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Generation and degradation of human endostatin proteins by various proteinases

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Abstract The angiogenesis inhibitor endostatin is a fragment of the NC1 domain of collagen XVIII. The generation of endostatin has been investigated only in murine hemangioendothelioma cell cultures and was ascribed to cathepsin L. Distinct endostatin-like fragments were detected in human tissues and serum. To identify proteinases able to generate such fragments, we incubated human NC1 with proteinases of all classes, including cathepsin L. Eleven out of 12 generate fragments with an N-terminus within the same 15 residue stretch as those occurring physiologically, indicating that this region is sensitive to many proteinases. None correspond to mouse endostatin. However, the efficiencies of these proteinases differed markedly. Some proteinases also proved to degrade endostatin, pointing to another regulatory loop of angiogenesis.

Key words: Endostatin; NC1; Collagen XVIII; Proteinase; Angiogenesis

1. Introduction

Angiogenesis is a process in which new blood vessels are formed by sprouting from existing ones. It is required during embryogenesis, tissue repair and tumor growth. A variety of proteinases have been reported to promote angiogenesis, but they have complex functions, which are far from being understood [1–5]. The discovery of natural antiangiogenic factors that correspond to fragments of larger proteins has led to the realization that some proteinases can also cause inhibition of angiogenesis. Examples of such fragments are angiostatin, a proteolytic fragment of plasminogen [6] and endostatin, a fragment of the carboxyl domain of collagen XVIII [7]. Both fragments have been shown to inhibit tumor growth and metastasis. Endostatin-containing fragments circulate in the plasma of apparently healthy people [8,9], suggesting that the proteolytic release of endostatin from collagen XVIII is a normal physiological process.

Collagen XVIII is the core protein of a heparan sulfate proteoglycan of vascular and epithelial basement membrane [10–15]. Its structure consists of a central region composed of 10 triple-helical domains, flanked by two non-triple-helical domains: NC11 at the N-terminal and NC1 at the C-terminal [11–15]. The structure of mouse NC1 consists of three regions: (1) an N-terminal region comprising approximately 60 residues, responsible for the trimerization of collagen XVIII polypeptides, (2) a central hinge region of approximately 70 residues and (3) a more compact 22 kDa C-terminal, endostatin region with about 180 residues [8]. Endostatin was originally detected in conditioned media of murine hemangioendothelioma (EOMA) cells as a fragment with the N-terminal sequence HTHQD, able to inhibit endothelial cell proliferation [7], migration [16,17], survival [18], as well as angiogenesis and tumor growth [7]. A recent study [19] showed that recombinant mouse NC1 itself does not inhibit FGF-2-induced chorioallantoic membrane angiogenesis, while endostatin does. This indicates that processing within the NC1 hinge region is needed to generate antiangiogenic effects. In mouse tissue and serum, endostatin-containing fragments of collagen XVIII resulting from proteolytic cleavage upstream in the hinge region have been detected ([8,20]; see also Fig. 2). In human sera, similar endostatin-containing fragments are present. There are indications that mouse endostatin-like protein fragments with extensions or deletions of a few residues at the N-terminus or C-terminus of the 22 kDa fragment are biologically active [17].

The proteinase that generates endostatin with the originally described N-terminal sequence HTHQD in EOMA cell cultures is cathepsin L [21], whereas metalloproteinases generate a fragment 8 kDa larger than endostatin in a parallel processing pathway [21]. Incubation of murine NC1 (mNC1) with porcine pancreatic elastase also produced endostatin with the N-terminal sequence HTHQD [22]. It is unlikely, however, that cathepsin L and elastase are the universal proteinases that generate endostatin or endostatin-like fragments from collagen XVIII, when considering the variability in the N-termini of endostatin-like fragments found in tissues, and the fact that the amino acid sequence around the cathepsin L/elastase cleavage site in mouse collagen XVIII is not well conserved amongst species.

We therefore examined the processing of recombinant human NC1 (hNC1) with a number of different proteinases. Our data show that cathepsins L, B and K, several matrix metalloproteinases (MMPs) and pancreatic elastase can generate endostatin-like protein fragments, although with different efficiencies. Moreover, our results demonstrate that some proteinases, including cathepsins L, B, D and K degrade endo-

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Abbreviations: MMP, matrix metalloproteinase; DTT, dithiothreitol; hES, human endostatin; mNC1, murine NC1; hNC1, human NC1

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statin or NC1 fragments very efficiently, whereas others do not.

