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THE ROLE OF NEUTRAL PROTEINASES IN CELLULAR INVASION

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CONTENTS

1. Introduction

The field of proteolytic enzymes is currently the scene of vigorous research in many laboratories in the world. The main stimulus for this is the growing body of evidence that the enzyme systems responsible for protein breakdown are virtully not only important for the normal health and development of living organism but also for pathological process of many disease.[143] The way in which the work of chemists and enzymologists has lead to the discovery of new classes of selected inhibitors for individual proteolytic enzymes. These agents are being used to investigate the role of the enzymes in normal physiology, and tested for usefulness as drugs in the control of disordered protein metabolism Many biological processes required the participation of proteolytic enzymes capable of degrading compound of the extracellular matrix. This degradation of intracellular and extracellular protein is very precisely controlled in the healthy state, and it is in the very area of control of biological process.

Many experimental evidences supports the hypothesis that proteolytic activity may, in fact, be required for cellular invasiveness.[36,59] According to this theory, cells are invasive by virture of their ability to secrete (or induce secretion of) proteinases capable of degrading the molecules which compose the barriers they must cross.[1,59,36,82,149]

In most mammalian tissues, structural barriers, including basement membranes, consist of a meshwork of tightly packed and highly cross-linked collagen fibils. These fibrils are imbeded in a viscoelastic ground substance whose major components are structured macromolecular complexes, i.e, proteoglycans, glycoproteins and elastin.[1,15,61,82,115,116,123,131]

Numerous reports of increased proteolytic activity associated with various disease; including inflammation, rheumatoid arthritis, emphysema and tumors which obtained either by extraction or tissue culture techniques, have strengthened the concept of a primary involvement of proteinases in all of these disease.[29,46,94,99,125,129,139,161,170]

In this review, I will consider the nature of these enzymes, role of enzymes in normal and pathological situation, the mechanism of the control of these enzymes and expectation of the inhibitors to use as therapeutic purpose of the disease related to these enzymes.

2. Classification and location of cellular proteases

The proteases are all of the peptide hydrolases in the Enzyme Commission's Subgroup 3.4.[69] Proteases devide into two classes, i.e., endopeptidases, and exopeptidases, and it was useful form of classification for several decades.

Exopeptidases can be allocated at least to their major classes on the basis of substrate specificity. Within these classes, substrate specificity is sometimes simple enough to form the basis of an acceptable name. Six enzymes belong to exopeptidase (peptidases) : 1) aminopeptidases, 2) carboxypeptidases, 3) dipeptidylpeptidases, 4) peptidyldipeptidases, 5) dipeptidases, 6) omega-peptidases.[5]

The endopeptidases are more commonly called proteinases, and five classes; 1) serine proteinases, 2) cysteine proteinases, 3) aspartic proteinases, 4) metalloproteinases, 5) unclassified proteinases. are recognized for the purposes of nomenclature.[4] The proteinases are unique among enzymes in that their classification depends on the calalytic mechanisms of their ac-

tive centers, which are revealed by the use of inhibitors rather than the substrates which are so important in the identification of other enzymes.[60] Human leukocyte neutral proteinases, collagenase, elastase and cathepsin G are classified as metallo-proteinases, serine-proteinases and serine-proteinases, respectively. In the case of elastase, reexammination of the classification might needs since experimental evidence showed that human leukocyte elastases showed both characteristics of serine-proteinases and metallo-proteinases.[79] Human leukocyte collagenases was found in the granules,[89] and elastase was also found in the PMN granules[78] and is localized in the azurophil granules.[26]

Release of PMN enzyme is specific, there is no accompanying release of cytoplasmic enzymes and agents which raise intracellular levels of c-AMP, inhibit secretion of enzymes without affecting phagocytosis, showing that enzyme secretion is not due to simply to leakage into the external medium duing phagocytosis. The secretion is thought to be stimulated by a chemotactic component of complement released during antibody-antigen interaction.[161]

3. Biological role of proteinases

1) Angiogenesis

Angiogenesis is the production of new blood vessels via migration and proliferation of endothelial cells from pre-existing capillaries. It requires invasion of the endothelial cell through the basement membrane of parent capillary and into the surrounding tissue and division of endothelial cells to supply the additional cells required for capillary extension.[140] Angiogenesis is important in number of physiological conditions, both normal and pathological, including vascularization of granulation tissue during wound healing, inflammation,[4,15,136] and vascularization during embryological development and tissue growth, and vascularization of grafts.[86] Angiogenesis is also important in the development and spread of tumors.[86,115,116] They believed that a tumor lacking a vascular supply is unable to grow until the tumor recruits capillaries to provide it nutrients and to remove its waste products.[18,34] The presence of a vascular network near a tumor also provide an exit route for metastasizing tumor cells.[34] In the initial stage in angiogenesis, basement membrane dissolution and interstitial tissue degradation required the production of degradative enzymes.[17] The activity of some malignatn cells to release tissue-destructive enzymes, particularly lysosomal hydrolases and collagenolytic enzymes, has also attracted attention as a passible factor in promoting tumor invasion.[117] Treatment of cultured endothelial cells with materials known to be angiogenic would induce the production of latent collagenase and plasminogen activator (PA).[17]

When cultured bovine capillary endothelial cells treated with angiogenic preparations; a lysate of human hepatoma cells, an extract of bovine retina, and conditioned medium from cultured mouse adipocytes, each of these angiogenic factors stimulated both PA and latent collagenase production several-fold over control levels. That this effect was specific was demonstrated by treating the cells with insulin, epidermal growth factor and retinoic acid. None of which induced proteinase production by these cells. This phenomenon was specific to capillary endothelial cells, ie angiogenesis occurs only from the microvasculature and not from large vessels.[51]

2) Tissue remodeling

Remodeling of tissues involves the localized breakdown of extracellular matrix and cell attachments and the topographical reorganization of cells. Several studies have shown that precisely controlled proteolysis should be occured in order for tissue remodeling to proceed. The controls include temporal and hormonal controls and also the presence of specific proteinased inhibitors, either produced locally or derieved from the blood.[143] In the absence of proper modulation, physiological remodeling events can quickly become pathological.

