Cathepsins and their endogenous inhibitors in clinical oncology

Primož Strojan

Institute of Oncology, Ljubljana, Slovenia

The invasion and metastasising of tumor cells is closely connected with the disintegration of basement membranes and extracellular matrix. The carriers of these processes are different proteolytic enzymes, among them also cathepsins – a group of ubiquitous lysosome proteinases. A correlation between the changed concentrations and/or activities of cathepsins in the tumor tissue and metastatic potential of tumors was demonstrated on different experimental models in vitro *and* in vivo. *The prognostic relevance of cathepsin D, particularly in breast cancer, and to a lesser extent also of cathepsin B, is nowadays widely studied in clinical oncology. The cells releasing cathepsins also produce their inhibitors. Stefins, cystatins and kininogens are endogenous inhibitors of cathepsin* **B**. *Their clinical relevance, either as therapeutic agents or prognostic factors, still remains unknown. For cathepsin* **D**, *an endogenous inhibitor has not been found yet.*

Key words: neoplasms; prognosis; cathepsins

Introduction

The behaviour of malignant tumors is typically determined by their ability to invade the surrounding tissues as well as by their potential to form metastases in different parts of the body, at a distance from the primary tumor. Both features are the result of a dynamic and complex process, known as metastatic cascade, i.e. the sequence of interrelated events including numerous interactions between the tumor and its host-organism.[1] In order to be able to form a new metastatic colony, an individual tumor cell or a group of these should successfully pass through each individual stage of the cascade; it should 1) leave the primary tumor, 2) invade the adjoining normal tissues, and 3) enter the blood circulation, which then can take it to the most distant parts of the organism. Once inside the target organ, tumor cell or a group of them must again pass through the vessel wall in order to enter into its new habitat and form a new, secondary colony.[2] In this transition, the cell crosses different tissue

Correspondence to: Primož Strojan, M.D.. Institute of Oncology, Dept. of Radiotherapy, Zaloška 2, 1105 Ljubljana, Slovenia. Fax: 386 61 1314180

compartments which form a mammalian organism. These are separated from one another by two types of extracellular matrix, which pose a few natural tissue barriers to the invading cell, i.e. the basement membranes and the interstitial connective tissue. The basic constituents of these structures are different proteins – particularly collagen, adhesive glycoproteins and proteoglycans.[3]

Disintegration of the extracellular matrix and the ensuing transition of tumor cells through it occur as a result of the activity exerted by different types of proteolytic enzymes which are produced and released onto the surface of cytoplasmic membrane or into its surroundings by the invading tumor cells as well as by host-cells.[4] Proteinases, i.e. the enzymes with endopeptidase activity, which are associated with these disintegration processes, are grouped into four classes with respect to the chemical nature of the groups responsible for the catalytic activity. These are 1) serine proteinases, 2) cysteine or thiol proteinases, 3) aspartic proteinases and 4) metallo-proteinases. The same cells that make up these enzymes also produce their inhibitors (Table 1).[3]

In a normal, non-malignant tissue, the activity of individual enzymes and their endogenous inhibitors is organised in the proteolytic cascade involved in

Table 1. Major classes of proteinases.

Class	EC number*	Examples	pH range for activity	Examples of protein inhibitor(s)
Serine	3.4.21.-	trypsin chymotrypsin plasmin plasminogen activator thrombin elastase cathepsin G	7-9	PAIs α2-antiplasmin α2-macroglobulin
Cysteine or thiol	3.4.22.-	cathepsin B cathepsin H cathepsin L	3-8	stefins cystatins kininogens
Aspartic	3.4.23.-	pepsin cathepsin D	2-7	pepstatin
Metallo-	3.4.24.-	collagenases gelatinases stromelysins	7-9 neutral neutral	TIMPs

*Based on the Nomenclature Committee of the International Union of Biochemistry (1992).
PAI - plasminogen activator inhibitor; TIMP - tissue inhibitor of metallo-proteinases.

numerous physiological processes such as tropho-blastic implantation, embryo morphogenesis, angio-genesis, wound healing, pathologic bacterial and parasitic invasions, etc.[2] The cascade activation is a complex process involving numerous interactions between enzymatically inactive pro-enzymes, active proteinases of different classes, and their inhibitors (Figure 1). Contrary to that, in tumor tissue the regulation of this cascade is altered: it is either

Figure 1. Activation of tumor associated proteinases: a complex pattern of events forming proteolytic cascade which involves enzymatically inactive pro-enzymes and active proteinases. Their action is counter-balanced by specific inhibitors (limited proteolysis).

uPA - urokinase-type plasminogen activator; PAI - plasminogen activator inhibitor; TIMP - tissue inhibitor of metallo-proteinase.

incomplete or wrong. This occurs as a result of the modulation of one or more mechanisms regulating the synthesis, transport and release of the involved enzymes and inhibitors, which further leads to chan-ges in their cell distribution and/or concentrations or activities, ending in the establishment of new, bizarre interrelations between them.[5]