2. Materials and methods

2.1. Proteinases

Mouse proMMP-9 was purified from culture media of transfected baby hamster kidney cells, by using gelatin-Sepharose and concanavalin A-Sepharose (Pharmacia) [23]. The catalytic domains of rabbit MMP-12 and MMP-14 were purified from *Escherichia coli* (unpublished). Recombinant proMMP-13 was provided by Dr. López-Otín (Oviedo, Spain). Human proMMP-2 was from Boehringer Mannheim. ProMMPs were activated with 4-aminophenyl mercuric acetate. Human cathepsins B and L and porcine pancreatic elastase were from Calbiochem, and human cathepsin D from Sigma. Recombinant human procathepsin K was purified from *Pichia pastoris* [24], and activated by acidic pH.

The molar concentrations of active MMPs [25], cysteine proteinases [26] and cathepsin D [27] were determined by titration with BB-94 (gift from Dr. van Wart, Roche), E64c (Sigma) and pepstatin A (Sigma), respectively.

2.2. Processing of hNC1

Recombinant human *N*-flagged NC1 [17,21] was incubated at 0.6 μM, at 37°C with 10 nM cathepsin L or 30 nM cathepsin K in 50 mM sodium acetate pH 5.5, 2 mM dithiothreitol (DTT), 5 mM EDTA; 50 nM cathepsin B in 50 mM sodium acetate pH 6.0, 2 mM DTT, 5 mM EDTA; 25 nM cathepsin D in 50 mM sodium citrate pH 3.3; 25 nM MMP in 50 mM Tris–HCl pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35, 50 μM $ZnSO_4$; 25 nM elastase in 50 mM sodium acetate pH 6.0. All incubations were at 37°C. They were stopped at the indicated times with 0.25 μM E64c for cathepsins L, B and K; 0.25 μM pepstatin A for cathepsin D; 0.25 μM BB94 for MMPs. Aliquots were then subjected to SDS–PAGE in 12% acrylamide (Pharmacia), under reducing conditions. Gels were stained with silver nitrate, or subjected to Western blot, using an anti-flag M2 monoclonal antibody (Sigma).

2.3. N-terminal amino acid sequencing

The NC1 cleavage products were separated in 12% acrylamide gels (Pharmacia), and electroblotted onto PVDF membranes (Applied Biosystems). Membranes were stained with Coomassie brilliant blue R-250. The bands of interest were excized, and the N-terminal amino acid sequence determined by Edman degradation on a Procise sequencer (Applied Biosystems).

2.4. Incubations of human endostatin (hES)

Recombinant hES (peptide starting at position H^{130}SHRF) [17], was incubated with proteinases in the same buffers as NC1. At the indicated times, aliquots of the mixtures were taken and analyzed by SDS–PAGE, as described above. In order to determine whether MMP activity was lost during the incubations, the level of activity was measured before and after the incubations, using the synthetic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 [28].

3. Results

3.1. Generation of hES proteins by a variety of proteinases

We incubated recombinant hNC1 with a wide range of proteinases at known molar concentrations of active enzyme, analyzed the reaction mixtures by SDS–PAGE (Fig. 1) and determined the N-terminal amino acid sequence of the bands of interest (Fig. 2). These proteinases included representatives of several classes of proteinases, i.e. cathepsins L, B and K from the cysteine proteinase family; cathepsin D from the aspartic proteinase family; MMP-2, MMP-3, MMP-9, MMP-12, MMP-13, MMP-14 and MMP-20 from the MMP family, and pancreatic elastase from the serine proteinase family.

