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New functional roles for non-collagenous domains of basement membrane collagens

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Summary

Collagens IV, XV and XVIII are major components of various basement membranes. In addition to the collagen-specific triple helix, these collagens are characterized by the presence of several non-collagenous domains. It is clear now that these ubiquitous collagen molecules are involved in more subtle and sophisticated functions than just the molecular architecture of basement membranes, particularly in the context of extracellular matrix degradation. Degradation of the basement membrane collagens occurs during numerous physiological and pathological processes such as embryonic development or tumorigenesis and generates collagen fragments. These fragments are involved in the regulation of functions differing from those of their original intact molecules. The non-collagenous C-terminal fragment NC1 of collagen IV, XV and XVIII have been recently highlighted in the literature because of their potential in reducing angiogenesis and tumorigenesis, but it is clear that their biological functions are not limited to these processes. Proteolytic release of soluble NC1 fragments stimulates migration, proliferation, apoptosis or survival of different cell types and suppresses various morphogenetic events.

Keywords

Basement membrane collagens; NC1 fragments; Angiogenesis; Morphogenesis

Introduction

The development of an extracellular matrix (ECM), a complex mesh of various proteins, was a crucial event in the emergence of multicellular organisms. Providing a mechanical support for the cells, the ECM also influences cell behavior. Its remodeling during physiological or pathological processes generates new signals, particularly between cells and the basement membranes (BMs). BMs are thin layers of specialized ECM associated closely with epithelial or endothelial cells, muscle fibers, adipocytes and peripheral nerves. Collagens type IV, XV and XVIII, together with laminins, nidogens, heparan sulfate proteoglycans (HSPG), fibulins, dystroglycan and other glycoproteins, are major constituents of BM (for a review, see Erickson and Couchman, 2000; Miosge, 2001; Schwarzbauer, 1999). Numerous mutations of the genes encoding these collagens are involved in human diseases (for a review, see Myllyharju and Kivirikko, 2001). Recently, the non-collagenous C-terminal domain (NC1), of these collagens has been the focus of numerous studies investigating its role in the molecular architecture of the BM as well as more subtle and sophisticated biological functions distinct from those of their original collagen, such as angiogenesis or branching morphogenesis.

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Angiogenesis is a complex and invasive process by which neovascularization occurs in adults. Because of its crucial role in tumorigenesis, much effort has been focused on developing and identifying anti-angiogenic molecules in the past decade with the aim of producing potential medical treatments. A search for endogenous angiogenesis inhibitors led to the discovery of endostatin, which is a non-collagenous C-terminal fragment of collagen XVIII (O'Reilly et al., 1997). These data emphasized the role of collagen fragments in regulating cell behavior and increased the interest around their potential biological functions. To date, half of the endogenous angiogenesis inhibitors described are cryptic fragments issued from proteolysis of large proteins, including angiostatin, a fragment of plasminogen, or vasostatin, a fragment of calreticulin, and six of them derive from collagens (for a review, see Cao, 2001; Marneros and Olsen, 2001). Besides angiogenesis, some other functions have been described for collagen fragments (Table 1).

Here, we focus on collagens IV, XV and XVIII because they have been recently highlighted in the literature. After a brief description of these collagens (for a review, see Olsen and Ninomiya, 1999; Sado et al., 1998), we discuss what is known about the biological functions of their NC1 fragments not only in angiogenesis but also in other processes. Indeed, although these fragments have become a focal point in tumor biology, it is clear that they are involved in other fundamental morphogenetic events.

Collagen type IV, XV and XVIII: genes, structure and physiological functions

Collagen type IV

Collagen IV regulates cell adhesion and migration and is highly conserved in vertebrates and invertebrates in terms of both its structure and its functional role in BM architecture (Blumberg et al., 1987; Netzer et al., 1998; Sarras et al., 1993). Six different polypeptides, α1 to α6, encoded by three sets of genes form collagen IV heterotrimers[†] (Filie et al., 1995; Momota et al., 1998; Soininen et al., 1988; Sugimoto et al., 1994).

Each α-chain is composed of three domains, a cysteine-rich N-terminal 7S domain, a central triple-helical domain and a globular C-terminal non-collagenous NC1 domain (Fig. 1A). The NC1 domain is involved in the assembly of α-chains to form the heterotrimers and the 7S domain is involved in the covalent assembly of four heterotrimers in a spider-shaped structure (Boutaud et al., 2000;Dolz et al., 1988;Tsilibary et al., 1990). Thus, collagen IV forms a complex branching network that serves as a scaffold for the BM (Yurchenco et al., 1987) (Fig. 1B). Although α1 and α2 chains are widely expressed and colocalize in numerous tissues, there is a temporal and spatial regulation of α3, α4, α5 and α6 expression in physiological as well as pathological processes (Dehan et al., 1997;Fleischmajer et al., 1997;Miner and Sanes, 1994;Shen et al., 1990;Tanaka et al., 1997).

Several human genetic diseases have provided insights into the physiological role of collagen IV. Mutations in *Col4a5*, *Col4a3* and *Col4a4* are involved in Alport syndrome, which is characterized by a defective glomerular BM and the subsequent development of glomerulonephritis (Mochizuki et al., 1994), whereas some deletions spanning the 5′ regions of the *Col4a5/Col4a6* cluster have been associated with Alport syndrome and diffuse leiomyomatosis, a benign smooth muscle tumor (Heidet et al., 1997; Zhou et al., 1993). The C-terminal region of the α3 chain has been identified as an autoantigen involved in Goodpasture syndrome, an immune disease characterized by glomerulonephritis and pulmonary hemorrhage (for a review, see Hudson et al., 1993; Kalluri, 1999). Mouse models for autosomal Alport

[†]α1 to α6 polypeptides are encoded by three sets of genes, *Col4a1/Col4a2*, *Col4a3/Col4a4* and *Col4a5/Col4a6*, mapping to human chromosomes 13, 2 and X, and mouse chromosomes 8, 1 and X respectively. Each pair of genes is organized head-to-head on the chromosome, and their expression is regulated by bidirectional promoters localized between the genes.

syndrome have been developed and phenocopy human disease (Cosgrove et al., 1996; Cosgrove et al., 1998; Lu et al., 1999; Miner and Sanes, 1996).

The existence of different α chains for collagen IV and their restricted tissue distribution determine the structural and functional specificity of BM. Moreover, the extremely high conservation of this molecule from lowest metazoans up to vertebrates identifies collagen IV as a key regulator of morphogenesis that is critical for the regulation of adhesion, migration and survival of different cell types.

Collagen type XV and XVIII

Collagens type XV and XVIII, identified as a chondroitin sulfate and heparan sulfate proteoglycan, respectively (Halfter et al., 1998; Li et al., 2000), are closely related non-fibrillar collagens that define the multiplexin subfamily (multiple triple helix domains with interruptions) (Abe et al., 1993; Oh et al., 1994a; Oh et al., 1994b; Rehn and Pihlajaniemi, 1994; Rehn et al., 1994). α1 (XV) and α1 (XVIII) chains organize as homotrimers. Each chain is divided in three subdomains that, as in collagen IV, include a C-terminal NC1 domain‡ (Fig. 2A). The genes encoding α1 chains of collagens XV and XVIII have been cloned and mapped to human chromosomes 9 and 21 and mice chromosomes 4 and 10, respectively (Hagg et al., 1997a; Huebner et al., 1992; Oh et al., 1994a).

