Review Articles

Proteolytic Enzymes as Biological Markers for Tumor Diseases: Review

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Nowadays, about one thousand proteolytic enzymes are isolated and purified from different organisms. According to the catalytic mechanism, the structure of the active center and their tertiary structure, proteases are divided into four main groups – aspartic peptidases, metallopeptidases, cysteine and serine peptidases. Most of them not only perform various physiological functions but are also involved in pathogenic mechanisms of different diseases. The aim of the present mini-review is to outline the main groups of proteases and individual enzymes typically used as biomarkers for tumor diseases as well as to designate certain enzymes that are promising for future application in oncology.

Key words: proteolytic enzymes, biological markers, tumor diseases.

General

In principle, an enzyme can be nominated as biomarker for a disease in case its expression and/or activity levels are different (higher or lower) in pathologically altered tissues in comparison to those in healthy tissues. In such cases, the enzyme levels might be used for diagnostic and/or prognostic purposes. On the other hand, an enzyme can be defined as a target for therapy, if it has been proven beyond any doubt (in experimental model systems) that restoration of its normal levels can slow down or even reverse the pathological process, i.e. it can improve the patient's condition. Therapeutic agents can be:

– At higher enzyme levels – specific enzyme inhibitors or antibodies, small interfering RNA, or other suppressive agents;

– At lower enzyme levels – specific activators, the enzyme itself or its encoding DNA.

The use of proteases as biological markers has long been controversial. The main objection has been related to the low specificity of those enzymes. For example, if a peptidase hydrolyzes a single amino acid (AA) from N- or C-terminal of peptides, usu-

ally it is active to several AAs, e.g. Ala, Met and Cys. If a peptidase hydrolyzes two AAs, one can compile a whole library of potential enzyme substrates in connection with the numerous combinations of 2 AAs. These facts inevitably lead to the assumption that proteases can be interchangeable - if one of them is inactive for some reasons, others can assume its functions. However, over the years a lot of data accumulate that the above assumption is not quite true. Thus, a genetic deficiency of certain proteases leads to the development of serious genetic diseases. Additionally, suppression or over-expression of a number of these enzymes invariably accompanies certain pathological processes and is actually an integral part of them.

Studies on the marker role of enzymes in tumor diseases usually start *in vitro* via comparing their expression/activity levels in tumor cell lines and normal cell lines/primary cell cultures of the same origin. Finding a difference in the enzyme levels suggests a potential marker role, particularly if the altered expression/activity level can be directly correlated with the pathological phenotype of the cells. To verify this, re-expression or inhibition of the enzyme and tracking the effect on the cells are performed. Studies *in vivo* require the use of experimental animals. Intravenous injection leads to a transport of tumor cells with blood and formation of secondary tumors initially in the lung and then in other organs. Subcutaneous injections of tumor cells or subcutaneous xenografts directly transfer the tumor mass in the animals. Determination of the enzyme as a marker requires a number of confirmatory experiments to refine its diagnostic/prognostic significance. Identification of the enzyme as a target for therapy is associated with further complex tests, including clinical trials.

Matrix metallopeptidases (MMPs)

Matrix metallopeptidases are zinc-dependent enzymes. Taken together, they can degrade almost all the components of the extracellular matrix (ECM) – structural proteins, soluble protein components and proteins of the cell surface. Controlled expression of MMPs is connected with a number of physiological processes requiring changes in ECM like fetal development, mammary glands involution after weaning, ovulation, etc. However, abnormal regulation of MMPs leads to different kinds of pathological processes including cancer. Together with two more families of metal-dependent proteases – ADAMs (a disintegrin and metalloproteinase) and ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs), they participate in the complete formation of the microenvironment in which tumor cells develop [10].

So far, 28 MMPs have been described. Depending on their substrate specificity, they are divided into 4 types: 1. Interstitial collagenases (e.g. MMPs-1, -8 and -13); 2. Gelatinases (e.g. MMPs-2 and -9); 3. Stromelysins (e.g. MMPs-3, -7 and -10); 4. Membrane-type MMPs (e.g. MT-MMP-4). The role of individual MMPs is different in different types of tumors [reviewed in 15]. Additionally, some of them are tumor-promoters at certain stages of the oncological disease but tumor-suppressors at other stages [22]. MMPs participate in practically all processes related to the tumorigenesis and tumor progression.

