting so far of four members, TIMP-1 to TIMP-4 [2]. TIMP-1 and TIMP-2 are multifunctional proteins, which not only exhibit a broad specificity of inhibition against the MMPs, but also function as growth factors and as inhibitors of angiogenesis [3]. The avascularity of articular cartilage and the characteristic resistance of this tissue to invasion by tumors and blood vessels have been attributed to the presence of anti-angiogenic factors [4], which are at the same time inhibitors of collagenase [5]. Such factors have been isolated from cartilage [6] and cultured chondrocytes [7,8], and found to belong to the TIMP family [2,8]. Cartilage neovascularization is necessary for its mineralization and osteophyte formation [9], a complicating feature

in osteoarthritis, the major cause of rheumatic disability in humans.

Here we show that the cysteine peptidase cathepsin B, an important mediator of tissue degradation in tumor invasion [10–13] and osteoarthritis [14,15], inactivates TIMP-1 and TIMP-2. This suggests a new adverse role for cathepsin B because it shares with the MMPs the same pathological sites of action.

2. Materials and methods

2.1. Preparation and assay of crude collagenase from human skin fibroblast

Normal human skin was processed by standard methods to isolate fibroblasts. The cells were plated at a density of 5×10^6 cells in 75 cm² culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, and cultured until they reached confluence. Thereafter the medium was removed and the cells washed with phosphate-buffered saline. Serum-free DMEM containing 10 ng/ml of human recombinant interleukin-1 β was then added. The conditioned media were collected after 48 h, cleared by centrifugation, and stored frozen at -20°C in aliquots. Acid-soluble calf skin collagen (Sigma, St. Louis, MO, USA) was acetylated with ¹⁴C-acetic anhydride and collagenase activity determined by a scaled-down variant of the diffuse fibril collagenase assay [16].

2.2. Collagenase inhibitory activity from cultured chondrocytes

Chondrocytes were prepared from pooled normal human knee and femoral head articular cartilage [15]. Primary cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 media containing 3% fetal calf serum. These conditions were sufficient for preserving the differentiated phenotype [15] during the experiment time as monitored by the secretion of cathepsin B, a marker of the dedifferentiated chondrocyte [17]. Adherent cells were rinsed twice with serum-free DMEM/F-12 and further cultured in this medium. After 48 h, the conditioned media were collected and fresh serum-free medium was added to the cells. The procedure was repeated twice at 48 h intervals. The collected media were immediately supplemented with 1 μM of 3,4-dichloroisocoumarin and 1 μM of *trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane (E-64), final concentrations, to block serine and cysteine endopeptidase activities. After exhaustive dialysis against doubly distilled water, the media were lyophilized. These lyophilisates were reconstituted in 0.05 M Tris-HCl buffer, pH 7.60, containing 0.2 M NaCl and 1 mM CaCl₂, and 100 μl portions were applied to a Superdex 75 HR 10/30 gel chromatography column (Pharmacia, Uppsala, Sweden) equilibrated and run with the same buffer at a flow rate of 0.5 ml/min in a fast protein liquid chromatography apparatus. Fractions of 250 μl were collected and assayed for collagenase inhibitory activity as described above. Fractions containing collagenase inhibitory activity from several runs were pooled, concentrated and washed against H₂O using an ultracentrifugal filter with a molecular mass cut off 10 kDa. Following 12.5% SDS-PAGE under both reducing and non-reducing conditions of the concentrated fractions containing collagenase inhibitory activity, proteins were transferred to nitrocellulose membranes. Immunoblotting was carried to reveal human TIMP-1 and TIMP-2. The primary antibodies were monoclonal mouse anti-human TIMP-1 antibodies and polyclonal rabbit anti-human TIMP-2 antibodies (NMI, San Diego, CA, USA). The antibodies to TIMP-1 preferentially recognized the antigen

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Abbreviations: CAM, (chick) chorioallantoic membrane; CA-074, N-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; DMEM, Dulbecco's modified Eagle's medium; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases

under non-reducing conditions, while those to TIMP-2 optimally bound the antigen under reducing conditions. The enhanced chemiluminescence immunoblotting, light emitting, non-radioactive method (ECL) from Amersham Life Science (Little Chalfont, UK) was used for detection of the immobilized primary antibodies conjugated with horseradish peroxidase-labeled secondary antibodies. The antibodies described above were also used for treating the enriched collagenase inhibitory fraction from human articular chondrocyte cultures, followed by precipitation with protein G-Sepharose (Pharmacia).