Collagen XV is highly expressed in heart, skeletal muscles and the placenta and moderately expressed in the adrenal gland, kidney and pancreas (Kivirikko et al., 1995; Muragaki et al., 1994). Its expression is associated with vascular, neuronal, mesenchymal and some epithelial BM, indicating a probable function in adhesion between BM and the underlying connective tissue stroma (Myers et al., 1996). Highly regulated during kidney, heart and lung development in the embryo, collagen XV colocalizes with collagen IV and is a component of continuous or fenestrated capillary BM, with the exception of the blood-brain barrier and liver and spleen sinusoids (Hagg et al., 1997b; Muona et al., 2002). For collagen XVIII several splicing variants exist: two isoforms have been described in humans (Saarela et al., 1998a) and three in mice (Rehn and Pihlajaniemi, 1995). Interestingly, these variants have specific expression patterns. The short isoform is expressed in various organs, whereas the long isoform is more specifically expressed in liver sinusoids and hepatocytes (Musso et al., 2001; Rehn et al., 1994; Saarela et al., 1998a; Saarela et al., 1998b). Transcription of collagen XVIII also occurs during adipogenesis (Inoue-Murayama et al., 2000) and a strong expression is observed in developing and post-natal eyes in various BM, except Descemet's membrane (Fukai et al., 2002).

The physiological roles of collagens XV and XVIII are not well understood. Mice lacking collagen XV show a higher sensitivity to exercise-induced muscle injury and progressive degeneration of skeletal muscles with collapsed capillaries and endothelial cell degeneration. Thus collagen XV might be involved in the survival and stabilization of muscle fibers and endothelial cells on the subjacent BM (Eklund et al., 2001). For collagen XVIII, a mutation affecting the short isoform has been recently associated with Knobloch syndrome, an autosomal recessive disorder characterized by high myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities and occipital defects (Sertie et al., 2000). Interestingly, mice lacking collagen XVIII develop the same ocular abnormalities (Fukai et al., 2002). Thus, collagens XV and XVIII regulate critical functions within specialized BMs.

‡As proposed by Olsen and Ninomiya (Olsen and Ninomiya, 1999), non-collagenous domains for collagen XV and XVIII are numbered as for collagen IV, starting from the C-terminal domain.

Structure, localization and receptors for C-terminal NC1 fragments of collagen IV, XV and XVIII

The NC1 domains of collagens IV, XV and XVIII have been isolated from invertebrate and vertebrate BM and identified as circulating fragments in serum. Collagen XV and XVIII share similar structures within their NC1 domains, which are organized into three different subdomains (Fig. 2B) (Sasaki et al., 1998; Sazaki et al., 2000). Endostatin, the C-terminal subdomain of NC1 (XVIII), was the first endogenous collagen fragment characterized with anti-angiogenic properties (O'Reilly et al., 1997). On the basis of its high degree of sequence similarity with collagen XVIII, the C-terminal subdomain of NC1 (XV), named restin (Ramchandran et al., 1999) or endostatin-like[§] (Sasaki et al., 2000), was also identified as an endogenous angiogenesis inhibitor.

For collagen IV, the NC1 domains from α1, α2 and α3 chains have also been identified as inhibitors of angiogenesis and named arresten, canstatin and tumstatin, respectively (Colorado et al., 2000; Kamphaus et al., 2000; Maeshima et al., 2000b). These NC1 domains have been implicated in the self-association of the heterotrimers (Boutaud et al., 2000; Timpl and Brown, 1996; Tsilibary et al., 1990). The recent crystal structure of the collagen IV NC1 domain showed that NC1 monomers fold into a novel tertiary structure comprising β-strands and two homologous subdomains, N and C. The trimers are assembled through unique three-dimensional domain swapping (Sundaramoorthy et al., 2002), and two trimers can be stabilized head to head by an uncharacterized covalent crosslink (Than et al., 2002).

The crystal structure of endostatin revealed a compact fold with a zinc-binding site and an extensive basic patch of 11 arginine residues, which explains the high affinity of endostatin for heparin. The overall structure of the endostatin domain is related to the C-type lectin carbohydrate-recognition domain, and the domains are present as dimers in the crystals (Ding et al., 1998; Hohenester et al., 1998; Hohenester et al., 2000). The structure of endostatin-like is very similar to that of endostatin (60% sequence identity) but lacks the zinc and heparin-binding sites (Sasaki et al., 2000).

Endostatin and endostatin-like fragments colocalize with collagen XV and XVIII in the BM of numerous organs, with the exception of the liver sinusoids, where endostatin-like but not collagen type XV staining is present (Miosge et al., 1999; Sasaki et al., 2000; Tomono et al., 2002).

What are the receptors for these fragments? Numerous integrins have been identified as major cellular receptors for NC1 fragments (Table 2). For endostatin two cell surface binding sites with K_d values of 18 pM and 200 pM have been described. The low-affinity receptor corresponds to glypicans, whereas the high-affinity receptor has not yet been identified. These receptors are not specific to endothelial cells; they are also present on epithelial cells (Karumanchi et al., 2001). Recently it has been shown that endostatin binds to VEGF-R2, a receptor involved in proliferation of endothelial cells. Besides integrins, two other types of receptors have been described for collagens, glycoprotein VI in platelets and discoidin domain receptors (DDR) in various cell types (for a review, see Vogel, 1999). But whether or not these interactions occur through NC1 domains of collagen IV, XV and XVIII is not known. Furthermore, several ECM proteins interact with these fragments (Table 2).

Taken together, these data suggest that NC1 fragments might be involved not only in the regulation of angiogenesis but also in other morphogenetic processes.

[§]For consistency, we will use the terms 'endostatin' in the case of collagen XVIII and 'endostatin-like' for collagen XV.

Proteolytic pathways generating NC1 fragments

For collagen IV the proteolytic pathways involved in generation of NC1 fragments are not clear, even though different fragments are detected in the serum. For collagens XV and XVIII, several different NC1 fragments have been extracted from tissue homogenates, and circulating forms have been isolated from human blood filtrates, suggesting that these fragments exist as physiological cleavage products (John et al., 1999; Sasaki et al., 1998; Standker et al., 1997). Endostatin originally was purified from conditioned medium of a murine hemangioendothelioma cell line (EOMA) as a 20 kDa fragment that binds to heparin (O'Reilly et al., 1997). In vitro studies with the same cell line have shown that the NC1 hinge domain contains cleavage sites for matrix metalloproteinases (MMPs) and cathepsin L. MMPs generate fragments of 30 kDa containing the endostatin domain, whereas cathepsin L directly and specifically releases the 20 kDa endostatin domain (Felbor et al., 2000). Other in vitro studies confirmed these results and shown that generation of endostatin is also mediated by elastase after a first processing of NC1 by MMPs (Ferreras et al., 2000; Lin et al., 2001; Wen et al., 1999). In corneal epithelial cells, MMP-7 generates a 28 kDa NC1 (collagen XVIII) fragment (Lin et al., 2001). Thus, several distinct proteolytic pathways may be involved in the generation of endostatin in various tissues. Although we have some clues for in vitro processing, the physiological pathways still need to be more extensively investigated (Fig. 2B).