Effects on the cell growth. Obviously, uncontrolled growth and division is a common feature of tumor cells. MMPs modify both the availability and functioning of growth factors in the cell microenvironment thus changing the balance between signaling molecules which control the cell growth. This may result in an appearance and distribution of tumor cells. For example, the transforming growth factor β (TGF-β) is important for maintaining tissue homeostasis and normally is considered a tumor-suppressor. However, during the tumor growth frequently mutations lead to changes in TGF-β receptor and tumor cells become insensitive to TGF-β. Accumulation of TGF-β

molecules in ECM whereas the tumor cells are insensitive to the factor is associated with an induction of tumor growth and metastasis [23]. MMP-9 is the main enzyme involved in the activation of TGF-β by hydrolyzing its inactive form. On the other hand, MMPs-2 and -14 hydrolyze TGF-β binding protein thus releasing additional active TGF-β in the ECM [6]. In this connection, the high levels of MMP-9 are accepted as a marker of poor prognosis.

Inhibition of apoptosis. Escaping apoptosis is another strategy for accumulation of cancer cells and tumor growth. MMPs accelerate the tumor spreading by blocking the receptor-dependent apoptosis. For example, MMP-7 participates in the formation of neoplastic lesions in the pancreas. This role of the enzyme has been supposed first by using immunohistochemistry [5]. The experiments have shown that the enzyme is not expressed in healthy pancreas. However, during the process of metaplasia from acinar to ductal cells, the metaplastic cells already express it and metaplastic ducts are highly positive for MMP-7. Later studies [9] have revealed the mechanism of MPP-7 involvement in pancreatic adenocarcinoma. The ligand of the Fas-receptor (FasL) which transfers pro-apoptotic signal, is a substrate of MMP-7. Through the hydrolysis of FasL by MMP-7, a soluble form of FasL is obtained, which is a powerful pro-apoptotic agent. On the other hand, by a yet unknown mechanism, MMP-7 induces the expression of anti-apoptotic proteins of Bcl2 family. Thus, the cells receive mixed signals leading to a selection of cells resistant to apoptosis. Those cells are involved in the formation of pancreatic intraepithelial neoplasia. This mechanism is activated in chronic pancreatitis which increases the probability of ductal adenocarcinoma. That is why overexpression of MMP-7 is regarded as a marker for pancreatic adenocarcinoma and resistance to chemotherapy [9].

Tumor vascularization. MMPs participate in angiogenesis by overcoming the physical barrier (degrading of ECM) and by generating pro-angiogenic factors. For example, the vascular endothelial growth factor (VEGF) is bound to ECM molecules and the free VEGF is generated through MMP-9 mediated degradation of collagen [3]. Active MMP-9 is secreted from neutrophils infiltrated in the tumor mass [24]. That is why the high number of infiltrated neutrophils is considered an indicator of intensive angiogenesis. Thus, a prolong treatment of experimental animals with an antibody selectively removing neutrophils from the blood leads to triple reduction of angiogenic lesions [24]. MMP-9 is identified as a target for therapy and it has been supposed to use its selective inhibitors along with radiotherapy in order to increase the positive effects of this treatment.

Regulation of adipocytes. Adipocytes are a part of the tumor stroma and contribute to the tumor progression by secreting products called adipokines. Certain adipokines control the expression and activity of MMPs. In turn, several MMPs regulate the growth and differentiation of adipocytes [30]. One of the most important MMPs influencing adipocytes is the so called stromelysine 3 (ST3). Mice that are deficient in the enzyme (knock-out mice) show a very quick differentiation of embryonal fibroblasts into adipocytes and greater weight and vice versa – treatment of those mice with recombinant ST3 results in a quick degradation of fats and regulation of body weight [1]. So, ST3 decreases pre-adipocytes differentiation and even reverses mature adipocytes into fibroblast-like cells. Accumulation of such cells modifies the tumor stroma so that it can meet the needs of the tumor cells. Therefore, the overexpression of ST3 in the tumor stroma is considered a marker of poor prognosis. The high ST3 gene expression is used to distinguish invasive ductal carcinoma of mammary gland from non-invasive ones.

