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The cell-elastin-elastase(s) interacting triade directs elastolysis

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1. ABSTRACT

Human elastases have been identified within serine, cysteine and metallopeptidase families. These enzymes are able to adsorb rapidly onto elastin, but they can also bind onto cell surface-associated proteins such as heparan sulfate proteoglycans, both interactions involving enzyme exosites distinct form active site. Immobilization of elastin at the cell surface will create a sequestered microenvironment and will favour elastolysis. Generated elastin peptides are potent matrikines displaying dual biological functions in physiopathology that are described in this review. Among properties, they are potent inducers of protease expression catalyzing collagenolysis or amplifying elastin degradation. The ability of unsaturated fatty acids and heparin(s) to control elastases action are delineated.

2. INTRODUCTION

Fragmentation of elastic fibers is a hallmark of cardio-vascular diseases (1, 2) and emphysema (3). It is also observed in skin during intrinsic ageing (4) and melanoma progression (5). These alterations are attributed to the action of proteases designated as elastases. Elastases are defined as endopeptidases that can generate soluble peptides from elastin (6). Such a definition excludes proteases able to cleave only a few peptide bonds within the polymer but lacking the ability to release peptides to appreciable extent. However, it needs to be delineated that within tissues, insoluble elastin is surrounded by a mantle of microfibrils constituted of fibrillins, emilins, fibulins, matrix-associated glycoproteins, … (7) that might confer some protection against elastolysis catalyzed by elastases. To that respect, initial proteolytic degradation of these

structural glycoproteins might be considered as a prerequisite for further elastin degradation. In human, endopeptidases exhibiting elastin-degrading activity have been identified in all enzyme families. Those enzymes lack any specificity and besides elastin, they hydrolyze other matrix macromolecules and most of them degrade an array of cytokines, chemokines and growth factors, thus participating in a myriad of biological functions (8-10).

The synergy mode of action of elastases, their rate of adsorption onto elastin as well as the localization of their catalytic action, i.e. extra-, peri- or intracellular, are among important factors directing elastolysis that are described here. Proteolysis of matrix components leads to the liberation of peptides that are named matrikines in keeping with their cytokine-like properties (11). Elastin peptides (EPs), i.e. elastokines, are produced by the action of elastases and are among the most potent matrikines modifying the phenotype of several cell types (12). The mechanisms underlying these effects are described and their implication in the progression of diseases as aneurysms and emphysema are analyzed. Finally, on line with the affinity of unsaturated fatty acids and heparin(s) for elastases, some perspectives in the control of elastolysis are elaborated.

3. HUMAN ELASTASES: DEFINITION, CLASSIFICATION AND PROPERTIES

3.1. Serine peptidases

The first elastase was isolated from porcine pancreas by Balo and Banga in 1949 (13). It was further established that this organ, in this particular species, contained two distinct endopeptidases named pancreatic elastase I (PE I, further designated as ELA I) and pancreatic elastase II (PE II, further named ELA II) (14). ELA I is silent in human pancreas which is the unique organ expressing ELA II, as revealed by blot hybridization studies (15). Analysis of human pancreatic cDNA library indicated that two elastases II were expressed in this tissue, exhibiting 90% homology in amino acid sequence. Recently, these two species ELA IIA and ELA IIB were expressed in *Escherichia coli*. Contrary to ELA IIA, ELA IIB is devoid of activity on DQ-elastin as well as on a synthetic elastase substrate, i.e. Glt-Ala-Ala-Pro-Leu-p-nitroanilide (16). ELA IIA displays a chymotrypsin-like specificity showing a P1 subsite preference for Met, Leu, Phe or Tyr. It is synthesized as a proenzyme stored in the acinar cells of the pancreas and further activated by trypsin in the duodenum. This enzyme probably contributes to the lysis of elastic fibers from pancreatic blood vessels, as observed during acute pancreatitis (6). Silencing of ELA I in human pancreas was attributed to mutations that inactivated both enhancer and promoter elements within its gene. Strikingly, a recent investigation evidenced the presence of ELA I mRNA in cultured human primary keratinocytes (17). The short length of its corresponding signal peptide probably did not allow enzyme secretion and its function inside the cell or at the keratinocyte plasma membrane remains to be clarified.

The azurophilic granules of human neutrophils sequester three elastolytic proteases namely neutrophil elastase (NE), cathepsin G (Cat G) and proteinase-3 (Pr-3) (18-20, for reviews). Their expression is limited to the earliest stages of myeloid differentiation and maturation of granulocytes or monocytes induces their down-regulation (21). NE is synthesized as a proenzyme containing an N-terminal dipeptide which can be cleaved in the post-Golgi organelles by dipeptidyl peptidase I, leading to enzyme activation through interaction of the free amino-terminus with the aspartic acid of the catalytic triade. Cat G and Pr-3, also named myeloblastin (18), are similarly activated and several NE, Cat G or Pr-3 isoforms containing complex N-linked oligosaccharide side chains have been identified; extent of glycosylation was not reported to modify the activity of those proteases. All three proteases participate intracellularly in the digestion of phagocytosized microorganisms and at sites of inflammation, they can hydrolyze a wide repertoire of matrix constituents besides elastin. Of note, these proteases are able to associate to neutrophil plasma membrane through interaction with negatively charged sulfate groups of glycosaminoglycans (NE, Cat G) or GPI-anchored surface receptors (22, 23).

Elastolytic activity susceptible to inhibition by serine protease inhibitors was also evidenced in plasma membrane preparations of smooth muscle cells from porcine aorta (24, 25). Further studies, on the basis of cDNA sequencing and immunological evidence, demonstrated that this enzyme shared common coding sequence with human adipsin (26). Table 1A summarizes several properties of serine elastases.

3.2. Cysteine peptidases

Four cysteine peptidases with elastolytic activity ((27-29), i.e. Cat L, Cat S, Cat K and Cat V (L2) were identified in clan CA, family C1 of proteolytic enzymes. Following signal peptide cleavage, proenzymes are modified by glycosylation, mannose phosphorylation and formation of disulfide bonds and further routed to lysosomes by the mannose-6-phosphate receptor (30, 31). Autocatalytic activation by limited proteolysis then leads to the formation of active enzymes (31). In some cases, Cat D or metallopeptidases can participate in zymogen processing. Generated propeptide domain, as found for serine elastases, can regulate enzyme activity (32). For instance, the propeptide domain of Cat V was shown to display potent inhibitory activity towards Cat V, but also to all three other cathepsins with K_i in the 10-100 nM range (33). Cat L is ubiquitously expressed in eukariotic cells while Cat S has a more restricted distribution, mainly in lymph nodes, spleen and macrophages. Also, Cat V, which shares 80% sequence identity with Cat L, was identified in thymus and testis, and more recently in monocytes-derived macrophages. Osteoclasts and, to a lower extent, myocytes and type II pneumocytes in the lung are main sources of Cat K. All cathepsins are produced by monocytes-derived macrophages at different stages of cell maturation. Importantly, Cat S is stable and still active at neutral pH and at its optimal pH of activity (pH ~ 5.5), it exhibits elastinolytic activity 4-fold greater than NE, as determined using Congo red-elastin as substrate (29).

