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Matricryptins derived from collagens and proteoglycans

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1. ABSTRACT

Controlled proteolysis of extracellular matrix components releases bioactive fragments or unmasks cryptic sites that play key roles in controlling various physio-pathological processes including angiogenesis, tissue remodeling, wound healing, inflammation, tumor growth, and metastasis. We review here the structure and mechanisms of release of *i*) the proteolytic fragments (matricryptins) cleaved from collagens, proteoglycans and glycosaminoglycans, and *ii*) the matricryptic sites existing in these molecules. The cell surface receptors and the signaling pathways they trigger to exert their biological activities is discussed with the major physio-pathological processes they control. Their involvement in autoimmune and inherited diseases is reported. Most matricryptins issued from collagens, proteoglycans and glycosaminoglycans exhibit anti-angiogenic and anti-tumor properties and their use as potential drugs and as potential disease markers is discussed. Perspectives for identifying the common structural features, if any, of the matricryptins and their use in combination with chemotherapy and radiotherapy in the treatment of cancer are presented.

2. INTRODUCTION

Controlled proteolysis of extracellular matrix components releases bioactive fragments or unmasks cryptic sites that play key roles in controlling various physio-pathological processes including angiogenesis, wound healing, inflammation, tumor growth and metastasis (1-4). Several matricryptins released from collagens (endostatin, arresten, canstatin, tumstatin, restin, vastatin) and from a proteoglycan (endorepellin) correspond to the

C-terminal domain of those molecules (5). Most of them exert their biological activities through signaling pathways mediated by integrins. We will focus in this review on matricryptins or matrikines existing in tissues, in biological fluids or released by cultured cells. We will not discuss synthetic peptides displaying biological activities that are not produced *in vivo*.

We will first define the terms matricryptins and matricryptic, and we will summarize the mechanisms leading to the release of matricryptins and to the exposure of cryptic sites. Then we will describe the major matricryptins derived from collagens, proteoglycans and glycosaminoglycans and the major patho-physiological processes they regulate, namely angiogenesis, tumor growth and metastasis, tissue remodeling wound healing, and inflammation. We will briefly introduce the autoimmune diseases they are involved in. In the last part of this review, we will discuss the matricryptins as potential drugs, matricryptic sites as therapeutic targets and matricryptins as potential markers of diseases.

3. WHAT ARE MATRICRYPTINS AND MATRICRYPTIC SITES?

3.1. Definition

The term matricryptin was proposed by Davis *et al.* (1) for enzymatic fragments of the extracellular matrix containing exposed matricryptic sites. This term refers specifically and is limited to biologically active extracellular matrix fragments that contain a cryptic domain that is not normally exposed in the intact molecule. Matricryptins are derived from molecular domains that are cryptic and enzymatic breakdown is required to expose the new biologically relevant activity. This is the major difference between matricryptins and matrikines that have been defined as peptides liberated by partial proteolysis of extracellular matrix macromolecules, which are able to regulate cell activities (6, 7). Matricryptic sites have been defined by Davis *et al.* (1) to be biologically active sites that are not exposed in the mature, secreted form of extracellular matrix molecules. They become exposed after structural or conformational alterations of the parent molecules. The appearance of matricryptic sites may provide important new signals to regulate biological processes such as cell migration, proliferation, differentiation, morphogenesis, survival, extracellular matrix assembly, and physio-pathological proccess such as angiogenesis, tumor growth and tissue repair.

3.2. Mechanisms of exposure

Mechanisms regulating the exposure of matricryptic sites within the extracellular matrix molecules include enzymatic breakdown, protein multimerization, adsorption, cell-mediated mechanical forces, and denaturation (1). Reactive oxygen species also expose cryptic epitopes as reported for epitopes associated with the autoimmune Goodpasture syndrome (8). In contrast, sugars may mask cryptic sites as reported for galactose that modulate the interaction of the triple-helical models of the α_1(IV) 1263-1277 sequence with melanoma cell CD44 (9). Due to space limitation, this review will focus mostly on matricryptins generated from collagens and proteoglycans by limited proteolysis. Matricryptins derived from fibronectin and laminins will be discussed in other chapters of this volume.

Several matricryptins released by proteolytic cleavage are C-terminal domains of collagens and of a proteoglycan (perlecan) found in the vascular basement membrane zone. They play a role in maintaining basement membrane integrity as shown for endostatin that is co-localized with perlecan in basement membranes (10). Endostatin overexpression specifically in the lens and skin leads to cataract and ultrastructural alterations in basement membrane (11). Endostatin is also a component of elastic fibers in vessel walls (12).

Various matrix metalloproteinases (MMPs) are involved in the release of matricryptins. Tumstatin is cleaved from the α_3 chain of collagen IV by MMP-9 (13). Fragments containing endostatin are cleaved from the NC1 domain of collagen XVIII by MMP-7 (14) and MMP-14 also referred to as MT1-MMP but not by MMP-2 or MMP-9 (15). Cathepsins B, L and V (16, 17) and pancreatic elastase (18) are also able to generate endostatin-containing fragments. MMP-2, able to degrade a number of extracellular matrix molecules, appears to act as a main 'decryptase' with MT1-MMP for collagen IV and laminin-332 (19). Hyaluronidase and heparinase that degrade glycosaminoglycans also release matricryptins. A matricryptin can exist under several forms depending on the presence of several cleavage sites in the parent molecule and on the enzyme cleaving the parent molecule. A matricryptin can also be further processed as it has been reported for the C-terminal matricryptin of perlecan, endorepellin, which is cleaved by Bone Morphogenetic Protein-1/Tolloid to generate the terminal laminin-like globular LG3 domain that possesses most of the biological activity on endothelial cells (20). MT-MMP1 cleaves syndecan-1 and this shedding stimulates cell migration (21).

The shedding of membrane proteins is a proteolytic process leading to the release of the extracellular domain of the proteins in the extracellular milieu. The shed ectodomain of membrane collagens XIII, XVII, XXIII, and XXV (22) and of syndecans 1-4 (21, 23) that are released from the membrane by enzymes belonging to the ADAM (a disintegrin and metalloproteinase), the MMP or the furin families might be considered as matricryptins because they exist under a membrane form and a soluble form corresponding to the shed ectodomain that exhibits biological activities regulating cell behavior upon shedding (22-25). They will not be discussed here due to space limitation. In the same way, synthetic peptides, resulting from cyanogen bromide cleavage and peptides not identified in tissue or biological fluids were not included in this review for the same reason.

Most matricryptins have been found in tissue or biological fluids. However more than 120 endogenous peptide inhibitors of endothelial cell proliferation and migration have been identified using a systematic

Table 1. Cryptic sites in collagens

Source of cryptic sites	Cryptic site(s)	Receptors	References
Collagen I	RGD sequence	αvβ3, α1β1 α2β1, α3β1	240, 241 31
	DGEA sequence	α2β1, α3β1	241
	α_2-CB3,5, [α_1-CB3] $_2$ α_1-CB3, α_1-CB8 α_2-CB4, α_1-CB7 α_1-CB8, α_1-CB3 α_1-CB6, α_1-CB2, α_1-CB4, α_1-CB5 (14 binding sites to fibronectin)		32
Collagen IV	HUIV26	αvβ3	242
	HU177		242

Cryptic sites of collagens I and IV and their receptors when identified. (CB: peptides issued from CNBr cleavage)

computational methodology based on bioinformatics (26). They include peptides derived from the α4 chain (tetrastatins), the α5 chain (pentastatins) and α6 chain (hexastatins) of collagen IV. The computational bioinformatic analysis was based on the hypothesis that there is an underlying sequence-based correlation between the activity of the known endogenous protein fragments and the predicted peptides (26).

4. MATRICRYPTINS FROM COLLAGENS

Cryptic sites are present in the triple helical domains of collagens. In addition, several collagens located in vascular basement membrane (IV, XVIII) or in basement membrane zones (XV, XIX) are cleaved by proteases that release matricryptins with anti-angiogenic and anti-tumor properties (25, 27-29). The most extensively studied matricryptin is endostatin that has been identified in 1997 in Judah Folkman's laboratory (30).

