Collagen XVIII/Endostatin Structure and Functional Role in Angiogenesis

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ABSTRACT. The angiogenesis inhibitor endostatin is a 20 kDA C-terminal fragment of collagen XVIII, a proteoglycan/collagen found in vessel walls and basement membranes. The endostatin fragment was originally identified in conditioned media from a murine endothelial tumor cell line. Endostatin inhibits endothelial cell migration in vitro and appears to be highly effective in murine in vivo studies. The molecular mechanisms behind the inhibition of angiogenesis have not yet been elucidated. Studies of the crystal structure of endostatin have shown a compact globular fold, with one face particularly rich in arginine residues acting as a heparin-binding epitope. It was initially suggested that zinc binding was essential for the antiangiogenic mechanism but later studies indicate that zinc has a structural rather than a functional role in endostatin. The generation of endostatin or endostatin-like collagen XVIII fragments is catalyzed by proteolytic enzymes, including cathepsin L and matrix metalloproteases, that cleave peptide bonds within the protease-sensitive hinge region of the C-terminal domain. The processing of collagen XVIII to endostatin may represent a local control mechanism for the regulation of angiogenesis.

Key words: angiogenesis/endostatin/collagen XVIII/neovascularization/neoplasm/review

Angiogenesis, neovascularization from preexisting vasculature, is crucial during embryonic development and all types of postnatal tissue growth and repair. The formation of new blood vessels is a complex multistage process that involves proteolytic degradation of basement membranes, loss of endothelial cell adhesion, proliferation and migration of endothelial cells into the surrounding stroma, and finally, re-adhesion of endothelial cells to form new capillary tubes. In adulthood, angiogenesis does not occur physiologically except under strictly controlled conditions. Inappropriate angiogenesis may be associated with different disorders either as a secondary phenomenon, or directly related to a syndrome.

The growth of neoplasms is associated with induced angiogenesis (Folkman et al., 1971). Without the formation of new blood vessels, tumors cannot expand beyond a few cubic millimeters, at which point diffusion of nutrients and waste products become rate limiting. Early investigations by Folkman et al. (Folkman et al., 1966) demonstrated that the formation of new blood vessels can be stimulated by soluble factors released by the tumor or host tissue, and that the rate of tumor growth correlates with the formation of new blood vessels. Neovascularization may also be a critical component of tumor metastasis since it enhances entry of tumor cells into the circulation by providing an increased density of immature vessels that have little basement membrane and fewer intercellular junctional complexes than normal mature vessels (Zetter, 1998; Weidner et al., 1991). Consequently, highly vascular tumors may have the potential to produce metastases at a higher rate than less angiogenic tumors. This has been documented in clinical studies showing a significant correlation between angiogenic factors in tumors and likelihood of metastases and poor survival (Weidner and Folkman, 1996; Zetter, 1998).

Proangiogenic substances have stimulatory effect on endothelial cells and induce angiogenesis. The first molecule definitely identified as a purified angiogenic growth factor was basic fibroblast growth factor (FGF-2), a tumor-derived endothelial cell growth factor (Shing et al., 1984). This discovery was followed by the identification of a large number...
of other angiogenic factors, of which the cytokines FGF-1, FGF-2 and VEGF (vascular endothelial growth factor) are the most widely expressed in normal adult organs. These growth factors are recognized on endothelial cells by transmembrane tyrosine kinase receptors coupled to intracellular signal transduction pathways.

In addition to producing angiogenic growth factors, tumors may produce angiogenic inhibitors (Folkman, 1995). Therefore, the growth rate of a tumor may be the result of the balance between positive and negative angiogenic effects. It is a clinical observation that surgical removal of a primary tumor often leads to rapid growth of metastases. A suggested explanation for this phenomenon is that the larger primary tumor may suppress the growth of metastases by releasing angiogenesis inhibitors into the system (Holmgren et al., 1995). Once the primary tumor is removed, the suppression disappears, allowing for more effective angiogenesis and rapid growth of metastases.