Table 1. Human elastases within peptidase families (from ref. 131)

Families	EC number	Gene locus	Molecular mass (active enzyme) (kDa)	Main cell type(s) (Others)	Subcellular localization	Inhibitors (endogenous)
A. SERINE ELASTASES						
Elastase I (ELA I)	3.4.21.36	12q13	26	Keratinocytes	Intracellular	$alpha_2M$, $alpha_1Pi$, elafin
Elastase II (ELA IIA)	3.4.21.71	1p36-21	26	Acinar cells		$alpha_2M$, $alpha_1Pi$, α_1ACT, SLPI
Neutophil elastase (NE)	3.4.21.37	19p13.3	29-33	Neutrophils (Monocytes, mastocytes)	Azurophilic granules	$alpha_2M$, $alpha_1Pi$, elafin, SLPI
Cathepsin G	3.4.21.20	14q11.2	28.5	Neutrophils (Monocytes)	Azurophilic granules (cell surface)	$alpha_2M$, $alpha_1Pi$, SLPI
Proteinase-3 (myeloblastin)	3.4.21.76	19p13.3	29-32	Neutrophils (Monocytes, mastocytes)	Azurophilic and specific granules	$alpha_2M$, $alpha_1Pi$, elafin
Smooth muscle cell elastase	3.4.21	Unknown	20-24	Smooth muscle cells	Plasma membrane	$alpha_2M$, $alpha_1Pi$
B. CYSTEINE ELASTASES						
Cathepsin L	3.4.22.15	9q21-22	28	Ubiquitous	Lysosomes	$alpha_2M$, cystatins, Ha-ras, MMC class II, cat V propeptide
Cathepsin S	3.4.22.27	1q21	24	Lymph nodes, (Macrophages, smooth muscle cells/atheroma)	Lysosomes	$alpha_2M$, cystatins, cat V propeptide
Cathepsin K	3.4.22.38	1q21	23.5	Osteoclasts (Epithelial cells, type II pneumocytes, macrophages, smooth muscle cells/atheroma)	Lysosomes	$alpha_2M$, cystatins, cat V propeptide
Cathepsin V	3.4.22.43	9q21	24	Thymus, testis, macrophages	Lysosomes (lamella granules and corneodesmosomes in skin)	Cystatin E/M, cat V propeptide
C. METALLOELASTASES						
Matrilysin-1; Pump-1 (MMP-7)	3.4.24.23	11q21-q22	19	Macrophages (epithelial cells)	Secreted (Plasma membrane)	TIMP-1, -2, -3, 4
Gelatinase A; 72-kDa type IV collagenase (MMP-2)	3.4.24.24	16q21-q13	62	Ubiquitous	Secreted (Plasma membrane)	TIMP-1, -2, -3, 4
Gelatinase B; 92-kDa type IV collagenase; type V collagenase (MMP-9)	3.4.24.35	20q12-q13	82	Macrophages, (PMNs, lymphocytes)	Secreted (Plasma membrane)	TIMP-1, -2, -3, 4
Macrophage elastase (MMP-12)	3.4.24.65	11q22.2-22.3	22	Macrophages	Secreted	TIMP-1, -2, -3, 4

Recently, the elastolytic activity of caspase-2, -3, and -7 has been documented. These enzymes have been localized at the surface of apoptotic vascular smooth muscle cells in culture, with caspase-2 being further released in the conditioned medium (34). The properties of cysteine-elastases are documented in Table 1B.

3.3. Metallopeptidases

Among the metallopeptidase superfamily, only four members are able to degrade insoluble elastin. These metalloelastases are Ca-containing Zn-endopeptidases belonging to the matrixin or matrix metalloproteinase (MMP) family (clan MB, family M10): macrophage metalloelastase (MMP-12), gelatinase B (MMP-9), gelatinase A (MMP-2) and matrilysin-1 (MMP-7) (35-39). Like several other members of the MMP family (10), and in contrast with the serine and cysteine elastases, they are first secreted as inactive proenzymes or zymogens. These enzymes are ubiquitous and their expression is regulated at both transcriptional and post-transcriptional levels. They must be activated before exerting their proteolytic activities and are inhibited by specific inhibitors of metalloproteinases, the tissue inhibitors of metalloproteinases, TIMPs (40). In order to become active, the interaction between the thiol-group of a cysteine in the prodomain and catalytic zinc in MMP active site must be disrupted. Such a "cysteine switch" can be induced by proteolysis within enzyme prodomain, oxidation or nitrosylation of the cysteine or conformational changes (10).

The human MMP-12 gene contains 10 exons and 10 introns (41) and encodes for a 54-kDa proenzyme which is processed to an active enzyme of 22 kDa. Unlike tissue macrophages from wild-type mice, macrophages from MMP-$12^{-/-}$ animals do not degrade insoluble elastin (42). The human MMP-9 gene contains 13 exons, 3 more than have been reported for the other members of this gene family (43). It encodes a proenzyme of 92 kDa which is activated as an 84-kDa species. Stromelysin-1 (MMP-3) has been considered as the most efficient enzyme in catalyzing proMMP-9 activation (44). Exons 5, 6, and 7 each encode one complete internal repeat which resembles the collagen-binding domains of fibronectin (38). The proenzyme noncovalently binds to TIMP-1 through its carboxylic-end domain (40). In contrast with the other MMPs, MMP-2 is constitutively expressed by various cell types in culture (45). Like MMP-9, the MMP-2 gene consists in 13 exons (46), and encodes a proenzyme of 72 kDa which can be noncovalently associated to TIMP-2 (11). Such proMMP-9:TIMP-1 and proMMP-2:TIMP-2 complexes appear to be unique in the MMP family and may

impede enzyme activation by MMP-3, as observed for proMMP-9:TIMP-1 complexes (44). On the contrary, ProMMP-2, as a complex with TIMP-2, is activated at the cell surface in a 62-kDa species by a transmembrane MMP, i.e. the membrane-type 1 MMP (MT1-MMP) (47). The three-extra exons encode the amino acids of the fibronectin-like repeats which have been found only in MMP-2 and MMP-9. These repeats confer their elastolytic activity to MMP-9 and -2 (48). The human MMP-7 gene contains 6 exons (49). It encodes for the smallest proMMP, with a size of 28 kDa, which is activated in a 19-kDa species (50). ProMMP-7 activation can be first triggered by several proteinases as plasmin, followed by autolytic cleavage at Glu_{77}-Tyr_{78} in the prodomain. Classification of MMP-7 as an elastase has been first based on its efficiency to degrade insoluble elastin *in vitro* (35). Surprisingly, MMP-7 appears to be the most potent metalloelastase *in vivo* although human macrophages close to fragmented elastin *in vivo* express proMMP-9, -12 as well as proMMP-7 (36, 37).