4.1. Matricryptins and cryptic sites of collagen I

Several cryptic sites have been identified in collagen I (Table 1). Partially denatured collagen exposes RGD-motifs that trigger binding of α5β1 and αv-integrins, which initiate cellular processes that stimulate osteoblast adhesion, spreading, motility and differentiation (31). 14 cryptic fibronectin binding sites of similar affinity have been identified in bovine collagen I treated with cyanogen bromide (32). Five were located on each of the α_1(1) chains and four on the α_2(I) chain. However, it should be noted that these cryptic sites were exposed by chemical cleavage and that they might not be exposed *in vivo*. Peptides of collagen I stimulate respiratory burst, granule exocytosis and cytokine secretion by human leukocytes (polymorphonuclear neutrophils or monocytes) for the detersion of inflammatory sites and then for the chemoattraction of various cell types needed for wound healing (33-35).

4.2. Chondrocalcin, the C-propeptide of collagen II

Chondrocalcin is identical to the C-propeptide of procollagen II, and plays a role in events leading to cartilage calcification (36, 37). It accumulates in calcifying cartilage and fetal bone. It may contribute to the anchorage of matrix vesicles to the extracellular matrix of calcifying cartilage through its binding to anchorin CII, a major component of matrix vesicles (38). Chondrocalcin has been found in serum and in synovial fluid, and is used as a marker of biosynthesis of collagen II in arthritic diseases (e.g. 39). Mutations in the C-Propeptide of procollagen II cause Stickler syndrome (40) and spondyloperipheral dysplasia (41). Specific mutations in the C-propeptide domain are associated with spondyloperipheral dysplasia and platyspondylic lethal skeletal dysplasia Torrance type that constitute a subfamily within the type II collagenopathies, distinct from other type 2 collagenopathies associated with mutations in the triple-helical domain of collagen II (42). Decreased collagen fibrillogenesis and accumulation of unfolded collagen chains inside the chondrocytes could contribute to the specific phenotype of mutations occurring in the C-propeptide (42).

4.3. Matricryptins and cryptic sites of collagen IV

The triple-helical domain of type IV collagen contains cryptic sites (Table 1). A series of monoclonal antibodies that bind to cryptic epitopes of collagen IV and that are differentially exposed during matrix remodeling and are key mediators of angiogenesis have been developed. Two cryptic angiogenesis regulatory epitopes have been identifed by monoclonal antibodies HUI77 and HUIV26. They have been detected within the basement membrane of angiogenic blood vessels and can be exposed by thermal denaturation or proteolytic cleavage. Ionizing radiation modulates the exposure of the HUIV26 cryptic epitope during angiogenesis and this might contribute to the mechanisms of angiogenesis inhibition by radiation (43). Proteolytic cleavage of collagen IV by MMP-9 results in the exposure of a cryptic site hidden within its triple helical structure (44). Exposure of this site is required for angiogenesis and tumor growth *in vivo*, and is associated with a loss of α1β1 integrin binding and the gain of αvβ3 binding (45). HU177 cryptic epitope could be exposed following tumor- and vascular smooth muscle cell–mediated proteolysis, ischemic injury and within the extracellular matrix of developing murine papillary membranes during angiogenesis. This second cryptic collagen epitope is present in both collagens IV and I and may be comprised of a peptide that includes the amino acid residues LPGFPG (46). It is selectively exposed within tumor blood vessel extracellular matrix, whereas little was associated with quiescent vessels (46).

Collagen IV is the source of several matricryptins or matrikines located in the C-terminal domains of α(IV) collagen chains (e.g. arresten, canstatin, tumstatin, and the NC1 domain of collagen IV) (see 28, 47-49 for reviews) (Table 2). Tetrastatins, pentastatins, and hexastatins are

Table 2. Collagen and proteoglycan matricryptins

Matricryptins	Parent molecule	UniProtBK identifier (http://www.uniprot.org/) of the parent molecule followed by the first and last amino acid numbers of matricryptins (human sequence)	3D structure (PDB identifier http://www.rcsb.org/pdb/)	References
Trimer C-propeptide (C3)	Collagen I	P02452, P08123		154, 155
Chondrocalcin	Collagen II	P02458		36
Arresten NC1 domain	α1(IV) chain Collagen IV	P02462 (1445-1669)	1LI1	53, 107
Canstatin NC1 domain	α2 (IV) chain Collagen IV	P08572 (1486-1712)	1LI1	53, 243
Tumstatin NC1 domain Oncothanin	α3(IV) chain Collagen IV Peptides 185-203 and 179-208	Q01955 (1426-1670)	Predicted secondary structure 246	53, 119, 244, 245
Tetrastatin-1	α4(IV) chain Collagen IV	P53420 (1514–1533) LPVFSTLPFAYCNIHQVCHY		247
Tetrastatin-2	α4(IV) chain Collagen IV	P53420 (1524–1543) YCNIHQVCHYAQRNDRSYWL		26, 247,
Tetrastatin-3	α4(IV) chain Collagen IV	P53420 (1628–1646) AAPFLECQGRQGTCHFFAN		247
Pentastatin-1	α5(IV) chain Collagen IV	P29400 (1516-1535) LRRFSTMPFMFCNINNVCNF		26, 165, 247
Pentastatin-2	α5(IV) chain Collagen IV	P29400 (1526–1545) FCNINNVCNFASRNDYSYWL		247
Pentastatin-3	α5(IV) chain Collagen IV	P29400 (1632–1650) SAPFIECHGRGTCNYYANS		247
Hexastatin-1	α6(IV) chain Collagen IV	Q14031 (1629–1647) ATPFIECSGARGTCHYFAN		247
Hexastatin-2	α6(IV) chain Collagen IV	Q14031 (1526–1545) YCNINEVCHYARRNDKSYWL		247
NC1 α6(IV)	α6(IV) chain Collagen IV	Q14031 (1467-1691)		145
α1(IV) 1263-1277	α1(IV) chain Collagen IV	P02462 (1263-1277) GVKGDKGNPGWPGAE	GVKGDKGNP GWPGAE	9, 248
α1(IV) CB3	α1(IV) chain Collagen IV			249, 250
α1(IV) 531-543	α1(IV) chain Collagen IV	P02462 (531-543) GEFYFDLRLKGDK		251- 253
Vastatin NC1 domain	α1(VIII) chain Collagen VIII	P27658 (572-744)	1O91	56
Restin C-terminal fragment	α1(XV) chain Collagen XV	P39059 (1212-1388)	1DY2	57
Frizzled module N-terminal fragment	α1(XVIII) chain Collagen XVIII	P39060 (329-446)		90, 91, 254
Endostatin C-terminal fragment	α1(XVIII) chain Collagen XVIII	P39060 (1572-1754)	1BNL	30,
Neostatin-7	α1(XVIII) chain Collagen XVIII	P39060 (1558-1754)		14, 15
Neostatin-14	α1(XVIII) chain Collagen XVIII	P39060 (1512-1754)		15
NC1 α1(XIX)	α1(XIX) chain Collagen XIX	Q14993 (1124-1142) NPEDCLYPVSHAHQRTGGN		92
Endorepellin Domain V	Perlecan	P98160 (3687-4391)		93

also part of the NC1 domain of the α_4, α_5 and α_6 chains of collagen IV (26) (Table 2). Tumstatin is generated by MMP-9 (13) and TIMP-1 (Tissue Inhibitor of Metalloproteinases-1) inhibits the production of tumstatin (50). Cathepsin S, a cysteine protease, regulates the levels of canstatin and arresten, but not of tumstatin, by degrading these matricryptins (51). The release of arresten and canstatin is mediated by MT2-MMP in developing tissues (52). Arresten and canstatin have been identified in developing tissues (52). Canstatin has been identified in other tissue extracts and in serum (51, 53), and tumstatin in renal carcinoma (54). An ELISA has been developed to measure tumstatin level in serum samples and tissue extracts (55).