Cathepsins

Cathepsins are ubiquitous lysosome proteolytic enzymes. They were named after the Greek word *"Kathepsin"*, which means "to digest" by Willstätter and Baumann in 1929. Cathepsins are present in all cells of mammalian organisms. Their concentrations varies with respect to individual types of cells and tissues, being particularly high in macrophages and in organs such as the kidney, spleen and liver. As to their chemical composition, these substances are glycoproteins, which – but for few exceptions – all belong to the group of endopeptidases. They take part in numerous physiological processes, such as e.g. intracellular protein turnover and posttranslation processing of some biologically important protein precursors (e.g. insulin and endorphin), as well as in the etiology of several pathological conditions, such as muscular dystrophy, arthritis, emphysema, multiple sclerosis and cancer.[6]

The synthesis of cathepsin precursors occurs on the membrane-bound ribosomes, wherefrom they are transferred cotranslationally into the lumen of endoplasmic reticulum, and proceed into Golgi apparatus where glycosylation takes place. After protein and carbohydrate parts of molecules have been modified, they are transported into lysosomes by means of receptors which recognise mannose-6-phosphate residues present on the precursor of lysosomal enzymes.[6,7]

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In cells, these enzymes are present prevailingly inside lysosomes. Beside proteinases, the lysosomes, in their acidic environment with pH ranging between 4.0 – 5.0, also contain a number of other hydrolytic enzymes such as nucleases, glycosidases, lipases, phospholipases, phosphatases and sulphatases. Their major function is to be involved in the controlled degradation of macromolecules, which may be of cellular or foreign origin.[8]

By now, there are 11 different cathepsins known, which differ from each other by their catalytic and molecular properties. They are assigned by letter designation from A to T. Due to the lack of firm evidence, the existence of cathepsins F, I, J, K, M, N, P and R is questionable.[9] The majority of cathepsins are active in acidic pH range whereas in neutral and alkaline pH values they are unstable. The molecular weight of active enzymes amounts to 14-650 kDa (Table 2).[6]

models, both *in vitro* and *in vivo*.[5] On the other hand, the clinical relevance of these enzymes is much less investigated. In terms of their value as prognostic factors in cancer patients, the most thoroughly studied are cathepsins D and B.

Cathepsin D

Cathepsin D is an aspartic endoproteinase with two asparagine groups positioned in its active site. In normal mammalian cells, it is initially synthesised as a precursor 52 kDa protein (pro-cathepsin D), which is transported prevailingly into lysosomes and processed through intermediate 48 kDa form to mature two-chain molecules, each with 34 kDa and 14 kDa respectively. There is only a negligible amount of pro-form accumulated or released from these cells. Inside lysosomes, cathepsin D is involved in the catabolic degradation of numerous intracellular and endocytotically imported proteins.[10] In the ran-

Table 2. Classification of lysosomal cathepsins.

Cathepsin*	EC number	IUB classification	M_r
A	3.4.16.1	Exopeptidase (serine type carboxypeptidase)	100-400.000
B	3.4.22.1	Endopeptidase (cysteine type)	25-29.000
B$_2$	3.4.18.1	Exopeptidase (cysteine type)	
C	3.4.14.1	Exopeptidase-dipeptidyl	200.000
D	3.4.23.5	Endopeptidase (aspartate type)	48.000
E	3.4.23.34	Endopeptidase (aspartate type)	100.000
F	3.4.99.-	Endopeptidase	50-70.000
G	3.4.21.20	Endopeptidase (serine type)	27-30.000
H	3.4.22.16	Endopeptidase (cysteine type)	26-28.000
L	3.4.22.15	Endopeptidase (cysteine type)	23-29.000
S	3.4.22.27	Endopeptidase (cysteine type)	14-30.000
T	3.4.22.24	Endopeptidase (cysteine type)	34.000

*Cathepsins F, I, J, K, M, N, P and R have not been cassified by the International Union of Biochemistry Committee on Nomenclature (1992). There is no substantional evidence that they exist.

The involvement of cathepsins in the proteolytic processes of extracellular matrix decomposition, as well as an association between changes in their concentrations, activities or distribution and the malignant potential of tumor cells have been confirmed by several studies carried out on different tumor

ge of pH 2.8 – 5.0, cathepsin D can effectively degrade denatured proteins while its activity against native molecules and synthetic low-molecular-weight substrates is limited. It has little or no enzymatic activity at a pH 7.0 or more, and its isoelectric point is between 5.5 – 6.5.[6] Unlike cysteine protei-

nases, cathepsin D is not affected by thiol compounds and thiol blocking reagents; it is effectively inhibited by pepstatin, a potent synthetic inhibitor of aspartic proteinases while endogenous protein inhibitor of cathepsin D in man has not been found yet.[10]