On the basis of Suc $(Ala)_3$ NA substrate hydrolysis, an elastase-like activity has been identified at the plasma membrane of human skin fibroblasts in culture. This activity resulted from the combined action of a 94-kDa metalloendopeptidase and of an aminopeptidase. The metalloendopeptidase displayed only a limited activity against insoluble elastin but it could extensively degrade skin oxytalan and elaunin fibers *in vivo* (51). The properties of metalloelastases are documented in Table 1C.

4. MECHANISM OF ELASTOLYSIS

4.1. Adsorption of elastases onto elastin

The adsorption of ELA I onto elastin was first shown by Hall and Czerkawski some 50 years ago (52) and since confirmed by several investigators (53-55). Interaction between either porcine proELA I or active enzyme was found to involve electrostatic interactions and esterification of carboxyl anionic groups in elastin impeded adsorption of enzyme and subsequent elastolysis (54, 55). Studies by B. Robert *et al* (56) evidenced that a maximal amount of 2 nmoles of ELA I could be adsorbed onto 1 mg of bovine ligamentum nuchae elastin. Such an enzyme concentration was calculated to occupy a surface area several fold higher than those determined by the Brunauer-Emmett-Teller method using ^{35}Kr as a probe; it suggested that initial adsorption of ELA I and limited elastolysis could extensively modify elastin structure, favouring higher interaction with the enzyme. Preadsorbed ELA I was about 3-fold more efficient than free enzyme in hydrolyzing elastin peptide bonds and its diffusion rate is limited to only 0.55% of enzyme leaving its substrate per minute.

Adsorption of serine elastases from leukocytes to elastin was also evidenced. Interaction of human NE with insoluble elastin was investigated by Baici *et al* (57) who developed a kinetic method in which progress curves from hydrolysis of synthetic elastase substrate was analyzed in the presence or the absence of the macromolecular substrate. Data indicated that the mechanism of elastolysis by this enzyme is similar to the general mechanism as proposed by Robert *et al* for ELA I. Adsorption of human NE on insoluble elastin first involved the fast formation of an encounter complex, which was followed by a step lasting several minutes before substrate hydrolysis resulting in the steady state release of EP. However, extent of adsorption was shown to vary markedly among the three leukocyte proteases: one milligram of elastin can be saturated by 0.4 nmole, > 0.5 nmole and 18 fmoles of NE, Cat G and Pr-3, respectively (58). These data indicate that interaction of an elastase with elastin could not be driven solely by electrostatic interactions since all serine elastases have isoelectric point in the 9.5 to 12 range (18) but differ extensively in their ability to bind to elastin. Also, importantly, extent of binding is not directly correlated to their potency to degrade elastin since Cat G, which interacts avidly with elastin, exhibits only low elastolytic activity as compared to ELA I or NE (59). Lonky and Wohl (60) provided evidence for the presence of two types of binding sites for NE onto elastin: a non productive one which might involve electrostatic interactions and a productive one which might require hydrophobic interactions. To that line, strong homology was observed between a hydrophobic sequence (VVG…A) in elastin receptor (S-Gal or EBP, see section 5-1) interacting with VGVAPG elastin peptides, and several elastases including ELA I and ELA II from different species and NE (61). Interactions of Cat K, L, and S with elastin were analyzed using the kinetic approach as developed for NE. These elastolytic cysteine proteases behave differently as compared to serine elastases since the presence of a lag phase in progress curves is not observed and tight complexes between enzyme and substrate are not formed. Kinetic data tend to support the contention that Cat K remains adsorbed on elastin, whereas Cat L could freely diffuse from its initial adsorbed state. In keeping with their proposed involvement in vascular diseases, the three cathepsins display the highest activity against aorta elastin, when compared with elastin from other tissue sources (62).

A very fast interaction between either proMMP-2 or proMMP-9 and elastin has been evidenced (63). However, contrary to NE, no saturation effect could be observed with active form of MMP-9 (63). In contrast, a maximal amount of 25 ng of proenzyme could be adsorbed onto 1 mg of elastin, a level which is 40-fold lower than NE. However, on a molar basis, the *in vitro* elastolytic activity of MMP-9 is approximately 30% of that of NE. Contrary to free enzyme, proMMP-9 bound to elastin could not be activated by proteases as plasmin or NE (63). Binding of proMMP-9 to type IV collagen was shown to induce enzyme activity towards synthetic and gelatin substrates, without modification of enzyme molecular mass (64). These data suggested that proMMP-9 interaction with substrate could disengage propeptide domain from active site, leading to an “open active site” with no autocatalytic cleavage. We noticed that proMMP-2 bound to elastin could be rapidly activated by low amounts of active MMP-2 indicating that elastin could act as a template for autoactivation of this enzyme. Such processing could be prevented by synthetic inhibitors and to a much lower extent by TIMP-2, (63). Binding of gelatinases to elastin involves fibronectin-like (FN-II) domains (65, 38, 39).

Indeed, MMP-2 or MMP-9 lacking FN-II domains do not interact with elastin and did not exhibit elastolytic activity (38, 39). Also, addition of recFN-II domains totally prevented binding of proMMP-9 to elastin while having no effect on MMP-12 adsorption onto this substrate. Recently, the interaction of MMP-12 with elastin has been studied by nuclear magnetic resonance (NMR). Data showed that the catalytic domain of the enzyme was able to bind directly to its natural substrate since affected nuclei (^{13}C, ^{2}H, ^{15}N) following enzyme-elastin interaction all belong to active crevice of MMP-12 (66).

Adsorption of elastases to elastin, as the initial step in elastolysis, has major impact on the control of these proteases by proteinase inhibitors. For instance, NE forming tight complexes with elastin was found partially refractory to inhibition in its adsorbed state by α_2 macroglobulin and α_1 proteinase inhibitor, probably due to steric hindrance (67-70). In contrast, cysteine elastases for which adsorption was followed by rapid desorption from elastin, are potently inhibited by cystatins (62). Our investigations also suggested that MMP-2 bound to elastin was still partially inhibited by TIMP-2, possibly indicating either a rapid desorption of this enzyme from elastin or difficult accessibility of adsorbed enzyme onto elastin by inhibitor. Clearly, additional experiments have to be performed to reach a final statement for each MMP.