Most known example of tissue remodeling is the post-partum involution of uterus. Muscle proteins, collagen, and elastin are the principle components of the uterus which must be degraded during involution.[162,163] The loss of nonfibrous protein in the involuting uterus occurs linearly, but the loss of fibrous protein is completed quickly.[162] Collagen turnover which is very slow in nomal state, is increased exponentially with a half life of only 25h during involution.[162]

There are two pathways of collagen breakdown during uterine involution. The first of these is the degradation of collagen by a classical vertebrate collagenase at neutral PH.[163] Progesterone blocks collagenase production and uterine involution.[163] The other pathway of collagen breakdown in the uterus involves lysosomal enzymes. Uterine macrophages phagocytize fragments of collagen, which are then presumably taken to lysosomes for degradation.[113, 111] Cathepsin B, cathepsin D, and collagenolytic cathepsin do degrade native insoluble collagen,[32] but this is no evidence involving neutrophil elastase and cathepsin G in degradation of native collagen in uterus involution [32]

3) Wound healing

Repair of a cutaneous wound requires both cell migration and tissue remodeling. Epithelial cell at the margin of the wound must become detached from their connective tissue base and migrate across the wound surface, and new fibroblasts must move into the center of the wound, laying down collagen and other extracellular matrix components and partially reserving them during wound contraction.[49] Grillo and Gross[49] found considerable collagenolytic activity in marginal wound tissue, and less activity in the granulation tissue. Both the epithelial and the mesenchymal portions of the wound tissues were able to lyse collagen gels when cultured separately. In unwounded skin, very little collagenolytic activity was observed, and all of it come from the epithelium, none from the mesenchyme.[49] Donoff et al[28] found two different collagenase from rabbit skin wound : one from the epithelium, and the other from the mesenchyme. These enzymes differed in their time of appearance, pH optimum, sensitivity to various inhibitors. Donoff et al, postulated that epithelial collagenase might aid in the cellular detachment and migration of epidermal cells at the wound margin, whereas the mesenchymal enzyme, which appeared only during active wound repair, might play a role in collagen remodeling.

Berman[9] explain the events of corneal ulceration as following. Severe corneal injury causes the corneal stroma and epithelium to secrete collagenase and the epithelium to secrete PA. Plasmin degrades the corneal basement membrane and block proper epithelial repair. The injured epithelium releases or causes the release of a chemotactic agent, bringing PMNs and fibroblasts into the injured area The influx of

PMNs and fibroblasts lead to ulceration because these all types release collagenase into the corneal stroma.

Corneal ulceration arises from healing processes which have gone wrong way for two reasons[143] (1) Because of the avascularity of the cornea, serum inhibitor of proteinases and collagenase, particularly α_2-macroglobulin, are not available to modulate matrix degradation because of steric limitations to their diffusion. In support of these notion is the finding that neovascularization of the injured area prevents ulceration,[91] and the proteinase inhibitor would be diffuse freely into the cornea and inhibits plasmin and collagenase effectively.[36] (2) Persistent epithelial defects resulting from unrepaired basement membrane "trap" the epithelium in a physiological state in which it secretes of PMN and fibroblasts into the wounded area. This leads to the generation of plasmin and large amounts of active collagenase, rather than the small, locally regulated amounts necessary for remodeling during repair. It provides a good illustration, along with cancer and arthritis, of how important the regulation of proteolytic events occuring in normal physiological situation is. When these controls are bad, pathologies develop.

4) Remodeling in development

The morphogenesis of organs during embryonic development generally involves changes in the relative positions of and relationships between cells. In order for this remodeling to occur, cells may break pre-existing attachments and either penetrate extracellular matrix locally, or degrade it in a path as they move along a surface.[151] Proteinases involve the formation of the partial endoderm in the early embryo.[151]

PA involves in the early morphogonesis and Strickland et al[50] hypothesize for the role of PA produced by partial endoderm cells throughout their lifespan : PA through the mediation of plasmin,[143] enabled the cells to migrate along the surface of the trophectoderm[36] and participated in the metabolism of Reichert's membrane as the embryo expanded.

Proteinases may be involved in the developmental remodeling resulting from induction, or in breaking down the basement membrane separating epithelium and mesenchyme, thus allowing the induction to take place, or both.[70]

5) Tooth eruption

The process of a decidual tooth is lost and replaced by a permanent tooth involves considerable remodeling of connective tissue, including bone, for a period of several weeks The roots of the decidual tooth must be resorbed, and the channel for the eruption of the permanent tooth must be enlarged. This channel is lined by the dental sac, which surrounds the entire erupting tooth, approaches the routs of the resorbing tooth, and sends a channel all the way to the gingiva.[164] Collagenase and a nonspecific proteinase capable of breaking down insoluble collagen of dental sac of erupting teeth.[164]

The collasgenase activity was Ca^{++}-dependent and was inhibited by tissue inhibitory factor. This implies that a closely regulated system of collagenolysis is operating during tooth eruption.

Collagenase activity arising in the dental sac diffused out of the sac and was concentrated in the routs of apposing decidual tooth and in the surrounding channel of eruption and channel enlargement to occur. The presence of collagenolytic activity in the area of the dental sac below the permanent tooth as well as above it

could be reasoned that tooth eruption resulted from a combination of eruption pathway formation above the tooth and bone remodeling below the tooth.