In malignant cells the processing and resulting compartmentization of cathepsin D is delayed and is different than in normal cells. This may be due to the decreased activities of processing proteinase(s) involved in cathepsin D maturation procedure and/ or to differences in the structure of pro-cathepsin D: namely, 52 kDa pro-enzyme released from tumor cells contains more acidic isoforms than that from normal cells, despite its almost identical amino acid sequence, and has a more acidic isoelectric point.[11] This could explain cytoplasmatic accumulation of pro- and intermediate enzyme forms by tumor cells as well as markedly increased proportion of secreted pro-enzyme, reaching up to 50 %. On the other hand, the increased secretion could be simply due to the increased cathepsin D gene expression, which saturates the limited number of manosa-6-phosphate receptor sites available, resulting in disruption of pro-enzyme molecule transport into lysosomes.[11,12,13] Generally, breast cancer cells produce 2-30-fold more cathepsin D than normal mammalian cells growing with the same rate.[12]

In human genome, cathepsin D gene is located at the extremity of the short arm of chromosome 11, close to the H-ras oncogene.[14] Its expression in estrogen receptor positive breast cancer cell lines is regulated by estrogens and growth factors.[15] This regulation is tissue-specific: in normal human endometrium, in rat uterus and in the Ishikawa human endometrial cancer cell lines, all of which contain functional estrogen and progesterone receptors in the same way as breast cancer cells, estrogens are unable to stimulate cathepsin D expression.[13,16] In *in vitro* conditions, both pro-enzyme as well as its mature forms stimulate the growth of hormone dependent estrogen-deprived cells of breast carcinoma.[17,18,19] This autocrine mitogenic activity of cathepsin D, however, does not imitate completely the stimulatory effect of estrogen, suggesting that other autocrine growth factors are also required. It can be either due to the direct effects of cathepsin D as a peptide growth factor[18,20] or due to its enzymatic activity. By its proteolytic activity, cathepsin D could play a role in the release of growth factors from precursors or from extracellular matrix and/or activation of their intra- or extracellular receptors, or it

could participate in supplying the cells with amino acids available for the formation of new protein molecules.[21] In a similar way, cathepsin D could be involved in the degradation of basement membrane and extracellular matrix components,[22] as well as in the processing and activation of cysteine proteinases, and thereby also in the initiation of proteolytic cascade.[23,24] Auto-activation of the secreted inactive 52 kDa pro-enzyme has only been demonstrated in *in vitro* conditions, at an acidic pH value.[11,12] Since *in vivo*, an acidic microenvironment is more frequently encountered within the cell (i.e. in endosomes and lysosomes) than out of them, it seems that the activation of the secreted pro-cathepsin D is associated with the internalisation of pro-enzyme molecules, together with its substrate, in the process of endo- or phagocytosis. This hypothesis is supported by the finding of large acidic vesicles, containing both mature cathepsin D molecules and endocytosed extracellular matrix, which were present in a much higher concentration in breast cancer cells than in normal breast tissue cells.[25]

With respect to its proteolytic and mitogenic properties, cathepsin D was widely studied as a marker with potential prognostic value. Since then, a number of clinical studies trying to establish a possible correlation between cathepsin D content in tumor tissue and patients survival have been published. The prevailing majority of these are concerned with breast cancer patients and have been carried out - or are under way – independently in several different countries (Table 3).[26-39] Despite the fact that assay types and methodology used for the determination of cathepsin D content in tumor tissue varied from one laboratory to another, the results obtained indicate that high enzyme concentrations are related to poor prognosis. The only exception in this respect is a study by Henry *et al.* which suggests a different, protective role of cathepsin D.[30] Furthermore, the majority of authors state that cathepsin D is a parameter, independent from other prognostic factors (tumor size, steroid receptor status, axillary lymph node involvement, pathohistological grade, S-phase). Cathepsin D was found to be more correlated with metastasising than with cell proliferation or local tumor invasion. Besides, there is also a cathepsin D assay, commercially available on the market, which is easy to perform and reproducible with a satisfactory degree of quality control. This is a solid-phase "sandwich" immunoradiometric assay (IRMA), developed at the University of Montpellier, France (ELSA-CATH-D kit, CIS bio inter-

Table 3. Clinical studies of prognostic significance of cathepsin D in breast cancer patients.