Biological functions of NC1 fragments and morphogenesis

Collagens IV, XV and XVIII, which are ubiquitously present in vascular and epithelial BM, are well conserved among different phyla. Interestingly, the biological functions of the NC1 domain also seem to be conserved throughout evolution. In the primitive invertebrate *Hydra vulgaris*, addition of NC1 (IV) alters morphogenesis, blocking cell aggregate development (Sarras et al., 1993; Zhang et al., 1994). In vitro, NC1 (IV) promotes axonal but not dendritic growth in sympathetic neurons from rat embryos (Lein et al., 1991), and hexameric NC1 supports attachment and migration of chicken neural crest cells. By contrast, intact dimers of collagen IV do not (Perris et al., 1993). These results imply that biological functions supported by NC1 domains are conformation dependent; proteolytic digestion of collagen IV may induce appearance of cryptic sites involved in new signals between cells and BMs.

A collagen homologous to type XV–XVIII collagens has been identified in *C. elegans*. Deletion of the NC1-encoding region of this gene (*cle-1*) causes defects in axon guidance and migration of neural and non-neural cells. This phenotype can be rescued by ectopic expression of NC1, but not endostatin (Ackley et al., 2001). These functional differences could be explained by NC1 being trimeric, whereas endostatin is monomeric. In analogous data, NC1 (XVIII) inhibits endothelial tube formation in Matrigel and stimulates cell motility of endothelial and non-endothelial cells. Monomeric endostatin has no effect by itself, but blocks the migration induced by NC1. The artificial oligomerization of endostatin stimulates cell motility (Kuo et al., 2001), indicating that proteolytic processes may regulate endogenous NC1 functions. These effects were not observed with the NC1 (XV) and endostatin-like domain. This lack of activity may not be surprising, since these molecules may have different spectra of activity depending on the identity of the motility signals.

More recently, murine endostatin has been shown to inhibit HGF-induced migration and branching morphogenesis of renal epithelial cells and the ureteric bud. These processes are dependent on the presence of glypican3. Ureteric bud expresses endostatin, and addition of neutralizing anti-endostatin antibodies enhances ureteric bud outgrowth and branching (Karihaloo et al., 2001).

Thus, high levels of endostatin or endostatin-like molecule may interfere with different pathways, downregulating morphogenetic processes. They may act as dominant-negative

ligands that interact with the same receptors as their native molecule and, thus, inhibit proliferation and migration, or they may interact with different receptors and induce apoptosis. They may also have a mechanical effect in interacting with the original collagen trimers and disrupt them, leading to the loss of anchors between BMs and cells. Differential degradation of NC1 thus constitutes a negative feedback loop.

Biological functions of NC1 fragments in angiogenesis and tumorigenesis

Tumstatin and other collagen IV NC1 fragments

Biological functions of collagen IV NC1 fragments have recently been extensively studied in mammals. A synthetic peptide encompassing residues 183–205 of α3 chain NC1 domain and containing an SNS triplet unique to α3 specifically inhibits activation of polymorphonuclear leukocytes (Monboisse et al., 1994). This peptide binds to a CD47/$\alpha_v\beta_3$ integrin complex, promotes adhesion and chemotaxis and inhibits proliferation of various human cancer cell lines (Han et al., 1997; Shahan et al., 1999a; Shahan et al., 1999b; Shahan et al., 2000). Indeed, direct interaction between α3NC1 and β3 integrin, independently of CD47, stimulates focal adhesion kinase (FAK) and PI 3-kinase phosphorylation (Pasco et al., 2000a). Furthermore, the inhibition of migration observed with melanoma and fibrosarcoma cells on native collagen IV or on the 185–205 α3NC1 peptide correlates with a decrease in expression of MT1-MMP and the β3 integrin subunit and a decrease in the level of activated membrane-bound MMP-2 (Pasco et al., 2000b). MMP-2 is involved in tumor progression and metastasis and its activation depends on MT1-MMP/TIMP-2 complexes (Itoh et al., 1998; Kinoshita et al., 1998). In vitro, α3NC1 fragment decreases the expression of MT1-MMP in a bronchial tumor cell line and the 185–205 α3NC1 peptide inhibits their invasion through $[\alpha1(IV)]_2$ α2(IV) collagen (Martinella-Catusse et al., 2001). Altogether, these data indicate that the ability of collagen IV to inhibit proliferation and regulate cellular adhesion and motility resides in the NC1 domain. The α3 (IV) chain has a specific interaction with invasive cancer cells, and, in contrast to α1 and α2 chains, which favor migration, α3 limits the invasive phenotype. Thus, in the context of tumor progression and metastasis, the presence of either the α3(IV) collagen chain or the α3NC1 fragment may negatively regulate the invasion process. Interestingly, in the lung, where α1, α2, α3, α4 and α5 (IV) collagen chains are expressed in normal alveolar BM, development of bronchioalveolar carcinoma correlates with a loss of α3, α4 and α5 chain expression and an increase in α1 and α2 chain expression (Nakano et al., 2001). The heterotrimer $[\alpha1(IV)]_2$ α2 is permissive for the invasion of different cancer cell lines and mediates pro-MMP-2 activation (Maquoi et al., 2000). By contrast, α1NC1 or α2NC1 monomers have some structural similarities to TIMP-1[¶] and inhibit MMP-2 and MMP-3 activity towards small synthetic peptide substrates in vitro (Netzer et al., 1998). Thus, the presence of different collagen chains or NC1 fragments during tumorigenesis might represent a key regulatory mechanism for the acquisition of an invasive phenotype.

