Proteolytic-antiproteolytic balance and its regulation in carcinogenesis

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INTRODUCTION

The conversion of normal cells into invasive cancers with metastatic potential is a process that involves several steps. These steps are manifested in distinguishable histological and temporal stages; for instance, normal tissue, hyperplasia with a high incidence of proliferating cells, dysplasia with the induction of angiogenesis before the emergence of frank tumors with metastatic potential. Analysis of the latter stages of tumor progression has resulted in a multi-step theory of carcinogenesis on the basis of genetic changes involving activation of oncogenes, inactivation of tumor suppressor genes and altered expression of tumor-associated molecules.

Requisite for neo-plastic cell and capillary or inflammatory cell invasion during carcinogenesis processes is the remodeling events that occur within the stroma or extracellular matrix (ECM). The ECM is a complex meshwork of collagens, fibrillar glycoproteins and proteoglycans that determines tissue architecture and conditions many biological activities. The ECM is involved in both normal and pathological processes. Components of the ECM provide a large variety of specific signals that directly influence cell proliferation, migration, morphology, differentiation, and biosynthetic activities. In addition, ECM plays an essential role in cell survival, since loss of adhesive contact, results in apoptosis termed anoikis. In tumors, alterations of the ECM might therefore lead to abnormal host and cancer cell functions and even to cancer progression. Perturbations in the production, deposition and degradation of matrix components have been observed in mammary carcinoma. Quantitative changes in matrix components may be related to an imbalance between their synthesis and degradation. Tumor cells may directly alter the adjacent matrix by changing the production of matrix proteins or proteolytic enzymes. Alternatively, the desmoplastic response may depend on specific interactions between tumor cells and host fibroblastic cells.

It is suggested that perturbation of the tissue microenvironment may be sufficient to induce tumor formation. Moreover, tumor cell invasion and metastasis also require destruction of the ECM during local invasion, angiogenesis, intravasation and extravasation. These processes are mediated by multiple degradative actions of proteolytic enzymes and these complex events need cooperation of different specificity proteases. There are four major groups...
of endoproteases: the aspartyl and cysteine enzymes (mainly cathepsins), which function at low pH and are involved mainly in intracellular proteolysis within lysosomes and serine and metal-dependent enzymes, which are active at neutral pH and responsible for extracellular proteolysis\[16\]. These enzymes can act directly by degrading ECM or indirectly by activating other proteases, which then degrade the ECM. They act in a determined order, resulting from the order of their activation. When proteases exert on other proteases, the end result is a cascade leading to proteolysis. Presumable order of events in this complicated cascade is that procathepsin B can be activated by cathepsin D and other proteases\[18\]. Once activated, cathepsin B may play an important role as an activator of other proteases. For example, active cathepsin B can activate pro-uPA, which is secreted as an inactive proenzyme\[19\]. Then active uPA can convert plasminogen into plasmin. However, cathepsin B and plasmin are capable of degrading several components of tumor stroma and may activate zymogens of matrix metalloproteinases (MMPs) (Figure 1)\[20\].

In the result of the above reactions, the first glycoproteins are degraded by cathepsins and plasmin and afterwards collagen is degraded by metalloproteinases. This order is a result of ECM composition. Glycoproteins surround collagen structures and protect them against proteolysis. Removing glycoproteins by plasmin enables collagen degradation by collagenases. The degradation of these components destabilizes ECM structure and enables neoplastic cells to migrate. Normal and neoplastic cells adhere to adhesive proteins localized on cell membrane surface junctions. There are also cell adherence to cell and cell to ECM adherence. Detachment of neo-plastic cells from the tumor is enabled by action of proteolytic enzymes degrading these proteins\[21\]. Free neo-plastic cells migrate in dense mesh of ECM due to proteolytic enzymes. Neo-plastic cells that cannot change their shape easily are particularly difficult to pass through ECM. The basement membrane also stands in the way of migrating neo-plastic cells. The main component of the basement membrane is collagen type IV. Leukocyte proteases participate in the process of protease activation and facilitate neoplastic cells passing through the basement membrane\[22\]. The contact of leukocytes with neoplastic cells enhances the synthesis of other proteolytic enzymes. Consequently, common action of different substrate specificity and localization in protease activation processes are completed and degradation of the ECM proteins and the basement membrane contribute to cancer development.

**LYSOSOMAL PROTEASES - ASPARTYL AND CYSTEINE PROTEASES**

Activation of cascade proteases participating in ECM degradation, in general, is induced by acidic proteases that are named cathepsins and collected mainly in lysosomes. In normal cells, only a small (5-10\%) quantity of cathepsins occurs in cytosol. However, tumor promotion is connected with disturbances in post-translational glycolization and phosphorylation of proenzymes in Golgi apparatus that prevents their transport to lysosomes. Consequently, a great majority of these proteases occur in cytosol of neoplastic cells\[23\]. The biological task of cathepsins is to degrade cellular and extracellular proteins and cathepsins, with different specificity, are complementary in their action on proteins. They cause total proteolysis degrading proteins of ECM and basement membrane components, as well as, participate in limited proteolysis, e.g., activating proenzymes and prohormones\[24-26\]. Aspartyl protease cathepsin D and many cysteine proteases participate in tumor development.