6) Host defence (Bacteriocidal)

Among other possible functions, elastase and cathepsin G in neutrophil leukocytes probably contribute to the essential function of neutrophils in combating bacterial infection.[11,143] Blondin et al have demonstrated that PMN elastase can lyse cell walls of autoclaved bacteria, when the latter are species whose peptidoglycan cross linkages contain amino acid sequences similar to those present in tropoelastin or in cross linkages of elastin.[73] In contrast, capthepsin G has been reported to be bacteriocidal owing to its cationic properties rather than to its proteolytic activity.[107,108,153] However, this same enzyme has also been found to digest outer membrane protein of Acinetobacter 199A and to potentiate the lytic action of lysozyme upon this organism.[153] The broad substrate range of both these enzymes also suggest that they could function in the digestion of bacterial protein.[11] After several experimental evidences were obtained using cell-free system and viable neutrophils, Blondin et al suggested that neutrophil elastase might participate in the breakdown of bacterial protein within PMN. Digestive vacuoles and PMN elastase participates in E. coli digestion once the microbes have been injected and killed by the leukocytes.[11]

Capthepsin G may also play a role in the digestion of E. coli proteins within living human PMN.[11] However, using cell-free system, granulocyte capthepsin G has bacteriocidal activity which is independent of enzymatic function, thermal inactivation of PMN capthepsin G does not affect the microbicidal potency of this substance.[107] Therefore, these two controversial result cannot explain the mechanism of bacteriocidal effect of capthepsin G.

4. Pathological role of neutrophil proteases

1) Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory joint disease in which joint function is irreversibly last. The course of the disease is commonly characterized by spontaneous exacerbations and remissions. The onset of disease usually appears as morning stiffness and pain in the joints of the hands or feet, followed by an obvious inflammatory process.[43] The synovium of the affected joint becomes inflamed and edematous, and large numbers of polymorphonuclear leukocytes (PMN) infiltrate the synovial fluid. The volume of synovial fluid increases and synovial lining cells become hyperplastic, as the result a swollen, multilayered synovial membrane is formed. This membrane evolves into an invasive, granulomatous "pannus" as it becomes vascularized and infiltrated with fibroblasts, lymphocytes, macrophages and plasma cells.[71,112,169] The collagen deposited in the proliferative phase appears to be derived largely from type III molecules, although some thick fiber bundles comprised of type I molecules are also observed. In addition, there occurs a release of prostaglandins as well as large amounts of proteolytic enzymes including collagenase, elastase and cathepsin G. As a result, areas of articular cartilage on which the proliferating pannus rests, undergo erosin through depletion of proteoglycan aggregates and degradation of collagen fibers. At this stage, the joint lesions are essentially irre-

versible.[43]

The progressive character of the proliferative process eventually leads to an invasion of the margins of the articular cartilage and ultimately the erosion of cartilage exposes the subchondral bone which may also be eroded. The exudative and proliferative process also result in increased quantities of synovial fluid is often turbid and cloudy. The seventy of clinical menifestation in the joints is related to the degree of cartilage and bone loss. Ultimately, area of necrosis and fibrinoid deposition are seen within the proliferating synovial tissue. Portions of the tissue however, may lead to the formation of fibrous adhesions between the eroded articular surfaces These bridges of connective tissue may eventually take the form of immobile junction, yielding permanent ankylosis.[343] Because of extensive tissue degradation seen in rheumatoid arthritis, the efforts of many laboratories have been focussed on defining the role of proteolytic enzymes in this disease. Endogenous enzymes capable of degrading proteoglycan and collagen have been found in the cartilage matrix of nonarthritis cartilage[92] and may be responsible for the normal turnover of the cartilage matrix. In pathological situation, including arthritis, these enzymes may become activated by exogenous factors or they may be released from inhibition by proteinase inhibitors normally present in the cartilage, either because these inhibitors become saturated by exogenous proteinase, or because they are present in reduced amounts. Because inflammation of the joint and arrival of large number of PMNs in the synovial fluid are early events in rheumatoid arthritis, PMN-derived enzymes have been implicated in the development of arthritis. Weissmann et al[158] demonstrated the induction of arthritis in rabbits by repeated intra-articular injections of rabbit leukocyte granules. These granules contains a number of enzymes; leukocyte elastase, cathepsin G, and collagenase. Elastase is able to degrade collagen and proteoglycans as well as elastin,[79,92,137,158] and cathepsin G is also able to degrade proteoglycans.[130,137,138,158] Other PMN proteinase including cathepsin D and B, are capable of degrading proteoglycans and collagen and found in arthritis joint.[130] However, because they are active only at acidic pH, these enzymes are thought to function intracellularly or pericullulary, but not extracellularly,[92] and their role in the pathogenesis of arthritis is not clear.

PMN collagenase and elastase may play an important role in at least the initial phases of arthritis.[54,57] The enzymes, collagenase and elastase in synovial fluid apparently derived from PMNs are able to degrade fibrils as well as collagen in solution.[54,57] The synovial enzyme is a typical vertebrate collagenase : a metalloproteinase, active at neutral pH, which cleaves native collagen fibrils to yield TC_A and TC_B fragments. These fragments are denatured at 37℃ and the same enzyme can degrade the denatured fragments to peptides of less than 10,000M.W., and the small fragments may be digested by elastase, cathepsin G.[79,138]

Synovial cells have also been shown to produce other enzymes, including gelatinase,[166] PA, and an unspecified caseinolytic activity.[33] However, the origin of gelatinase and caseinolytic activity is not clear because the gelatinase and caseinolytic activity have also shown in PMN leukocyte enzyme preparations, too (unpublished).