4.2. *Ex vivo* degradation of elastin by elastases

Data from the literature indicate that, according to *in vitro* studies, the potency of elastases towards elastin could be classified in the following decreasing order: Cat V > Cat K > Cat S > ELA I > NE > MMP-7 > Cat G > MMP-2 = MMP-9 > MMP-12 > Pr-3 > vascular elastase (62). Such a classification is rather misleading since it does not take into account local pH and the stability of enzymes for instance. In addition, due to the lack of specificity of these enzymes, other matrix macromolecules could act as competitive substrates and thus could alter the susceptibility of elastin to elastases. In a more general way, enzyme activity, *in vivo*, is controlled by substrate availability, concentration, and affinity. In order to evaluate the elastolytic activity of a given protease, we developed an *ex vivo* assay in which enzyme is deposited onto skin tissue section and elastolysis quantitated by computerized morphometric analysis (71). Skin contains low amounts of elastin (2-4%), as compared to other organs like aorta and lung and thus elastolysis will reflect strong affinity of the protease for elastin. Skin sections were overlaid with increasing concentrations of NE, Cat G, MMP-2 or-9 for 18-20 h at pH 8.0 and extent of elastolysis or collagenolysis was quantitated following staining with polyphenolic catechin dye (elastic fibers) or red syrius (collagen fibers), respectively. Data indicate that NE, as well as Cat G, were able to degrade elastin fibers in this *ex vivo* assay (71). Similarly, as observed *in vitro*, Cat G appeared to be a less potent elastolytic enzyme as compared to NE (59). On a molecular basis, MMP-9 was more efficient in degrading elastin than NE but elastolysis reached a plateau phase with only 50% hydrolysis. In contrast, MMP-2, which degrades elastin to a similar extent as MMP-9 *in vitro*, displayed only a weak *ex vivo* elastolytic activity but instead extensively degraded collagen fibers (72). As a whole, these experiments emphasized the high affinity of MMP-9 for elastin in a natural microenvironment. Cat G, NE and MMP-9 can be released simultaneously from polymorphonuclear neutrophils (PMNs) under inflammatory conditions. *In vitro* assay using human lung elastin as substrate or *ex vivo* assay using skin sections demonstrated that Cat G was able to stimulate the activity of NE. A 2-fold synergestic effect was observed when 1.1 nM of NE and 1.5 nM Cat G were applied onto tissue section (71). A 3.6 higher stimulatory influence was noticed for a 2.5 MMP-9/NE ratio (unpublished data).

4.3. Cell-directed elastolysis

Contrary to enzymes sequestered within secondary granules as MMP-9, serine elastases, i.e. NE, Cat G and Pr-3 are not, or to a very low extent, secreted by PMNs. Instead, following priming of cells with cytokines or following activation by chemoattractants, an average amount of 200 ng of either NE or Cat G / 10^6 cells was recovered at the cell plasma membrane. Further investigations evidenced that binding of NE and Cat G to PMNs was saturable and reversible with K_D and binding sites averaging 4.10^{-7} and 10^7 per cell, respectively. Exposure of PMNs to cytokines and chemoattractant also led to a 5-6-fold increase in Pr-3 concentration at the plasma membrane. Similarly as observed following adsorption of NE to elastin, immobilization of the enzymes to the cell surface confers them resistance to naturally occurring proteinase inhibitors (19, 73-75).

Several investigations also pinpointed that matrix degradation, including elastic fibers, by macrophages was confined to the cell surface. Pericellular proteolysis catalyzed by this cell type might originate from *i*) quantum proteolysis and local saturation of proteinase inhibitors; *ii*) cellular metabolism by activated cells and lactate production which drops pH by 1-2 units allowing cysteine elastases (Cat L, S, and V) to be active in the pericellular environment; *iii*) the participation of cell-surface bound proteases (75). Initial *in vitro* experiments demonstrated that elastolysis, following macrophage-elastin interaction, could be attributed to cysteine elastases Cat L and Cat S when cell culture medium was lacking plasminogen. Supplementation of culture medium with plasminogen significantly enhanced elastin degradation and plasmin-activated proMMPs, mainly MMP-7, were found to act in concert with cysteine elastases in elastin degradation (36, 37). Recently, peritoneal macrophages isolated from wild type, MT1-MMP$^{-/-}$, MMP-2$^{-/-}$, MMP-9$^{-/-}$ mice were activated with IFNγ and LPS and cultured in the presence of [^{3}H]-elastin. Surprisingly, since the soluble form of MT1-MMP is devoid of elastolytic activity, MT1-MMP-deficient macrophages demonstrated a 64% decrease in their ability to degrade [^{3}H]-elastin, while the elastolytic activity of MMP-2 and MMP-9-deficient macrophages was unchanged (76). However, it needs to be considered that elastin binding to the cell surface could favor elastolysis by a template mechanism similar to the one we described for collagenolysis (11). Insoluble elastin was previously shown to interact with human skin fibroblasts via a cysteine-rich glycoprotein designated as elastonectin (77, 78); such a