**Aspartyl protease**

Cancer cells are characterized by over-expression and secrete a large proportion of cathepsin D\[27\]. Over-expression of cathepsin D has been described both at mRNA and at protein levels\[28-30\]. Cathepsin D is synthesized in an inactive form as procathepsin D, which has no proteolytic activity\[31\]. It is auto-activated in an acidic environment (pH<5) in acidic intracellular vesicles or activated during the action of other proteases\[32\]. Secreted procathepsin D could also be activated extracellularly in sufficiently acidic milieu. The extracellular pH in tumors is generally more acidic than that in corresponding normal tissue\[33\]. Above transformations lead to the formation of an active mono-chain form and then to the formation of an active bi-chain form. No difference has been shown in the composition and amino acid sequence of cathepsin D in normal and neoplastic tissues. The observed qualitative differences concern only the enzyme structure depending on post-translation processes\[34\]. But there are differences in distribution of cathepsin D, i.e., it is found not only in the lysosomes of cancer cells but also in the cytosols as well\[35-37\]. The significant increase in cytosols and in the neo-plastic cells has been shown in cases of breast cancer\[38\]. Moreover, the correlation between high cathepsin D expression in cytosols and poor survival in node-negative breast cancer is observed\[39,40\]. Marked expression of cathepsin D takes place in carcinomas of uterus, ovary, lung, intestines and many other organs\[41-43\]. Related
increase of cathepsin D activity and concentration is also observed in the fluids of the body cavities and in the blood serum of patients with carcinomas\textsuperscript{[12-37]}. Cathepsin D as an endopeptidase degrades many intracellular and endocytosed proteins, as well as, ECM proteins and proteins of the basal epithelium. Cathepsin D participates in limited proteolysis and activates cysteine procathepsins B and L; it also degrades and makes inactive their active forms\textsuperscript{[40]}. In addition, it inactivates cysteine proteases inhibitors - cystatins\textsuperscript{[50]}. It has been shown that human cathepsin D stimulates tumor growth by acting directly or indirectly as a mitogenic factor on cancer cells independently of its catalytic activity\textsuperscript{[59]}. Cathepsin D stimulates proliferation and tumor angiogenesis of cancer cells. It was suggested that cathepsin D stimulates angiogenesis by releasing ECM-bound bFGF or pro-cathepsin D is responsible for generation of specific inhibitor of angiogenesis angiotatin\textsuperscript{[14,48]}. Now it is speculated that cathepsin D may stimulate endothelial cell growth via a paracrine loop, acting as a protein ligand, by directly or indirectly triggering a yet unidentified cell surface receptor\textsuperscript{[51,41]}. Moreover, it has been documented that cathepsin D influences apoptosis and mediates apoptosis induced in cell lines by various agents, i.e., INF-γ, Fas/APO, FNF-α and oxidative stress\textsuperscript{[42,43]}. However, now it is suggested that proteolytic action of cathepsin D may provide some protection against apoptosis \textsuperscript{[59]}. Thanks that it participates in the process of neo-epithelial growth and metastasis. Moreover, until now an endogenous cathepsin D inhibitor has not been found, the reason why it can work in each condition is still unknown.

**Cysteine proteases**

The second step in proteolytic cascade may be linked with different lysosomal cysteine proteases, including cathepsins B, L, H, C, S, F, K, O, V, W and X\textsuperscript{[64]}. The promotion role of these cathepsins is connected with their proteolytic effects on basement membrane and interstitial stroma\textsuperscript{[65]}. In normal cells, these cathepsins are regulated at every level of their biosynthesis including transcription, post-transcriptional processing, translation, post-translational processing and trafficking, thus maintaining their normal function in cell metabolism. In tumor cells, misregulation of cathepsins at one or more of these levels results in increased mRNA and protein expression, increased activity and altered intracellular distribution\textsuperscript{[49]}. Among the lysosomal cysteine proteases, cathepsin B has been the most extensively investigated due to its important role in cancer progression\textsuperscript{[45-48]}. Increased expression together with enhanced secretion and cell surface association of cathepsin B is found in different types of tumor cells, especially in their more malignant variants\textsuperscript{[65-67]}. Studies have revealed that enhanced production and release of this cathepsin in tumor cells lead to tumor cell growth, invasion and metastasis\textsuperscript{[68,69]}.

It has been found that cathepsin B mRNA, protein level and activity in tumors and cancer cell lines, especially with high metastatic potential, are increased\textsuperscript{[100]}. Over-expression of cathepsin B mRNA has been reported in several human tumors including tumors of brain, colon, prostate and thyroid\textsuperscript{[52-53]}. However, increased expression of cathepsin B, in pre-malignant lesions, suggests that this enzyme may play a role in the transformation of pre-malignant lesions to malignant tumors\textsuperscript{[50]}. Moreover, it has been found that cathepsin B expression often increases specifically at the invasive edge of tumor cells\textsuperscript{[59]}, because granules containing cathepsin B, in normal tissues, are localized perinuclear while during tumor progression they move to the inner basal surface of plasma membrane\textsuperscript{[70]}. The redistribution of cathepsin B to the basal membrane in cancer cells occurs coincidently with degradation of the underlying basement membrane. It is very important for degradation of the surrounding ECM in tumor progression, because tumor cell invasion involves local proteolysis.