Study	Assay	Group	Number of patients	Median F/U (mo)	Cut-off*	Univariate p-values DFS	Univariate p-values OS	Multivariate p-values DFS	Multivariate p-values OS	Note
Thorpe et al., 1989 (26)	ELISA	Pre and Peri-MP Post-MP	242 154	48 67	78 24	0.06 0.039	0.3 0.089	0.029 0.032	n.r. n.r.	
Spyratos et al., 1989 (27)	IRMA	All N-	122 68	55	45/70	n.r. n.r.	n.r/0.04 n.r.	0.001/<0.001 <0.01/0.001	n.r. n.r.	
Tandon et al., 1990 (28)	WB	N-	188	64	75	<0.0001	0.0001	0.0003	0.0001	quantifie only 34 kDa form
Romain et al., 1990 (29)	IRMA	All N+	85 46	<58	30	0.08 NS	0.02 0.02	NS n.r.	0.02 n.r.	
Henry et al., 1990 (30)	IHC	All N- N+	94 62 32	<60	++/+ vs. -	<0.05 NS <0.025	<0.1 NS <0.025	n.r n.r n.r	n.r n.r n.r	protective role of cathepsin D
Namer et al., 1991 (31)	IRMA	All N- N+	413 246 166	84	35	NS NS 0.02	0.03 NS 0.008	n.r n.r 0.03	0.02 n.r 0.009	
Granata et al., 1991 (32)	IRMA	N- N- and ER+	199 148	87	40	NS 0.02	NS 0.01	NS n.r	NS n.r	
Duffy et al., 1992 (33)	IRMA	All N- N+	331 141 149	48 51 44	40	<0.05 NS NS	<0.01 NS NS	n.r n.r n.r	n.r n.r n.r	
Kute et al., 1992 (34)	IRMA EA	N- N-	139 138	29 29	63 52	0.0001 0.0031	0.0004 0.0013	0.0263 0.22	0.0044 0.0005	
Pujol et al., 1993 (35)	ELISA	All N- N+	123 64 59	59	20	0.01 0.07 0.009	0.03 n.r n.r	0.02 n.r n.r	NS n.r n.r	
Isola et al., 1993 (36)	IHC	N-	262	<96	++ vs. +/-	<0.0001	<0.0001	<0.0001	<0.01	
Seshadri et al., 1994 (37)	IRMA	All N- N+	858 491 367	31	25	<0.05 NS 0.005	n.r n.r n.r	0.0067 NS 0.037	n.r n.r n.r	
Gion et al., 1994 (38)	IRMA	All	266	39	31	0.0003	0.0222	<0.001	<0.001	
O'Donoghue et al.,IHC 1995 (39)	IRMA	Tu cells Stromal cells	103	>60	++ vs. +/-	n.r 0.0001	NS 0.0086	n.r n.r	n.r n.r	

F/U – follow up; DFS - disease free survival; OS - overall survival; ELISA - enzyme-linked immunossorbent assey; IRMA - immunoradiometric assay; WB – Western bloting; EA -enzymatic activity; IHC - immunohistochemistry; MP - menopausal; N - axillary lymph node; ER - estrogene receptor; NS – non-significant; n.r. - not reported.
*Cut off values for ELISA, IRMA and WB are presented in pmol/mg proteins.

national, GIF-sur-Yvette, France).[40] It quantifies the total enzyme concentration (52kDa, 48kDa and 34 kDa forms) present in cytosols of tissue samples. The latter is also used for the determination of steroid receptor concentrations; this altogether provides a more detailed information on biological properties of tumors. Regarding the above mentioned, cathepsin D already fulfils most of the criteria that should be considered in introducing a new prognostic marker for routine clinical use. However, a number of questions and dilemmas are still open, among them also some which have been posed only recently, by studies just completed: 1) an optimal cut-off value should be selected, which would reliably distinguish between patients with favourable and those with poor prognosis; 2) to find out which form of enzyme is the most potent marker for survival; 3) to establish which type of cells in the tumor is actually overexpressing the enzyme; and 4) to determine its prognostic relevance with respect to menopausal status, steroid receptor status and axillary lymph node involvement. Similarly, it should be investigated how tumors with high cathepsin D concentrations respond to adjuvant therapies: the controversial results reported in the literature, referring to the subpopulation of patients with negative axillary lymph nodes, may be due to different implementation of adjuvant therapies. Only well-controlled randomised clinical studies using an accurately defined and standardised methodology will be able to provide answers to the questions posed. Until then, the routine use of cathepsin D as a prognostic marker with decisive influence on the selection of type or aggressiveness of treatment in individual patients remains unjustified and controversial, despite the promising results obtained so far.