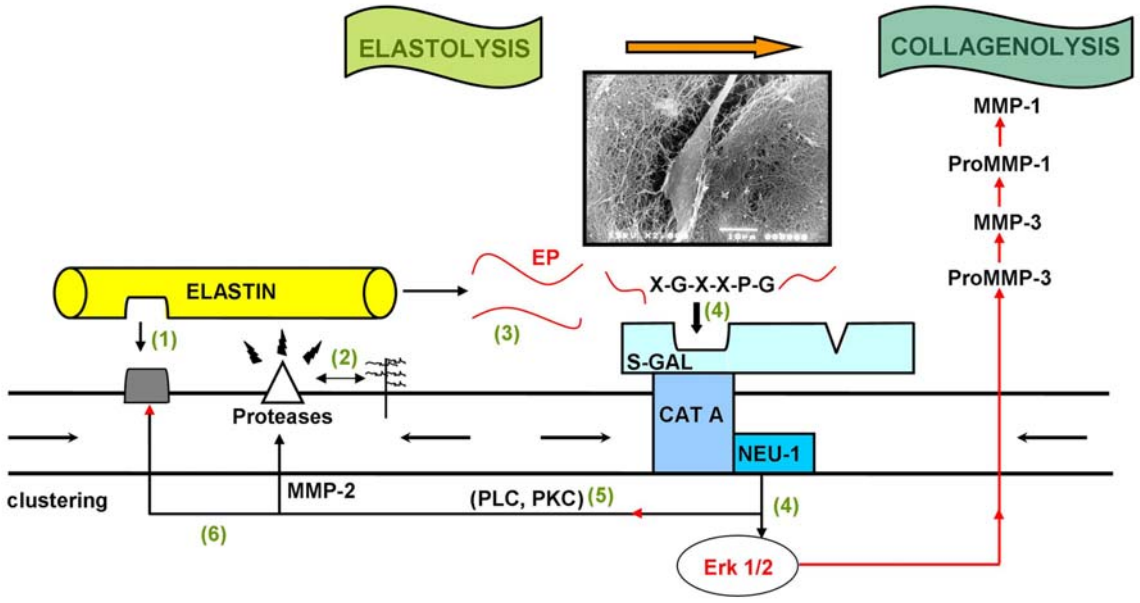

Figure 1. Fibroblast-directed elastolysis induces collagenolysis. (1) Insoluble elastin interacts with fibroblast via plasma membrane-associated protein(s) (elastonectin, S-Gal, galectin-3, …); (2) clustering allows the degradation of elastin by proteases as fibroblast elastase, MMP-2, MT1-MMP (?), …with heparan sulfate proteoglycans possibly controlling such process; (3) production of elastin peptides (EP); and (4) binding to S-Gal and signal transduction through Neu-1, leading to Erk 1/2 activation, upregulation of MMP-3 and MMP-1 expression and collagenolysis; (5,6) in turn, S-Gal occupancy by EPs can stimulate MMP-2 expression (via PLC, PKC) and binding of elastin to fibroblasts, thus mediating an amplifying proteolytic loop. Encard: Fibroblasts were cultured in 3D Collagen lattices in presence of EP. Collagenolysis could be observed at the pericellular environment.

binding could position elastin for degradation by membrane-bound proteases such as fibroblast elastase or MT1-MMP which otherwise have no or low elastase activity, probably due to their low adsorption to the polymer. Local degradation of insoluble elastin would result in the exposure of matricryptic sites or the release of elastin peptides. These peptides are able to bind to a receptor complex (see chapter 5) and through activation of protein kinase C (PKC) and MAP kinase could stimulate the expression of not only MMP-2 (79) but also stromelysin-1 (MMP-3) and interstitial collagenase (MMP-1), further increasing elastolysis but also collagen degradation (80). In addition, EPs were shown to potentiate elastin binding to cells, thus creating an amplifying loop in proteolysis (Figure 1). The participation of MT1-MMP that prepares collagen fragments to be engulfed by fibroblasts through urokinase-type plasminogen activator receptor associated protein (uPARAP)/Endo 180 has been reported (81). By analogy, it may be hypothesized that this enzyme similarly prepares elastin to be endocytosed by macrophages or other cell types. Recently, immunostaining experiments evidenced the intracellular localization of elastin in lysosomes following incubation of fibroblasts with DQ-elastin. Intracellular degradation of elastin was attributed to Cat K since fibroblasts did not express either Cat L or Cat S (82)

Once secreted, (pro)metalloelastases can be recruited at the cell surface to be activated and/or to exert their proteolytic activities. We already mentioned that proMMP-2 is activated at the cell surface as a 62-kDa species by a transmembrane MMP, MT1-MMP (47). ProMMP-2 first binds to the C-terminal domain of TIMP-2. The proMMP-2:TIMP-2 complex then localizes to cell surface through interaction of the TIMP-2 N-terminal domain with catalytic domain of one MT1-MMP molecule, allowing subsequent proMMP-2 activation by a neighbouring MT1-MMP unoccupied by TIMP-2 (11). ProMMP-9 also binds to cell surface, through interaction with the transmembrane glycoprotein CD44 (83). Furthermore, binding to PMN membrane confers to active MMP-9 a resistance to inhibition by TIMP-1 (84). Similarly, MMP-7 can localize at the cell surface, through binding with CD44v3 and/or cholesterol sulfate which renders it refractory to its natural inhibitor TIMP-2 (85). Thus, it appears that once localized at cell surface, secreted MMPs become out of control by TIMPs, whereas MT-MMPs are still inhibited by TIMPs (86). Conversely, the membrane-associated reversion-inducing-cysteine-rich protein with kazal motifs (RECK) inhibits both MMP-9 and MMP-2 activities and pro-MMP-2 activation by MT1-MMP (87). In addition, a receptor-mediated endocytosis process participates in regulation of two metalloelastases, MMP-2 and MMP-9 (88). The low-density lipoprotein receptor-related protein (LRP)-1 mediates the endocytosis and catabolism of both MMP-2 (89, 90) and MMP-9 (91), while LRP-2/megalin mediates the clearance of MMP-9 (92).

5. ELASTIN PEPTIDES AS POTENT MATRIKINES

5.1. The elastin-receptor complex

The earliest investigations indicated that fragments of type IV collagen, laminin and elastin display potent chemotactic activity for PMNs. Corresponding peptides as well tropoelastin avidly bind (K_D = 8 nM) to a 67-kDa plasma membrane-associated protein, first designated as EBP for "Elastin Binding Protein". EBP was then identified as an inactive alternatively spliced variant of β-galactosidase (S-Gal) (93) which forms a ternary complex with cathepsin A (Cat A) and neuraminidase-1 (Neu-1) at the surface of several cell types (94, 95, 13). Data from our studies revealed that the interactions between elastin peptides (EPs) and S-Gal involved an X-G-X-X-P-G consensus peptide sequence adopting a type VIII β-turn conformation (80), repeated 28 fold in tropoelastin but also present in other matrix macromolecules (5). The alternative splicing of β-galactosidase primary transcripts leads to the introduction of a unique 32-mer. Within this sequence, the 14-mer VVGSPAQDEASPLS sequence was found to interact with VGVAPG elastin peptide with K_D value of 515 nM, and could also antagonize several biological effects of EPs (see below). Circular dichroism, NMR spectroscopy associated with theoretical methods, i.e. Monte Carlo computation and molecular dynamics simulations, evidenced that a type I β-turn at the QDEA sequence highly favoured interaction between EPs and S-Gal. Interaction between the two partners was stabilized by three hydrogen bonds involving Q and E residues of S-Gal and elastin backbone atoms (61).