Although, a few studies indicate a correlation between cathepsin B mRNA over-expression and tumor invasion, numerous studies have been focused on cathepsin B expression at the level of protein and activity. Cathepsin B protein and activity levels have been found to be higher in many human tumors including tumors of breast, cervix and ovary, colon, stomach, glioma, lung and thyroid\textsuperscript{[68,69-71]}. Cathepsin B like other cathepsins is synthesized as a proenzyme and activated in prelysosomal acidic vesicles prior to its delivery to lysosomes. Procathepsin B can be activated by cathepsin D, elastase and cathepsins G, uPA or tPA\textsuperscript{[40]}. Mature cathepsin B is localized in lysosomal and participates in intracellular proteolysis. However, in cancer cells significant increase of cathepsin B in cytosol has been observed\textsuperscript{[60,61]}. Increased secretion of proenzymes, as well as, mature enzymes by human colorectal carcinoma cell lines and hepatomas and human liver, colorectal and lung cancers have been reported\textsuperscript{[62-64]}. Moreover, secretion of procathepsin B can occur from cells that do not exhibit an increase in mRNA levels, indicating that this secretion is probably due to altered intracellular trafficking and distribution of this enzyme\textsuperscript{[68]}. Another indication that tumor cells secrete cathepsin B is the increased serum level of cathepsin B in patients with hepatocellular carcinoma, prostate cancer and melanoma\textsuperscript{[66-68]}. In addition, cathepsin B has also been found in other body fluids surrounding tumors, such as bronchoalveolar lavage fluid of lung cancer patients or cerebrospinal fluid from patients with leptomeningeal metastasis\textsuperscript{[65,69]}. Cathepsin B can affect extra-cellular connective matrix directly causing its proteolytic degradation or indirectly via activation or amplification of other ECM-degrading proteases. This cathepsin may act on the contact regions of tumor cells and basement membrane or interstitial stroma. These places are often acidified by tumor cells, that are conducive to activation of secreted precursors to active forms that degrade the protein components of basement membranes and the interstitial connective matrix including laminin, fibronectin, elastin, and various types of collagen\textsuperscript{[71-72]}. Digestion of fibronectin by cathepsin B results in exposure of the CS-1 sequence, which is within the alternatively spliced type III connecting segment (IIICS) of fibronectin and recognized by the integrin receptor, αβ\textsuperscript{[57]}. In this way, cathepsin B may not only be involved in extra-cellular degradation but may also be linked to cellular signal transduction events.

Cathepsin B indirectly enhances proteolysis by activating the urokinase-type pro-plasminogen which can subsequently activate the plasmin-metalloproteinases proteolytic
pathway. Moreover, cathepsin B may change the balance between metalloproteinases and their inhibitors and directly activates some of the MMPs - interstitial procollagenase (proMMPs-3) and prostromelysin-1 (proMMPs-2)\(^2\), directly cleaves and inactivates some of the MMP inhibitors TIMP-1 and TIMP-2\(^7\). In such a way, cathepsin B assists tumor cells in their detachment from ECMs and metastasis. Moreover, during proteolytic breakdown of ECMs, some ECM-bound growth factors such as bFGF, EGF, TGF-β, IGF-I and VEGF may be liberated and become bioavailable for growth modulation of receptor-partner expressing tumor and stroma cells\(^7\).

It has been proved that another cysteine protease, cathepsin L, has similar proteolytic properties and action in tumor progression to cathepsin B\(^8\). The highest level of cathepsin L is seen in most of the tumors and transformed cell lines possessing the highest malignancy\(^9\). The cathepsin L gene may be activated by a variety of growth factors and activated oncogenes\(^10\). It has also been shown that procathepsin L possesses proteolytic activity and can act like a growth factor or a progression factor on cell proliferation and is involved in differentiation processes\(^8\)\(^,\)\(^9\). Moreover, procathepsin L degrades both fibronectin and laminin while cathepsin L degrades types I and IV collagens, fibronectin and laminin.

Other cysteine proteases like cathepsins H and K participate in carcinogenesis. Cathepsin H is easily distinguished from other endosomal cysteine proteases by its unique aminopeptidase activity\(^9\). There is growing evidence that the expression of cathepsin H increases in disease states including breast carcinoma, melanoma, glioma, and lung, colorectal, prostate and other carcinomas\(^4\)\(^,\)\(^5\). It seems that the over-expression of cathepsin H in tumor progressions is not well understood. A possible function of cathepsin H in tumor progression is its ability to degrade fibrinogen and fibronectin, like a growth factor or a progression factor on cell proliferation and is involved in differentiation processes\(^8\)\(^,\)\(^9\). Moreover, procathepsin L degrades fibronectin and laminin while cathepsin L degrades types I and IV collagens, fibronectin and laminin.

SERINE PROTEASES

During the past decades, the important role of ECM, in direct digestion, has been attributed to plasminogen activator system, which is composed of pro-activators, plasminogen, their cell surface receptors and activation inhibitors and antiplasmins. There are two types of plasminogen activators: the urokinase-type (uPA) and the tissue type (tPA). These activators are coded by two different genes\(^11\). However, they are quite similar in structure. Both are capable of catalyzing the conversion of inactive zymogen plasminogen to active protease plasmin. There is evidence that the primary role of tPA is to generate plasmin for thrombolysis, while it is the uPA that generates plasmin in events involving degradation of ECM. In consequence uPA participates in cancer invasion and metastasis\(^12\). uPA is released from cells as a zymogen form of pro-uPA, which is converted to active form by plasmin, present in trace quantities in ECM. Elastase and cathepsin B are also pro-urokinase activators\(^7\). UPAR, a surface receptor, binds to pro-uPA with high affinity\(^13\). Coincident binding of pro-uPA and plasminogen to UPAR and free lysine groups, on cell surfaces strongly enhance plasminogen activation\(^14\). This situation facilitates pro-uPA by plasmin and activation of plasminogen by urokinase. Because plasmin is produced in the second reaction, the activation behaves like a closed cycle reaction. Two inhibitors control plasminogen activation. There are two main inhibitors of plasminogen activators, PAI-1 and PAI-2, while plasmin is inhibited by α2-antiplasmin\(^15\). These inhibitors belong to the serpin super-family. The other serpins, proteinase nexin-1 (PN-1) and protein C inhibitor can also inhibit uPA and tPA at physiologically relevant rates, though they are not specific for plasminogen activators and react more slowly with these proteases than PAI-1 and PAI-2\(^16\)\(^,\)\(^17\). Active plasmin can degrade most ECM proteins such as fibronectin, vitronectin and fibrin, a notable exception being native collagen\(^18\). It can also indirectly promote matrix degradation through activation of some but not all pro-metalloproteinases\(^7\). Plasmin probably has functions unregulated in matrix degradation, e.g., activation of proforms of cytokines and growth factors such as pro-TGF-β\(^19\). In addition, activation of proteolysis by plasminogen activator system has been reported in several human malignancies and is believed to contribute to tumor cell mobility and invasion\(^20\)\(^,\)\(^21\).