2) Inflammation

Inflammation is defined as "a localized protective response elicited by injury or destruc-

tion of tissues, which serves to destroy, dilute or wall off both the injurious agent and injured tissue". For the best description of inflammation process, we go back to Cohnheim.[23] "The first thing you notice in the exposed vessel is a dilatation which occurs chiefly in the arteries, then in the veins, and least of all in the capilaries…" According to Cohnheim's description; it is characterized in the acute form by the classical signs of pain, heat, redness, swelling, and loss of function. Histologically, it involves a complex series of events, including dilatation of arteries, capilaries, and venules with increased permeability and blood flow; exudation of fluids, including plasma proteins; and circulating cells migration into the inflammatory focus.[23,152] The circulating cells that are important in inflammation include neutrophils, monocytes, eosinophils, lymphocytes, basophils, and platelets The main connective tissue cells are the mast cells, which intimately surround blood vessels, and the connective tissue fibroblasts. The extracellular connective tissue consists of basement membrane, the various type of collagen, elastin and proteoglycans. Fibronectin and laminin are glycoprotein that, together with some types of collagen (Ⅳ, and Ⅴ) are present in basement membrane.[152] When we look at a piece of inflammed tissue under a microscope, the most striking and characteristic thing is the presence of leukocytes. This cells come from the blood.[152]

Both macrophages and PMN leukocytes are recruited from the circulation to site of inflammation by specific stimuli. The inflammatory stimuli and suppressants, in vivo and in vitro, have linked the effects of these agents to their ability to modulate the synthesis and secretion of PA, collagenase, and elastase by these cells. The mechanisms regulating the release of collagenase by the fibroblast and eosinophil are unclear. For the neutrophil, most inflammatory stimuli (phagocytosis, immune complexes) result in rapid release of proteinases. The same is true for alveolar macrophages from experimental animals.[159] Proteolytic enzymes released by inflammatory cells initiate the digestion of nonviable tissue, fibrin, and denatured proteins and proteoglycans and may activate mediators of inflammation.[139] At least five neutral proteinases have been identified in human neutrophil; i.e., collagenase, elastase, cathepsin G, gelatinase and plasminogen activator.[139] Collagenase is active against collagen types Ⅰ, Ⅱ, and Ⅲ, and elastase is active against not only elastin but also fibronectin, collagen type Ⅲ & Ⅳ, and proteoglycans. Given its broad range of activity extending to many proteins in addition to these associated with extracellular matrix, it is likely that neutrophil elastase is active against laminin and other extracellular attachment molecules.[139]

Cathepsin G can degrade a number of proteins including fibronectin[159] and laminin[124] in vitro, it may not have a direct role in extracellular matrix degradation since it is minimally released from viable cells even when the other azurophil granule contents (including elastase) are liberated.[134] Perhaps cathepsin G serves intracellular functions that influence degradation of extracellular matrix.[139] Some evidences, including my laboratory (unpublished data) suggests that elastin is degraded by a concerted action of elastase and cathepsin G more rapidly than by elastase acting alone.[16]

In contrast to elastase and cathepsin G, collagenase and gelatinase have restricted substrate specificities.[25] Since they are formed in specific granules, however, they are released readily and more completely in response to chemotactic fac-

tors and other soluble stimuli then are the proteinases found in the azurophil granules[172]; this suggests that collagenase and gelatinase may serve important functions during cell migration in vivo.[139]. However, leukocyte proteinases including elastase and cathepsin G might also play a pathological role in inflammation.[70] It is, however, well known that inflammatory exudates like synovial fluids contain plasma proteins and therefore plasma inhibitors which may block the activity of these proteases. Leukocyte enzymes can therefore not play a pathological role if they are unable to overcome this high inhibitory power.[10]

3) Pulmonary emphysema

Emphysema is a condition of the lung characterized by abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls.[171,21]

Emphysema can be induced by injecting the bronchial arteries with concentrated chloropromazine solution to form ischemis injury.[31] The mechanism of ischemic theory of emphysema is not clear yet. Oxidant gases including NO_2 and O_3 also cause the emphysema,[14,98] and the mechanism of injury was believed to involve an inflammatory intermediate. Other irritant gases, including phosgene ($COCl_2$), chloride gas, and cigarette smoke, and cadmium salt are very strong emphysema inducers.[8,81,128] The most interested one is that habitual cigarette smoking is probably the single most important etiologic co-factor in the production of human emphysema.[81] Emphysema is caused by cigarette smoking accompanied alterations of increased pulmonary recruitment of cells which can release elastin-degrading proteases in tissues,[63,68] and also decreased activity of lung elastase inhibitors.[19,40,74,77]

〔Enzyme induced emphysema〕: Papain which is a proteolytic enzyme mixture derived from the fluid and latex of the papaya tree causes emphysema in animal.[52] It was difficult to relate papain-induced disease to human emphysema since papain is plant derived. Therefore many laboratories attempted to prove induction of emphysema with mammalian products like leukocytes. The alveolar macrophage,[159] lung fibroblast,[42] neutrophil[83] and eosinophil[67] are potential cellular sources of collagenase with the lower respiratory tract of man. With the notable exception of the neutrophil, collagenase release from these cells appears, to depend upon de novo protein synthesis of the enzyme.[41]

In contrast, the bulk of the neutrophils collagenase activity exists in a preformed state that is stored after synthesis.[89] Neutrophil collagenase is readily released into the extracellular spaces in response to a variety of phagocytotic and inflammatory stimuli.[172] For the neutrophil, most inflammatory stimuli result in rapid collagenase release. The same is true for alveolar macrophages.[159] For human alveolar macrophages, however, collagenase release appears to be constitutive, i.e., unaffective by stimulation.[42]

In contrast to the collagenases, the elastase are neutral proteases that attack all of the connective tissue structural proteins of alveolar structures.[41,75,96] Studies of alveolar macrophage and neutrophil elastases have demonstrated that while both are capable of cleaving elastin, they are different enzymes[159,42] i.e., the elastase of the human neutrophil has classified as a serine-dependent active site and does not require free divalent cations for its elastolytic activity.[121] According to my preliminary ex-

perimental result, human PMN leukocyte elastase shows not only serine proteases activity but also shows the metalloenzyme activity which can be inhibited by metal chelators.[79] Therefore it need more careful and detail biochemical studies on this enzymes to see the role of this enzyme in emphysema.