As shown previously [21], cathepsin L processed hNC1 to a

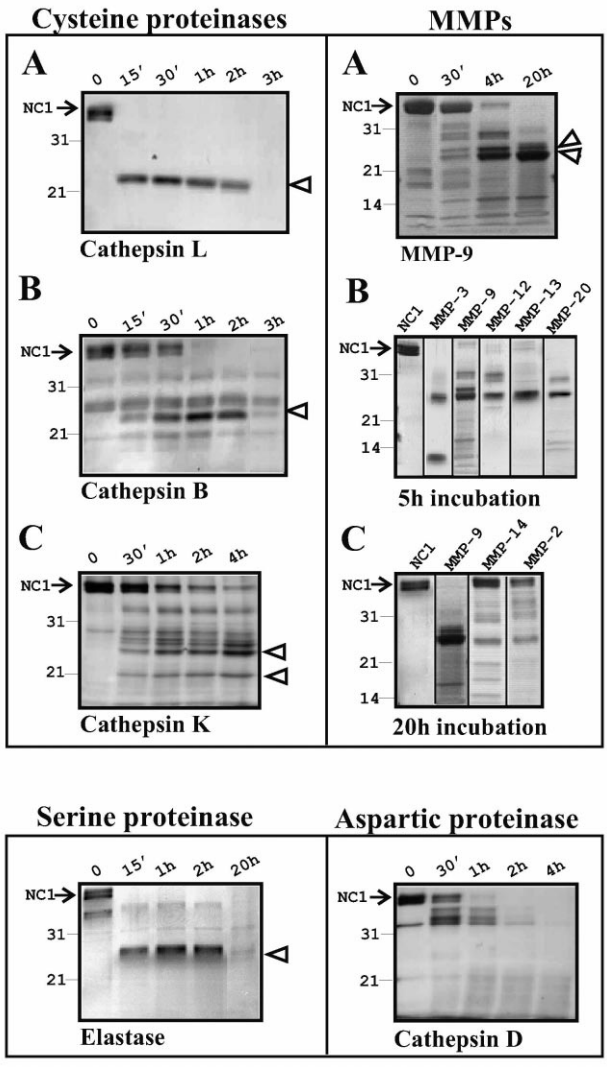

Fig. 1. Processing of recombinant hNC1 by proteinases. hNC1 was incubated for the indicated times with various proteinases as explained in Section 2, and the reaction mixtures were analyzed by SDS–PAGE. Migration distances of molecular mass markers and NC1 are indicated on the left. Empty arrowheads on the right indicate the proteolytic products subjected to amino acid sequencing.

25 kDa fragment with the N-terminus L^{119}RPARPTSPPAHS, which is 11 residues longer than the cathepsin L-generated murine fragment defined as endostatin. In this paper, we will refer to this cathepsin L-generated human peptide as 'L-h-endostatin'. In our assays, cathepsin L completely processed NC1 to L-h-endostatin within 15 min. Cathepsin B also generated the same L-h-endostatin fragment (Figs. 1 and 2), but complete processing required about 1 h, when using an enzyme concentration five times higher than in cathepsin L assays. Cathepsin K, also generated L-h-endostatin, but complete processing required about 5 h incubation, when using an enzyme concentration three times higher than in cathepsin L assays. Cathepsin K generated additional proteolytic fragments. The major products below 25 kDa corresponded to N-terminal fragments of NC1, as revealed by Western blot using an anti-flag M2 antibody, and by N-terminal sequencing (data not shown).

All seven MMPs tested processed hNC1, and generated a

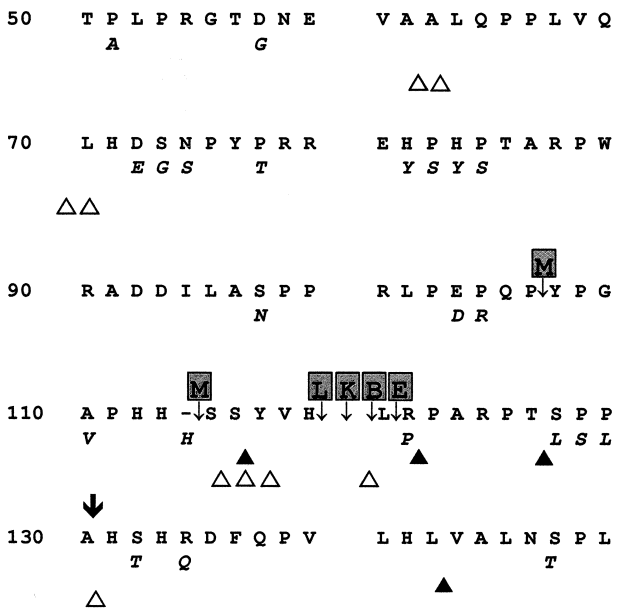

Fig. 2. Identification of proteolytic cleavage sites in the NC1 domain of human collagen XVIII. The amino acid sequence of hNC1 is from [31]. Substitutions in mouse NC1 are indicated below in italics [11]. The figure shows the N-terminal sequence of endostatin-like fragments detected in human plasma (full triangle) and in mouse (empty triangle) [8,9], as well as the cleavage sites for cathepsin L (L), cathepsin K (K), cathepsin B (B), MMP-9 (M) and pancreatic elastase (E) reported in this work. The thick arrow indicates the N-terminus of the originally described mouse endostatin.

very similar pattern of fragments in SDS–PAGE. The major accumulating product was a peptide of about 25 kDa, as well as a slightly longer peptide in the case of MMP-9 (Fig. 1). The latter two peptides proved to have an N-terminal extension of 5 and 12 residues, respectively, when compared to L-h-endostatin (Fig. 2). Peptides smaller than 25 kDa corresponded to N-terminal fragments of NC1 as determined by Western blot and N-terminal sequencing (data not shown). Significant differences in the efficiency of hNC1 cleavage were observed amongst these seven MMPs. MMP-3, MMP-9, MMP-12, MMP-13 and MMP-20 completed the reaction in about 5 h (Fig. 1). In contrast, under the same experimental conditions, MMP-2 and MMP-14 led to only little accumulation of endostatin-like fragments after 20 h incubation.