METALLOPROTEINASES

Metalloproteinases (MMPs) are a family of secreted or
transmembrane proteins that are capable of digesting ECM and basement membrane components under physiological conditions. Based on their structure and substrate specificity, they can be classified into different groups of closely-related members. Collagenases degrade fibrillar collagen; gelatinases are particularly potent in degradation of nonfibrillar and denatured collagen (gelatin), stromelysins prefer proteoglycans and glycoproteins as substrates, membrane-type MMPs (MT-MMPs) and others. They share a catalytic domain with the HEXGH motif responsible for ligating zinc, which is essential for catalytic function. In contrast to soluble MMPs, MT-MMPs possess a transmembrane domain at their COOH terminus, resulting in cell surface localization. MMPs are also characterized by a distinctive PRCGVPD sequence in the prodomain that is responsible for maintaining latency in the zymogens. MMP family members differ from each other in structure by the presence or absence of additional domains that contribute to activities, such as substrate specificity, inhibitor binding, matrix binding and cell-surface localization.

MMPs are generally produced by cells at very low levels. However, when either physiological or pathological circumstances require remodeling of ECM, increased expression levels of the enzymes can be induced. The activity of MMPs is tightly regulated by the action of activators or proenzymes and inhibitors. Transcriptional regulation of MMP genes is mediated by an AP-1 regulatory element in their proximal promote region. MMP gene transcription is induced by a variety of extracellular stimuli, such as cytokines (IL-4 and IL-10), growth factors such as TGF-α, bFGF, and TGF-β and cell-cell or cell-matrix interactions. Binding of these stimulatory ligands to their receptors triggers a cascade of intracellular reactions that are mediated through at least three different classes of mitogen-activated protein kinases: extra-cellular signal-regulated kinase (ERK), stress-activated protein kinase/Jun N-terminal kinases (SAPK/JNks) and p38. Activation of these kinases culminates in the activation of a nuclear AP-1 transcription factor, which binds to the AP-1 cis element and activates the transcription of the corresponding MMP gene. Next to the AP-1 element, other transcription factors such as ETS are involved in the regulation of MMP gene expression during tumor cell invasion. Expression of ETS-1 has been demonstrated in stromal fibroblasts adjacent to invading tumor cells and in endothelial cells during tumor vascularization. In general, the ERK1/2 cascade is activated by mitogenic signals, resulting in phosphorylation of various substrates including Elk-1 and subsequent activation of c-fos transcription. SAPK/JNks and p38 are activated by cytokines (THF, IL-1) and cellular oxidative stress resulting in phosphorylation of c-Jun and ATF-2, which then induce c-jun transcription.

Constitutively active mutants of Raf-1 and MEK1 transform fibroblasts in vitro and in vivo, activation of the ERK1/2 pathway has been observed in renal and breast carcinomas. However, blocking the pathway ERK and ERK 2, by a specific chemical inhibitor PD98059, inhibits the expression of MMP-1 and MMP-2 by SCC cells, and their invasion.

### Table 1 Proteases participating in degradation of ECM components

| Protease family | Protease | Protease function | Protease inhibitors |
|-----------------|----------|------------------|--------------------|
| Aspartyl protease | Cathepsin D | Degradation of ECM components | Conversion of cysteine proteases into cathepsins |
| Cysteine proteases | Cathepsins B, L, H, K | Degradation of ECM components | Conversion of pro-MMPs into MMPs |
| Serine proteases | Plasmin | Degradation of ECM components | Activation of uPA |
| | Urokinase-type plasminogen activator (uPA) | Degradation of ECM components | Conversion of inactive elastase into elastase |
| | Tissue-type plasminogen activator (tPA) | Degradation of ECM components | Conversion of plasminogen into plasmin |
| Neutrophil serine proteases | Elastase | Degradation of ECM components | Conversion of plasminogen into plasmin |
| Matrix metalloproteinases | Cathepsin G | Degradation of ECM components | α2-antiplasmin, α2-macroglobulin |
| Collagenases [MMP-1, 8, 13] | Degradation of collagens, laminin, gelatin, | TIMP-1, 2, 3, 4 |
| Stromelysins [MMP-3, 10] | Activation another pro-MMPs into MMPs |
| Gelatinases [MMP-2, 9] | Degradation of collagens, laminin, gelatin, | α2-macroglobulin |
| Membrane-type [MMP-14, 15, 16, 17, 24, 25] | Degradation of proteoglycans, laminin, fibronectin, gelatin, collagens IV, elastin, tenasin, nidogen |
| Others [MMP-7, 11, 12, 19, 20, 23] | Degradation of proteoglycans, laminin, fibronectin, gelatin, collagens IV, elastin, tenasin, αI -antiproteinase, amelogenin |
MMP expression has been shown in a variety of tumor types including carcinomas of lung, colon, breast and pancreas. In addition, the plasma and urine levels of MMPs are elevated in patients with cancer compared with healthy subjects. MMPs in tumor tissues are produced not only by malignant tumors but also by stromal fibroblasts and inflammatory cells. These cells may produce cytokines and proteins that induce the production of MMPs by surrounding cells, creating extracellular networks of MMP secretion and activation. Furthermore, analyses of cellular components derived from primary tumor tissues or their corresponding lymph node metastases demonstrated that expression of MMPs is increased in the metastatic tissue, indicating that MMP expression is a component of the metastatic process. In addition, there is clinical evidence that over-production of these molecules confers a poor prognosis in patients with a variety of malignancies.