4.4. Vastatin, a matricryptin of collagen VIII

A single study reports that vastatin (Table 2), the NC1 domain of the α_1 chain of collagen VIII, inhibits bovine aortic endothelial cell proliferation and causes cell apoptosis (56).

4.5 Restin, a matricryptin of collagen XV

Collagen XV contains a C-terminal fragment released by proteolytic cleavage (57). This 22-kDa fragment is called restin (or endostatin-XV, Table 2) and is highly homologous (61% sequence identity) to the C-terminal domain of collagen XVIII referred to as endostatin (cf. the next paragraph). Both matricryptins are issued from collagens belonging to the same collagen subfamily, the multiplexins (multiple triple helix domains and interruptions) and their overall fold is very similar with two α helices, 16 β strands and two conserved disulfide bridges (58). However restin does not contain a zinc-binding site in contrast to endostatin (58). Restin-containing fragments of different sizes have been detected in tissues by Western blotting (58).

4.6. Matricryptins of collagen XVIII

Endostatin (Table 2) has been identified in 1997 in Judah Folkman's laboratory (30) and has been extensively reviewed (59-64). Endostatin raised high expectations for antiangiogenic therapy of experimental cancer because drug resistance does not develop in the first studies (65). It contains zinc-binding site and although conflicting results have been reported on the role of zinc in anti-angiogenic and anti-tumoral properties of endostatin (reviewed in 62), zinc increases the binding of endostatin to heparan sulfate by approximately 40% as well as its antiproliferative effect on endothelial cells stimulated by fibroblast growth factor-2 (FGF-2) (66). However, endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding (67). Zinc-dependent dimers have been observed in crystals of human endostatin (68). Zn(II)-binding largely stabilizes the structure of endostatin at physiological pH and engineering an extra zinc-binding peptide to the N-terminus of human endostatin makes this molecule more stable and cooperative in the presence of Zn(II) (69).The N-terminal fragment of endostatin also binds copper(II) with very high affinity (70). Endostatin is centered on a seven-stranded β-sheet. One side of the β-sheet contains the α1 α-helix and the other side is covered by short β sheets, loops, and a second helix, α2 (68, 71, 72). Endostatin is one of the three subdomains of the NC1 domain of collagen XVIII that is composed of a trimerization domain and a hinge region in addition to endostatin. Several proteolytic cleavage sites exist in the hinge region of the NC1 domain and they lead to the release of endostatin-containing fragments differing in molecular size (24-30 kDa) and in N-terminal sequences. These fragments are generated by MMP-3, -7, -9, -13 and -20. Several components containing endostatin (22–38 kDa) have also been detected in murine tissues (73, 74) and tumors. Endostatin is found in tumor stromal components, including vessel walls, basement membranes, extracellular spaces, and tumor cells (75). Endostatin and several fragments containing endostatin (24-38 kDa) are secreted by tumor cells (73, 76). Two C-terminal fragments of collagen XVIII containing the endostatin sequence, neostatin-7 (28 kDa) and neostatin-14 (23 kDa) generated by MMP-7 and MMP-14 cleavage respectively have been described (14, 15). It should be noted that a fragment issued from the digestion of fibulin-1 by cathepsin D, and inhibiting endothelial cell proliferation, has been also named neostatin (77).

Mechanical factors are able to stimulate fibroblasts to release increased concentrations of endostatin (78). Tamoxifen exposure of highly hormone responsive ovarian cancer cells decreases proliferation, and increases MMP-9 activity leading to increased levels of endostatin both in cell culture *in vitro* and in solid tumors of nude mice (79). Hypoxia induces increase in endostatin in murine aorta and lung (74). Endostatin expression is up regulated in ischemic kidneys under the form of a 28-kDa fragment (80) and in the neuroblastoma cell line SK-N-SH (81). In contrast, hypoxia down-regulates endostatin production by human microvascular endothelial cells and pericytes (82). Endothelial endostatin release is also induced by general cell stress and modulated by the nitric oxide/cGMP pathway (83) and p53 expression greatly enhances the processing of full-length collagen XVIII to endostatin (84). p53 induction leads to an increase in endostatin levels in the Soas-2 human osteosarcoma cell line (85). Hypocrellin photochemotherapy induces endostatin release in glioma cells, and reduced it in macrophages and endothelial cells (81). Endostatin is present in serum, urine (73), in leg ulcer fluid, where most of it is bound to glypican-1 (86), in broncho-alveolar fluid (87), and in ocular fluid (88). Endogenous endostatin and related peptides are also widely distributed in murine tumors (75) and peri-infarct neurons express endostatin during the early stage of cerebral ischemia (89).

Collagen XVIII exists as three amino terminal end variants with specific amino terminal polypeptide modules. The variant 3 contains a 110-residue sequence with 10 cysteine residues, homologous with frizzled proteins belonging to the family of G-protein-coupled membrane receptors (90). This variant is proteolytically processed into a cell surface 50 kDa glycoprotein precursor containing the N-terminal frizzled module (Table 2). In human liver cancers, collagen XVIII is processed to frizzled cysteine-rich domain-containing polypeptides (91).

4.7. Matricryptin of collagen XIX

The NC1 domain of collagen XIX (Table 2) has anti-tumor and anti-angiogenic activities. It strongly inhibits the invasive capacities of tumor cells and the expression of MT-1 MMP and VEGF (92).

5 MATRICRYPTINS FROM PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

5.1. Endorepellin

Endorepellin, named from its repulsive activity against endothelial cells, is derived from the C-terminus (domain V) of perlecan (Table 2). It is comprised of three laminin-like globular (LG1-LG3) modules interspaced by four epidermal growth factor EGF-like repeats (93, see 94, 95 for reviews), and has a higher molecular weight than the other matricryptins (85 kDa *versus* ~ 20-30 kDa). Most of the anti-apoptotic and fibrogenic activity of endorepellin is mediated by the C-terminal LG3 module. Enzymes of the bone morphogenetic protein-1/Tolloid family of metalloproteinases cleave LG3 from recombinant endorepellin and from endogenous perlecan in cultured mouse and human cells (20). Caspase-3 activation triggers extracellular cathepsin L release and endorepellin proteolysis (96). Apoptotic endothelial cells release LG3 that induces resistance to apoptosis in fibroblasts (97).

5.2. Hyaluronan fragments

Hyaluronan (HA) is a ubiquitous glycosaminoglycan that can have a molecular mass of several millions Dalton (98). Enzymatic degradation of hyaluronan by hyaluronidases 1 and 2 during tissue remodeling gives rise to fragments and oligosaccharides of various size (from 4 up to 1000 saccharides) that trigger cellular responses (proliferation, migration, cytokine synthesis) in a size-dependent manner (reviewed in 99, 100). Reactive oxygen species accumulated at sites of

Table 3. Receptors of collagen and proteoglycan matricryptins

Matricryptin	Receptors at the cell surface	Reference(s)
Arresten	Cell surface proteoglycans, α1β1, α3β1, α2β1, αvβ3	106-109
Canstatin	αvβ3, αvβ5, α1β1	111, 163
Tumstatin	CD47, αvβ3 αvβ5, α3β1, α6β1	53, 112, 113, 118, 119, 244, 255
NC1 α6(IV)	αvβ3	53
Neostatin-7	VEGF-R3	149
Endostatin	α5β1, αvβ3, αvβ5, Glypicans 1 and 4, Nucleolin,VEGF-R1, VEGF-R2	105, 121-124, 128
Endorepellin	α2β1	132

tissue injury may provide a mechanism for generating hyaluronan fragments *in vivo* (100). These fragments synergize with reactive oxygen species to activate the innate immune system and further promote reactive oxygen species, generation of hyaluronan fragments, inflammation, tissue injury, and ultimately fibrosis (101). In addition, hyaluronan fragments play a role in angiogenesis, chondrogenesis, wound healing, cancer and infection (99). 12-mers of hyaluronan inhibit the sequestration of *Plasmodium falciparum*-infected red blood cells in the placenta. We will focus in this review on the effects of hyaluronan fragments on angiogenesis, tumor growth and wound healing.