2.3. Angiogenesis assays

On day 3 of development, fertilized chick embryos were removed from their shells, placed in plastic Petri dishes, and maintained in a humidified incubator under 5% CO₂ at 37°C. On day 5 of development, 6 µl of the samples to be tested (or the same volume of sterile water as control) was applied to a sterile Whatman filter (3 mm diameter) and placed on the surface of the growing chorioallantoic membrane (CAM). After 48 h exposure of the CAMs to samples, the filters were removed, results evaluated under a binocular microscope and the images registered digitally. The response was evaluated as positive or negative depending on the presence or absence of newly formed small capillaries using 10 eggs for each experiment.

2.4. Degradation of TIMPs by cathepsin B

The mature form of cathepsin B was affinity-purified from human kidney [18] and was free of other enzymes as ascertained by electrophoresis, specificity tests with substrates and inhibitors, and N-terminal sequencing. Cathepsin B (containing the pro-form and the single-chain form) was also prepared from conditioned media of dedifferentiated human articular chondrocytes by gel chromatography [12] followed by ion exchange chromatography on DEAE-Sepharose (Pharmacia). Human recombinant, non-glycosylated TIMP-1 whose Asn-30 and Asn-78 were mutated to Ala was expressed in CHO K1 cells and purified on MacroPrep 50Q anion exchange resin (Bio-Rad), Green A Dymatex (Amicon) and Sephacryl S-200 (Pharmacia). Recombinant human, glycosylated TIMP-1 was expressed in CHO K1 cells and purified using anti-(human TIMP-1) affinity chromatography [19]. Human TIMP-2 was purified from the medium of uterine cervical fibroblasts [20]. The incubation solutions in 50 mM sodium acetate buffer, 2 mM dithiothreitol, 2 mM EDTA, pH 5.50, contained cathepsin B (5 mg/ml final concentration) and recombinant wild-type or non-glycosylated TIMP-1 (final concentrations in different experiments 100–160 µg/ml), or the same cathepsin B concentration and TIMP-2 (final concentrations in different experiments 25–57 mg/ml). Incubation was performed at 30°C, reaction was stopped at time intervals by making the solution 2 mM with iodoacetic acid, and the progressive disappearance of the original protein band followed by SDS-PAGE. After 5 h of incubation the original proteins were completely digested giving a mixture of peptides. Control experiments included the addition of the cathepsin B-specific inactivator CA-074 at a final concentration of 1 mM. MALDI-TOF spectra of TIMP-1 and TIMP-2 peptides were accumulated using a Voyager Elite instrumentation (PerSeptive Biosystems, Framingham, MA, USA) with α -cyano-4-hydroxy-cinnamic-acid (5 mg/ml in 50% acetonitrile and 0.1% trifluoroacetic acid in water) as the matrix. Samples were analyzed in pulsed extraction reflectron mode using an acceleration voltage of 20 kV, a pulse delay time of 75 ns, a grid voltage of 55% and a guide

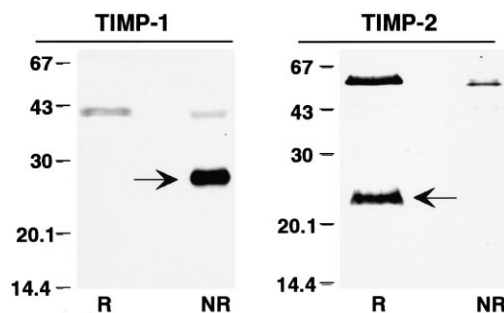


Fig. 1. Presence of TIMP-1 and TIMP-2 in the conditioned media of human articular chondrocytes. Concentrated spent culture medium was chromatographed on Superdex 75 HR, and fractions containing collagenase inhibitory activity in the molecular mass range 18–30 kDa were pooled and concentrated. After 12.5% SDS-PAGE under reducing (R) and non-reducing (NR) conditions, immunoblot analysis was carried out with antibodies to TIMP-1 and to TIMP-2. Numbers and arrows indicate the molecular masses (kDa) of standards and the TIMP bands, respectively. The bands at M_r higher than 30 000 were due to non-specific binding of the secondary antibody.

voltage of 0.05%. Peptide sequences matching the determined molecular masses were calculated with the software PAWS (Protein Analysis WorkSheet) of ProteoMetrics (<http://www.proteometrics.com>).