Initiation of neoplastic progression (epithelial–mesenchymal transition - EMT). The initial process of tumor invasion is accompanied with a loss of cell-cell adhesion and cell polarity of epithelial cells and acquiring of mesenchymal phenotype, i.e. EMT.

All the MMPs able to degrade E-cadherin and β-catenin and/or activate TGF-β, participate in EMT. Especially important for this process is stromelysin 1 (ST1). Induced expression of ST1 in a primary culture of normal cells from mammary gland leads to a degradation of E-cadherin and β-catenin, detachment of the cells, loss of differentiation and increase of the cell migration, i.e. the cells acquire a tumor phenotype [20]. MMP-7 is also involved in EMT especially in the pancreas, uterus, mammary gland and prostate. Its enhanced expression in glandular epithelial cells results in an initiation of tumor progression. This enzyme is a subject of a great scientific interest due to the increased number of cases of adenocarcinomas. The serum levels of MMP-7 have a diagnostic value in pancreatic ductal carcinoma [9].

Tumor invasion and metastasis. Secondary tumors or metastases are the main cause for fatal outcomes in cancer. Invasion and metastases require that the cells overcome many physical barriers with the help of MMPs. Interstitial collagenases are the MMPs mostly involved in these processes. That is why they are the main marker enzymes for tumor invasion and the central targets for therapy. For example, MMP-1 is usually expressed in the invasive front of many types of advanced tumors [31]. It is expresses both by tumor and stromal cells. The appearance of this enzyme in the border parts of the tumor is connected with the most aggressive and metastatic phenotype.

Another interstitial collagenase that plays important role in the tumor invasion is MMP-13 [reviewed in 28], which is expressed in the invasive front of squamous cell carcinomas of the mammary gland, head and neck. Actually, the enzyme is present only in malignant lesions but not in non-malignant. In that respect, it is useful as a prognostic marker for the above types of tumors.

One more MMP directly involved in the tumor growth and metastasis is the membrane-type 1 MMP (MT1-MMP). It is usually expressed in the invadopodia and provides the cells with a great potential for migration by cleaving the ECM components and activating other MMPs [reviewed in 36]. The increased expression of MT1-MMP at the border membranes between the tumor and stroma is considered a poor prognostic marker for squamous cell carcinomas, some glyomas and neuroblastomas.

Kallikrein-related peptidases (KLKs)

Kallikrein-related peptidases or kalikreins are serine-type soluble proteases which, like MMPs. are directly involved in tumorigenesis and tumor progression. Till now, 15 members of KLK-family are identified, most of which are already used as diagnostic/prognostic markers of tumor diseases. Normally, KLKs participate in a number of physiological processes like regulation of the blood pressure, contractions of the smooth muscles, neutrophis' chemotaxis, etc. KLK2, 3, 5 and 11 are expressed in the prostate and seminal plasma, where they take part in the liquefaction of the sperm. Certain KLKs support the normal skin physiology, activate peptide hormones and growth factors and participate in myelination and demyelination in the central nervous system [25].

Altered expression of KLKs has been detected in many types of adenocarcinomas [8]. For example, the expression of KLK3 in the prostate is applied for differentiation between malignant and non-malignant lesions [4]. Also, KLK3 takes part in the attachment of metastatic cells to the bones thus favoring the development of bone metastases [29]. Actually, in most cases KLKs act as proteolytic cascades (PCs) both in healthy and pathologically altered tissues. PCs are highly organized sequences of proteolytic enzymes, which activate each other and operate coordinately to transfer a specific signal. PCs are activated when a well-directed and secure (from physiological point of view) signal transfer to the target cells is needed. The best studied PCs are those of the blood

coagulation, caspases at apoptosis and PCs of the innate and acquired immunity. Kallikreins` PC of the liquefaction of the sperm before ejaculation as well as the one of the physiological skin peeling, are also well known. Amongst the pathological kallikreins` PCs the best known is the one in prostate cancer. It results in increased angiogenesis, suppression of the immune reactions and acceleration of the tumor growth. Pathological PCs are used for the development of more accurate multifactorial methods for diagnosis and prognosis of cancer. Such a method has been introduced for diagnosis of prostate cancer, based on kallikreins' PC [8].