5.3. Heparan sulfate oligosaccharides

Tumor cell surface heparan sulfate contain cryptic bioactive sequences that either promote or inhibit tumor growth and metastasis *in vivo*. Heparinase I, which cleaves at the highly sulfated regions of heparan sulfate, releases heparan sulfate fragments that promote tumor growth *in vivo*. In contrast, heparinase III, that cleaves at the undersulfated regions of heparan sulfate chain, releases heparan sulfate fragments that inhibit primary tumor growth by ~ 70% (102). Heparin octasaccharides inhibit angiogenesis *in vivo* (103) but since they are synthetic oligosaccharides, they will not be discussed in this review.

6. MATRICRYPTIN RECEPTORS AND SIGNALING

Cell recognition of matricryptic sites appears to be an important component of a broad cell and tissue sensory system to detect and respond to environmental cues generated following varied types of tissue injury (104). Most matricryptins bind to integrins and some of them also bound to heparan sulfate chains (Table 3, Figure 1). Since it has been shown that several integrins regulating angiogenesis (α5β1, αvβ3 and αvβ5) bind to heparan sulfate (105), the cross-talk between integrins and cell surface proteoglycans requires further investigation to determine its impact on the mechanisms used by matricryptins to exert their anti-angiogenic and anti-tumor activities. Furthermore as detailed below, different cell types may bind to matricryptins *via* different cell surface receptors (106).

6.1. Matricryptins of collagen IV

Heparan sulfate proteoglycans might act as receptors or co-receptors for arresten (107) but most of the studies have focused on signaling mediated by integrins. The anti-angiogenic activity of arresten is mediated through the α1β1 integrin by its inhibition of FAK/c-af/MEK/ERK1/2/p38 MAPK activation in endothelial cells that lead to inhibition of hypoxia inducible factor (HIF-1 α) and VEGF expression (108). The pro-apoptotic effect of arresten on endothelial cells is also mediated by the α1β1 integrin and is due to a decrease in the amount of anti-apoptotic molecules of the Bcl-family, Bcl-2 and Bcl-xL (109). TSV-40-immortalized human glomerular epithelial cells bind to both tumstatin and arresten, with preferential binding to tumstatin, through the α3β1 and α2β1 integrin receptors, whereas HPV-16-immortalized human proximal tubular epithelial cells and primary human mesangial cells bind almost exclusively to arresten via the α3β1/αvβ3 and α1β1/α2β1 receptors, respectively (106).

Canstatin inhibits the phosphorylation of Akt, focal adhesion kinase (FAK), mammalian target of rapamycin (mTOR), eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), and ribosomal S6 kinase (110). It induces Fas-dependent apoptosis in endothelial cells (110). Canstatin exerts its pro-apoptotic activity on endothelial and tumor cells *via* procaspase-9 cleavage mediated through cross-talk between αvβ3 and αvβ5 integrins. Canstatin triggers two distinct apoptotic pathways in endothelial cells through the αv-integrin-FAK/phosphatidylinositol 3-kinase (PI3K)/caspase-9 pathway and the Fas/Fas ligand/caspase-8/caspase-9 cascade leading to cleavage of procaspase-3 (111). Arresten and canstatin provide transcriptional feedback to increase MT-MMP, collagen IV, and proproliferative gene expression *via* β1-integrin signaling, influencing collagen IV proteolysis and synthesis during branching morphogenesis (52).

The signaling mechanisms induced by tumstatin and their implication in the control of tumor growth have been recently reviewed (48, 49). The peptide encompassing residues 185–203 of tumstatin has first been demonstrated to inhibit tumor cell proliferation *via* a CD47/integrin-associated protein, and αvβ3 integrin driven mechanism (112). The 185–206 peptide of tumstatin stimulates FAK and phosphatidylinositol 3-kinase (PI3K) phosphorylation in melanoma cells (113). Tumstatin inhibits the migration of tumor cells and the activation of membrane-bound MMP-2 by decreasing the expressions of MT1-MMP and the β3 integrin subunit (114). In endothelial cells, the anti-angiogenic activity of tumstatin is mediated through αvβ3 integrin. Tumstatin inhibits activation of FAK and PI3K, protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR), and prevents the dissociation of eukaryotic initiation factor 4E protein (eIF4E) from

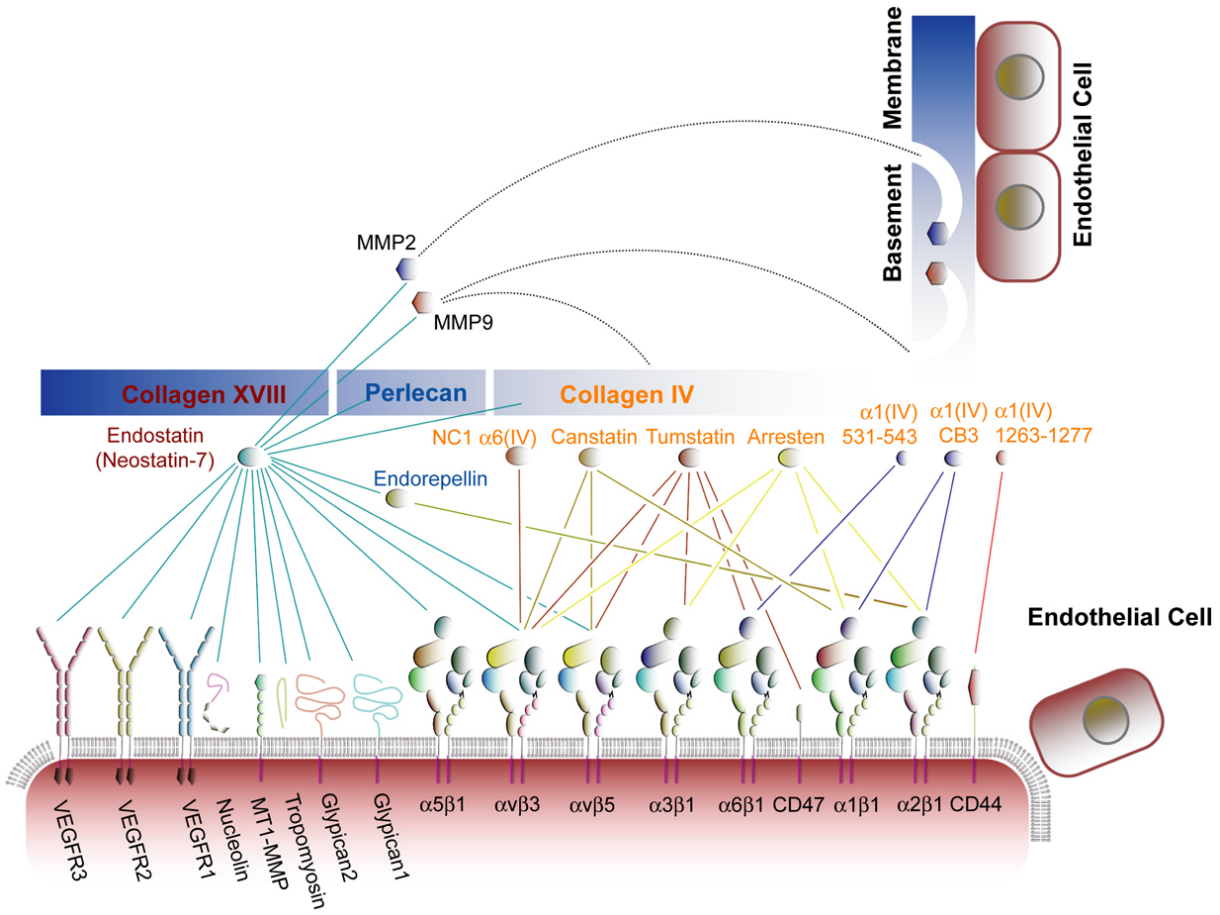

Figure 1. Matricryptins and cryptic sites regulating angiogenesis. Legend: Matricryptins and matricryptic sites from collagens IV, XV, XVIII and perlecan and their receptors at the surface of endothelial cells (CB: CNBr peptide, MMP: Matrix metalloproteinase, MT-MMP: Membrane type-matrix metalloproteinase, NC1: Non collagenous, VEGFR: Vascular endothelial growth factor receptor).