It needs to be emphasized that S-Gal, together with Cat A and Neu-1, acts as a chaperone for tropoelastin, impeding its intracellular coacervation; the S-Gal/Cat A/Neu-1 complex also directs elastogenesis (95). It is recycling at the cell plasma membrane, and at this location, it behaves as a receptor for EPs generated by the action of elastases. There is a wealth of available data on the myriad of biological effects triggered by S-Gal-EP interaction (12, 94) and recent data illustrate the importance of Neu-1 activation in several of those effects (96). S-Gal occupancy by EPs was shown 1) to mediate the migration of inflammatory cells; 2) to stimulate angiogenesis *in vitro* and *in vivo*; 3) to act as a survival factor for endothelial cells and fibroblasts and 4) to induce a TH-1 response in lymphocytes. Initial data by Jacob *et al* indicated that low amounts of EP, in the 0.1-1 µg/ml range, strongly increased calcium influx and inhibited calcium efflux, by a calmodulin-dependent pathway in fibroblasts and aorta smooth muscle cells in culture (97). To that line, it has been recently shown that treatment of aortic smooth muscle cells and fibroblasts by EPs induced the up-regulation of typical bone proteins such as Cbfa-1, osteocalcin and alkaline phosphatise (98, 99). Therefore, these elastokines might be considered as procalcifying agents in S-Gal expressing cells of mesenchymal origin.

Although emphasis has been made here on S-Gal-mediated effect, proteins other than S-Gal exhibited affinity for EPs. As an example, VGVAPG as well as VAPG can interact with three surface receptors on melanoma cells, namely S-Gal, $alpha_V beta_3$ and galectin-3; furthermore, galectin-3, which contains GXXPG motifs, was found mainly responsible for the induction of cancer cell chemotaxis as well as CXCR-4 and CXCL-12 up-regulation following EP supplementation to the culture medium of melanoma cells (100). Also, EPs which do not contain G-X-X-P-G consensus sequence proved to exhibit biological effects. The pentapeptide VPGVG could induce the proliferation of smooth muscle cells (101) and the nanopeptide GFGVGAGXP was described as a potent chemotactic agent for macrophages (102). Finally, VGVGVA, PGVGVA and GVGVA, all containing a VGV core peptide sequence, induced a dose- and endothelium-dependent vasorelaxation (103).

5.2. Dualism in elastin peptide properties

Elastin is a long-life protein with an estimated half-life of 70 years; intuitively, it was assumed that the generation of EP following elastin degradation by elastases might constitute a strong signal for tissue repair (11). The plasma membrane-associated ternary elastin receptor complex was shown to direct elastic fiber assembly. EP, in turn, can accelerate several steps of skin repair following injury since they can promote the migration of keratinocytes, the formation of neovessels and the differentiation of fibroblasts to a myelofibroblast-type (7). Accordingly, several EP-containing scaffolds were designed and found to improve wound healing. Such a beneficial influence of EP administration is not limited to skin but may also concern infarcted myocardium. Mizuno *et al* (104) evidenced that implantation of genetically modified Cos-7 cells for containing a 600 bp fragment of tropoelastin gene directly in the scar following heart ischemia in rats attenuates scar expansion and heart dysfunction. Such an EP was found to accumulate in the extracellular matrix at the implantation site. Since corresponding peptide contained several GXXPG sequences, it could be hypothesized that binding to its cognate receptor influences the survival of cardiac cells. Using the *ex vivo* rat heart ischemia Langendorff model, we demonstrated that administration of $(VGVAPG)_3$ elastin peptide (660 nM), either under pre-conditioning or post-conditioning conditions, could improve several heart contractile parameters; such a treatment also decreased creatinine kinase release and heart necrosis area. Lactose, as well as a 14-mer peptide corresponding to a specific S-Gal sequence following β-Gal splicing, totally suppressed these *ex vivo* EP effects, thus indicating the involvement of elastin receptor. Interaction between EPs and S-Gal proved to trigger the PI_3 kinase/Akt/Erk 1/2 transduction pathway in endothelial cells and cardiomyocytes but also in heart tissue following ischemia (105). Of note, cascade activation of these kinases, designated as RISK for "Reperfusion Injury Salvage Pathway", is considered as the leading pathway directing cell survival.

However, as previously mentioned, EPs at concentration as low as 1 nM are potent inducers of angiogenesis and monocyte chemotaxis and thus might act as inflammatory mediators (12). For instance, infusion of the rat abdominal aorta with VGVAPG elastin peptide induced a significant 26% increase in vessel diameter.

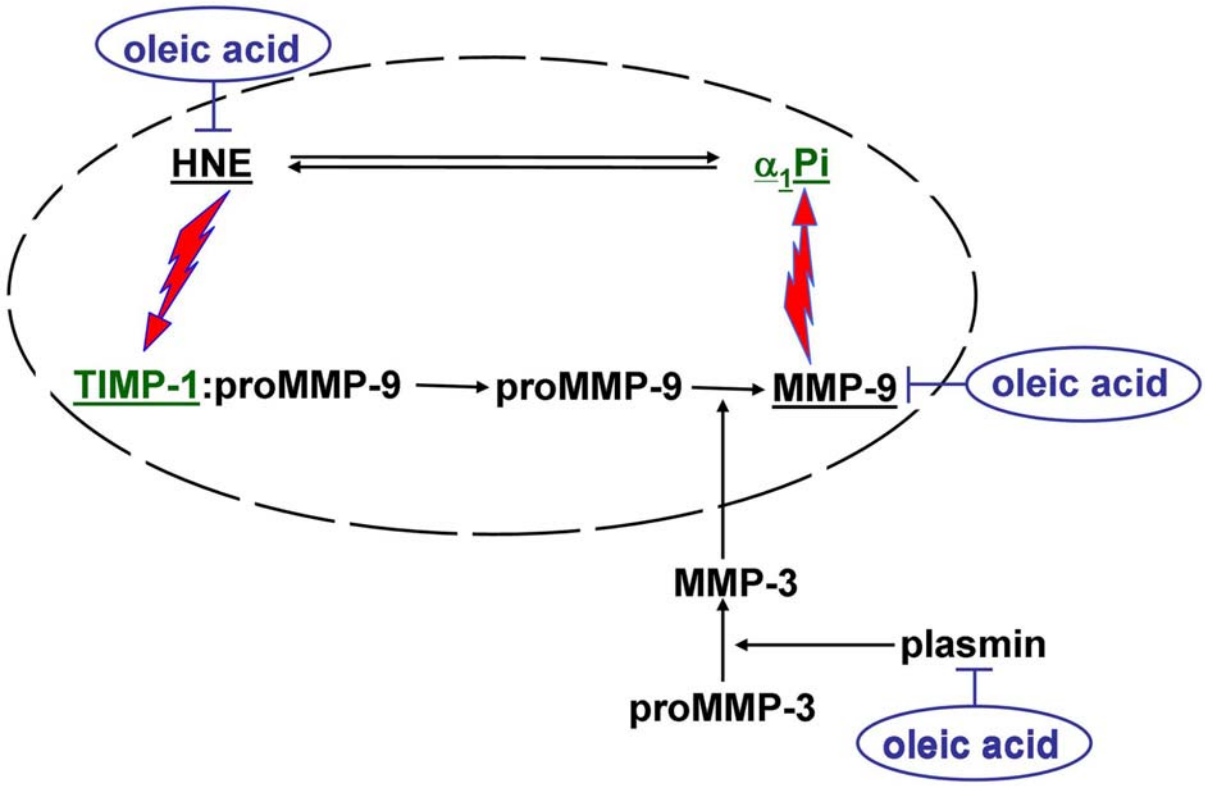

Figure 2. An elastin-degrading mechanism and its control by oleic acid.