Human alveolar macrophage elastase is metalloenzyme which is inhibited by metal chelating agents.[12] In contrast, the neutrophil carries large amounts of elastase in a preformed state and rapidly releases this elastase in response to inflammatory stimuli.[43]

〔Other proteases〕 : In addition to collagenase and elastase, there are other proteases capable of destroying some of the proteins that make up the connective tissue framework of the alveolar structures. For example, pepsin can hydrolyze the collagens type I and Ⅲ, trypsin attacks type Ⅲ,[102] Cathepsin B attacks the protein portion of proteoglycans,[76] trypsin destroys fibronectin.[53] The relevance of such proteases to chronic inflammatory disease of the lower respiratory tract is unknown. Neutrophil carries cathepsin G (a nonspecific neutral protease that can hydrolyze elastin), and this engyme may play a role in disorders of the alveolar structure. Author can demonstrate that cathepsin G attacks the collagen (unpublished) and inhences the collagenase and elastase activity.[137,138] However, we have to do a lot of work to investigate the exact role of cathepsin G in inflammatory diseases.

Current concepts of the role of proteolysis in human lung disease derive from the theory that maintenance of normal alveolar structure is dependent on the existence of a homeostatic balance between the connective tissue-specific proteinases, i.e., elastase, collagenase and cathepsin G, and the antiproteinases that inhibit these proteases. In this scheme, disease result from an imbalance between the connective tissue proteases and antiproteases such that expression of connective tissue proteolysis is permitted.[43] This can occur in two ways : either from an excess of proteases or from a relative deficiency of antiprotease, α_2 macroglobulin, α_1-trypsin inhibitor[109] and other low molecular weight of inhibitors.[115] Detail of the this topic will be discussed in later chapter. Facilitation of PMN elastase activity by oxidative inactivation of α-1 PI has been proposed as a mechanism for lung injury in cigarette smokers. Specially interested development was that application of recombinant DNA technology to the production of mutant forms of α-1 PI. For example, a molecule identical in all respects to the native inhibitor except for the substitution of valine for methionine in the active site (position 358) has been cloned in yeast (S. Rosenberg's observation). This variant of α-1 PI is an active inhibitor of PMN elastase and it resists oxidative inactivation. "Valine α-1-PI" may therefore have considerable therapheutic potential for controlling tissue injury caused by the synergism between PMN elastase and PMN oxidant like cigarette smoke.[72]

4) Tumor invasion

Theoretically, locomotion and lytic action of cells, whether normal or neoplastic, should be two highly interconnected functions. Whereas physiologic cell migration within the organism may only require inconspicuous lytic effects at the molecular level, it is well known that cancer cell infilteration is often associated with lytic action easily recognizable at the cellular level.[149] Detachment makes cancer cells mobile, which implies that they can be carried away.[17]

The essential step for single cancer cell is to

become motile or capable of active translocation is not an automatic consequence of detachment of the single cell state.[149] In the body, cancer cells do not migrate along preformed pathways or do so only to a minor extent. They are constantly faced with barriers, among them the structural manifestations of extracellular matrix.[149]

Cancer cells can overcome the barriers physically through the momentum of their propulsion, at the same time making full use of their capacity for shape adaptation; or they can overcome the barrier chemically by lossening or dissolving it with the help of enzymes.[149]

[Mechanisms of tumor invasion]

A) Mechanical pressure : The pressure theory of invasion proposes that as a tumor expanded in size; it exerts pressure on surrounding tissues.[30] The increased pressure causes rapidly proliferating tumor cells to force their way into alternative locations within host tissue.[30] However, the pressure theory has limitation since small tumor cell clusters, seperated from the primary tumor, do not appear to generate increased pressure, yet may be highly invasive.[150] The theory also fail to explain the lack of metastases from some very large, bulky tumors. Considering all of the evidence, pressure probably play a minor role in the process of tumor invasion in most cases.[116]

B) Proteolysis of host extracellular matrix : Numerous cross-links and intermolecular bonds between martrix macromolecules are thought to be responsible for the local confinement of many cell types.[170] Tumor invasion may therefore require the destruction and solubilization of the tumor-surrounding extracellular matrix.

Utilizing enzyme immunolocalization techniques, it has been shown that lysis of extracellular matrix is restricted to multiple, small area of the tumor invasion zone.[48,169,170] Such localized bursts of enzyme activities may be triggered by favorable conditions in the microenvironment, appropriate pH, accumulation of catalytic factors (metal ions) or, depletion of enzyme inhibitors.[50] They may explain the usually time consuming process of invasion in most clinical sittings, and may exemplify the fact that solid tumors are composed of numorous, heterogenous tumor cell subpopulations which differ from each other with respect to their invasive potentials.[116]

Enzymatic lysis of host tissues during tumor invasion may not required the total destruction and dissolution of tissue barriers.[115] It may be sufficient to lower the level of molecular organization to reduce the physical resistance of host tissue to invasion.[149]

Nondestructive, tumor-induced lysis consists of a reversible, temporary, and focal loosening of host tissues, which facilitates the invasion of some neoplasms.[115] Example of this mechanism is the contraction of liver sinusoidal endothelial cells prior to metastatic invasion by blood-borne cancer cells.[27]

Collagenase : Collagenase is elaborated by various normal and neoplastic cells; whereas its limited production by cells in normal tissues suggests a role in collagen remodeling processes, its more frequent occurrence in malignant tissues suggests an important role in pathological collagen resorption[116] Tumor cells release collagenolytic enzymes as an aid to their migration and penetration of the surrounding collagenous extracellular matrices, basement membrane.[116] This concept is supported by the correlation of high levels of enzyme activity with the clinical aggressiveness of some human and animal malignancies[94]