**Endostatin is an endogenous angiogenesis inhibitor derived from collagen XVIII**

The first isolated inhibitor to support the theory of a negative factor released by a primary tumor and suppressing the growth of metastases was angiotatin, found in the urine of mice carrying low-metastatic Lewis lung carcinomas (O’Reilly et al., 1994). The molecule angiotatin represents an internal fragment of plasminogen that may be produced by metalloelastase from tumor-infiltrating macrophages (Dong et al., 1997).

Endostatin was the next factor to be identified as an endogenous angiogenesis inhibitor (O’Reilly et al., 1997). This molecule also derives from a larger protein molecule, but while angiotatin was discovered in an in vivo model, endostatin was isolated from the conditioned media of a non-metastatic murine hemangioendothelioma cell line, EOMA. When applied to bovine capillary endothelial cells that had been stimulated with FGF-2, the conditioned media of EOMA cells inhibited proliferation of the growth factor stimulated cells (O’Reilly et al., 1997).

Analogous to many other angiogenesis inhibitors, endostatin has an affinity for heparin. Experimental studies show that recombinant endostatin made in baculovirus-infected insect cells specifically inhibits the proliferation of endothelial cells in a dose-dependent fashion similar to that seen for endostatin purified from EOMA cell culture media. Recombinant endostatin from bacteria is largely insoluble, but still efficient in arresting tumor growth in vivo after injection into mice (Boehm et al., 1997; O’Reilly et al., 1997). Intermittent therapy with recombinant bacterially produced endostatin reduces several experimental tumors, including Lewis lung carcinoma, to a dormant state. No sign of drug induced resistance has been reported and, in the original study, the treatment dormancy appeared to persist even when therapy was discontinued (Boehm et al., 1997).

Purification and partial amino acid sequencing of endostatin from EOMA culture media showed that endostatin is a fragment of collagen XVIII (O’Reilly et al., 1997). The first cloning and sequencing of collagen XVIII (Abe et al., 1993, Oh et al., 1994; Rehn and Pihlajaniemi, 1994) demonstrated that this collagen represents a special type of collagen, belonging to a subfamily that has been designated the multiplexin family (multiple triple-helix domains and interruptions) (Oh et al., 1994). The multiplexins, including collagens XV and XVIII, have a domain organization that differs from other known collagen types by non-triple-helical regions interrupting several triple-helical domains (Fig. 1). In contrast to many other collagens that are rigid and inextensible with one large triple-helix domain, the interruptions of collagen XVIII probably allow for flexibility between triple-helical regions. Within the non-triple-helical regions are several ser-gly-containing sequences that conform to consensus sequences for glycosaminoglycan attachment sites in proteoglycan core proteins. Recent studies (Haltter et al., 1998) have demonstrated that collagen XVI-...
II, in fact, is a proteoglycan containing heparan sulfate side chains.

**How is endostatin generated?**

The proteolytic mechanisms by which endostatin is cleaved from collagen XVIII are currently being elucidated (Fig. 2). Structural analyses of the recombinant 38 kDa C-terminal NC1 domain of murine collagen XVIII suggest that it consists of three major segments: a 5 kDa N-terminal trimerization domain, a central protease-sensitive hinge region and the C-terminal 22 kDa endostatin domain (Fig. 2) (Sasaki et al., 1998). Felbor et al. (Felbor et al., 2000) examined the murine hemangioendothelioma cell (EOMA) culture from which endostatin was originally isolated. EOMA cells produce collagen XVIII, the precursor of endostatin, as well as proteolytic activities responsible for generation of endostatin. Using a panel of class-specific protease inhibitors it was shown that endostatin generation by EOMA cells could be completely inhibited by cysteine protease inhibitors. EOMA cells secrete large amounts of procathepsin L that gets activated to cathepsin L in a slightly acidic medium. Cathepsin L in turn generates endostatin from collagen XVIII. EOMA cells also secrete matrix metalloproteases (MMPs) which produce a larger, 30 kDa endostatin containing fragment in a parallel processing pathway (Felbor et al., 2000).

In a separate study Wen et al. (Wen et al., 1999) found that the production of endostatin in EOMA cell culture appeared to be inhibited in the presence of the elastase inhibitor elastatinal. It is unlikely, however, that elastase activity is a major factor in the generation of endostatin in EOMA cell culture, since it was not possible to purify the protease from EOMA cells or EOMA cell supernatant. Instead, elastase purified from a porcine pancreatic extract was used to generate endostatin from recombinant murine NC1 in vitro (Wen et al., 1999).