3. Results

3.1. Enrichment of a collagenase inhibitory fraction from articular chondrocytes

Conditioned media of primary cultures of human articular chondrocytes were fractionated by gel chromatography. Fractions showing collagenase inhibitory activity were found in the molecular mass range 18–30 kDa. After SDS-PAGE under both non-reducing and reducing conditions of pooled fractions containing collagenase inhibitory activity, immunoblotting with anti-human TIMP-1 and TIMP-2 antibodies revealed the presence of both MMP inhibitors. The anti-human TIMP-1 antibodies recognized a band with an apparent M_r of about 27 000 under non-reducing conditions (Fig. 1), which corresponds to glycosylated TIMP-1, whereas no epitopes were recognized after reduction of the antigen. Conversely, the anti-human TIMP-2 antibodies reacted with the partially purified collagenase inhibitory activity preferentially under reducing conditions and detected a band at 24 kDa (Fig. 1). The apparently higher molecular mass of TIMP-2 under reducing conditions (24 kDa) instead of the expected

Table 1
Peptides recovered after complete digestion of human TIMP-1 by human cathepsin B

Mass (D)	Matching peptides
5004.2 ± 1.5	T[26–68]S; H[77–120]Y
2919.9 ± 0.9	V[24–47]K
2576.4 ± 1.0	Y[120–143]T; T[128–150]Q
2378.5 ± 0.7	Q[36–55]A
2025.6 ± 0.8	A[51–69]V; L[152–169]R

The peptide molecular masses were determined by MALDI-TOF spectrometry, and peptide fragments matching the masses were calculated with the PAWS program. The sequence below (amino acids 1–184) represents the whole molecule of human TIMP-1.

1	CTCVPHPQT AFCNSDLVIR AKFVGTPENV QTTLYQRYEI KMTKMYKGFQ	50
51	ALGDAADIRF VYTPAMESVC GYFHRSHNRS EEFLIAGKLQ DGLLHITTCs	100
101	FVAPWNSLSL AQRRGFTKTY TVGCCECTVF PCLSIPCKLQ SGTHCLWTDQ	150
151	LLQGSEKGFQ SRHLACLPRE PGLCTWQSLR SQIA	184

value of 22 kDa under non-reducing conditions has been reported [8].

3.2. Anti-angiogenic properties of TIMP-containing fractions from articular chondrocytes

To determine if the collagenase inhibitory fraction from conditioned media of human articular chondrocytes was capable of inhibiting angiogenesis *in vivo*, the CAM assay was used. Fig. 2b shows a significant inhibition of embryonic neovascularization in the zone underneath the filter. This effect was observed in all of the eggs tested, while control CAMs developed vascular zones (Fig. 2a). Inhibition of capillary formation by human recombinant TIMP-1 and purified TIMP-2 was tested under the same conditions. TIMP-1 had no appreciable effects in inhibiting neovascularization of the CAM (Fig. 2c) in 80% of the eggs tested, and in 20% of them a moderate effect was seen. In contrast, TIMP-2 inhibited angiogenesis in all of the samples tested (Fig. 2d), thus reinforcing the concept that it is a major inhibitor of vascularization [2]. TIMP-1 and TIMP-2 seem to play different roles in angiogenesis. While the primary function of TIMP-1 is inhibition of MMPs used by endothelial cells for tissue invasion (this property may barely be observable in the CAM assay), TIMP-2 mainly acts as an inhibitor of endothelial cell proliferation [2]. The collagenase inhibitory fraction from chondrocyte conditioned media was separately treated with antibodies against TIMP-1 and TIMP-2 and then subjected to CAM assays (not shown). Immunoprecipitation of TIMP-1 completely abolished the already modest anti-angiogenic activity, while treatment with antibodies against TIMP-2 inhibited vascularization in 50% of the eggs and weakened the process in the remaining samples. The lack of total precipitation of TIMP-2 was due to the fact that the antibodies to this protein preferentially reacted with reduced TIMP-2. However, this experiment qualitatively shows that, in the conditioned media of articular chondrocytes, TIMP-2 was a species able to inhibit angiogenesis.