Concentration and/or activity of cathepsin D was also measured in other types of cancer. However, its prognostic significance was generally not studied. A higher concentration of cathepsin D (from 1.5 to 3-fold) was established in laryngeal carcinoma tissue[41] as well as in other types of head and neck tumors[42,43] or their regional metastases,[42] as compared to the adjoining normal tissue of the same patients. None of these studies was able to confirm a correlation between tumor concentrations of the enzyme and the already established clinical and pathohistological prognostic factors. Métayé *et al.* reported a 3-fold higher concentration of cathepsin D measured in 14 samples of thyroid carcinoma tissue than in 7 samples of normal glandular tissue and in 6 samples of benign thyroid nodules. The level of cathepsin D in primary tumors correlated with their size. A similar increase in the enzyme concentration was observed in the tissue of toxic adenomas (8 samples) and in the tissue samples from 7 patients with Grave's disease.[44] Letto *et al.* assessed the level of cathepsin D activity in 67 surgical samples of colorectal carcinoma and in matched paired sets of normal mucosa; the enzyme activity measured in tumor tissue were 1.3-fold higher that in normal mucosa of the same patients.[45] The enzyme activity values found by Tumminello *et al.* in tumor tissue samples from 21 patients with colorectal carcinoma were 1.6-times higher than the respective values measured in normal mucosa of the same patients. A higher activity was observed in the tissue of Dukes' stage A tumors compared to Dukes' B and C, as well as in tumors smaller than 5 cm. There were no differences in cathepsin D concentration between tumor tissue and paired normal mucosa.[46] Cytoplasmic expression of cathepsin D in the cells of gastric adenocarcinoma was studied immunohistochemically in 62 patients by Theodoropoulos *et al.*. Increased expression correlated with early tumor stages (I and II), well- and moderately differentiated carcinomas, positive status of estrogen receptors and a better survival of patients at 36 month.[47] Increased plasma concentrations of cathepsin D assayed in 20 patients with primary hepatocellular carcinoma as well as in 7 patients with liver metastases were reported by Brouillet *et al.*. The values were significantly higher than those established in a group of 56 healthy controls or in 48 breast cancer patients.[48] In a group of 72 patients with primary ovarian carcinoma, Scambia and co-workers reported a worse 3-year progression-free survival for patients with high tumor concentrations of cathepsin D. In 12 patients with metastases in the omentum, the enzyme concentrations measured in the metastatic deposits were 2-fold higher than those found in the primary tumors. Cathepsin D status retained an independent prognostic value for progression when assessed in the multivariate analysis.[49] A correlation between cathepsin D concentration, the grade of tumor differentiation, and the depth of myometrial invasion was also observed in 26 patients with endometrial carcinoma: a significantly higher increase in enzyme level was associated with a higher pathohistological grade and deeper invasion.[50]

Cathepsin B

Cathepsin B belongs to the class of cysteine or thiol proteinases. It has cysteine as essential catalytic group bound to its active site.[50] Human gene

for cathepsin B is localised on the chromosome 8.[52] As all known proteinases, it is first synthesized as a high-molecular-weight inactive precursor with a molecular mass of 37 kDa, which changes into its enzymatically active mature form in the course of posttranslation processing. The molecular mass of the latter ranges between 23-28 kDa; it is found in the cell – depending on species and tissue of origin – as a single- (28 kDa), double- (23 kDa and 5 kDa) or as both single and double chain forms.[51] It is optimally active at a pH of about 6.0 and is poorly active or inactive within the range of neutral and alkaline pH values, depending on the nature of the substrate[51] and the stage of enzyme maturity.[53] As endopeptidase, it exerts an effect on numerous proteinic substrates – also on the components of the extracellular matrix,[54] and has the potential of activating the precursors of some collagenases[55] and urokinase-type plasminogen activator.[56] Thiol reagents and chelators are required for its activation, but it can be activated also by pepsin,[6] cathepsin D[23,24] and metallo-proteinases.[24,57] It has been demonstrated that enzyme activation can also occur as a result of its autocatalytic activity.[58] Cathepsin B is irreversibly inhibited by thiol blocking reagents, and reversibly by leupeptin and other peptide aldehydes, α2-macroglobulin and members of cystatin superfamily (i.e. stefins, cystatins and kininogens). The homology of amino acid sequences suggests its common evolutionary origin with other cysteine proteinase class members.[51]

Unlike in normal cells, where cathepsin B molecules are found prevailingly in lysosomes, in tumor cells a great proportion of the enzyme is found on the cytoplasmic membranes.[59-62] As pro-enzyme, it can also be released into the slightly alkaline surroundings of the extracellular space, and once activated extracellularly, it may be stable in its active form due to the presence of large protein substrates such as extracellular matrix proteins.[53] Correlation between the enhanced activity, mRNA level, the rate of membrane-bound cathepsin B and/or the quantity of high-molecular-weight enzyme forms released into the surroundings on one side, and malignancy of different tumor types of epithelial as well as mesenchymal origin on the other, has been proved in different *in vitro* and *in vivo* experimental models. It seems, however, that this correlation is of qualitative rather than quantitative nature.[63] Furthermore, the reduced inhibitory capacity of human sarcoma derived stefin A, an important intracellular

inhibitor of cathepsin B from cystatin superfamily, has been demonstrated too, as a probable result of changes in its structure.[64] This indicates that the activity of cathepsin B in malignant tumors is regulated at many different levels. Its alterations could be attributed to the modulation of synthesis, activation, processing and intracellular transport of enzyme molecules and/or changes in the inhibition by endogenous inhibitors.[51]

An increased concentration and/or activity of cathepsin B molecules was measured in various types of human malignant tumors: carcinomas of the breast,[65-67] colon and/or rectum,[45,68,69] stomach,[70,71] liver,[72] lung,[73-75] uterine cervix,[76] head and neck carcinomas,[43] gliomas,[77] and tumors of the hypophysis.[78] There has been a correlation established between the measured tumor concentration and/or activity of cathepsin B, and individual clinical and pathohistological tumor properties; in some tumors, a correlation with treatment outcome and/or survival was found as well. It should be pointed out that the results of these studies are much less established and conclusive than those referring to cathepsin D and the survival of breast cancer patients.