4Ebinding protein 1 (115, 116). Tumstatin inhibits NF-κB-mediated signaling leading to inhibition of COX-2 expression, which in turn results in downregulation of hypoxia-induced VEGF/FGF-2 expression. This anti-angiogenic effect is mediated by α3β1 integrin (117), that transdominantly inhibits integrin αvβ3 activation (118). Tumstatin is also able to bind to α6β1 integrin on endothelial cells (119). The C-terminal part of tumstatin, encompassing to the 183 - 232 sequence, inhibits *in vivo* melanoma progression by triggering an intracellular transduction pathway, which involves a cyclic AMP-dependent mechanism. A decrease in cyclin D1 expression is associated with an increase of the intratumor cyclic AMP (cAMP) level (120).

6.2. Matricryptins of collagen XVIII

Endostatin does not bind to syndecans at the surface of endothelial cells, but to glypicans 1 and 4 *via* their glycosaminoglycan chains (121). Since the same residues are crucial for endostatin interaction with heparan sulfate and αvβ3 or α5β1 integrins, it seems unlikely that endostatin binds simultaneously to both integrins and glypicans at the cell surface (105). However, we have shown that heparan sulfate is able to bind integrins, and this suggests the possible existence of ternary complexes comprising endostatin, heparan sulfate and integrin at the endothelial cell surface (105).

Endostatin binds to α5β1 and αvβ3 integrins at the surface of endothelial cells (122). Endostatin also associates with caveolin-1, and activates Src *via* a tyrosyl phosphatase-dependent pathway in human endothelial cells (123). The binding of endostatin to α5β1 integrin inhibits the focal adhesion kinase/c-Raf/MEK1/2/p38/ERK1 mitogen-activated protein kinase pathway (116). Endostatin interferes with another pathway by inhibiting vascular endothelial growth factor (VEGF)-mediated signaling *via* direct interaction with KDR/Flk-1. Endostatin blocks VEGF-induced tyrosine phosphorylation of KDR/Flk-1 and activation of ERK, p38 MAPK, and p125(FAK) in human umbilical vein endothelial cells (124). VEGF(121)-stimulated ERK activation is blocked by endostatin. ERK1/2 phosphorylation is regulated by endostatin *via* the protein phosphatase 2A (125).

Endostatin, the C-terminal fragment of collagen XVIII, is a potential inhibitor of Wnt signaling (126). The N-terminal domain of collagen XVIII binds to Wnt3a *in vitro* and suppressed Wnt3a-induced stabilization of β-catenin. Expression of the frizzled domain inhibits Wnt/β-catenin signaling in colorectal and liver cancer cell lines, downregulating major cell cycle checkpoint gatekeepers cyclin D1 and c-myc and reducing tumor cell growth (91).

Endostatin is internalized by endothelial cells (127, 128) and by AIDS-related Kaposi's sarcoma cells where it initiates activation of the transcription activating factors nuclear factor-κB and activating protein 1. Endostatin co-localizes to tropomyosin microfilaments and inhibits cytokine-mediated Kaposi cell migration and invasion (129). Endostatin treatment of endothelial cells induces tyrosine phosphorylation of Shb, an adaptor protein implicated in angiostatin-induced apoptosis, and formation of multiprotein complexes (127). Endostatin binds *via* its heparin-binding domain to the cell surface nucleolin, which mediates the antiangiogenic and antitumor functions of endostatin (128,130). Nucleolin mediates endostatin internalization in endothelial cells and endostatin inhibits FGF-2 stimulated phosphorylation of nucleolin (128).

6.3. Endorepellin

Endorepellin disrupts α2β1 integrin function that plays a key role in experimental and developmental angiogenesis (131). The binding of endorepellin to α2β1 integrin on endothelial cells triggers a signaling cascade that leads to the disassembly of the actin cytoskeleton and focal adhesions (132). Endorepellin increases intracellular cAMP level, accompanied by concurrent activation of protein kinase A, sustained activation of FAK and transient activation of p38 MAPK and the small chaperone heat shock protein 27 (5,94). The Src homology-2 protein phosphatase-1 (SHP-1) is a mediator of endorepellin angiostatic activity (133). The C-terminal fragment of endorepellin, LG3, interacts with the α2β1 integrin receptor of fibroblasts and triggers integrin-dependent signaling events leading to the inhibition of apoptosis of fibroblasts through PI3K-dependent pathways leading to down-regulation of the pro-apoptotic protein Bim-EL and up-regulation of the anti-apoptotic protein Bcl-xL (,97, 134). Endorepellin is internalized by tumor-derived endothelial cells causing a redistribution of α2β1 integrin that colocalizes with endorepellin to punctate deposits in the perivascular region (135).

6.4. Hyaluronan fragments

Several signal transduction pathways are initiated by various sizes of HA fragments binding to cell surface HA receptors such as CD44 and Receptor for HA-Mediated Motility (RHAMM, CD168) (99). They include Raf-1 kinase, MAP kinase, and extracellular signaling kinases such as ERK-1 activated by hyaluronan oligosaccharides, regulation of Erb2 phosphorylation and signaling in cancer cells and activation of an NF-κB/I-κB alpha auto-regulatory inducing transcription of MMP-9 and MMP-12 (99). Hyaluronan fragments (10–15 disaccharides) induce tyrosine phosphorylation of p125(FAK), paxillin, and p42/44 ERK in endothelial cells (100).

Oligosaccharides of hyaluronan induce angiogenesis through distinct CD44 and RHAMM-mediated signaling pathways involving Cdc2 (Cdk1) for CD44 and gamma-adducin for both receptors (136). The stimulation of angiogenesis by hyaluronan oligosaccharides is initiated by RHAMM mediated signal pathway, but not by CD44, in wound healing (137). Signaling from hyaluronan degradation products also involves other signaling pathways through Toll-like receptors, TLR-2 and TLR-4 (see 100 for review), that are critical components in the innate immune response. The regulatory effect exerted by hyaluronan, whatever its molecular size, on NF-κB activation may depend upon the interaction between HA and Toll-like receptor-4 (TLR-4) (138). Hyaluronan fragments stimulate endothelial recognition of injury through TLR-4 (139) and low molecular weight hyaluronic acid obtained by biosynthesis (< 200 kDa) increases the self-defense of skin epithelium by induction of β-defensin 2 *via* TLR-2 and TLR-4 (140).

7. PHYSIO-PATHOLOGICAL PROCESSES CONTROLLED BY MATRICRYPTINS

Matricryptins and cryptic sites issued from the same molecule might have different biological roles. Cleavage of several α chains of collagen IV releases C-terminal fragments exhibiting anti-angiogenic properties, whereas the cryptic site demasked within collagen IV triple helix by proteolytic cleavage due to MMP-9 is pro-angiogenic (44, 45). Major physio-pathological processes regulated by matricryptins include angiogenesis and tumor growth. Matricryptin cleavage may be regulated by the same mechanism as reported for endostatin and tumstatin. The increased extracellular release of both matricryptins results from p53-dependent up-regulation of α(II) prolyl hydroxylase, p53 playing a role in the regulation of angiogenesis (84). The anti-angiogenic activity of endostatin and tumstatin mediate in part their tumor suppression activity (141). Anti-angiogenic matricryptins do not always act in synergy. Indeed, endorepellin binds to endostatin but it counteracts its anti-angiogenic effects (93).