Contrary to ELA I administration, animals did not develop aneurysm, but demonstrated intense angiogenesis that led to prominent vasa vasorum, a site of inflammatory cell infiltrates, a main feature of aneurismal disease (106). The elastin degradation peptide hypothesis in abdominal aortic aneurysm (AAA) progression has been further validated by studies showing that the monocyte chemotactic activity in AAA extracts could be suppressed by lactose as well as by BA-4 monoclonal antibody (107)

Invalidation of MMP-12 was described to protect mice from cigarette smoke emphysema. Such an effect was not attributed to overall impaired migration of monocytes since instillation of MCP-1 resulted in a significant increase in alveolar macrophages in the MMP-12$^{-/-}$ mice. It was evidenced that EPs, present in MMP-12$^{+/+}$ mice but absent in MMP-12$^{-/-}$ lung homogenates were responsible for monocyte chemotaxis in this animal model of emphysema (108). As questioned by these investigators, MMP-12 might not be directly involved in the production of EPs displaying chemotactic activity for monocytes. Recently, mapping of MMP-12 cleavage sites in tropoelastin and insoluble elastin from human skin or lung has be analyzed by HPLC separation and mass spectrometry characterization of hydrolysates (109, 110, 35). Eighty-six sites have been identified following hydrolysis of tropoelastin by MMP-12 and small peptides containing the GXXPG consensus sequence could be isolated. However, using insoluble elastin, only amino acids encoded by exons 5, 6, 26 and 28-31 are susceptible to cleavage by MMP-12. These domains contain only a few GXXPG consensus sequences which bind to S-Gal and only one peptide presenting this motif was identified using static nano ESI in hydrolysates of elastin from human skin or lung. In contrast, hydrolysis of lung elastin by ELA I and Cat K generated several of these peptides. Interestingly, small peptides were undetected in MMP-9-degraded elastin. Overall these experiments indicate that elastases, even belonging to the same clan, processed elastin differently and importantly, released different peptides which might display distinct biological functions.

Identification of the target protein substrate for a given elastase *in vivo* is rather a difficult task. Mice-deficient in any elastase often display similar phenotype. MMP-7$^{-/-}$, as well as NE$^{-/-}$, are both protected from macrophage accumulation in the lung and development of airspace enlargement upon exposure to cigarette smoke (108). In a different setting, mice deficient in either MMP-9 or NE are protected from blister formation in a bullous pemphigoid model. Elegant studies indicated that MMP-9 was acting upstream in the mechanism by degrading α1 proteinase inhibitor, thus leaving free NE to cleave collagen XVII (111). Several MMPs can hydrolyze naturally occurring serine elastase inhibitors but, in turn, NE can inactivate TIMP-1 bound to proMMP-9. Activation of proMMP-9 could then be catalyzed by MMP-3 (112). These reciprocal actions of elastases on proteinase inhibitors might constitute the key control elements in elastolysis and drive, together with the production of EP,

several elastic tissue diseases (113): emphysema, coronary artery disease and aortic aneurysm (Figure 2).

6. ROLE OF FATTY ACIDS AND HEPARIN(S) IN THE CONTROL OF ELASTASES

The search for substances able to interact with enzyme exosites, i.e. binding sites involved in enzyme-substrate interaction, constitutes a novel approach to control proteolysis (114). As we reported, the two gelatinases MMP-2 and MMP-9 contain fibronectin-like (FN-II) domains which are critical for collagen but also for elastin binding and design of peptides which can interact with such modules could be of value for inhibiting collagenolysis or elastolysis. As an example, the alpha1 (I) 715-721 sequence within CB7 type I collagen fragment was identified as a ligand for FN-II domains and was found to inhibit MMP-2-driven gelatinolysis (115). We documented that long-chain fatty acids containing one or several unsaturations were able to inhibit the activity of gelatinases both on synthetic and natural substrates, *in vitro* and *ex vivo* (116). Zinc chelation was not found as the main determinant in such inhibitory effect and mixed-mode of inhibition of oleic acid was observed with full-length gelatinase A, with K_i equal to 4.3 microM. Deletion of FN-II domains decreased by one log the MMP-2 inhibitory capacity of oleic acid and inhibition in such a case was classified as purely competitive. Our recent unpublished investigations also showed that such a fatty acid could similarly interfere with the activity of other MMPs including MMP-7 and MMP-12 displaying elastolytic activity (manuscript in preparation). It suggested that S'1 subsites of these enzymes could possibly accommodate long-chain fatty acids with their carboxylic-end moiety chelating the zinc active site. In contrast, gelatinases can additionally bind to FN-II domains; surface plasmon resonance analysis indicated that oleic acid could interact with FN-II domains with $K_D = 41$ µM. Binding of fatty acids is not restricted to MMPs, since they can also interact with kringle domains in plasmin and interfere with its amidolytic activity and its capacity to activate proMMP-3 (117). Besides, oleic acid, as well as other unsaturated fatty acids like parinaric acids were reported to inhibit NE activity (118, 119). The *cis* configuration of the double bonds, the chain length as well as the carboxylate end group were found determinant in such a property and binding of parinaric acids to NE involved two affinity modes: a high one ($K_i = 48$ nM) characterized by partial non competitive inhibition and a lower one ($K_i \sim 1$ µM) resulting in competitive inhibition. Arginine side chains have been involved in the binding of several proteinases to substrate; in NE, the side chains of Arg_{117} and Arg_{217} are located in proximity to the hydrophobic extended substrate domain and situated at 10-15 Å distance of Ser of the active site. It was then hypothesized that the carboxyl end of fatty acid could interact with these positively charged residues while unsaturation would form pi-pi interactions with Phe-Ala residues near the active site of NE (119), a contention which we have recently confirmed by molecular docking (Moroy *et al*, in preparation).