Lah *et al.* have reported 18.5-times higher cathepsin B activity measured in the breast cancer tissue of 50 patients as compared to the relevant values measured in normal breast tissue of the same patients. There has been no correlation established with pathohistological grade, axillary lymph node involvement, hormone receptor status, and relapse free survival.[67] Similar results of cathepsin B activity measurements in 90 matched pairs of breast carcinoma and normal breast tissue samples were reported by Gabrijelčič *et al.*. Besides the enzyme activity, authors also measured total enzyme concentration in the serum and tissue cytosol: the latter was found to be approximately 3.3-fold the concentration measured in the serum of healthy controls, while the relevant cytosol concentrations were 8.8-fold higher, respectively. Higher enzyme concentrations were found in the tumor tissue cytosols from patients without axillary lymph node involvement and a higher pathohistological grade. In this study, patients' survival was not considered among the parameters observed.[65] A similar negative correlation between cathepsin B concentration in tumor cytosols of 62 breast carcinoma patients and their lymph node status, as well as the status of hormone receptors, was reported by Budihna *et al.*. Besides, patients with cathepsin B tumor concentrations up to 23 mg/g proteins were found to have worse re-

currence-free survival at 54 months than those with higher tumor concentrations. In the multivariate analysis, besides axillary lymph node status, only cathepsin B proved to be an independent prognostic factor.[79] On the contrary, Thomssen *et al.* reported a better 5-year recurrence-free survival in patients with lower cathepsin B concentrations (< 1092 ng/mg proteins). In this study of 167 breast cancer patients tumor concentrations of cathepsin B were 11.3-fold higher than those measured in benign breast tissue, and no correlation to established prognostic factors were found. The relevance of cathepsin B as independent prognostic factor for recurrence-free or overall survival of those patients was not confirmed by the multivariate analysis.[80]

Analysing cathepsin B activity in paired tissue samples from 27 patients with colorectal carcinoma, Sheahan *et al.* registered 1.4-fold higher activity in tumor tissue as compared to the adjoining normal mucosa. The highest enzyme activity was established in a group with Dukes A stage of the disease.[68] Similar findings were reported by Leto *et al.*: cathepsin B activity measured in carcinomatous tissue from 67 patients was 1.4-fold higher than that found in normal mucosa, the increase being evident in patients with Dukes A stage of disease only. There was no correlation with either clinical or pathohistological prognostic factors established.[45] Contrary to that, Campo *et al.* found that the elevated cathepsin B expression correlated with advanced stages of disease. The expression of enzyme was found to be negative in all 15 samples of normal mucosa and 17 samples of benign adenomas. However, in a group of 28 patients with early, non-metastatic tumors (stages I-II), the expression was negative in 6, low in 17 and high in 5 patients. In 41 patients with advanced, metastatic carcinomas (stages III-IV), the expression was negative in 3, low in 17 and high in 21 patients. Lower overall survival at 84 months of follow up correlated with high cathepsin B expression in all cancer patients, whereas after stratification by stages, the correlation was established only for those with advanced disease.[69]

After having compared 33 match pairs of gastric carcinoma and the adjoining normal mucosa, Watanabe *et al.* measured 3-fold higher cathepsin B activity in tumor tissue samples. The enzyme activity was significantly, i.e. 1.9-fold higher in poorly differentiated adenocarcinomas than in well or moderately differentiated tubular adenocarcinomas.[70] Plebani *et al.* reported the results of their cathepsin B measurements in paired tissue samples of 25 pa-

tients with gastric cancer. The concentrations found in tumor tissue were twice as high as those measured in normal mucosa. Higher enzyme concentrations were also found in the tissue of patients with regional or hepatic metastases vs. those without metastases, in poorly or moderately differentiated vs. well differentiated tumors, and in diffuse vs. intestinal tumor types. At 27 months, the survival of patients with cathepsin B tumor concentrations below the cut-off value of 265 ng/mg proteins was better than that of those with higher enzyme concentrations.[71]