Synthesis of collagen IV by vascular BM is a prerequisite for angiogenesis (Maragoudakis et al., 1993; Haralabopoulos et al., 1994). Moreover, α1 and α2 NC1 induce adhesion and spreading of endothelial cells (Koliakos et al., 1989; Tsilibary et al., 1990). On the basis of these observations and the data previously described, several groups have focused their attention on potential anti-angiogenic properties of NC1 fragments. Kalluri's group identified anti-angiogenic activities for α1, α2 and α3 NC1, named arresten, canstatin and tumstatin, respectively (Colorado et al., 2000; Kamphaus et al., 2000; Maeshima et al., 2000b). In vitro, these molecules inhibit endothelial cell proliferation and migration. Tumstatin seems to be the most efficient. None of the whole NC1 fragments inhibited the proliferation of cancer cell lines,

[¶]The homology of NC1 domain and TIMP-1 has been described using sequence-based approaches (Netzer et al., 1998) but it should be noted that recent X-ray crystallography data, which found that the structure of NC1 domain is unlike any other protein of known structure (Sundaramoorthy et al., 2002; Than et al., 2002), does not report this homology.

as observed with the 185–205 α3NC1 peptide, which indicated that this effect is dependent on partial degradation of the NC1 domain. Arresten, canstatin and tumstatin molecules inhibit angiogenesis: in vitro, they block the formation of tubular structures by mouse aortic endothelial cells embedded in Matrigel; in vivo, they block the recruitment of capillaries in Matrigel plugs and inhibit the growth of large and small tumors in mouse xenograft models. Brooks' group generated similar NC1 fragments and described the anti-angiogenic effects of α2, α3 and α6 NC1 in chorioallantoic membrane (CAM) assays (Petitclerc et al., 2000). However, none of these NC1 fragments inhibited proliferation of cancer cell lines or endothelial cells in vitro.

In contrast to the results shown for arresten, no inhibitory effects were observed for the α1NC1 fragment. Although identical mammalian expression systems were used for the production of the fragments in both cases, these discrepancies may be attributed to the use of different endothelial cell types and different tumorigenesis models (xenografts in mice versus development of tumor on chorioallantoic membranes).

What are the cell surface receptors involved in these functions? It is clear that integrins are key targets of NC1 (Table 2). Tumstatin binds $\alpha_v\beta_3$ integrin in an RGD-independent manner and interacts through two different sites, one site, composed of tumstatin residues 54–132, is involved in the anti-angiogenic effect, whereas the other, composed of residues 185–203, is involved in the anti-proliferative activity on cancer cell lines (Maeshima et al., 2000a;Maeshima et al., 2001b;Shahan et al., 1999b). Adhesion of endothelial cells (HUVEC or C-PAE) to tumstatin also seems to occur through $\alpha_6\beta_1$ integrin binding (Maeshima et al., 2000b). Although it is well known that the central triple-helical domain, as well as the NC1 domain of collagen type IV, interacts with cells via $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (Eble et al., 1993;Setty et al., 1998), these new results indicate that NC1 domains support novel integrin-mediated cellular interactions involved in the regulation of angiogenesis. Interestingly, collagen IV also contains cryptic integrin-binding sites. During angiogenesis these sites are exposed and induce a switch in integrin recognition, with a loss of $\alpha_1\beta_1$ binding and a gain of $\alpha_v\beta_3$ binding (Xu et al., 2001), which might be due to denaturation and concomitant degradation of collagen IV by MMPs such as MMP-2 (Eble et al., 1996).

In the case of tumstatin, a peptide composed of residues 45–132 of α3NC1 fragment is sufficient to inhibit in vitro and in vivo angiogenesis by increasing apoptosis of endothelial cells and is tenfold more active than endostatin. Because of the involvement of this fragment in Goodpasture syndrome, deletion of the Goodpasture epitope (residues 45–54) has been done, and the anti-angiogenic properties are preserved (Maeshima et al., 2000a). The effects of tumstatin are independent of disulfide bonds and are located in a 25-residue peptide (residues 74–98) (Maeshima et al., 2001a; Maeshima et al., 2001b). Apoptosis induced specifically in endothelial cells by this peptide is associated with inhibition of cap-dependent translation through negative regulation of mTOR signaling and depends on the presence of β_3 integrin (Maeshima et al., 2002).

Thus, NC1 domains of collagen type IV exhibit specific regulatory subdomains controlling adhesion, proliferation or apoptosis of various cells. The specificity of these subdomains for endothelial or cancer cells is very interesting, particularly in the case of α3NC1. Indeed, the recently published crystal structure of the collagen IV NC1 domain reveals a 3D structure with two homologous subdomains, N and C, with the major difference between these subdomains for each chain occurring in the region composed of residues 86–95 in the N subdomain and 196–209 in the C subdomain. Curiously these regions overlap two sequences identified previously as having anti-angiogenic activity and cancer cell anti-proliferative effects, respectively. It will be interesting to identify more precisely the integrin-binding sites on these NC1 domains. Indeed, their interactions with integrins might be involved in the disruption of

the contacts between endothelial cells or tumor cells and the basement membrane, leading to apoptosis of these cells. They might be also involved in the disruption of the C-terminal association that occurs during the assembly of a collagen IV network and induce disorganization of this matrix, thus disturbing migration, proliferation or survival of the cells. Moreover, the unique properties of the tumstatin NC1 domain in regulating neovascularization are very interesting and suggest a promising new family of integrin-dependent angiogenesis inhibitors.

NC1(XV), NC1(XVIII), endostatin and endostatin-like fragments

Since its discovery by O'Reilly and co-workers in 1997, endostatin has been the object of extensive research, trials and controversy in the angiogenesis field. In vitro, endostatin inhibits proliferation of bovine capillary endothelial cells but not cancer cells. In vivo, it inhibits angiogenesis on CAM assays and growth of various primary tumors. Moreover, no signs of toxicity, drug resistance or regrowth of tumors are observed as long as mice are treated (O'Reilly et al., 1997). Another striking effect is that endostatin when administrated on repeated cycles allowing the tumor to re-grow between each of them is still efficient and induces a prolonged dormancy of the tumor without resistance after two to six cycles, depending on the tumor model (Boehm et al., 1997). Thus far, multiple studies have demonstrated anti-angiogenic properties of endostatin in pathological models of tumorigenesis (Bergers et al., 1999; Blezinger et al., 1999; Boehle et al., 2001; Dhanabal et al., 1999a; Kisker et al., 2001; Sorensen et al., 2002; Yoon et al., 1999), choroidal neovascularization (Mori et al., 2001) and arthritis (Matsuno et al., 2002; Yin et al., 2002). However, in a controlled angiogenic process such as wound healing, endostatin does not affect the overall neovascularization. Ultrastructural analysis demonstrated some abnormalities in vessel maturation, but the blood vessel density is not affected (Bloch et al., 2000; Berger et al., 2000).

Tumorigenesis is not affected in mice lacking collagen XVIII, indicating that even if endostatin is detected as a circulating molecule, the physiological levels may not be sufficient to decrease tumor progression (Fukai et al., 2002). By contrast, collagen XVIII is required for normal regression of hyaloid vessels and for anchoring vitreal collagen fibrils to the retina inner limiting membrane. Thus, collagen XVIII may act as a gatekeeper, inducing regression of vessels in non-permissive territory for angiogenesis. Although endostatin is efficient in reducing choroidal neovascularization, a role for endogenous endostatin has still to be fully demonstrated.