Aminopeptidase N (APN; EC 3.4.11.2)

In the group of aminopeptidases – enzymes which hydrolyze single AA from the N-terminal of peptides, most studies are directed to the marker role of aminopeptidase N, also known as CD13. APN is a membrane-bound, Zn-dependent peptidase hydrolyzing neutral AAs. The enzyme is normally expressed in many tissues and organs but in the development of tumor diseases its expression increases, which allows to be used as a biological marker for diagnostic purposes.

About 20 years ago, it has been found that phages expressing the motif Asp-Gly-Arg (NGR) selectively bind to the endothelium of growing capillaries and the receptor of this motif is APN [26, 27]. The high expression of APN in tumor capillaries can be applied for *in vivo* monitoring of the tumor growth and angiogenesis by using non-invasive techniques like e.g. fluorescence-mediated tomography. APN is used as a diagnostic marker for carcinomas of the mammary gland, colon, pancreas, thyroid gland and non-small cell carcinoma of the lung [reviewed in 35]. More than that, several anti-cancer strategies are developed, based on the overexpression of APN in tumors [reviewed in 35], the most promising of which is the delivery of medications directly into the tumor by means of NGR-peptide and more particularly by its cyclic analogue with two cysteine residues at both ends – CNGRC. This peptide can be used for precise delivery of TNFα, which potentiates the effect of chemotherapeutic agents. Attempts have been made for a directed delivery of chemotherapeutics, toxic medicines and small interfering RNAs. Those methods are still at experimental stage but they are promising for the development of more effective therapies.

Fibroblast activation protein alfa (FAP-α; EC 3.4.21.B28)

FAP-α is a membrane-associated serine-type protease belonging to the family of post-proline cleaving proteases. It is also called F19-antigene after the name of the monoclonal antibody by which the enzyme has been discovered. Another trivial name of FAP-α is Seprase (from **s**urface **e**xpressed **pr**ot**ease**). Its soluble form entering the blood after shedding from cell surface is known as **A**nti**P**lasmin **C**leaving **E**nzyme (APCE). FAP-α is usually absent from normal adult tissues. In healthy adults it can be find only in single reactive fibroblasts, glucagon producing A-cells in the pancreas and individual endometrial cells [reviewed in 37]. On the other hand, lots of data show elevated expression in the fibroblasts of the tumor stroma as well as in tumor cells in several types of cancer. In fact, more than 90% of carcinomas and many types of sarcomas are FAP-α-positive [reviewed in 12 and 14]. Thus, the enzyme should be very convenient for diagnostic purposes. However, in some types of tumors it could be a tumor-promoter [19] and in others - a tumor suppressor [33, 34]. For example, in ductal mammary gland carcinomas and in some melanomas its expression is considered a good prognostic marker. Oppositely, in

ovarian carcinomas the high enzyme levels are regarded as a marker for poor prognosis. Similarly, in colorectal carcinoma the enzyme occurrence is a sign for the existence of metastases in the lymph nodes. The truth is that although many scientific groups work on the application of FAP-α as a biological marker in oncology, very little is still known about the mechanisms of the enzyme participation in tumor progression. All the same, FAP-α is very important for the development of novel therapies. For example, an experimental anti-tumor vaccine, based on FAP-α has been already proposed [21].

Dipeptidyl peptidase IV (DPPIV;EC 3.4.14.5)

DPPIV is a membrane-associated serine peptidase, cleaving off dipeptides from the N-terminal of peptides and small proteins. Together with FAP-α it is a member of the family of post-proline cleaving enzymes. DPPIV is considered a potential marker for thyroid carcinoma [16]. It is believed that DPPIV possesses a tumor-suppressor function in non-small cell lung carcinoma, prostate cancers, some melanomas, etc. That is why in those types of cancers the enzyme has a very low to lacking expression [2, 33]. For example, studies on DPPIV activity levels in tumor lung cells in comparison with normal lung-derived cells reveal that in normal cells it is about ten times more active than in tumor cell lines [34]. Additionally, our own results show a lack of activity in tumor cells in cryo-sections of lung squamous carcinoma and high activity in the surrounding stroma [7]. The re-expression of DPPIV in tumor cells by the use of a plasmid vector leads to a number of changes, showing a conversion of the cells to a normal phenotype – decrease of the cell growth, increase of the adhesion and loss of migration ability, re-expression of CD44 and FAP-α, which are known to inhibit the tumor growth and dissemination in lung carcinoma [34]. Obviously, the decreased DPPIV expression in lung carcinoma is a part of the tumor phenotype of the cells and vice versa - restoration of the enzyme activity can lead to a suppression of the tumor growth. The above results are promising for the development of novel anti-cancer therapies.