At the protein level, biological activity of MMPs is determined by the action of activators of proenzymes and inhibitors. In general, MMPs are secreted in latent forms (as proenzymes) and require specific proteolytic activation in the extracellular matrix by furin-like proteases. This activation step serves as a regulatory element. MMP activity is further regulated by MMP inhibitors (TIMPs) and expression of molecules, which present the active enzyme on the cell surface. However, most MMPs are converted into active forms in a specific multistep activation process that is known as the “cytotoxic switch”. A conserved unpaired cysteine residue in the prodomain forms a coordinate bond with zinc ion at the active site. Cleavage of the prodomain results in opening of the active site by disruption of the zinc—cytotoxic bond and is followed by loss of the amino-terminal prodomain.

For most MMPs, proteolytic activation is initiated in the extracellular space by serine proteases such as plasmin and urokinase plasminogen activator, neutrophil elastase or by other members of the MMP family members, especially MMP-2 and MMP-13 by MT1-MMP cleavage of the proenzymes. Activation of MMPs can also be induced by non-proteolytic compounds as thiol compounds including oxidized glutathione and hypochlorous acid. Therefore, MT-MMP expression is another regulating factor for MMP activation. Little is known about the activation induced by other factors including other MMPs, cytokines, trypsin, serine proteases, reactive oxygen species, hyposia, c-erbB ligands, and leukocyte elastase. Some activated MMPs can further activate other proMMPs. MMPs containing a furin-like recognition domain (RXKR) in their propeptides are activated intracellularly in the trans-Golgi network by a group of calcium-dependent transmembrane serine proteases (furin/PACE/kex-2). Proteolytically active MMPs may be localized on the cell surface by binding to membrane molecules, which leads to a more direct ECM degradation. Furthermore, functionally active MMP-2 can also bind to integrin α,β, while proteolytically active MMP-9 can associate with CD44.

Active MMPs are inhibited by various protease inhibitors such as non-specific protease inhibitors (α1-antiprotease and α2-macroglobulin) and a family of specific tissue inhibitors, TIMPs. There are currently four members of the TIMP family (TIMP-1, -2, -3, and -4) that have common inhibitory effect on MMPs. TIMPs share several structural similarities between each other such as the N-terminal MMP binding site, 12 cysteine residues within the conserved domain which form disulfide bonds. TIMPs are found to be involved in connective tissue turnover and remodeling. They bind to MMPs by forming non-covalent complexes, resulting in MMP inhibition. TIMPs bind to either proMMPs or active MMPs, thereby inhibiting the autolytic capacity of active proteases. TIMP-1 and TIMP-2 regulate activation of proMMP-9 and proMMP-2, respectively. TIMP-1 inhibits the activity of most MMPs, with the exception of MT1-MMP and MMP-2. TIMP-2 also inhibits the activity of most MMPs, except for MMP-9. TIMP-3 inhibits the activity of MMP-1, -2, -3, -9 and -13 and human TIMP-4 inhibit the activity of MMP-2, -9, and TIMP-5.

The role of MMP family in tumor development is further complicated by the balance of these proteins in relation to TIMPs. TIMPs have various antioncogenic functions and the expression of TIMPs has been associated with less aggressive tumor behavior and favorable prognosis in patients with cancer. For example, the exposure of mouse fibroblasts to TIMP-1 and TIMP-2 could inhibit oncocytic transformation by oncogenic viruses, whereas administration of recombinant TIMP-1 to mice injected with B16F10 melanoma cells could reduce the number of pulmonary metastases. TIMPs bind to either α1-antiprotease, or a family of specific tissue inhibitors, TIMPs. There are currently four members of the TIMP family (TIMP-1, -2, -3, and -4) that have common inhibitory effect on MMPs. TIMPs share several structural similarities between each other such as the N-terminal MMP binding site, 12 cysteine residues within the conserved domain which form disulfide bonds. TIMPs are found to be involved in connective tissue turnover and remodeling. They bind to MMPs by forming non-covalent complexes, resulting in MMP inhibition. TIMPs bind to either proMMPs or active MMPs, thereby inhibiting the autolytic capacity of active proteases. TIMP-1 and TIMP-2 regulate activation of proMMP-9 and proMMP-2, respectively. TIMP-1 inhibits the activity of most MMPs, with the exception of MT1-MMP and MMP-2. TIMP-2 also inhibits the activity of most MMPs, except for MMP-9. TIMP-3 inhibits the activity of MMP-1, -2, -3, -9 and -13 and human TIMP-4 inhibit the activity of MMP-2, -9, and TIMP-5. It is clear that balance between active MMPs and TIMPs appears to be critical for MMP activity and for ECM degradation.