Ex-vivo, endostatin decreases and stabilizes microvessel formation in rat aortic or human vein ring angiogenesis assays (Kruger et al., 2000; Ergun et al., 2001). In vitro, endostatin inhibits basal and FGF2 or VEGF-induced proliferation and migration of different endothelial cell types (Dhanabal et al., 1999b; O'Reilly et al., 1997; Taddei et al., 1999; Yamaguchi et al., 1999; You et al., 1999). The circulating form purified from human plasma lacks 12 N-terminal residues and does not inhibit proliferation (Standker et al., 1997); so the anti-proliferative activity of endostatin requires the full-length fragment but is independent of its zinc- or heparin-binding capacity (Yamaguchi et al., 1999). Soluble endostatin induces endothelial cell apoptosis by altering Bcl-2 expression (Dhanabal et al., 1999c) and inducing tyrosine phosphorylation of the protein adaptor Shb (Dixelius et al., 2000).

The molecular targets of endostatin are not yet clear. In endothelial cells growing exponentially, endostatin mimics serum deprivation in downregulating the transcription of genes involved in proliferation, apoptosis and cell migration. In the presence of serum, endostatin affects only migration of endothelial cells (Hanai et al., 2002; Shirichi and Hirata, 2001).

Inhibition of migration and survival are the most constant functions reported for endostatin. It is clear that migration of endothelial cells involves assembly and disassembly of focal

adhesions in concert with integrin signaling, and endostatin seems to interfere effectively with both systems. Immobilized endostatin promotes and soluble endostatin inhibits $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin-dependent endothelial cell migration and survival (Rehn et al., 2001). Depending on the cell type and the growth factor environment, inhibition of migration with soluble endostatin correlates with an increase or a decrease in focal adhesion and actin stress fiber formation (Dixelius et al., 2002; Wickstrom et al., 2001). Inhibition of VEGF-induced migration induces eNOS dephosphorylation (Urbich et al., 2002). Furthermore, endostatin induces a downregulation of the urokinase plasminogen activator system (Wickstrom et al., 2001), indicating that the signals induced by endostatin not only modify the cytoskeletal architecture and survival signals, but also affect pericellular proteolytic activity.

Interestingly, as observed in the case of collagen IV NC1 fragments, some functions of endostatin may interfere with MMP signaling (for a review, see Egeblad and Werb, 2002). Indeed, endostatin has been reported to inhibit endothelial and cancer cell invasion through Matrigel. This effect seems to be mediated by its association with pro-MMP-2, which inhibits MMP-2 activation (Kim et al., 2000). Recent data have shown a direct interaction of endostatin with the catalytic domain of MMP-2 (Lee et al., 2002). MMP-2 is very inefficient in generating endostatin fragments from collagen XVIII (Ferreras et al., 2000), but it may be one of the endostatin key targets for downregulating expression or activation of other MMPs and proteases.

A direct interaction between endostatin and VEGFR2 has been described previously (Kim et al., 2002). This interaction may be due to the basic character of endostatin, similar to the interaction demonstrated between VEGFR2 and the transactivator protein Tat of HIV-1 (Albini et al., 1996). Thus endostatin may act essentially by interfering with the binding of VEGF to its receptors, VEGF-R2 as well as VEGF-R1[**].

Molecular data are more limited for the related endostatin-like molecule. Inhibition of FGF2-induced migration, but not proliferation, of endothelial cells has been reported. In a model of renal cell carcinoma xenograft, a reduction of the tumor growth was observed but, in contrast with endostatin, endostatin-like did not induce any regression (Ramchandran et al., 1999). However, in CAM assays, only endostatin-like and NC1(XV) inhibited angiogenesis induced by VEGF, whereas angiogenesis induced by FGF2 was inhibited only by endostatin and NC1 (XV) (Sasaki et al., 2000). Thus these fragments might have different inhibition properties depending on their angiogenic environment and motility signaling, as discussed in the previous paragraph. These data correlate with previous observations showing that VEGF and FGF2 mediate their effects through different integrins, αvβ5 and αvβ3, respectively (Friedlander et al., 1995).

Conclusions and perspectives

The detailed biological functions of these different NC1 collagen fragments are not completely identified, but the similarities within the pathways they affect are troubling (Table 3). As immobilized substrates, NC1 fragments from collagen IV, XV or XVIII can induce migration, proliferation or survival depending on the cell type; however, soluble forms act in the opposite way, inhibiting proliferation, migration and inducing apoptosis (Fig. 3). This balance between negative and positive pathways may be part of a highly controlled regulatory mechanism. In the context of an invasive process such as angiogenesis or branching morphogenesis, the cells need to migrate and proliferate, and these processes are dependent on protease activity. In the first steps of these processes, partial degradation of the matrix may unmask some cryptic sites exposing NC1 immobilized domains that bind to cell surface integrins and activate different

[**]The authors mentioned also an interaction with VEGF-R1 (Kim et al., 2002).

pathways depending on the integrin pattern. Sustained proteolysis of the extracellular matrix may release high local concentration of soluble fragments that then act as dominant-negative molecules. They can inhibit proliferation and migration, allowing stabilization of new vessels or tubular structures or induce complete regression of these new tubules through the activation of apoptotic pathways.

Interestingly, the activities of these fragments are dependent on the activation of integrins or proteoglycans, such as glypicans in the case of endostatin. The integrin $\alpha_v\beta_3$ is a common ligand for collagen IV, XV and XVIII NC1 fragments and endostatin. In mice lacking β_3 integrin, tumor angiogenesis as well as VEGF or hypoxia-induced angiogenesis are enhanced, suggesting a role for this integrin in limiting angiogenesis in vivo (Reynolds et al., 2002). The anti-angiogenic activities of different NC1 fragments dependent on binding to β_3 integrin might also support this hypothesis. In that case, the NC1-fragment–β_3-integrin interaction might be a key regulator of this negative feedback. Another feature in the biological activities of these collagen fragments is the downregulation of gene expression: the collagen IV α3NC1 domain as well as endostatin decreases the expression of various genes involved in cell cycle regulation, migration and survival. However these activities seem to be correlated with environmental factors, and the anti-angiogenic activities of various fragments may depend on the specificity and the concentrations of growth factors locally released.

Besides β3 integrin, another common target emerging for these fragments is MMP-2. A direct interaction with the catalytic domain has been shown in the case of endostatin. For endostatin-like and the collagen IV α3NC1 domain, such an interaction has not been demonstrated, but the three molecules induce a decrease in MMP-2 membrane-bound activity, which might be part of the negative feedback loop mentioned previously. A decrease in the basal level of pro-MMP-2 activity is also observed in collagen-XV knockout mice (Eklund et al., 2001). Moreover, several MMPs are involved in the generation of the fragments themselves. Thus, in a process such as angiogenesis, proteolytic activity of MMPs might be part of a biphasic regulation: proangiogenic in early steps, critical for the rupture of basement membrane and migration of the endothelial cells, and anti-angiogenic in late steps, generating endogenous inhibitor fragments.

Endogenous inhibitors or activators derived from larger precursor proteins now appear to be a common theme in the context of remodeling processes. If endostatin or tumstatin have received increased attention recently because of their strong anti-angiogenic potential, it is clear that their activity is not specific for microvascular endothelial cells, but this work has opened new interest in the potential cryptic biological functions of these ubiquitous collagen molecules. Thus, these studies might be interesting not only in the context of cancer but also, considering the large number of diseases linked to collagens, in numerous other human disorders.