In general, packed properly into fibers, the native triple-helical region of the interstitial collagens (Type Ⅰ, Ⅱ, and Ⅲ) is remarkably impervious to enzyme attack. However, vertebrate collagenases can attack the native triple-helical collagen. These enzymes are narrowly defined by their ability to cleave native triple-helical collagen at the approximate 3/4~1/4 point between the NH_2-and-COOH termini of molecule.[116] Collagenase attack at Gly-Leu or Gly-Ile sequence along the chain.[50, 56] This region appears to be unstable, due in part to decreased number of hydorxproline residues.[116] Other locations along the triple helix with these amino acid sequences are not cleaved by collagenases. The action of vertebrate collagenase on collagen fibers is considerably slower than its action on soluble molecules.[167] After cleavage of the triple helix by collagenase, the resultant 3/4 and 1/4 length fragments are thermally unstable at 37℃ and denature.[116] At physiological temperature the weak hydrogen bonds that stabilize the helix are by themselves insufficient to maintain this conformation; thus approximately 1/2 of the monomeric molecules are denatured at 37℃. When polimerized into fibers, as it is the case for the majority of collagen molecules in vivo, the denaturation temperature is increased significantly above body temperature However, an enzyme which can cleave the telopeptide region, between a cross-link and the beginning of a triple helix, may release collagen monomers from fibers. This would cause significant helix denaturation.[116]

Elastase : Elastase may be localized in tissue by using the double immunofluorescence method.[65] Tumor elastolytic activity has been demonstrated in extract of human breast carcinoma[95] and in media of several human breast carcinima cell lines.[80] Elastase may also be involved in physiological and pathological processes associated with acute arthritis, pulmonary emphysema, acute inflammation, corneal ulceration, damage to the internal elastic lamina of arteries and to the kidney basement membrane and cancer invasion.[4, 5, 21, 78, 81, 116]

Human leukocyte elastase behaves differently from pancreatic elastases. For example, its action on the oxidized chain of insulin is directed more toward valine than alanine as the residue contributing the carboxyl group of the cleaved bond, the most susceptible bonds being Val-12 and Val-18[121], i.e., His^{10}-Leu-Val-Glu-Ala^{14} and Tyr^{16}-Leu-Val-Cys-Gly^{20}. Human leukocyte elastase is much less active on fibrous elastin and on succinyl-$(Ala)_3$-p-nitroanilide than the pancreatic elastase is.[7]

After sequential purification of the human neutrophil elastases by gell filtration and High performance liquid chromatography, three different elastases, which have been named as three isozymes, showed M.W. : 29,700, 28,200 and 26,400 as determined by gel electrophoresis in sodium dodecyl sulfate,[79] and these three elastases might be different enzymes since we cannot identify these enzymes as isozyme which should be followed the definition.[79] Isozymes are defined as a multiple molecular-forms of an enzyme occuring within a single species as a result of the presence of more than one structural gene (commission on biological nomenclature of IUPAC-IUB 1977). Until the genetic relationships between these various forms of elastases are clarified, these enzymes should be called as three different "forms" or "species" of the enzyme.[79]

Substrate specificity of elastase : The enzyme has a very broad specificity against protein substrates. It has been reported to hydroly-

se elastin, collagen, proteoglycan, azo-casein.[144] Elastase solubilizes insoluble collagen, being as active or even rather more active, against type Ⅱ collagen of bovine articular cartilage than against type I collagen of bovine achilles tendon. This is in marked contrast to mammalian collagenases which are much more active against type I collagen than type Ⅱ.[166] Analysis of the reaction products resulting from the digestion by elastase of collagen in solution shows that the enzyme cleaves the extra-helical cross-link region of the collagen fibrils, causing release of single chain which then be futher degraded.[143] It is presumably the cleavage of the cross-link regions which allow elastase to solubilize insoluble collagen, converting is into a form susceptible to degradation by the specific mammalian collagenases.[143]

Proteolytic activity of elastase showed much higher in mixture of elastase and cathepsin G (which will be discussed later) than elastzse alone does (unpublished observation). Therefore, these might be enhanced proteolytic activity of three enzymes; elastase, collagenase, and cathepsin G, in cancer invasion.[138] Elastase is active in neutral pH and is activated by various ions, Ca^{++}, Mg^{++}, Zn^{++}, Na^{+}, K^{+}, Li^{+} [79,143]

Capthepsin G : This enzyme has been shown to hydrolyze haemoglobin and fibrinogen,[135] casein, azocasein, collagen, and proteoglycan.[143] Capthepsin G attacks the core protein degrading it to fragments containing 5-12 condroitin sulfate side-chains, and also digests the larger of the two link proteins.[143]

The enzyme solubilizes collagen, being more active against type Ⅱ than type I collagen.[143] The specificity of cathepsin G against the insulin B chain is similar to, though not identical with, that of porcine chymotrypsin C.[13] Capthepsin G is active against a variety low molecular weight substrates, all of which are also substrates of chymotrypsin.[143] Cathepsin G has a pH optimum of 7.5-7 8 with azo-casein and B_z-DL-Phe-2-ONap.[145]

Plasminogen activator may also involve in tumor invasion, growth, and fibrinolysis.[116] Plasminogen activator (PA) converts the zymogen plasminogen to the active proteinase plasmin, which in turn can degrade fibrin, fibronectin, lominin, and other protein substrates.[20,105] It can also serve as an activatior of other proteolytic zymogens, such as latent collagenase.[20] Therefore PA can also generate substantial proteolytic activity within the cellular microenvironment by means of well-established cascade mechanisms.[20,105] The following properties of malignant tumors have been correlated with increased PA activity and the generation of plasmin : anchorage independent growth; induction of cell division; cell migration; cytoskeletal and cell surface alteration; tumorigenicity of viral transformations in nude mice; and invasion and metastatic potential of various cell lines.[38,47]

5) Arteriosclerosis

Arteriosclerosis is a very complex disease which is probably generated by a variety of factors. One of the characteristic features of this disease is the fragmentation of elastin fibers within the media of arterial walls. This suggests that elastolytic enzymes might intervene in the origin and/or development of arteriosclerosis.[10]