Apart from the above proteolytic systems participating in enhanced degradation of interstitial matrix observed during cancer development, neutrophil proteases may also take part in this process. It has been shown that in particular chronic inflammation neutrophils may create an environment that supports tumor promotion. In such a situation proteases of accumulated neutrophils may be significant factors for ECM degradation. Neutrophil granules contain a large family of over 20 enzymes such as metalloproteinases, collagenase, gelatinase and elastase and cathepsin G, but has the greatest potential to act as a mediator of tissue destruction. However, the ECM is protected from degradation by the major plasma protease inhibitors and there is a critical balance between the enzymes and their inhibitors.
secretory leukoprotease inhibitors are the main plasma protease inhibitors that effectively regulate extracellular neutrophil elastase and cathepsin G. However, proteolytic-antiproteolytic balance directly depends on reactive oxygen species (ROS) generated by activated neutrophils. It is known that inflammatory process and different carcinogens stimulate phagocytic cells including neutrophils, monocytes and macrophages, which undergo a respiratory burst to generate both superoxide anion and hydrogen peroxide. The source of superoxide generation in these cells is a membrane-bound NADPH oxidase that remains dormant until activated by a complex cascade of signal transduction. The combined activities of NADPH oxidase and myeloperoxidase in phagocytes leads to the production of hypochlorous acid, one of the strongest physiological oxidants. In addition, it has been documented that the generation of superoxide anion, hydroxyl radical, hydrogen peroxide, singlet oxygen and hypochlorous acid is enhanced in cancer cells. Moreover, many exogenous chemicals can be activated to radical intermediates, which can serve as electrophiles or participate in redox cycling processes of reactive oxygen generation. In addition, both exogenous and endogenous agents can interact with various cellular receptors, such as the Ah receptor and protein kinase C leading to increased oxidation in cells. In such a case ROS may disturb cellular metabolism and directly or indirectly influence proteolytic-antiproteolytic balance of cancer tissue.

It is known that ROS may indirectly act as a second messenger. Some cytokines (TNF, IL-1) and growth factors (bFGF, VEGF) are capable of producing ROS in target cells and the produced ROS further participate in mediating the effects of cytokines and growth factors. ROS may also serve as common signaling molecules regulating the activity of transcription factors, NF-κB and AP-1, in response to cytokines and other stimuli. Oxidants can also stimulate transcription of other transcription factor genes such as c-jun, c-fos and c-myc in various cell types. The regulation of gene expression by these factors will ultimately lead to a series of cellular changes such as proliferation, growth suppression, differentiation, senescence and apoptosis. These transcription factors are involved either directly in the induction of the expression of MMP genes (by binding to enhancer regions in these genes) or indirectly in modulating the expression of proteins which ultimately affects proteinase release. ROS may also regulate cellular components other than transcription factors.

The most important factor for proteolytic-antiproteolytic balance is that ROS influences protease activation and activity and distribution of enzymes and their inhibitors. It has been shown that ROS can activate procoagulase. However, changes in activity and distribution of enzymes and their inhibitors are connected with direct oxidative modification of cell components.

**Effects of ROS on cell components**

Reactive oxygen species as chemically reactive molecules can modify most cell components such as lipids, nucleic acids, carbohydrates and proteins. The residues of amino acids occurring in proteins are less vulnerable to action of ROS than that of free amino acids. Most amino acids present in proteins undergo oxidative transformation to radicals, but aromatic and sulfhydryl groups containing amino acids are the most susceptible. Susceptibility of amino acid residues to ROS also depends on their position in the polypeptide chain. Unstable amino acid radicals are formed at the first stage of ROS action and then undergo transformation yielding stable products. Other amino acid residues of proteins can also undergo oxidative transformation under formation of peroxides that are mainly generated by the aliphatic amino acid radicals. Alteration in the primary structure of proteins brings about changes in the secondary and tertiary structures and results in denaturation, aggregation or fragmentation of protein molecules (Figure 2). The protein structure modification mainly depends on the kind and concentration of acting ROS.

In addition, ROS can react with membrane phospholipids forming hydroperoxides and reactive small-molecule aldehydes with a longer lifetime. Thus, these aldehydes can be considered as the secondary lipid peroxidation transmitters.

A marked increase of malondialdehyde and 4-hydroxynonenal concentrations has been found in cancer cells. It is proved that 4-hydroxynonenal can react with sulfhydryl groups of cysteine present in polypeptide chains and with lysine, histidine, as well as other amino acid residues of proteins. In this way, covalent lipid-protein bond residues are formed. Lipid radicals formed by peroxidation can also react with each other forming lipid-lipid covalent bonds. These reactions provoke changes in membrane structure, uncovering phosphatidylycerine on its surface and modify membrane permeability.

An important factor preventing lipids from peroxidation is α-tocopherol, which reduces polyunsaturated fatty acid radicals and acts as a chain reaction terminator. In addition, physicochemical action of α-tocopherol on biological membranes has a stabilizing effect and protects them from liberation and activation of endogenic phospholipases, which accelerate lipid peroxidation by hydrolyzing the lipid membrane PUFA. Free fatty acids destabilize the membranes and undergo oxidation more readily than their esterified forms present in the membrane.
α-Tocopherol can form more stable protective complexes with free PUFA. Stability of such complexes increases with the number of unsaturated bonds of PUFA. α-Tocopherol stabilizes PUFA-rich membranes by decreasing their permeability to small ions and small molecules. However, decreased α-tocopherol content in cancer tissues makes the biological membrane protection against destructive ROS action impossible.

