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Lysosomal Cysteine Proteinases as Mediators of Inflammation and Tumor Spread: Control of their Extracellular Proteolytic Activity

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SUMMARY. **The lysosomal cysteine proteinases cathepsin B and L have been implicated in proteolytic pathomechanisms of inflammation and tumor spread. Data obtained with ex vivo samples of blood plasma and inflammatory secretions as well as tumor homogenates suggest that both enzymes are sufficiently stable at neutral to alkaline pH to remain active for a limited time in the extracellular space. Whereas cathepsin L should be proteolytically active only under conditions of local inhibitor deficit, cathepsin B is unique in its loose binding to endogenous protein inhibitors and its ability to dissociate as active enzyme from its inhibitor complexes.**

A major group of proteinases responsible for intracellular protein degradation are the cysteine proteinases cathepsin B, H, L, and S (see Barrett et al.[1] for review). Normally they act within lysosomes, but it is known that they can be released by tumor cells[2] and active phagocytes[3] into the extracellular space. Limited cleavage and inactivation of various functionally important proteins by cathepsins L and B has been demonstrated in vitro[1,4–9] suggesting that these cysteine proteinases may be involved in destructive proteolytic processes occurring in vivo during severe inflammation and at certain stages of tumor cell invasion and metastasis.

After it had been discovered that the lysosomal cysteine proteinases are rapidly inactivated by exposure to neutral or alkaline pH and/or oxidizing conditions as well as by complex formation with protein inhibitors[1], extracellular proteolysis by the discharged enzymes seemed questionable. Therefore we have studied in more detail the activity of cysteine proteinases and their interaction with endogenous protein inhibitors in ex vivo samples obtained from patients with inflammatory diseases and, more recently, in tumor extracts. Some of the results presented in this communication may help to understand the role of cysteine proteinases in tumor-associated proteolysis.

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MATERIALS AND METHODS

Clinical studies on inflammatory diseases were performed as described.[10–14] The design of the clinical study on breast cancer patients, extraction of tumor samples and protein determination have been reported by Jänicke et al.[15]

Cathepsin B antigen was determined with a commercial ELISA (Medor, D-8036 Herrsching); cathepsin B activity at 30°C was measured with the fluorogenic substrate Z-Arg-Arg-NH-Mec (0.15 mM) and the specific inhibitor E-64 using the assay described earlier.[11,13] One unit of enzyme activity (1 U) is the amount of enzyme catalyzing the turnover of 1 μmol substrate per min under the conditions of the assays.

Previously published procedures were used for the determination of inhibition constants and rate constants of complex formation,[16] for dilution experiments[11,13] and measurement of resorufin-casein proteolysis.[14] Cathepsin B (human liver) and cathepsin L (human kidney), human stefin A, and human stefin B were obtained from Medor (Herrsching).

RESULTS AND DISCUSSION

Cysteine Proteinases in Inflammation

Proteolysis seems to play an important role in the response of the organism to inflammatory stimuli like

tissue destruction due to multiple trauma and major surgery or invasive microorganisms and endotoxins in sepsis. In this respect, the lysosomal serine proteinase elastase, and the cysteine proteinases cathepsin L and B of polymorphonuclear (PMN) granulocytes and monocytes/macrophages are supposed to be potent mediators and/or effectors of tissue damage when they are discharged extracellularly by activated phagocytes.

A presumable inflammatory effector role proved especially true for PMN elastase because the destructive potency of this proteinase could be convincingly demonstrated not only in vitro but also in vivo at least on vital humoral proteins in relation to the development of organ dysfunctions in traumatized patients (*see* Jochum et al.[10] for review). In local inflammatory secretions, such as the epithelial lining fluid (ELF) of the shock lung of a polytraumatized patient, the major portion of the proteolytic activity measurable by resorufin-casein proteolysis can be suppressed by the addition of α_1-proteinase inhibitor indicating that it is mainly due to PMN elastase (Fig. 1). However, in the course of the same respiratory distress syndrome also the cysteine proteinase cathepsin B is released into the ELF, but not strictly parallel with PMN elastase.

We have collected plenty of evidence that cathepsin B is discharged from monocytes/macrophages during local and systemic inflammation and that the cathepsin B activity of blood plasma and local secretions (ELF, peritonitis exudate, synovial fluid) correlates with the severity of inflammation and the clinical manifestation of organ dysfunction.[10–14]

Whereas PMN elastase is usually determined in the form of its complex with α_1-proteinase inhibitor, cathepsin B is detectable by its enzymatic activity using sensitive fluorogenic peptide substrates and the specific inhibitor E-64 (*see* Assfalg-Machleidt[11,13] for details). So far no cathepsin L activity has been found extracellularly because this cysteine proteinase is very tightly bound to the protein inhibitors (*see below*).

Typically, extracellular levels of released cathepsin B are 1–2 orders of magnitude lower than those of simultaneously discharged PMN elastase (Table 1), with the exception of the perfusion fluid of liver transplants where enormous amounts of cathepsin B seem to originate from hypoxic cells of the reticuloendothelial system.[17]

According to in vitro experiments, the non-specific proteolytic potency of cathepsin B is rather limited compared to that of cathepsin L (Table 2). Cathepsin L is a potent elastase comparable to PMN elastase[5] and is able to inactivate α_1-proteinase inhibitor effectively[4] suggesting that lysosomal cysteine and serine proteinases may cooperate in proteolysis-induced pathomechanisms of inflammation. Cathepsin H, predominantly an aminopeptidase, has almost no detectable effects on proteins.