**What is the structural basis for the activity of endostatin?**

Recombinant mouse endostatin from transfected human kidney cells shows a distinct binding to heparin (Hohenester et al., 1998). Three-dimensional analysis of the 22 kDa endostatin fragment by x-ray crystallography at 1.5 Å resolution revealed a compact molecule (Hohenester et al., 1998). A large basic patch of arginine residues constitutes the heparin binding site. This site was recently shown to be involved in the inhibition of induced angiogenesis (Sasaki et al., 1999) in the chick embryo chorioallantoic membrane (CAM) assay. In another study, heparin-binding did not play any role in the ability of endostatin to inhibit the chemotactic migration of human umbilical vein endothelial cells (HUVECs) towards VEGF (Yamaguchi et al., 1999). It is likely, therefore, that endostatin may inhibit angiogenesis by both heparin-dependent and heparin-independent mechanisms. At picomolar concentrations, heparin-binding plays no role (Yamaguchi et al., 1999), but at concentrations several orders of magnitude higher, heparin-binding may well be critical.

Analysis of the structure of human endostatin revealed a zinc ion near the N-terminus (Ding et al., 1998). It was initially reported that zinc-binding was essential for the antangiogenic mechanism of endostatin (Boehm et al., 1998) but later studies have failed to confirm the relationship between zinc binding and inhibition of endothelial cell migration or angiogenesis (Yamaguchi et al., 1999; Sasaki et al., 1999). A recent study found a structural heterogeneity in the zinc-binding site between two different crystal forms of mouse endostatin and concluded that zinc is likely to play a struc-

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**Fig. 2.** Diagram showing the C-terminal NC1 domain of collagen XVIII with three endostatin molecules attached to the trimerization domain through a hinge region with several proteolytic sites. Numbers above the diagram indicate amino acid residues that define the boundaries of the three regions.
What is the function of the collagen XVIII/endostatin system?

Angiogenesis is initiated by various cytokines such as FGF-2 and VEGF. At later stages of angiogenesis the process becomes dependent on platelet derived growth factors and transforming growth factor-1 (Beck and D’Amore, 1997; Risau, 1997). The inducers of angiogenesis usually bind to cell surface heparan sulfate proteoglycans. In fact, FGF-2 and VEGF require the proteoglycans as cofactors in order to efficiently induce angiogenic signalling. Since collagen XVIII is the core protein of a heparan sulfate proteoglycan in vascular and epithelial basement membranes, the physiological role of collagen XVIII in relation to endothelial cells may be that of a positive cofactor for angiogenesis, possibly together with perlecan (Olsen, 1999). During sprouting angiogenesis, endothelial cells release proteolytic enzymes, including MMPs and cathepsin L, that cleave peptide bonds within the protease-sensitive hinge region of the NC1 domain of collagen XVIII, whereby endostatin fragments are released (Fig. 3). Such fragments in turn may be local inhibitors of angiogenesis.

The collagen XVIII/endostatin system may thus have evolved as a “sensor” of proteolytic activities associated with angiogenesis and serve as a negative control by release of antiangiogenic fragments (Fig. 3). Although definitive data on the activities of various endostatin-like fragments are not available, it is likely that fragments slightly larger or smaller than endostatin, have a similar antiangiogenic activity. One argument is the fact that the cleavage sites for endostatin in human and mouse collagen XVIII are not identical (Sasaki et al., 1999). Consistent with this observation is the finding that the fragment generated from human recombinant NC1 protein by human cathepsin L is precisely 11 amino acid residues longer than mouse endostatin (Felbor et al., 2000). Recent data also show that recombinant endostatins with various peptide epitopes added to the N-terminus are equally active and that the deletion of five amino acid residues from the N-terminus of murine endostatin has no effect on activity in vitro or in vivo (Yamaguchi et al., 1999).

In summary, current data on the collagen XVIII/endostatin system suggest that during endothelial activation, the production of proteolytic enzymes leads to release of antiangiogenic fragments that serve as local inhibitors of angiogenesis (Fig. 3).

References


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(Received for publication, March 31, 2000 and accepted, March 31, 2000)