The non-enzymatic mechanisms protecting cells against lipid peroxidation are completed by two GSH-dependent enzymatic systems. α-Tocopherol is involved in the first system, in which a GSH-dependent α-tocopherol radical reduction to α-tocopherol takes place. The second system probably consists of glutathione transferase coupled with glutathione peroxidase and reduces toxic lipid hydroperoxides to less reactive alcohols. Antioxidant action of these mechanisms during cancer development may be questionable because of different data about GSH content in cancer cells.

A consequence of the above-discussed changes is increased fluidity of cell and lysosomal membranes. This observation is supported by transfer of lysosome enzymes to cytosol. Action of ROS on cell organelle membrane components can provoke changes in function of Na⁺/H⁺ exchangers possibly resulting in acidosis of the cell content, which favors the action of lysosomal proteases. These processes can bring about activation of proteolytic processes in cytosol.

The literature data concerning other experimental models confirm that ROS reactions with proteins and lipids of lysosome membranes are possible and indicate that oxidation reactions catalyzed by iron ion are probable in lysosomes. Lysosomes are particularly vulnerable to oxidation stress because they contain an active oxidation mediator, i.e., the iron ion. The iron ion is present in lysosomes due to endocytosis of various metalloproteins like ferritin and cytochrome and their degradation in organelles. Hydrogen peroxide is formed as a result of oxidation and activation of inflammatory enzymes. If not decomposed by catalase or peroxidase, it diffuses through the membranes to the lysosomes and takes part in intralysosome reactions catalyzed by iron ion. Hydroxyl radical is formed in such reactions and chain oxidation processes are initiated, resulting in increased permeability of the lysosome membrane or even fragmentation and liberation of the lysosome content to cytosol including proteolytic enzymes.

Oxidative modification of biologically active proteins results in changes in their activity. In most cases, it results in their inactivation, particularly if the oxidative modification occurs with amino acid residues, which are essential for a specific biological activity. It was documented that cysteine proteases, in particular, are susceptible to oxidative modification. On the other hand, participation of ROS is needed for some proteins to fulfill their biological function. It particularly applies to metalloproteases, which are also activated by ROS.

**Perturbation of proteolytic-antiproteolytic balance by ROS**

Physicochemical and biological properties of proteins may be changed during cancer development. ROS generated, during this process, can directly react with proteins including enzymatic proteins and provoke their modification, which can result in activity changes of enzymatic proteins including proteases and their inhibitors.

In physiological conditions, equilibrium between proteases and their inhibitors exists in the organism. Cancer development is followed by a temporary decrease in activity of proteolytic enzymes in cancer cells with simultaneous increase in activity of proteases and decrease in activity of inhibitors in blood serum. The changes mainly occur both in the enzymes containing seryl groups in their active center (cathepsin G and elastase) and in the serine protease inhibitors like α₁-macroglobulin and α₁-antiprotease. As the changes are temporary, it can be supposed that post-translational protein modification only occurs and its biosynthesis remains unchanged while the activity of de novo synthesized enzymes is unmodified. The modifications induced by ROS are involved in direct chemical reactions with proteases and their inhibitors. Inactivation of biologically active proteins can also be caused by their reaction with lipid peroxidation products. In the case of cathepsin B, it is the effects of thiol-ether type adduct formation by sulfhydryl groups of the active center of the enzyme and 4-hydroxynonenal. Modifications of the proteolytic enzyme structure and their inhibitors, caused by ROS, result in a shift of proteolytic-antiproteolytic equilibrium. However, the equilibrium perturbation enhances formation of ROS. It is considered that activation of ROS-producing granulocytes and macrophages is controlled by serine protease inhibitors, decrease activity of which is observed in cancer cases. Active NAD(P)H oxidase synthesized as proenzyme is necessary for ROS formation in granulocytes. The activation is induced by serine protease. However, activity of this protease group is changed in the case of cancer. In such a case, production of inhibitors of active NAD(P)H oxidase is inhibited by these enzymes and ROS production is indirectly controlled by them. Formation of superoxide anions by granulocytes is inhibited by α₁-proteinase inhibitor and α₁-antichymotrypsin. Activity of granulocyte elastase is inhibited by hydrogen peroxide. It is suggested that another mechanism may underlie the control of oxidative activation of granulocytes by inhibitors.

**Figure 3** Effect of reactive oxygen species on proteolytic–antiproteolytic balance.
It was documented that the activity of main plasma protease inhibitors such as $\alpha_1$-antiprotease and $\alpha_2$-macroglobulin are decreased in carcinogenesis\textsuperscript{219,241-243}. These inhibitors as well as plasminogen activation inhibitor (PAI-1) and leukocyte protease inhibitor (SLPI) contain methionine in their inhibition center and are most susceptible to inactivation by oxidants\textsuperscript{244,246}. Unlike them, the inhibitors containing residues of other amino acids in their inhibition center (antithrombin III, PAI-2, PAI-3) are relatively unsusceptible to oxidative inactivation\textsuperscript{246,249}. $\alpha_1$-antiprotease and PAI-1 are particularly susceptible to the action of oxidants. The $\alpha_1$-antiprotease molecule contains eight methionyl residues\textsuperscript{252}. The methionine molecule occupying the position 358 is situated in the inhibition center\textsuperscript{253}. The action of oxidants causes oxidative modification of two methionyl residues out of eight including residues of the inhibition center\textsuperscript{256,257}. Oxidants acting on the $\alpha_2$-antiprotease molecule neither modify the radicals of other amino acids nor provoke molecule fragmentation\textsuperscript{254}. The oxidant that probably inactivates $\alpha_1$-antiprotease in \textit{in vivo} conditions is chlorate (I) (ClO\textsuperscript{-}) formed in myeloperoxidase-H\textsubscript{2}O\textsubscript{2}-Cl\textsuperscript{-}\textsuperscript{259}. Hydrogen peroxide generated in cancer cells can also oxidize methionine of the $\alpha_1$-antiprotease molecule.