The role of tissue elastases in the pathogenesis of arteriosclerosis is more likely than pancreatic elastase is sine the possibility of intestinal absorption of proteases is extremely low and serum antitrypsin and α_2-macroglobulin are extremely potent elastase inhibitors. Horneback et al have found that there is a positive correlation between the concentration of human

arterial elastase and the degree of atheromatosis.[66] On the other hand, platelet elastase may be considered as a factor which accelerates the evolution of the arteriosclerotic plagues. In this respect, it is worthwhile noticing that cholesterol-induced arteriosclerosis in rabbits is ameriorated by a treatment which reduces the blood platelet number.[22]

A striking feature of arteriosclerosis is the deposition of various substances into the arterial wall. Such compounds may interact with the elastic fibers and may increase their susceptibility to elastase attack. Therefore it might be specculated that elastase constitutes an aggregating rather than an initiating factor of arteriosclerosis.[10]

5. Control of neutral proteinases

A large number of naturally occurring proteinase inhibitors serve to control the endogenous proteinases, i.e., limit their reactions locally and temporally.[6,62] They may also inhibit protenases introduced into the body by infective and parasitic agents. It seems obvious that a disturbance of proteinase/proteinase inhibitors balance can lead to proteinase-mediated tissue destruction, i.e., tumor invasion,[116,149] emphysema,[41,43] arthritis,[109,165] arteriosclerosis,[10] and probably other pathological conditions.

1) Endogenous inhibitors.

(a) Plasma proteinase inhibitors : Proteinase inhibitor comprise 10% of the human plasma proteins[62] At least eight inhibitors, α_1-antitrypsin, α_1-antichymotrypsin, α_2-macroglobulin, inter-α-trypsin inhibitor, α_2-plasmin inhibitor, α_1-anticollagenase, C-1-inactivator, antithrombin Ⅲ, α_2-thiolproteinase inhibitor, has been isolated and characterized to date.[62]

α_2-Macroglobulin has the broadest spectrum of inhibition[55,146] reacting with proteinases of all classes. It effects the very rapid clearance of proteinases from the plasma.[111] Due to its large size, it diffuses poorly into tissues and secretions, thus, its main role may be policing the invascular space.[6]

α_1-Proteinase inhibitor is another defensive inhibitor, and it is the largest molar concentration in plasma and displays a broad spectrum of inhibition on serine proteinases.[103] On the other hand, α_1-antichymotrypsin is a very specific chymotrypsin inhibitor.[62] The importance of α_1-PI and α_1-antichymotrypsin in the defence against tissue destruction is the fact that both are "acute phase protein", thus increasing during inflammatory responses.[88] β_1-anticollagenase (β_1AC) is another highly specific proteinase inhibitor, controlling only the action of mammalian collagenases.[168] α_2-Thiol PI may be important in controlling the action of cathepsin B and other thiol proteinases.[132] Inter-α-trypsin inhibitor (IαI) does not play a major defensive role by itself,[147] and it is not clear the role of this inhibitor yet. Elastases are primarily inhibited by α_1-PI and collagenases by β_1AC.[168] Although α_2-macroglobulin is the predominant inhibitor of cathepsin G in plasma,[110] in terms of protecting tissues, α_1-antichymotrypsin is probably the more important inhibitor due to its ability to diffuse more easily into the extravascular space.[153]

The ability of plasma proteinase inhibitors to prevent tissue destruction is determined not only by their presence in plasma and their affinities for proteinases, but most importantly by their ability to diffuse into the extravascular space.[62] A firm indication that certain plasma proteinase inhibitors play a role in

protecting tissues from proteolytic attack is derived from the fact that proteinase/plasma proteinase inhibitor complexes are found regularly at the sites of tissue destruction.[6] In synovial fluid of patients with rheumatoid arthritis, one generally find an excess of free α_1-PI and α_2-M together with their complexes.[3,110,142] Since neither α_1-PI nor α_2-M can penetrate cartilage their actions seem to be confined to the synovial fluid.[35] In purulent sputum from patients with chronic bronchitis, an excess of free proteinases, together with saturated plasma proteinase inhibitors, is found.[97]

A role of proteinase inhibitors in the pathogenesis or the prevention of certain disease states can also be infered from an association of altered plasma levels with disease. Three aspects are; (1) altered plasma proteinase inhibitor levels causes or facilitates a disease, (2) altered plasma proteinase inhibitor levels as a result of disease associated consumption, and (3) altered levels as a nonspecific defence mechanism i.e., acute phase.

(b) Tissue inhibitors : In addition to the plasma proteinase inhibitors which are called to sites of injury and tissue destruction (inflammation), the tissues themselves produce or at least store proteinase inhibitors which are ready to contain possible proteolytic attacks.[6] Cartilage, muscle, mucous secretion, lung, bone, tendon, skin, spleen, aorta, uterus, brain contain proteinase inhibitors which are different molecular weight and specificity to substrates.[6]

The presence of proteinase inhibitors in cartilage is thought to be the reason why cartilage is rarely invaded by tumors.[85,87] It should be noted that cartilage is also resistant vascularization,[87] which is a prerequisiste for tumor growth[37] and thus also for tumor invasion.[116] In human cartilage a low molecular weight inhibitor against trypsin and collagenase has been identified and named as "anti-invasive factor (AIF)".[85,87] AIF has not only anti-protease activity but also anti-proliferative activity,[115] and Kuettner's group suggested that AIF might act as local regulators for some of the major mechanistic pathways by which tumor cells are thought to invade host tissues and metastasize to distant sites. In addition AIF may inhibits tumor neovascularization.[115]

In muscle, proteinase inhibitors not only defend against proteolytic injury but are probably involved in the continuous process of remodeling and restructuring of this tissue.[6]

Human mucous secretions are rich in a low molecular weight acid-stable proteinase inhibitor.[133] It inhibits strongly the leukocytic elastase and cathepsin G, but not the collagenase.[6]

2) Exogenous inhibitors

If the assumption that tissue destruction results when the balance between proteinase and the endogenous inhibitors is upset in favor of the enzymes, is correct, it follows that tissue damage may be reduced or prevented by the introduction of exogenous inhibitors. In order to be useful in an in vivo situation, such inhibitors must fulfill at least fine criteris; a) specificity; b) efficiency/potency; c) low toxicity; d) lack of antigenicity; e) bioavailability.