3.3. Inactivation of TIMPs by cathepsin B

Upon incubation of human TIMP-1 and TIMP-2 with hu-

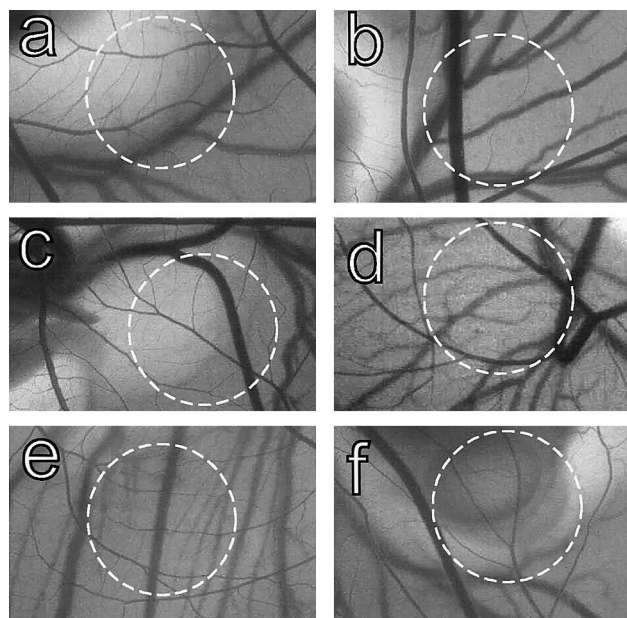


Fig. 2. Anti-angiogenic activity demonstrated with the CAM assay. The dashed circles (3 mm diameter) show the area where a sterile Whatman filter, impregnated with 6 µl of sample, was placed on the fifth day of development of a fertilized chick embryo. The filter was removed after 48 h for evaluation. a: Control with water. b: Collagenase inhibitory fraction from the spent culture medium of human articular chondrocytes. c: 5.2 µg of human recombinant TIMP-1. d: 0.7 µg of human purified TIMP-2 (the fuzzy visible capillaries represent microvessels which are out of focus and belong to another, deeper level). e: Fractionated collagenase inhibitory activity from the spent medium of articular chondrocytes after incubation with cathepsin B. f: 0.7 µg of cathepsin B-treated human purified TIMP-2. Capillary growth is visible in panels a, c, e, and f, but not so in panels b and d.

man kidney cathepsin B the two inhibitors were degraded with extensive fragmentation in a time-dependent manner. Both non-glycosylated and wild-type recombinant human TIMP-1 were equally susceptible to proteolysis by cathepsin B. The reaction products were analyzed by MALDI-TOF. The frag-

Table 2
Peptides recovered after complete digestion of human TIMP-2 by human cathepsin B

Mass (D)	Matching peptides
6957.0 ± 0.7	S[75–136]I; D[77–138]C
4355.9 ± 1.0	C[128–164]F
4039.6 ± 1.2	G[37–71]V
3531.7 ± 1.0	T[110–139]Y; S[111–140]I; T[112–141]S
2717.6 ± 0.8	E[127–149]M
2714.3 ± 0.9	S[111–133]C; I[157–180]G
2658.4 ± 0.8	L[118–139]Y; N[119–140]I
2133.7 ± 0.6	S[25–43]I
1629.4 ± 0.5	W[148–160]H
1581.5 ± 0.5	I[50–62]F; M[124–137]P
798.5 ± 0.3	Q[123–129]K
758.9 ± 0.3	L[85–92]G
757.0 ± 0.3	G[92–98]I

The peptide molecular masses were determined by MALDI-TOF spectrometry, and peptide fragments matching the masses were calculated with the PAWS program. The sequence below (amino acids 1–194) represents the whole molecule of human TIMP-2.

1	CSCSPVHPQQ AFCNADVIR AKAVSEKVD SGNDIYGNPI KRIQYEIKQI	50
51	KMFKGPEKDI EFIYTAPSSA VCGVSLDVGG KKEYLIAGKA EGDGMHITL	100
101	CDFIVPDTL STTQKKSLNH RYQMGCECKI TRCPMIPCYI SSPDECLWMD	150
151	WVTEKNGINH QAKFFACIKR SDGSCAWYRG AAPPKQEFELD IEDP	194

ments derived from TIMP-1 and TIMP-2 and their molecular masses are shown in Tables 1 and 2, respectively. Two of the peptides produced by cathepsin B cleavage of TIMP-1, V[24–47]K and Q[36–55]A, could unequivocally be assigned, while three other fragments each matched two peptides with masses within the experimental error. Among these, and to mention the N-terminal cleavage sites only, peptide L[152–169]R can be generated by hydrolysis between two Leu residues, a cleavage motif known from cathepsin B degradation of thyroglobulin [21]. Peptide T[26–68]S is generated by cleavage after a Val-Gly sequence, representing the subsites P2-P1 [22], respectively, a motif that is also known from thyroglobulin cleavage by cathepsin B [21]. Peptide A[51–69]V derives from cleavage between residues 50 and 51, having Gln at P1 and Leu at P2', a major site of cathepsin B cleavage in the insulin B-chain [23]. Also hydrolysis between Ser and His (peptide H[77–120]Y) has been recorded as a major site of cleavage in the insulin B-chain [23]. TIMP-2 digestion resulted in more extensive fragmentation, with eight clearly defined peptides and five more peaks matching different peptides. For many of these, cleavage sites corresponded to known P1-specificities of cathepsin B, such as Leu (peptides D[77–138]C, S[111–140]C, N[119–140]I, S[111–133]C), Ser (peptides T[112–141]S, L[118–139]Y), and Gln (peptides I[50–62]F, M[124–137]P) [21,23]. CA-074, a cathepsin B-specific inactivator [24], totally prevented degradation of both TIMP-1 and TIMP-2, indicating that their cleavage was only due to cathepsin B and not to other endopeptidases possibly present as contaminants. Proteolysis by cathepsin B is generally complex as a result of the combined endo- and exoproteolytic activities of the enzyme [25]. Due to this property, cathepsin B cleaves the cartilage proteoglycan, aggrecan, producing a fragment terminating in the sequence VDIPEN, that was previously considered an exclusive feature of the MMPs [26].