Molecular docking simulations also pinpointed the importance of several clusters of Arg residues at the interdomain crevice in directing the strong interaction of *N*- and *O*-sulfated groups of heparin with NE (120). Our initial investigations revealed that oversulfated hexadecasaccharides such as heparin derivatives were able to inhibit rat NE in the 40 nM to 100 nM range (121). Extent of inhibition was inversely correlated with the chain length of these oligosaccharides and increased with their over-*O*-sulfatation. Inhibition was classified as tight-binding, hyperbolic and mixed, competitive or non-competitive, depending upon the enzyme species and type of heparin derivatives (120-123). The strong inhibition of NE by low molecular mass heparin has been recently further documented with K_i values ranging from 3.3 to 30 nM with 2-3 molecules of heparin interacting with enzyme (120, 122). The use of chemically modified heparin as well as a series of heparin-derived oligosaccharides confirmed the requirement of extent of sulfation (particularly 6-*O*-sulfate) and chain length (12-14 saccharide units) in inhibitory potency (120). Activity of Cat G was also repressed by heparin and derivatives. Mechanism of inhibition appeared similar but with higher K_i value. Such data might reflect the different charge distribution in the channel leading to the active site of both enzymes (122, 123).

A large amount of data evidenced that cell surface heparan sulfate proteoglycans act as receptors for many soluble heparin-binding ligands, such as matrix macromolecules and growth factors. They also function as docking molecules for neutral proteinases. Such an enzyme anchoring may *i*) increase the concentration of proteases in the pericellular environment, *ii*) direct their activation which could improve cell migration or *iii*) cluster substrate, activator and zymogen in specific sites at the cell plasma membrane (75) (Figure 1). As a result, proteolysis at the cell surface can occur even in the presence of excess inhibitors (74, 84). We already mentioned that NE, as well as Cat G, bind to heparan and chondroitin sulfate proteoglycans at PMN plasma membrane but macrophages also express high capacity, low affinity binding sites for these enzymes (22).

Several MMPs, including MMP-2, -7 and -9, were also described to bind to heparin with MMP-7 demonstrating the highest affinity (124). Molecular modeling revealed the presence of several tracks of Arg and Lys residues in MMP-7 as binding sites for heparin. These residues present a similar distribution as those of Cobra cardiotoxin M3 which interacts potently with heparin ($K_D = 20$ nM). MMP-7 affinity was similar with $K_D = 5$-10 nM, as determined by affinity electrophoresis (124). At the cell surface, candidate binding proteoglycans include GPI-anchored glypican, epican and syndecan(s) on the basolateral cell surface. Binding of the PEX domain of ProMMP-2 to heparin was reported to trigger zymogen activation (125). Recent investigations by Ra *et al* (126) showed that heparin, as well as chondroitine-4 and -6 sulfate glycosaminoglycans could potently enhance proMMP-7 activation but also selectively directed proteolysis of biological substrates. Accordingly, cell surface proteoglycans could increase both proMMP-7 activation but also vectorisation of proteolysis. Whether such a mechanism also applied to elastolysis required further studies. We already documented the pivotal

function of the MT1-MMP/TIMP-2/proMMP-2 triade in directing elastolysis and collagenolysis, thus favoring melanoma progression (127). Recently, MMP-2 was described to bind to heparan sulfate side chains of syndecan-2 in Lewis lung carcinoma cell line (128). Importantly, removal of heparan sulfate side chains with heparitinase-I promoted proMMP-2 activation while overexpression of syndecan-2, in turn, suppressed proMMP-2 activation and formation of metastases. Surface plasmon resonance analysis confirmed the high affinity of heparin for MMP-2, with a K_D value equal to 61.7 nM. Therefore, binding of heparan sulfate side chains to hemopexin-like domains of proMMP-2 could impede TIMP-2 interaction through its C-terminus domain, and inhibit the formation of the ternary complex required for proMMP-2 activation. .Action of Heparin is not solely limited to serine and metalloelastolytic enzymes since it can either destabilize the zymogen form of cathepsin L through interaction with an heparin binding motif in the prodomain of enzyme (129) or enhance cross-class serpin inhibition of this cysteine protease (130). The anti-metastatic property of heparin is well established since decades. Besides its anti-heparanase and anti-coagulant effects, heparin as well as derivatives lacking anti-coagulant property could either dislodge PGs-linked elastases from pericellular environment or by mimicking heparan sulfate proteoglycans interfere with their activation or control by natural occurring inhibitors.

7. CONCLUDING REMARKS

Three important steps: *i*) adsorption of enzymes on to elastin; *ii*) binding of enzymes to cell surface; and *iii*) interaction of generated elastin peptides with cognate receptor(s) need to be considered at aims to control the deleterious action of elastases in elastic tissue diseases. Since elastases, regardless of family, possess a wide range of substrate specificities, elastin, targeting is required for not interfering with other enzyme functions which might be beneficial. This approach necessitates a better understanding of elastases-elastin interactions to define, as initiated for collagenase-collagen interactions, the corresponding domains involved in both enzymes and substrate. The use of heparin, heparin derivatives or other sulfated compounds to dissociate elastases from the cell surface microenvironment, to modulate their activation, or to direct their proteolytic action is promising but again structure-function relationships need to be addressed. Finally, the deleterious actions of elastin peptides in emphysema, aneurismal disease and cancer progression has to be controlled. As mentioned, small peptides (such as V14) encompassing the specific S-Gal sequence, could strongly interfere with several, but probably not all, biological properties of EPs. An analogous motif was identified in serine elastases, and, of note, it can strongly interact with VGVAPG domains in elastin. Therefore, binding of such S-Gal peptide to elastin might somewhat inhibit elastase interaction; it might be further chemically modified with compounds displaying wide range of inhibitory capacity towards elastases such as unsaturated fatty acids, to confer protection of elastin against elastases.

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Abbreviations*:* EP: elastin peptide, ELA: elastase, NE: neutrophil elastase, Cat: cathepsin, Pr-3: proteinase-3, MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; MT-MMP, membrane-type MMP;

EBP: elastin binding protein, FN: fibronectin, NMR: nuclear magnetic resonance, PMN: polymorphonuclear neutrophil, IFN: interferon, LPS: lipopolysaccharide, PKC: protein kinase C, MAP: mitogen-activated protein, UPARAP: urokinase-type plasminogen activator receptor associated protein, RECK: reversion-inducing -cysteine-rich protein with kazal motifs, LRP: low-density lipoprotein receptor-related protein, Gal: galactosidase, Neu: neuraminidase, PLC: phospholipase C.

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