7.1. Angiogenesis

Angiogenesis occurs in physiological (wound healing, menstrual cycle, embryogenesis) and pathological (rheumatoid arthritis, psoriasis, macular degeneration, diabetic retinopathy, and tumorigenesis). Several cryptic proteolytic fragments of extracellular matrix molecules exhibit anti-angiogenic properties including endostatin, arresten, canstatin, tumstatin, and vastatin (3, 27). Tumor growth being angiogenesis-dependent (142), numerous studies have investigated the clinical potential of antiangiogenic cryptic fragments of extracellular proteins as anti-tumor drugs (143, 144). Matrikines issued from collagen IV (arresten, canstatin, tumstatin, the NC1 domain of the α_6 chain, 48, 145), VIII (vastatin), XV (restin), and XVIII (endostatin, see 62 for review), are endogenous inhibitors of angiogenesis. They inhibit the proliferation and migration of endothelial cells and several of them induce endothelial cell apoptosis. They are thus of potential therapeutic interest in all the pathological situations involving angiogenesis.

Tumstatin is an endothelial cell-specific inhibitor of protein synthesis (115) and its anti-angiogenic activity is localized in the 54 -132 sequence. Angiogenesis is a feature of remodeling in asthma and the tumstatin level is markedly decreased in the airways of patients with asthma. It inhibits airway hyperresponsiveness and angiogenesis and is of potential use as a therapeutic agent in asthma (146) and in diseases that are characterized by angiogenesis and tissue remodeling.

Restin inhibits the migration of endothelial cells *in vitro* but has no effect on the proliferation of these cells (57). Restin and the NC1(XV) domain inhibit angiogenesis in the chick chorioallantoic membrane assay. Both reduced stimulation by VEGF levels close to background (58).

Mice lacking collagen XVIII and its proteolytically derived product endostatin show delayed regression of blood vessels in the vitreous along the surface of the retina after birth (147). Endostatin has a biphasic effect on the inhibition of endothelial cell migration and proliferation *in vitro* and on the inhibition of tumor growth *in vivo* (148). Neostatin-7, a fragment of collagen XVIII containing endostatin, regulates FGF-2-induced corneal lymphangiogenesis (149). Angiogenesis is perpetuated in arthritis and endostatin abrogates arthritis and suppresses pannus formation and joint destruction in rodent models (150).

Endorepellin inhibits angiogenesis by altering the endothelial cytoskeleton, which is essential for the ability of cells to migrate and form capillary-like structures, in a Ca^{2+}-dependent, heparan sulfate-independent fashion (5). It can act in a paracrine function on sprouting endothelial cells, either locally or distantly (95). A proteomic analysis of endorepellin-treated human endothelial cells show that β-actin, calreticulin, and chaperonin/Hsp60 (heat shock protein 60) are down-regulated and vimentin and the β subunit of prolyl 4-hydroxylase (protein disulfide isomerase) are up-regulated in response to endorepellin treatment (151). These proteins represent potential target areas involved with endorepellin action. Perlecan is essential for the integrity of somitic muscle and developmental angiogenesis in zebrafish and endorepellin mediates most of these biological activities (152). Endorepellin is present in the upper proliferating zone of fetal growth plate suggesting that it might counteract blood vessel formation in cartilage (153).

The trimer carboxyl propeptide of collagen I produced by mature osteoblasts is chemotactic for endothelial cells (154), and induces directional migration and metalloproteinases in breast cancer cells (155) and VEGF-A and CXCR4 (C-X-C chemokine receptor type 4) expression in breast carcinoma cells (156). It promotes rapid vascularization of the tumors while does not affect mitotic and apoptotic indexes and overall tumor growth. It has an early promoting effect in the acquisition by the tumors of prometastatic phenotype (157).

The regulation of angiogenesis by hyaluronan depends on its molecular size. High molecular weight hyaluronan inhibits angiogenesis, whereas hyaluronan oligosaccharides (3-25 disaccharides) promote angiogenesis by stimulating endothelial cell proliferation, migration and tube formation (reviewed in 99). The study of several hyaluronan of defined size showed that hyaluronan hexasaccharide, octasaccharide and decasaccharide, but not tetrasaccharide, are proangiogenic (158). Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses (159). Oligosaccharides of 3-10 disaccharide units (1350-4500 Da) increase the synthesis of collagens I and VIII by endothelial cells (160).

7.2. Tumor growth and metastasis

Proteolytic exposure of matricryptic sites or matricryptin release regulates tumor cell growth and invasive properties (161). Matrikines issued from collagen IV (arresten, canstatin, tumstatin, the NC1 domain of the α_6 collagen IV chain, 28, 47, 48, 145) inhibit tumor growth. *In vivo* mice showing decreased circulating levels of tumstatin due to deficience in MMP-9 showed also accelerated tumor growth (13). Mice deficient in endostatin show increased tumor growth when implanted with cancer cells that do not produce collagen XVIII (144).

Melanoma progression is controlled by matrikines from basement membrane proteins that regulate the proteolytic cascades leading to cancer cell dissemination and metastasis. Tumstatin inhibits melanoma cell proliferation, migration and invasion by decreasing MMP production and activation (reviewed in 28, 47, 120). The NC1 domain of the α_6(IV) chain suppresses the growth of subcutaneously transplanted Lewis lung carcinoma and of spontaneous pancreatic insulinomas that develop in the Rip1Tag2 mice (145). Canstatin inhibits M21 melanoma tumor growth within full thickness human skin and exhibits a dose-dependent inhibition of tumor growth in nude mice. Canstatin also retards the growth of pancreatic cancer (162). Furthermore it enhances cellular senescence in tumor cells *in vivo* and *in vitro* (163). A peptide encompassing amino acids 69 to 98 of tumstatin has direct growth-suppression activity dependent on Akt/mTOR activation in tumor cells. Indeed, direct growth suppression of glioma multiforme cells by this peptide occurs only if the cells express the αvβ3 integrin and have a functional PTEN/low levels of Akt (164). Pentastatin-1, issued from the α_5 chain of collagen IV, inhibits tumor growth *in vivo* in a breast xenograft model (165), and in a small cell lung cancer xenograft model (166). The cryptic epitope HU177 regulates endothelial and melanoma cell adhesion *in vitro* and angiogenesis *in vivo* (167).

Systemic administration of restin suppressed the growth of tumors in a xenograft renal carcinoma model (57). Endostatin inhibits the proliferation of several tumor cell lines including HT 29 and C51 cell lines from human and murine colon adenocarcinoma (66). It inhibits migration and invasion of head and neck squamous cell carcinoma cells. Endostatin activates the transcription factors NF-κB and AP-1 and down-regulated the gene expression of several pro-migratory molecules (168).

Endostatin inhibits human tongue carcinoma cell invasion and intravasation. This anti-tumor effect might be explained, at least in part, by the inhibition by endostatin of activation and activities of tumor-associated pro-MMPs, such as pro-MMP-2, -9, and -13 (169) and MT1-MMP (170). Other matricryptins inhibit matrix metalloproteases. Arresten inhibits matrix metalloproteinase-2 activation and FGF-2-induced proliferation in murine retinal endothelial cells (171), whereas the NC1 domain of collagen XIX inhibits MT1-MMP expression and activity (92).

Endorepellin specifically targets the tumor vasculature, inhibits tumor angiogenesis, enhances tumor hypoxia, and leads to a statistically significant decrease in tumor metabolism and mitotic index in two human tumor models in mice (135). In contrast to other angiogenesis inhibitors, endorepellin does not increase the apoptotic index of human tumor cells *in vivo* (135).

6.9-kDa hyaluronan fragments (36 mers) promote tumor cell motility in a CD44-dependent manner (100). Hyaluronan fragments modulate growth and cell survival and sensitize multidrug resistance breast cancer cells to cytotoxic drugs by decreasing p-Akt as well as Pgp activity in a CD44-dependent way (172). They inhibit cell viability and induce apoptosis of human and murine osteoblastic osteosarcoma cell lines. *In vivo*, intratumoral injection of hyaluronan octasaccharides results in significant suppression of the formation of distant lung metastasis (173).

7.3. Tissue remodeling and wound healing

Tissue remodeling and repair includes angiogenesis and several studies have focused on the role of anti-angiogenic matricryptins on these processes. Implications of matricryptins and matricryptic sites for cutaneous cancers and skin repair and for the control of tissue injury in the cardiovascular system have been reviewed (104).