The pancreatic serine elastase cleaved hNC1 at one position closer to its C-terminal, compared to L-h-endostatin (Fig. 2). The reaction was completed within 15 min like in cathepsin L assays, but using an elastase concentration twice as high as cathepsin L.

The aspartic proteinase cathepsin D cleaved NC1 very quickly, but only transient products larger than 34 kDa were detected (Fig. 1).

3.2. Proteolytic degradation of endostatin

Fig. 1 shows that cathepsins L and B not only generated L-h-endostatin from hNC1, but also degraded it quickly, and after 3 h incubation, only small amounts of L-h-endostatin were left. In contrast, cathepsin K was much slower in degrading the generated L-h-endostatin, which remained in the reaction media for longer than 5 h.

MMP-3, -9, -12, -13 and -20 produced endostatin fragments

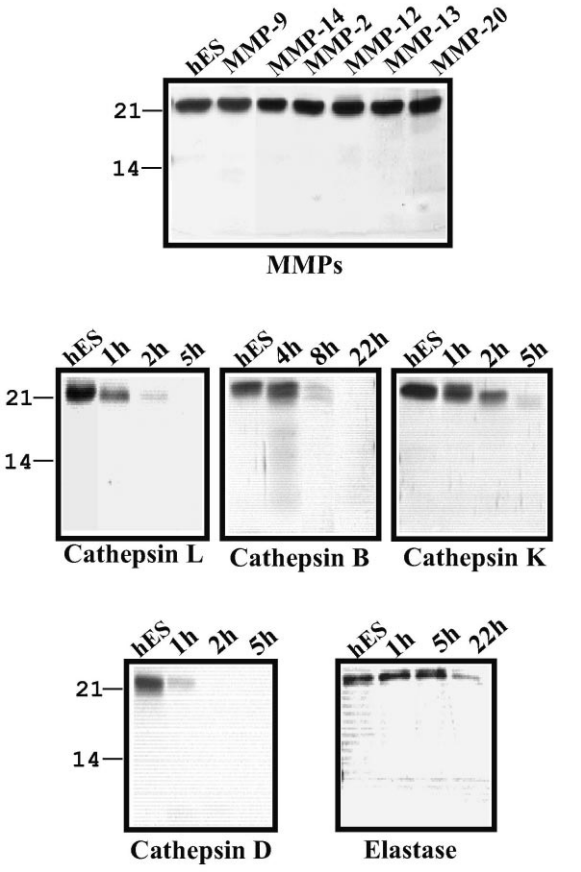

Fig. 3. Incubation of recombinant hES with proteinases. 2.5 μM hES was incubated with 25 nM MMP for 22 h, or with 25 nM cathepsins L, K, D and pancreatic elastase or 50 nM cathepsin B for the indicated times. Control samples containing only hES were run in parallel. Molecular mass markers are indicated on the left.

that accumulated after 20 h incubation, suggesting that MMPs cannot degrade them. Elastase was able to remove the endostatin peptide that it had generated, but it was detected only in the 20 h assays. In addition, the efficiency of these proteinases in endostatin degradation was directly investigated by incubating them with hES [17] (Fig. 3). Again a wide range of efficiencies was observed, supporting the data of Fig. 1. Cathepsins L and D were the most efficient and removed completely endostatin in 1–2 h. Cathepsins B and K also degraded endostatin, but incubations over 5–10 h were

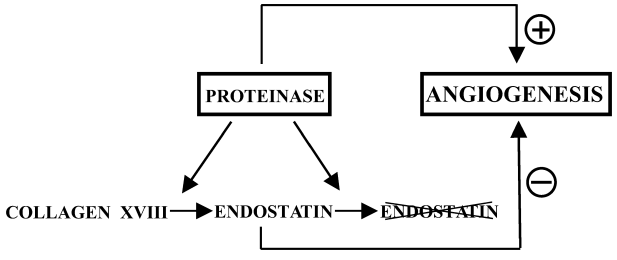

Fig. 4. Mode of action of proteinases in the control of angiogenesis. The overall effect of a proteinase in angiogenesis is determined by its relative efficiency to promote angiogenesis and to increase the concentration of antiangiogenic factors (e.g. endostatin, angiostatin). The latter depends on its relative efficiency to generate and degrade the antiangiogenic factor.

required to complete the reaction. The MMPs appeared totally inefficient in degrading endostatin, even upon 22 h incubation. The pancreatic elastase was not efficient, and incubations over 22 h were required to detect a decrease in endostatin levels.