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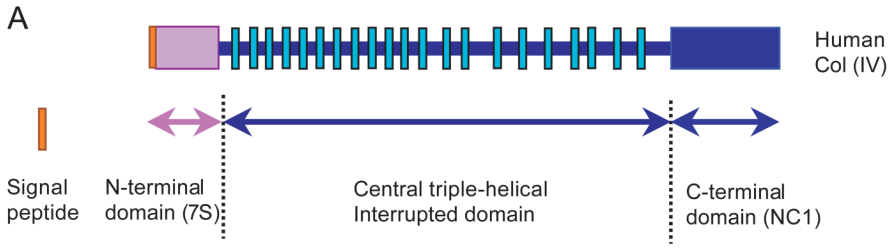

Chain	N-terminal 7S domain	Central domain	C-terminal NC1 domain	Interruptions
α1	143 aa	1271 aa	228 aa	21
α2	157	1303	227	26
α3	13	1398	231	26
α4	26	1396	230	25
α5	15	1416	228	23
α6	29	1407	227	25

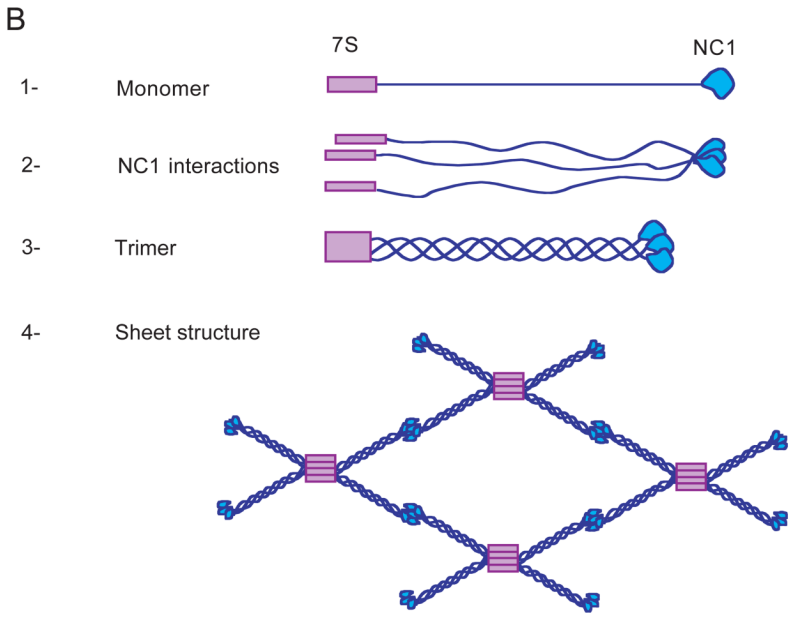

Fig. 1.

(A) Linear structure of human collagen IV α chains. Six different genes encode collagen IV α chains; each polypeptide is composed of three distinct domains: a cysteine-rich N-terminal 7S domain, a central triple-helical domain with multiple small interruptions (green boxes) and a globular C-terminal non-collagenous NC1 domain. The NC1 and central triple-helical domains are of an equivalent size, whereas the 7S domains are shorter in the case of α3, α4, α5 and α6 compared with α1 and α2. On the basis of sequence homology these different chains can be divided in two groups, the α1-like (α1, α3, α5) and the α2-like (α2, α4, α6). Assembly of collagen IV α chains. (B) The assembly of trimers is dependent first on the association of the NC1 domains, then the triple-helical structure forms and 7S domains are covalently

associated. Four trimers interact through their 7S domains in a spider-shaped structure, and two trimers interact head to head through their NC1 domains, forming a sheet structure. Several trimers can also lace together along their triple-helical domain, thickening the structure (Timpl et al., 1981).

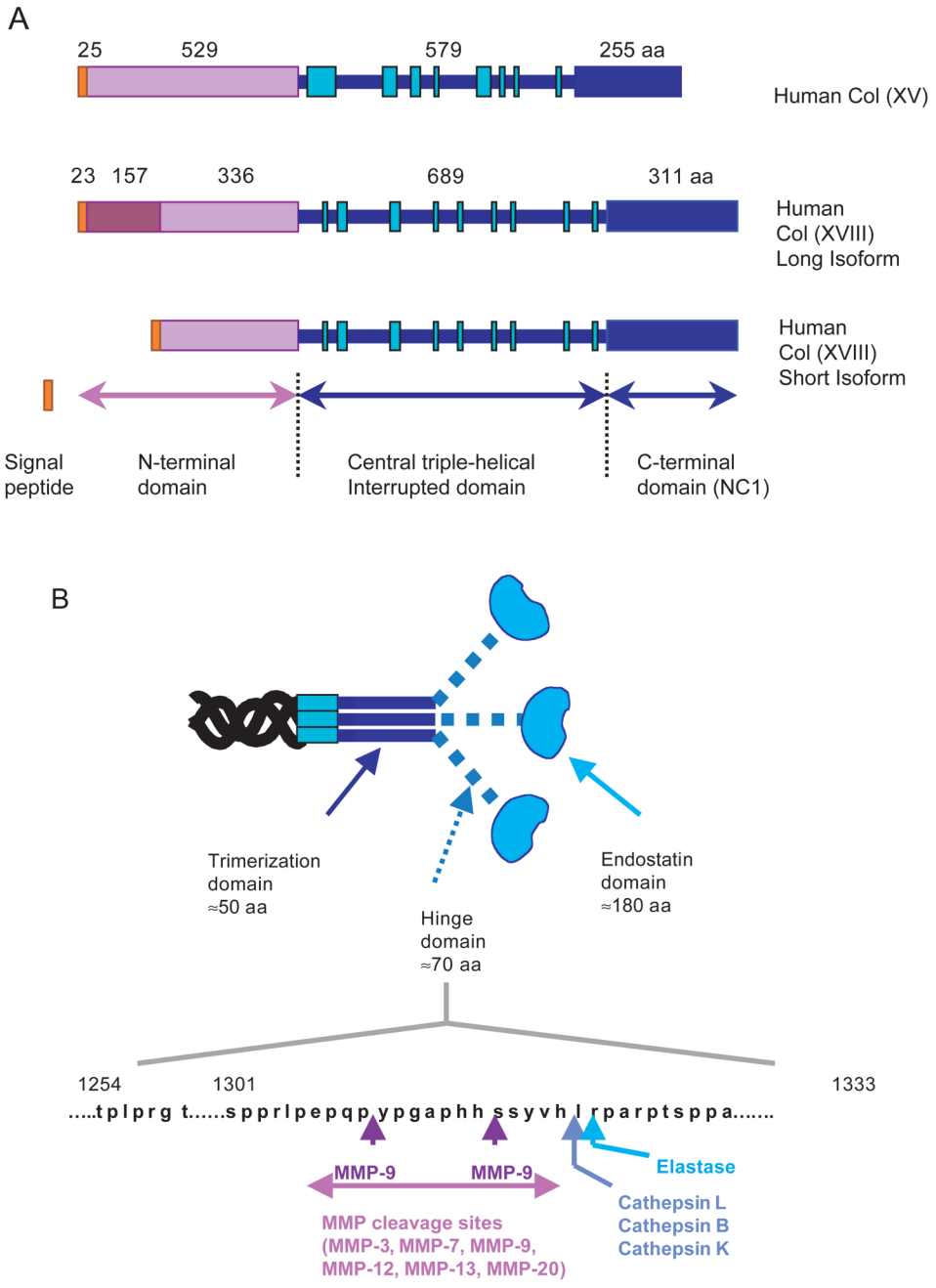

Fig. 2.