After incubation of both TIMPs and of the partially purified collagenase inhibitory activity from conditioned media of human articular chondrocytes with human kidney cathepsin B, their ability to inhibit MMPs was completely lost. The same result was obtained using a cathepsin B preparation from conditioned media of dedifferentiated human articular chondrocytes. CAM assays were performed with TIMP-1, TIMP-2 and with enriched conditioned medium from chondrocyte cultures containing both TIMPs after treatment with either human kidney cathepsin B or cathepsin B prepared from conditioned media of dedifferentiated human articular chondrocytes. In all cases, the anti-angiogenic activity of the samples was completely lost. The effect of this treatment on the chondrocytic anti-angiogenic activity and TIMP-2 with chondrocyte cathepsin B is shown in Fig. 2e,f, respectively. The undisturbed growth of capillaries in the area underneath the filter confirms that none of the peptides generated by cathepsin B digestion of TIMP-1 and TIMP-2 retained the anti-angiogenic properties of the corresponding intact molecules. If CA-074 was added to the incubation solutions of both cathepsin B preparations with TIMPs, their anti-angiogenic potential was fully retained. Cathepsin B alone had no influence on capillary growth in the CAM assay.

4. Discussion

Destruction of important antagonists of neovascularization may have a direct impact in conditions characterized by the

concomitant presence of MMPs and cathepsin B in the pathological scenario. Two representative examples are tumor growth and invasion [10–13,27,28] and osteoarthritis [14,15,29]. Cathepsin B has been localized in microvascular endothelial cells around human glioblastoma and prostate carcinomas, a feature which may play a role in the malignancy of these tumors [28].

Neovascularization is required for the initiation of cartilage mineralization [9]. The process of endochondral ossification during the growth of osteophytes is characterized by an initial invasion of cartilage by vascular connective tissue, followed by osteoclastic resorption. Erosion on one side and formation of cartilage on the other result in the outgrowth of marginal osteophytes [30]. In normal articular cartilage, angiogenesis factors are balanced in their action by vascularization inhibitors, with the result that cartilage is efficiently protected from invasion by capillaries. This balance is obviously disturbed in osteoarthritic cartilage, as shown histologically by the invasion of new capillaries penetrating the subchondral plate and the deeper zones of cartilage. We demonstrated active cathepsin B at sites of remodeling in osteoarthritic cartilage and in osteophytes of human osteoarthritic joints [14,15]. The high levels of interstitial collagenase at sites of vascular invasion, as well as in the osteoblasts and some osteoclasts of human osteophytic bone, together with the low levels of TIMP-1 in the same zones [31], immediately provide a rationale for our present results: cathepsin B would be responsible for shifting the balance between MMPs and TIMPs through inactivation of the latter components. Membrane type 1 MMP acts as a pericellular fibrinolysin during the neovascularization process [32]. This enzyme is not inhibited by TIMP-1, but is regulated by TIMP-2 and TIMP-3 [33]. Thus, TIMP-2 inactivation by cathepsin B favors the action of this enzyme also.

The importance of our present results goes beyond cartilage angiogenesis. They provide a rationale for the presence and role of cathepsin B in many pathological situations pointing to novel, deleterious roles of this aggressive enzyme. Besides a direct participation in tissue destruction, cathepsin B enhances the activity of the MMPs by destroying their inhibitors, even in the absence of mechanisms capable of MMP upregulation, and at the same time behaves as an angiogenesis-stimulating factor. Indeed, cathepsin B secreted by dedifferentiated articular chondrocytes [17] is the same species produced by invasive tumors [12], and corresponds to the proenzyme, which undergoes extracellular activation [25].

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