Ebert *et al.* have established a 4.5-fold higher cathepsin B activity in 65 lung tumor tissue samples as compared to the normal lung parenchyma. The activity was found to be insignificantly higher in adenocarcinomas than in other histological tumor types. The highest cathepsin B activity levels were measured in lung metastases. There was no correlation with stage of disease or pathohistological grade established. Elevated activity above the cut-off value of 1674 μU/mg proteins was related to lower survival rates of the patients at 8 months.[75] Higher cathepsin B activity found in the tissue of lung adenocarcinomas as compared to squamous cell carcinomas was reported by Krepela *et al.*[73] and by Lüthgens *et al.* who compared adenocarcinoma cathepsin B activity to the activity measured in squamous cell and small cell carcinomas.[74] In the group of 142 patients with primary lung adenocarcinoma, Inoue *et al.* registered immunohistochemically increased cathepsin B expression in the tumor tissue of cases with stage III and IV of disease as compared to that in cases with stage I, which also correlated with worse overall 5-year survival rates. In a multivariate analysis, cathepsin B expression proved to be an independent prognostic factor associated with death due to disease.[81]

After having compared 53 matched pairs of head and neck carcinoma and adjacent normal tissue, Kos *et al.* found 5.4-fold higher cathepsin B concentration in tumor tissue samples. There was no correlation with clinical and pathohistological prognostic factors established, whereas patients' survival was not included among the parameters observed.[43]

In a study by Hirano *et al.*, serum cathepsin B levels and its urinary excretion were reported to be significantly higher in a group of 7 patients with distant metastases from a variety of cancers than in the control non-cancer patients (11 samples) or in cancer patients without distant metastases (7 sam-

ples). Six weeks after completed radical curative operation the enzyme concentration in the group without distant metastases decreased to the control values. However, in the group of cancer patients with distant metastases after resection of primary tumor, both serum and urine enzyme concentrations were still high – as before surgery. In the group without distant metastases, for all of the resected specimens of cancer tissue, cathepsin B concentrations were significantly, 1.8-times higher than those in normal tissue.[82]

Endogenous cathepsin inhibitors

Endogenous, i.e. physiological inhibitors of proteinases naturally present in tissues, appear always to be proteins. They are involved in the control mechanisms responsible for intra- and extracellular protein breakdown, thus protecting the cell against adverse endo- and exogenous proteolysis. The compilation of new knowledge and information on these substances contributes to better understanding of their role and importance in the process of tumor rise and its consecutive spread. By preserving the delicate balance that exist between tumor cells, extracellular-matrix-bound growth factors and cytokines, and constituents of the matrix, these inhibitors may exert a marked cytotoxic effect on the primary tumor as well as on the existing metastatic lesions. This ability assigns them the role of potential therapeuticals and/or prognostic indicators in all conditions where proteolytic degradation represents the pathophysiological basis for clinical manifestation of disease, thus also in cancer.[83] It seems, however, that it will take long before these substances become a part of the routine therapeutic tools for cancer treatment, considering that all the studies are still carried out at a preclinical, i.e. laboratory level. The same applies to their prognostic value, since until now no reports on this issue could be found in the available literature.

Probably, in view of future clinical use, the most promising of endogenous inhibitors are those which suppress the activity of cathepsins B and D. Considering that an endogenous inhibitor of cathepsin D in man is not known yet, these are – for the time being – restricted only to the inhibitors of cysteine proteinases. Based upon the evolutionary and structural similarities, they constitute a single protein superfamily of cystatins. This is subdivided into three families: stefins (family I), cystatins (family II) and kininogens (family III). There is yet a group of non-inhibitory proteins (family IV) including histidine-rich glycoproteins and α2H-glycoproteins (Table 4). The members of the first three families,

Table 4. Cysteine proteinase inhibitors of cystatin superfamily in human.

Family		Examples	Distribution	M_r
Name	No.			
Stefins	I	stefin A stefin B	intracellular	11.000
Cystatins	II	cystatin C	extracellular	13.000
Kininogens	III	HMW-kininogen LMW-kininogen	extracellular	120.000 68.000

HMW – high molecular weight; LMW – low molecular weight.

capable of inhibitory activity, differ from one another with regard to their binding affinity and binding ratio for different cathepsin molecules; in all of them binding is strong though competitive and reversible. Contrary to kininogens and cystatins, which occur at relatively high concentrations in various biological fluids, stefins can be found prevailingly inside the cells. The presence of the molecules of cystatin superfamily inside as well as outside the cells renders them to serve as a "reservoir" for cysteine endopeptidases: they bind the enzymes when released from lysosomes in order to transport and deposit them at other sites in the cell or organism.[84-86]

The measurements of the concentrations and/or activities of cysteine proteinase inhibitors gave controversial results, and the existing literature on these topics is very scarce. Thus, the total activity of inhibitors measured in tumor tissue was found to be either lower,[67] equal[68] or higher[87] than in the adjoining normal tissue. This variability indicates that it is indispensable to determine the contribution of each individual member of cystatin superfamily to their total inhibitory potential. While the role of cystatins and kininogens in the process of the development and spread of malignant tumors has not been extensively studied yet, the involvement of stefins in these processes is more investigated.