(A) Linear structure of human collagen XV and XVIII α1 chains. The α1 chains of collagen XV and XVIII are structurally homologous; they define a new collagen subfamily, the multiplexin family, on the basis of their central triple-helical domain with multiple long interruptions (green boxes). They are also characterized by a long non-collagenous N-terminal-domain-containing Thrombospondin sequence motif with two splicing variants in human collagen XVIII and a long non-collagenous globular C-terminal domain or NC1 domain. (B) Functional sub-domains of human NC1(XVIII) and protease cleavage sites. The NC1 domain contains three functionally different subdomains: these domains consist of a N-terminal non-covalent trimerization domain necessary for the association of trimers, a hinge domain

containing multiple sites sensitive to different proteases and an endostatin globular domain covering a fragment of 20 kDa with anti-angiogenic and anti-branching morphogenesis activities. Numerous enzymes can generate fragments containing endostatin. Cathepsin L and elastase are the most efficient, but in contrast to MMP cleavage leading to accumulation of endostatin, cathepsin L and B degrade the molecule [cleavage sites are indicated according to the data published by Ferreras (Ferreras et al., 2000)].

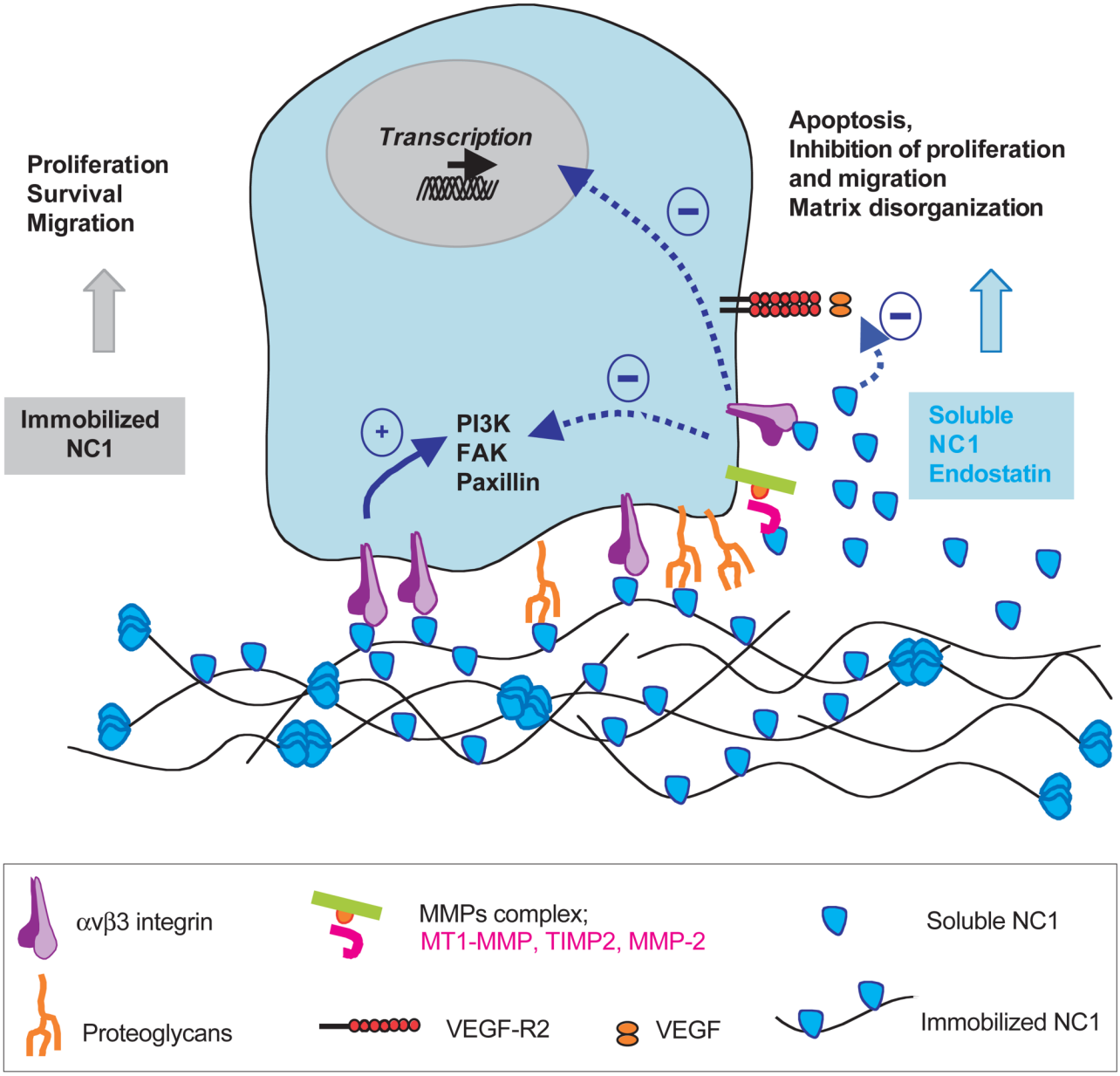

Fig. 3.

Biological activities of immobilized versus soluble NC1 and endostatin fragments. Immobilized NC1 domains from collagen IV, XV and XVIII induce proliferation, survival and migration of different cell types. These effects correlate with an increase in PI3K, FAK and paxillin phosphorylation. By contrast, soluble NC1 or endostatin fragments bind to various receptors on the surface of the cells and decrease phosphorylation of PI3K, FAK and paxillin. Endostatin as well as NC1(IV) induces a decrease in the transcription of different genes. Endostatin interacts with VEGF-R2 and thus decreases the binding of VEGF to its receptor; endostatin also interacts directly with MMP-2, inhibiting the activation of the enzyme. Taken together, these soluble fragments act in the opposite way to their original molecules, negatively

regulating the proliferation and the migration of different cell types and inducing apoptosis and extracellular matrix disorganization.