Endostatin does not affect murine cutaneous wound healing, and does not induce a significant decrease in breaking strength of cutaneous wounds in mice (174). Endostatin treatment reduces the number of functional blood vessels and the matrix density in the granulation tissue, but does not significantly affect the overall wound healing process in mice (175). However, in another study, endostatin-treated mice showed a significant delay in wound healing. Granulation tissue formation and wound vascularity were significantly decreased, but reepithelialization was not effected (176). Overexpression of endostatin leads to delayed wound healing in mice, whereas the lack of collagen XVIII accelerates cutaneous wound healing (177). Endostatin participates in cardiovascular remodeling by suppressing aberrant left ventricular remodeling and heart failure after myocardial infarction. Endostatin may thus have a cardioprotective effect (178). Tumstatin prevents glomerular hypertrophy in the early stage of diabetic nephropathy (179).

The topical application of a pool of hyaluronan oligosaccharides ranging from 2 to 10 disaccharides promote excisional wound healing through enhanced angiogenesis *in vivo*. This treatment promotes collagen deposition and fibroblast proliferation and may be useful in acute wound repair (180). Skin atrophy, often associated with delayed wound healing, is reversed by hyaluronan fragments through a CD44-dependent mechanism. This effect is dependent upon the size of the fragments. Fragments with molecular weight ranging from 50 up to 400 kDa, but not smaller or higher fragments, induce keratinocyte proliferation (181).

7.4. Inflammation

Matricryptins play a role as mediators of inflammation by exhibiting chemotactic activity for inflammatory cells, enhancing phagocytic functions, and inducing immune responses and changes in gene expression of inflammatory cells (see 182 for review). Fragments of collagen I and IV exhibit chemotactic activity for inflammatory cells (182). The acetylated Pro-Gly-Pro tripeptide, derived from proteolytic cleavage of collagen I *in vivo*, is a potent neutrophil chemoattractant and utilizes CXC chemokine receptors CXCR1 and CXCR2 (183). The N-terminal 7S domain of collagen IV chains has neutrophil chemotactic activity, whereas a tumstatin peptide suppresses neutrophil activation (184). The 185-203 sequence of tumstatin protects basement membrane against damage by polymorphonuclear neutrophils (185).

At sites of inflammation, hyaluronan is degraded into fragments that induce chemokines and cytokines, thereby augmenting the inflammatory response. The effect of hyaluronan on the inflammatory response is related to its molecular size. High-molecular-weight hyaluronan has anti-inflammatory activity while smaller hyaluronan fragments have proinflammatory activity (138). Low molecular weight hyaluronan (<500 kDa) induces inflammatory responses in inflammatory macrophages. Hyaluronan fragments, but not native high-molecular-weight hyaluronan, can stimulate the production of inflammatory mediators by many cell types (see 100 for review). Fragments of hyaluronan increase the expression of MMP-12, plasminogen activator inhibitor and stimulate the production of several cytokines, including macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, monocyte chemoattractant protein-1, macrophage inflammatory protein-2, interleukin-8, and interleukin-12 by macrophages (182). Hyaluronan fragments induce MMP-9 and MMP-13 expression in tumor cells, MMP-9 transcription being mediated *via* NF-κB (186). They also induce macrophage metalloelastase (187) and nitric oxide synthase (188) in macrophages. HA fragments can influence dendritic cell maturation (100).

7.5. Autoimmune and inherited diseases

Some proteolytic fragments generated from collagens trigger autoimmune diseases. An autoantigen generated from proteolytic cleavage of collagen I is associated with autoimmune uveitis (189).

Goodpasture syndrome is an autoimmune disease of the kidneys and lungs mediated by antibodies and T-cells directed to cryptic epitopes hidden within basement

membrane hexamers rich in α_3(NC1) domain (tumstatin) of collagen IV (8). Residues 17-31 and 127-141 of tumstatin are two conformational epitopes of anti-glomerular basement membranes antibodies leading to Goodpasture syndrome. These autoantibodies bind to α_3(IV) collagen in glomerular basement membrane, and cause progressive glomerulonephritis and pulmonary hemorrhage (190). Goodpasture autoantibody epitopes are cryptic because they are structurally sequestered by adjacent non-collagenous domains of α_4 and α_5 chains of collagen IV. The epitope sequestered inside the hexamer becomes exposed and binds with the Goodpasture antibody upon dissociation of the hexamer formed by two trimeric NC1 domains in the collagen IV network found in tissues into its subunits (191, 192). Mucosal tolerance can be induced in an animal model of Goodpasture's disease by nasal administration of an immunodominant peptide from the N-terminus of tumstatin (pCol 24-38,193). This opens new perspectives for treating patients with Goodpasture's disease.

The C-terminal part of the α_3, α_4 and α_5 chains of collagen IV contain polymorphisms and mutations that have been found in patients with Alport syndrome (194-197). All known variants in the COL4A5 gene and their clinical significance are recorded in a database (http://www.arup.utah.edu/database/ALPORT/ALPORT_welcome.php).

Endostatin contains a D104N polymorphism (D1437N in the sequence of the α1 chain of collagen XVIII). Individuals homozygous for the D104N polymorphism in the COL18A1 gene have a high risk of occurrence of sporadic breast cancer (198). This polymorphism may influence the age of onset of acute myeloid leukemia (199). It was initially thought to predispose for the development of prostatic adenocarcinoma (200). However, more recent studies show that the D104N polymorphism does not appear to be associated with increased risk to develop androgen independent prostate cancer (201), with breast cancer susceptibility or severity (202), or with lung cancer susceptibility (203). This variant has been found in patients with Knobloch syndrome, an autosomal recessive disorder characterized by high myopia, vitreoretinal degeneration with retinal detachment, and congenital encephalocele. This polymorphism may represent a pathogenic mutation (204).

8 MATRICRYPTINS, CRYPTIC SITES AND THERAPEUTICS

8.1. Matricryptins as potential drugs

Endostatin has raised very high expectations as antiangiogenic therapy of cancer because it induces tumor dormancy in experimental model of cancer and does not induce significant drug resistance (65). Endostatin inhibits 65 different tumor types and modifies 12% of the human genome to downregulate pathological angiogenesis without side-effect (62). Its potential use in cancer therapy has been extensively reviewed (62, 63, 205, 206). Due to the poor clinical responses observed in phase I and II trials, recombinant endostatin (Endostatin^{TM}, EntreMed Inc) was terminated at phase II trials (63). Trial design, administration regimen and patient selection might not have been optimally designed to assess the effect of endostatin that might cause only tumor stabilization (143). A derivative of recombinant endostatin has been designed with an additional metal chelating sequence (MGGSHHHHH, or Zn(II)-binding peptide) at the N-terminus that enhances endostatin solubility and stability (207). This derivative called Endostar has been approved for the treatment of non-small-cell lung cancer in 2005 by the State Food and Drug Administration in China. It exerts its antiangiogenic effect *via* blocking VEGF-induced tyrosine phosphorylation of KDR/Flk-1 of endothelial cells (208), and suppresses invasion through downregulating the expression of matrix metalloproteinase-2/9 in MDA-MB-435 human breast cancer cells (209). Endostar induces apoptotic effects in HUVECs through activation of caspase-3 and decrease of Bcl-2 (210).

Several other derivatives of endostatin have been prepared to extend its half-life (see 64 for review). Modification of endostatin by low molecular weight heparin increases its half-life (64). PEGylation enhances the stability of recombinant endostatin and improves its antitumor activity as shown with N-terminal mono-PEGylated endostatin (211). Endostar has also been PEGylated (212). Another modification linking the Fc region of an IgG molecule to endostatin significantly increases endostatin half-life. Furthermore, the amount of antitumor dose of Fc-endostatin was ~ 100 times less than endostatin (213). Therapeutic efficacy of Fc-endostatin (213) and of endostatin (148) exhibits a biphasic dose-response curve. A 27-amino-acid peptide corresponding to the N-terminal zinc binding domain of endostatin and exhibiting the anti-angiogenic and anti-tumor properties of endostatin might be of drug candidate too (214).