(a) synthetic inhibitors

Major classes of small molecular inhibitors are : reversible inhibitors, transition analogs, affinity labels, and miscellaneous.[6] Of the reversible-type inhibitors trifluoroacetyl oligopeptides,[90,91] the corresponding peptide

anilides, and peptide carbazates[118,119] are the most widely investigated. Peptides and peptide anilides probably represent "poor" competitive substrates or perhaps resemble to structure of natural protein or peptide proteinase inhibitors.[6] Peptide carbazates contain an "aza amino acid" in which the α-methine group is replaced by a nitrogen. The intermediate formed with carbazates during enzymatic reaction is considerably more slowly deacylated due to the adjacent nitrogen atom than the normal acyl enzyme.[118] Other peptide derivatives include a peptide hydroxamic acid may serve as an inhibitor of mammalian collagenase.[106] Some amides and guanidines are potent inhibitors for serine proteinases; kallikrelins, trypsin, plasmin, thrombin, however, none are known to interact with tissue destroying enzymes.[6] Transition state analogs find for more tightly then their corresponding substrates, due to the fact that they form complexes at the catalytic site of the enzymes.[84] Boronic acids and aldehydes form tetrahedral adducts to serine proteinases and are thus good inhibitors. It would appear that changing the R-group to satisfy the specificity requirements of a given proteinase should result in both potent and specific inhibitors.[81]

Affinity labels are irreversible inhibitors which contain a group that reacts covalently with a constituent or group at or near the active site. Oligopeptide-chloromethyl ketone with very high degrees of specificity and reactivity towards human leukocyte elastase[122] and cathepsin G[120] have been synthesized. Chloromethyl ketones also inhibit thiol proteinase. Diazomethyl ketones, with high specificity and reactivity against thiol enzymes,[141] sulfonyl fluorides[195] and organophosphates,[104] are also showed irreversible inhibitions on serine proteinase, thiol proteinases and elastases. Gold sodium thiomalate and pentosan polysulfate also inhibit human PMN leukocyte elastases 40% and 60% respectively.[2]

(b) Microbial proteinase inhibitors

Actinomycetes produce a number of small molecular weight compounds which inhibit proteinase.[6,39] Leupeptin, antipain, chymostatin, elastatinal, pepstatin, phosphoramidon, elasnin, bestatin are the examples of microbial proteinase inhibitors.

3) Effects of proteinase inhibitors

(a) Antitumor effects : Proteinase inhibitor has been known as effective inhibitors of both tumorigenesis[155] and tumor invasion.[116] Cartilage-derived antiinvasive fector (AIF) contains the following anti-invasive activities[115]; (1) proteinases (collagenase and elastase[unpublished data]) inhibitory activity; (2) antiproliferative and anti-migratory activities directed against endothelial cell (anti-angiogenetic activity); and (3) tumor growth inhibitory activity. ε-Amino-caproate inhibits tumor promotion but not induction.[155] Leupeptin and bestatin inhibit both growth and metastasis of several types of experimental tumors.[155]

(b) Effects on emphysema : α_1-PI, elastinal (microbial elastase inhibitors) shows very effective against emphysema in animal.[173] In addition, a number of synthetic elastase inhibitors, including a chloromethyl ketone[148] and peptide carbazate[100] have been shown to be effective in experimental emphysema. Other elastase inhibitors have been pattented as potential therapeutic agents in emphysema.[164,101]

(c) Antiinflammation : Peptide aldehydes have

been shown to inhibit carrageenin-induced edema,[156] and aprotinin has been shown to inhibit a variety of experimentally induced edema reactions, however, its action is mostly due to its inhibition of kinin system[160] and perhaps to other pharmacological actions, e.g., on the vascular system, or its effect on leukocyte degranulation.[160]

It has been suggested that some clinically used antiinflammatory agents may act by inhibiting proteinases.[153] However, the inhibition occurs only at unreasonably high concentrations of the agents tested, making an anti-proteolytic effect at therapeutic dosages unlikely.[6]

6. Conclusion

The production of a variety of proteinases accompanies the invasive behavior of a large diversity of cell types in both normal and pathological situation. The inference that proteinases play a causal, obligatory role in cell invasivenes, however, is still based on circumstantial evidences since proteases production has been monitored as a result of invasive events, rather than the reverse. Even though there are some critical opinion interpretation of experimental evidences using experimental animals and in vitro experiments, these experimental approaches are still the most resonable and available ways on scientific medical research. One promising approach for obtaining more direct evidence for the interpretation that proteinase production is required for cells to be invasive would rely on in vitro and/or in vivo assays in which cell invasion is blocked with purified natural proteinase inhibitors. This example is cartilage extract anti-invasive factor (AIF) which has been shown to block both neovascularization and tumor cell invasion.[87,87] Once purified, these inhibitors could be introduced into assays of neovascularization or tumor cell invasion through cellular and non-cellular barries.[87] In any experimental system, the proteinases should be inhibited specifically and completely, while other parameters of the system remain unchainged. In addition, the assay must be complex enough to measure cell invasiveness, no simply cell movement or cell division, yet defines enough that the cell type producing the proteinase is known.

Another experimental approach that would satisfy the criteria would be to use monospecific antibodies raised against each of the enzymes that to be involved and capable of fully neutralizing enzymatic activity.

If we can demonstrate that specific proteinases can be shown to be responsible for the ability of cells to be invasive, and if the invasive behavior of cells can be prevented with specific proteinase inhibitors or with specific antibodies directed against individual proteinases, this would have exciting therapeutic implications for cancer, arthritis and diabetic retinopathy. Synthetic inhibitors, purified proteinase inhibitors and specific antibodies might be targeted to the cells causing the pathology, so that metastasis, cartilage erosion, and neovascularization, could be prevented.

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