Tripeptidyl peptidase I (TPPI; EC 3.4.14.9)

TPPI is a lysosomal serine protease hydrolyzing a large number of tripeptides from the N-terminal of polypeptides and unfolded proteins. The enzyme is essential for the neuronal function - its genetically determined deficiency leads to the hereditary neurodegenerative disorder classical late-infantile neuronal ceroid lipofuscinose (LINCL), connected with severe symptoms and early death of affected individuals [32]. On the other hand, TPPI is widely distributed in many peripheral organs and tissues such as the spleen, kidney, liver, pancreas, lungs, male and female reproductive organs [18]. Therein, it is supposed to play a role not only in physiological but also in pathological processes like tumorigenesis and tumor growth. The mechanisms of the enzyme involvement in tumors are not elucidated yet. Nevertheless, TPPI has been proposed as a marker for breast carcinomas, since its activity during the tumor development is about 20 times higher than that in the normal tissue [13]. Increased TPPI levels has also been found in other types of cancer like carcinomas of the lower esophagus, colorectal carcinomas, thyroid adenocarcinoma, liver cancer, meningioma, mesothelioma, etc. [17]. Using enzyme histochemical method in a rare malignant epithelial neoplasm, i.e. pancreatic acinar cell carcinoma, we found an increased TPPI activity in tumor acinar cells with varying grades of differentiation (**Fig. 1B**), whereas in healthy pancreas the enzyme is absent in the pancreatic acini (**Fig. 1A**).
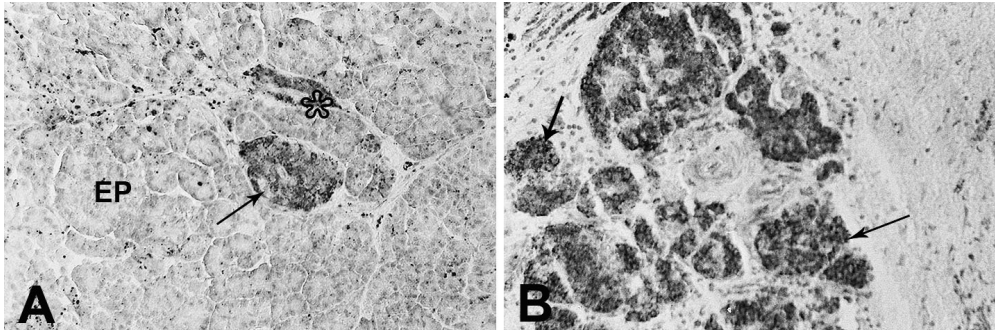
Fig. 1. TPPI activity in normal human pancreas (A) and in human pancreatic acinar cell carcinoma (B). A – TPPI in Langerhans islands (arrow) and in pancreatic ducts and stromal elements (asterisk); lack of the enzyme expression in the exocrine pancreas (EP). B – High TPPI activity in the tumor acinar cells at various grades of differentiation (arrows). A, B – 200×

In fact, all the studies on solid tumors show substantially elevated TPPI expression in the areas of tumor invasion into the adjacent tissues and/or in metastases, which is typical for the enzymes degrading ECM. However, the enzyme is lysosomal and is active only at pH about 4.5. On the other hand, secretion of TPPI pro-enzyme from cells over-expressing it has been already documented. In the ECM it binds to polyanionic glycosaminoglycans such as dextran sulfate, heparan sulfate and chondroitin sulfate B, which exert a protective effect on the enzyme molecule [11]. They not only protect TPPI from heat- or alkaline pH-induced degradation but also facilitate the enzyme activation at pH up to 6.0. These findings may explain the possible TPPI role as an extracellular (matrix) protease in tumor diseases.

In conclusion, many proteolytic enzymes are involved in tumorigenesis, tumor progression and metastasis. Some of them are already used as valuable markers for the diagnosis/prognosis of tumor diseases. Additionally, certain proteases are identified as promising targets for therapy. Those enzymes potential for application in oncology is huge and opens infinite possibilities for future research in the field of tumor diseases.

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