Table 1

Biological functions of various collagen fragments

Collagens	Fragments	Biological functions
Col I	Trimer carboxyl propeptide	Chemotactic for endothelial cells (Palmieri et al., 2000)
	Various fragments	Chemotactic for dermal fibroblasts and peripheral monocytes (Albini and Adelmann-Grill, 1985; Malone et al., 1991)
Col II	Various fragments	Inhibit collagen synthesis and MMP secretion by chondrocytes (Jennings et al., 2001)
	C-propeptide or chondrocalcin	Cartilage calcification (Alini et al., 1992; Van der Rest et al., 1986)
Col IV	NC1 and 7S domains	Inhibit morphogenesis in *Hydra vulgaris* (Sarras et al., 1993)
	NC1 domain	Promote axonal growth (Lein et al., 1991)
	α1 NC1, arresten	Anti-angiogenic (Colorado et al., 2000)
	α2 NC1, canstatin	Anti-angiogenic (Kamphaus et al., 2000; Petitclerc et al., 2000)
	α3 NC1, tumstatin	Anti-angiogenic (Maeshima et al., 2000; Petitclerc et al., 2000)
	α6 NC1	Anti-angiogenic (Petitclerc et al., 2000)
Col VIII	NC1 domain vastatin	Angiogenic (Xu et al., 2001)
Col XIV	N-terminal fragment	Chemotactic for neutrophils (Nakagawa et al., 1999)
Col XV	NC1	Anti-angiogenic (Sasaki et al., 2000)
	Restin	Anti-angiogenic (Ramchandran et al., 1999; Sasaki et al., 2000)
Col XVIII	NC1	Induce migration of neural and non-neural cells in *C. elegans* (Ackley et al., 2001)
	Endostatin	Anti-angiogenic (O'Reilly et al., 1997) Inhibit branching morphogenesis (Karihaloo et al., 2001)

Table 2

Interaction of specific receptors and other proteins with NC1 fragments of collagens IV, XV and XVIII

Protein interactions	NC1 fragments
Receptors	
Integrins	
$\alpha_1\beta_1$ and $\alpha_2\beta_1$	All NC1 (IV) fragments
$A_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$	Endostatin (Rehn et al., 2001)
$\alpha_6\beta_1$ and $\alpha_v\beta_3$	α3NC1(IV) or Tumstatin (Maeshima et al., 2000b)
$\alpha_3\beta_1$ and $\alpha_v\beta_3$	Canstatin (Kamphaus et al., 2000)
Glypican3	Endostatin (Karumanchi et al., 2001)
VEGF-R2	Endostatin (Kim et al., 2002)
ECM proteins	
Fibulin1, fibulin2, nidogen2	Endostatin and endostatin-like (Sasaki et al., 2000)
Perlecan, laminin1	NC1(XV) and NC1(XVIII) (Sasaki et al., 2000)
MMP-2	Endostatin (Kim et al., 2000; Lee et al., 2002)
Intracellular proteins	
Tropomyosin	Endostatin (MacDonald et al., 2001)

Table 3

Functions of NC1 and NC1 fragments of collagen IV, XV and XVIII

	Induction	Inhibition
Collagen IV		
Immobilized NC1	Adhesion and chemotaxis of cancer cell lines (α3) (Han et al., 1997; Shahan et al., 1999a; Shahan et al., 2000) FAK and PI3K phosphorylation (α3) (Pasco et al., 2000a)	
Soluble NC1	(α1 and α2 chains) Axonal growth of sympathetic neurons (Lein et al., 1991)	Hydra regeneration (Sarras et al., 1993) Activation of leukocytes (a3) (Monboisse et al., 1994)
	Adhesion and migration of chicken neural crest cells (α1 and α2) (Perris et al., 1993)	Proliferation and migration of cancer cell lines (α3) (Han et al., 1997; Shahan et al., 1999a; Shahan et al., 2000; Pasco et al., 2000b)
	Adhesion and spreading of endothelial cell (α1 and α2) (Koliakos et al., 1989; Tsilibary et al., 1990)	MMP-2 and MMP-3 activation (α1, α2 and α3) (Netzer et al., 1998; Pasco et al., 2000b)
	Apoptosis of endothelial cells (54–132 aa peptide α3) (Maeshima et al., 2001a)	Proliferation and migration of endothelial cells (α1, α2 and α3) (Colorado et al., 2000; Kamphaus et al., 2000; Maeshima et al., 2000a; Maeshima et al., 2001a) In vitro angiogenesis (α1, α2 and α3) (Colorado et al., 2000; Kamphaus et al., 2000; Maeshima et al., 2000a) Chorioallantoic membrane angiogenesis FGF2-induced (α1, α2 and α6) or VEGF-induced (α2) (Petitclerc et al., 2000) Tumor angiogenesis (α1, α2 and α3) (Colorado et al., 2000; Kamphaus et al., 2000; Maeshima et al., 2000a; Petitclerc et al., 2000) Protein synthesis cap-dependent (54–132 aa peptide α3) (Maeshima et al., 2002)
Collagen XV		
Soluble monomeric endostatin-like	Reduction of tumor growth (Ramchandran et al., 1999)	Migration of endothelial cells FGF2-induced (Ramchandran et al., 1999) Chorioallantoic membrane angiogenesis VEGF-induced (Sasaki et al., 2000)
NC1 trimer		Chorioallantoic membrane angiogenesis FGF2 and VEGF-induced (Sasaki et al., 2000)
Collagen XVIII		
Soluble endostatin	Apoptosis of endothelial cells (Dhanabal et al., 1999b)	Proliferation and migration of endothelial cells FGF2 or VEGF-induced (O'Reilly et al., 1997; Yamaguchi et al., 1999; You et al., 1999; Taddei et al., 1999; Dhanabal et al., 1999a)
	Tumor regression (O'Reilly et al., 1997; Boehm et al., 1997)	Tumor angiogenesis (O'Reilly et al., 1997; Boehm et al., 1997; Bergers et al., 1999; Blezinger et al., 1999; Boehle et al., 2001; Dhanabel et al., 1999b; Kisker et al., 2001; Sorensen et al., 2002; Yoon et al., 1999)
	Down-regulation of gene transcription (Shirichi and Hirata, 2001; Hanai et al., 2002)	Choroidal neovascularization (Mori et al., 2001)
	G1 arrest of endothelial cells (Hanai et al., 2002)	Arthritis (Matsuno et al., 2002; Yin et al., 2002)
	In vitro stabilization of microvessels (Ergun et al., 2001)	Microvessel formation in rat aortic or human vein ring angiogenesis assay (Kruger et al., 2000; Ergun et al., 2001) Chorioallantoic membrane angiogenesis FGF2-induced (Sasaki et al., 2000) Migration of neural and non-neural cells in *C. elegans* (Ackley et al., 2001) Migration and branching morphogenesis of renal epithelial cells (Karihaloo et al., 2001) FAK phosphorylation, focal adhesion and actin stress fibers formation growth factor induced (Dixelius et al., 2002; Wickstrom et al., 2001) Endothelial and cancer cell invasion through Matrigel (Kim et al., 2000)
Immobilized		
Monomeric endostatin	Endothelial cell spreading, FAK phosphorylation (Rehn et al., 2001)	
NC1 trimer	Cell motility of endothelial and non-endothelial cells (Kuo et al., 2001)	Endothelial tube formation in Matrigel (Kuo et al., 2001)