Experimental approaches and clinical trials focusing on delivery of endostatin have been reviewed (215). Anti-angiogenic gene therapy of cancer has been reviewed (216, 217). A phase I clinical trial of an adenovirus-mediated endostatin gene named E10A has already been carried out in patients with solid tumors showing mild antitumor effects (218). Conditionally-replicating adenovirus is currently being developed as anti-tumor therapeutics in solid tumors including pancreatic cancer, and a new virus carrying the gene sequence coding for canstatin has been successfully designed for selective replication in tumor cells. Its replication leads to decreased microvessel density and increased cancer cell apoptosis *in vitro* and *in vivo* (219). Biodegradable poly(lactide-co-glycolide) microspheres have been used to deliver Endostar *in vitro* and *in vivo* (220). CHO cells expressing endostatin encapsulated into a polytetrafluoroethylene semi-permeable membrane (Theracyte system) deliver endostatin continuously at a high level (221). This should improve the efficacy and potency of the antitumor therapy.

Arresten, canstatin, and tumstatin have not been investigated intensively in clinic so far. Preclinical trials show that canstatin gene electrotransfer combined with radiotherapy has a better efficacy over irradiation alone, as

a result of strong vessel disorganization and dramatic increase of tumor cells apoptosis in two xenografted human carcinomas from mammary and prostate origin in nude mice (222). Treatment using the canstatin- or TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand)-expressing vector significantly suppresses tumor growth in human xenograft tumor models, but their combination lead to a greater antitumor activity than either treatment alone (223). The combination of TRAIL and canstatin appears thus to be a promising approach for the gene therapy of breast tumors.

A preliminary study carried out in mice bearing S180 tumors shows that the gene delivery of tum-5, the fragment encoding amino acids 45-132 of tumstatin, may be an effective strategy for cancer therapy (224). The addition of a NGR motif, a marker of angiogenic endothelial cells, to the 45-132 sequence of tumstatin increases its anti-angiogenic and anti-tumor properties (225). However, the epitope recognised by the pathogenic auto-antibodies for Goodpasture's syndrome is localized in tumstatin sequence and its potential effect has to be carefully evaluated when considering the use of tumstatin as a drug (143).

Anti-angiogenic inhibitors may improve the efficacy of anti-tumoral treatment by normalizing tumor vasculature and microenvironment. Recombinant human endostatin improves anti-tumor efficacy of paclitaxel by normalizing tumor vasculature in a murine model of Lewis lung carcinoma (226).

8.2. Cryptic sites as therapeutic targets

Selective targeting of angiogenic cryptic collagen IV epitopes may represent an effective antiangiogenic treatment strategy. Indeed primary melanoma induces detectable changes in systemic levels of HU177 cryptic epitope shedding. A subset of patients with nodular melanoma may be more responsive to treatment with D93 (TRC093), a humanized monoclonal antibody directed to the HU177 site that is currently undergoing a phase I clinical trial in patients with advanced cancers (167).

8.3 .Matricryptins as potential markers of disease

Several matricryptins have been detected in serum and a number of studies have investigated their possible use as diagnostic, pronostic, or follow-up markers. Serum endostatin level has been investigated in patients with various cancers and in patients with coronary heart disease (227). Pretreatment serum endostatin as a prognostic indicator in metastatic gastric carcinoma (228). Endostatin is elevated within the plasma and bronchoalveolar lavage fluid of patients with acute lung injury and its plasma level correlated with the severity of injury (87). The expression of endostatin is increased in patients with osteosarcoma and may be used as a pronostic marker of the disease progression and of responsiveness to therapy (229). The C-terminal domain of endorepellin (LG3) has been detected in various tissues and biological fluids (urine, blood, amniotic fluid) and might be a marker of vascular injury (see 95 for review). Circulating LG3 levels are reduced in patients with breast cancer suggesting that they might be a useful biomarker for cancer progression and invasion (230).

Circulating levels of cryptic collagen IV epitope HU177 can be detected in the sera of melanoma patients. A significant correlation was observed between HU177 serum concentration and nodular melanoma histologic subtype (167). Furthermore increased shedding of HU177 in the serum of primary melanoma patients is associated with poor prognosis (231).

9. PERSPECTIVES

The extracellular matrix is not the single reservoir of cryptic sites or cryptic fragments. Intracellular, blood or exogenous proteins (e.g. from milk) also give rise by limited proteolytic cleavage to biologically active fragments termed crypteins and collectively designed as the cryptome (232). Besides the extracellular matrix, the sources of cryptic peptides are frequently proteins involved in endocrine signaling and the complement cascade.

Most matricryptins bind to different receptors to exert their biological activities and several matricryptins are able to bind to the same integrin receptors. Canstatin, tumstatin, the NC1 domain of the α_6(IV) chain and endostatin all bind to the $\alpha v\beta 3$ integrin. In order to understand the complex interplay between all these matricryptins regulating angiogenesis and tumor growth, a global, integrative, approach is needed. The first draft of the interaction network of endostatin has been established (233), and the building of the interaction network of the other matricryptins will allow us to identify cross-talks between signaling pathways and molecular mechanisms underlying the anti-angiogenic and anti-tumor activities of those matricryptins. The study of the effect of matricryptins on a single cell type (*i.e.* endothelial cells) has provided valuable insights into signaling pathways underlying their anti-angiogenic activities. However, different matricryptins target the same integrin receptor and are released by the same cells. One challenge is thus to integrate these data in a single network to understand how all the anti-angiogenic matricryptins and cryptic sites work together to regulate angiogenesis *in vivo*. Davis (104) proposes that two classes of pattern recognition receptors, scavenger receptors and toll-like receptors are critical receptors for matricryptic sites and matricryptins that could be functionally relevant during tissue injury response as shown for hyaluronan fragments. Despite the fact that a number of matricryptins exhibit the same biological activities and share some receptors, no structural features associated with cryptic sites or matricryptins have been identified so far. Endostatin and tumstatin share 14% amino acid homology for example (3). A common feature of antiangiogenic matricryptins might be a cross-β structure, stacked β-sheets composed of non-twisted β strands, which is present in amyloidogenic polypeptides (234). Cross-β sheets are present in endostatin (235), and amyloid endostatin induces endothelial cell detachment by stimulation of the plasminogen activation system (236). However, the presence of this structure in other antiangiogenic

matricryptins derived from collagens and proteoglycans remains to be investigated. Another challenge would be to be able to predict the release of matricryptins and/or the existence of cryptic sites in extracellular proteins. The investigation of intrinsic disorder might be a useful approach for this purpose.

Regarding the use of matricryptins as anti-tumor drugs, it seems that tumors might escape from matricryptins, including endostatin and tumstatin, by up-regulating multiple proangiogenic factors (237). Anti-angiogenic matricryptins might thus be used in combination with other anti-angiogenic agents or with conventional anticancer therapies such as chemotherapy and radiotherapy as reported for canstatin (222, 238) and endostatin (239).

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Abbreviations: BCL-2: B cell CLL/lymphoma-2, cAMP: Cyclic adenosine monophosphate, cGMP: Cyclic guanosine monophosphate, COX: Cyclooxygenase, EGF: Epidermal growth factor, ERK1/2, Extracellular signal-regulated kinase , FAK: Focal adhesion kinase, FGF: Fibroblast growth factor, KDR: Kinase insert domain receptor, Flk-1: Fetal liver kinase 1, MEK: Map-Erk kinase, p38: MAPK p38 mitogen-activated protein kinase, MMP: Matrix metalloproteinase, MT-MMP Membrane-type matrix metalloproteinase, NC1: Non collagenous domain 1, PTEN: Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity: protein phosphatase, SV40: Simian virus 40, TLR: Toll-like receptor, VEGF: Vascular endothelial growth factor

Key Words Extracellular matrix, Matricryptic sites, Matricryptins, Collagens, Proteoglycans, Review

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