4. Discussion

The present study shows that many different types of proteinases, all tested at similar concentrations of active enzyme, cleave human collagen XVIII's NC1 within a region of about 15 residues, thereby releasing endostatin proteins. This finding has several interesting implications.

First, the previous studies showing that cathepsin L and elastase generate precisely the fragment initially defined as endostatin, concerned cleavage of mouse NC1 [21,22]. In contrast, the present data are the first identifying multiple proteinases processing hNC1. We show that cathepsin L and pancreatic elastase generate endostatin proteins that are 11 and 10 residues longer than mouse endostatin, respectively. Cathepsins B and K cleave hNC1 at the same site as cathepsin L. The respective cleavage sites into mouse and hNC1 are as expected from the non-conserved amino acid sequence of mouse and hNC1, and from the preference of these proteinases for substrates with large hydrophobic or non-polar residues at P2 position [29], i.e. Val for YVH ↓ LRP in hNC1, and Leu in SLA ↓ HTH in mNC1. The sequence corresponding to the latter in hNC1 is PPA HSH, where P2 is Pro, a small non-polar amino acid, and accordingly, cleavage does not occur.

Second, it is of interest that endostatin fragments of similar length are present in human tissues and plasma ([8,9]; Fig. 2). The cleavages found in our test tube experiments are thus relevant to those occurring under physiological situations. They further support conclusions from a structural study, that the central hinge region of NC1 is especially sensitive to proteolysis [8]. It should be emphasized that the collagen XVIII fragment initially defined as endostatin has neither been detected in humans nor generated in our test tube assays. The existence of a unique biologically relevant endostatin molecule corresponding strictly to the initial definition may thus be questioned. Consistent with this view is the observation that endostatin-like fragments similar to those described in this study inhibit endothelial cell migration. The removal of five residues or the addition of a flag-tag at either the N-terminal or C-terminal ends of recombinant hES does not affect its activity in endothelial cell migration assays [17]. However, the molecular mode of action of endostatin or endostatin-like molecules is unknown, and it has not yet been shown exactly what portion of the endostatin peptide is required for biological activity. The latter is difficult to assess experimentally, as it is not possible to control the molecular transformations the peptide may undergo after its addition to culture media, or in in vivo situations.

Third, although 11 of the 12 proteinases tested generated endostatin-like fragments, they do this with different efficiencies. Cathepsin L and elastase are clearly the most efficient. Cathepsin B, MMP-9, MMP-3, MMP-12, MMP-13, and MMP-20 appear 4 to 20 times less active, while MMP-14 and MMP-2 appear at least 100 times less active compared to cathepsin L and elastase. The efficiency of MMP-14 and MMP-2 may, however, be significantly higher in tissues, since they may reach high local concentrations on the cell plasma membrane. Therefore, even lower activities in test tube assays may be relevant and should not be overlooked.

Finally, our data point to the fact that the actual concentration of endostatin-containing fragments in a tissue will not depend only on the efficiency of proteinases to generate them, but also on their ability to destroy them. Cathepsins L and B generate endostatin fast, but also degrade it efficiently. It is therefore expected that these cathepsins will generate high levels of endostatin only if the endostatin can diffuse away from the more acidic environment in which it is generated [21]. MMPs generate endostatin-like fragments quite efficiently, but do not degrade them.

These observations should be interpreted in the general context of angiogenesis. A wide variety of proteinases appear necessary for angiogenesis. They are involved in endothelial cell proliferation, migration, invasion through connective tissue barriers, and release of growth factors from the extracellular matrix [1–5]. Collagen XVIII is a major protein of the matrix surrounding endothelial cells, and is therefore directly available to proteinases for the generation of endostatin proteins controlling angiogenesis in a feed-back mechanism [21]. The overall effect of a proteinase will thus depend on its relative efficiency in promoting angiogenesis, and in allowing accumulation of endostatin-like fragments (Fig. 4). This mechanism should be relevant to many situations where angiogenesis is occurring, since basement membrane collagen XVIII is widely distributed, and proteolytic release of endostatin is not restricted by a requirement for specific proteinases. This mechanism also applies to the release of angiostatin from the widely distributed plasminogen [30].

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