In man the stefin family comprises stefin A and stefin B. These are small single-chain non-glycosilated proteins with a molecular weight of about 11 kDa.[84] Genes for family I proteins are located on human chromosome 3[52,88] and do not include secretory signal sequences.[89] This is consistent with the fact that stefins are generally found in the cell cytoplasm,[90-92] although they have also been iso-

lated from the extracellular fluids.[93] They are heat resistant and stable in neutral and alkaline pH range. Although quite similar structurally – 51 % of stefins A and B structure is identical, they differ from one another with respect to their immunological properties which enable immunohistochemical studies of their cellular and tissue distribution.[84] While the presence of stefin B in different tissues is relatively uniform,[84,94,95] stefin A is abundant primarily in various types of epithelial cells and in some cell types of the lymphoid tissue.[96-102] This suggests a possible role of stefin B as the protector of cells against uncontrolled activities of endogenous cysteine proteinases, and the involvement of stefin A in the immunological processes protecting epithelial and lymphoid tissues from invading bacteria and parasites or their (i.e. external) cysteine proteinases[84,92,99] Functional differences between both stefins, which may also be of physiological importance, lye in their inhibitory capacity for individual cathepsins, as well as in their resistance to proteolytic degradation by aspartic proteinase cathepsin D: stefin A is a better inhibitor of cathepsin B than stefin B is, and shows a higher resistance to cathepsin D. Both stefins exert a stronger inhibitory effect on the molecules of cathepsins L and H than of cathepsin B.[84,103,104]

There are several findings implicating stefin A in the process of malignant progression more than any other member of the cystatin superfamily. When determining the total activity of cysteine proteinase inhibitors in 50 matched pairs of breast carcinoma and normal breast tissue, Lah *et al.* found lowered inhibitor activity in carcinoma as compared to normal tissue in two thirds of their patients. In this group, a correlation was established between lower inhibitor activity, higher pathohistological grade and negative hormone receptors, as well as significantly higher relative increase in Cathepsin B and L specific activity between tumor and normal tissue than in the group with unchanged or elevated activity of the inhibitors studied. In the same study, lower mRNA concentrations of stefin A were measured in carcinomatous than in normal breast tissue samples and correlated with the total activity of cysteine proteinase inhibitors.[67] Reduced immunohistochemical staining for stefins A and B in lymphoma and esophageal carcinoma tissue was reported by Järvinen *et al.*;[92] in the latter it could be associated with the dedifferentiation and malignant transformation of epithelial cells. A similar observation applies to squamous cell carcinomas of the human uterine cervix,[105,106] skin,[107] lung,[108] as well as for prostatic

adenocarcinoma;[109] there also, stefins A expression was related to cell proliferation and dedifferentiation, suggesting to be an important factor in maintaining cell differentiation. In the case of prostatic adenocarcinoma, the authors even suggest stefin A to be used as a marker in histologic differential diagnosis of malignant and benign lesions, especially in the detection of small carcinomatous foci in the prostate.[109] A high concentration of stefin B along with an unusually low concentration of stefin A – approximately 20-times lower that that found in normal epithelial tissue – was measured in ovarian carcinoma tissue by Kastelic *et al.*. The authors hypothesise that stefin A is down-regulated in malignant ovarian carcinoma.[110] A reduced inhibitory capacity of human-sarcoma-derived stefin A against different cysteine proteinases was reported by Lah *et al.*. It appears to be due to a higher inhibition constant of stefin A for the inhibition of these enzymes, which indicates that endogenous inhibitors of tumor origin exert different inhibitory properties than those originating from normal tissues.[64]

Conclusion

Introducing of new prognostic markers into routine clinical practice enables us to differentiate more precisely between prognostically more and less favourable forms of disease, and thus also influence treatment planning. It is important that patients with favourable prognosis are spared from too aggressive therapy, and vice versa, that those with worse prognosis receive sufficient treatment. The role of cathepsins and their endogenous inhibitors in the development of cancer is indicated particularly from their involvement in the proteolytic processes leading to invasion and dissemination of tumor cells. Their concentrations and/or activities in tumor tissue or body fluids can also be of prognostic value.

By now, it has been generally accepted in clinical oncology that high cathepsin D concentrations in breast cancer tissue should be regarded as indicator of worse prognosis. The prognostic relevance of the enzyme in different subgroups of these patients, as well as in patients with tumors of other sites, is less clear. Likewise, the prognostic value of cathepsin B has also been unclear, while that of other cathepsins has not been extensively studied at all.

The role of endogenous cathepsin inhibitors – cystatins in clinical oncology could be double: they could function as therapeuticals and/or prognostic factors. In view of the fact that most studies in this

field have been carried out on different experimental models only, while clinical trials – rare as they are – involve small series of patients, the question of clinical importance of these inhibitors remains to be solved.

Acknowledgement

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