$\alpha_1$-antiprotease being a polyvalent inhibitor also inhibits the activity of leukocyte cathepsin G and elastase forming inactive and stable complexes of 1:1 stoechiometry\textsuperscript{253,256}. In this way, it prevents possible damage to tissues caused by those proteases\textsuperscript{259}. Activity of these enzymes is manifested if the mentioned inhibitor is inactivated\textsuperscript{257}. Cathepsin G and elastase exhibit substrate specificity and can degrade elastin, collagen, proteoglycans, as well as, the complement immunoglobulin, fibrinogen, basic proteins, and other proteins. Cathepsin G is particularly aggressive in its action on proteins as it degrades them not only directly but also by activating procollagenases\textsuperscript{258}.

It has been suggested that increased activity of proteases in blood serum in cases of cancer, particularly the activity of cathepsin G and elastase, is due to increased transport from cells and reduced inactivation of inhibitors whose activity is decreased\textsuperscript{284}. Existing protease-inhibitor interdependence can also suggest inactivation of inhibitors by proteases. $\alpha_1$-antiprotease is known to be inactivated by sulphydryl proteases and metalloproteinases that are also activated by ROS\textsuperscript{259,260}. Such an action is evidenced for granulocyte collagenase\textsuperscript{259,260}, endothelium vessel stromelysin and collagenase\textsuperscript{259,261}. These proteases split the peptide bond of Phe\textsubscript{162} with Leu\textsubscript{163}, which are present in the $\alpha_2$-antiprotease loop exposed to the exterior molecule in which the inhibition center is present. Split-out $\alpha_1$-antiprotease peptide fragments are found in synovial fluid of patients with rheumatic arthritis\textsuperscript{262}, indicating that $\alpha_1$-antiprotease can be inactivated both by oxidative modification and by proteolytic degradation\textsuperscript{260,261}. Such a situation can occur particularly in the case of increased activity of proteases and has been observed in cancer development.

Changes in the proteolytic-antiproteolytic system lead to the shift of equilibrium in favor of proteases. Such a situation can lead to destruction processes because components of proteolytic-antiproteolytic system penetrate from blood to extracellular space\textsuperscript{264} and an uncontrolled proteolysis can occur if activity of protease inhibitors is lower and activity of proteases is higher as it occurs in cancer development. It should be noted that proteolysis of ECM proteins may also be enhanced by oxidative modification of protein substrates and their increased susceptibility to protease action.

\section*{FINAL REMARKS}

In conclusion, oxidative stress observed in tumor development may cause imbalance of proteolytic-antiproteolytic system leading to enhanced proteolysis and destruction of ECM proteins and metastasis. The general principles of the mechanisms underlying proteolytic degradation of the ECM in cancer are becoming clear and the complexity of this process is revealed. Matrix degradation appears to be important for both cancer invasion and metastasis. It is accomplished by a cooperative interaction between several proteases. Effective protease action depends on many agents including inhibitors. The ability of TIMPs to inhibit tumor growth suggests that MMPs are more important for cancer development than normal physiological activities in adult host\textsuperscript{257,265}. Thus, development of more specific protease inhibitors may represent tractable chemotherapeutics for human cancer. As a result, many pharmaceutical companies have designed novel drugs that variably block MMP activity. Gene delivery of TIMPs into malignant cells may also be a potent way of inhibiting the tumor invasion and in consequence prolonging survival\textsuperscript{266}. Furthermore, an effective way of inhibiting the expression of MMPs may be blocking of signaling pathways mediating activation of MMP transcription.

Proteolytic-antiproteolytic balance also depends on the influence of other endogenous and exogenous compounds e.g., ROS, which is why the view on anti-cancer therapy pays attention to the possibilities connected with this fact. It has been mentioned above that cancer development accompanies enhanced generation of ROS, which can react with cell components and additionally influence proteolytic-antiproteolytic balance in neoplastic and surrounding cells. In this connection, approaches toward therapeutic intervention against ROS damage include administration of radical scavenger compounds, use of novel drugs that increase cellular production of constituent antioxidants or pharmacological agents that modify the intracellular transport of antioxidants. Strategies to modify the cellular effects of ROS on cancer lead to novel approaches of gene therapy in which the antioxidant proteins can be expressed in specific tissues. Reducing tissue-damaging effects of ROS may have a relevance to cancer patients by ameliorating normal tissue damage\textsuperscript{257}.

The opposite point of view will be, prolonged accumulation of ROS is beneficial to overcoming neoplastic changes because they can kill cancer cells. Thus, inhibition of superoxide dismutase responsible for ROS elimination may provide a novel way in cancer therapy. Cancer cells are more dependent on superoxide dismutase to eliminate the toxic superoxide radicals and become more sensitive to superoxide dismutase inhibitors. In such case it is possible that inhibition of superoxide dismutase may preferentially...
kill malignant cells through a ROS-mediated mechanism.

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