Until now, a destructive effect of cysteine proteinases has not been demonstrated in vivo, but we were able to show that limited proteolysis of immunoglobulin G (IgG) in peritonitis exudates is partially due to E-64-sensitive cysteine proteinases.[14] Very recently, the

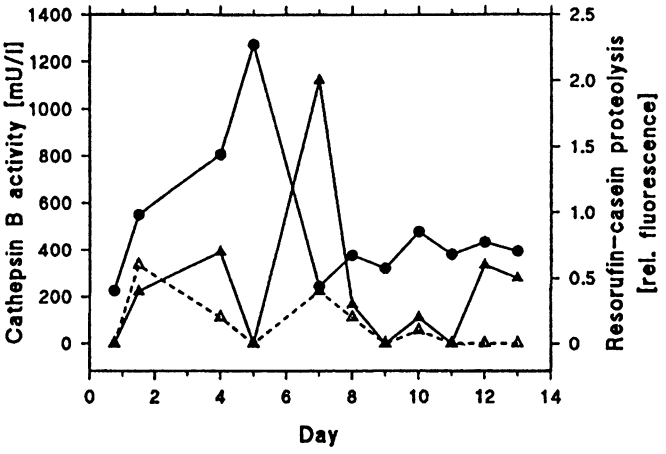

Fig. 1 Cathepsin B activity (filled circles) and non-specific proteolysis of resorufin-casein (filled triangles) in bronchoalaveolar lavage fluid of a polytraumatized patient suffering from an adult respiratory distress syndrome (ARDS). Open triangles, resorufin-casein proteolysis in the presence of α_1-proteinase inhibitor (45 μM).

Table 1 E-64-sensitive cysteine proteinase activity. Peak values measured in blood plasma and inflammatory secretions. The concentrations of the α_1-PI-elastase complex determined by ELISA are included for comparison

Sample	*Activity U/l*	*Cathepsin B equivalent* ng/ml*	*α_1-PI-elastase ng/ml*
Blood plasma			
normal	0.09	1.1	120
polytrauma, sepsis	0.8	10	1400
Synovial fluid			
traumatic arthritis	0.8	10	9000
Bronchoalveolar lavage fluid			
shock lung (ARDS)	10	120	2000
Peritoneal exudate			
diffuse peritonitis	50	600	120000
Liver perfusate			
liver transplants	500	6000	1400

*calculated on the basis of a specific activity of 80 U/mg[11]

Table 2 Resorufin-casein proteolysis by lysosomal proteinases

Enzyme	*pH*	*Relative proteolytic activity per μg of enzyme*
PMN elastase	7.4	1.0
Cathepsin L	5.5	1.1
Cathepsin B	5.5	0.1
Cathepsin H	5.5	< 0.01

restricted proteolytic potency of cathepsin B (as compared to cathepsin L) has been explained by the existence of an 'occluding loop' in the cathepsin B structure making its endopeptidase activity highly

dependent on the conformation of the substrate proteins.[19]

Cysteine Proteinases of Tumor Cells

The lysosomal cysteine proteinases cathepsin B and L have been suggested to play a role in tumor cell invasion and spread, either directly by cleaving extracellular matrix proteins or indirectly by activating other proteinases.[2] Cathepsin B and L have been shown in vitro to degrade type IV collagen, laminin and fibronectin at both acid and neutral pH.[9] Both enzymes are able to activate the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) which is secreted by tumor cells and can bind to receptors on the tumor cell surface.[20,21] In this cascade mechanism the lysosomal cysteine proteinases may function as effective mediators of tumor-associated proteolysis.

There have been many reports on increased production and secretion of cysteine proteinases by cultivated tumor cells[2], but relatively few data are available on the role of these proteinases in clinical cancer. Therefore we started investigate this problem within a follow-up study with patients undergoing surgery for breast cancer.[15] Preliminary results obtained with part of the patients included in this study indicated that the tumor extracts (n = 53) contained elevated levels of cathepsin B antigen (1563 ± 1066 ng/mg of tissue protein; mean ± S.D.) compared to benign controls (n = 5; 281 ± 156 ng/mg). The broad distribution of cathepsin B levels (Fig. 2) suggests that the cathepsin B content of tumor cells may represent a novel differentiating prognostic factor that will have to be evaluated and correlated with the other factors of the study. This view is supported by results of flow cytometry with cathepsin B antibodies showing increased cathepsin B content along with increased number of uPA receptors on the tumor cells (*see* N. Chucholowski et al., this issue).

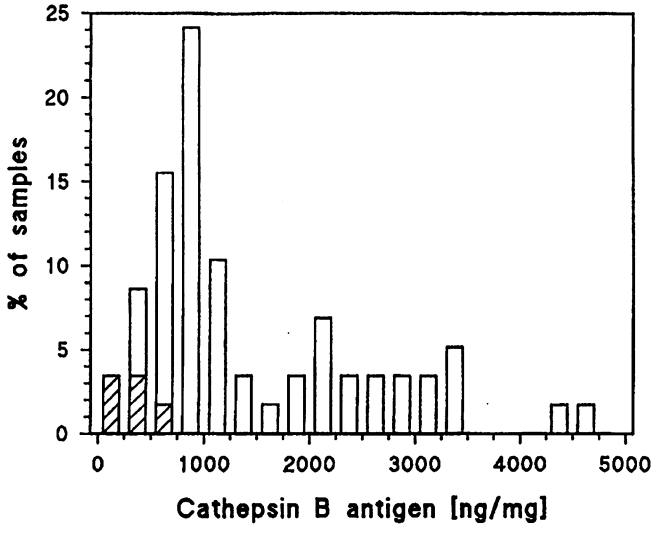

Fig. 2 Distribution of cathepsin B antigen concentration in homogenates of breast cancer tissue (n = 53) and normal breast tissue controls (n = 5; hatched areas) as determined by ELISA.

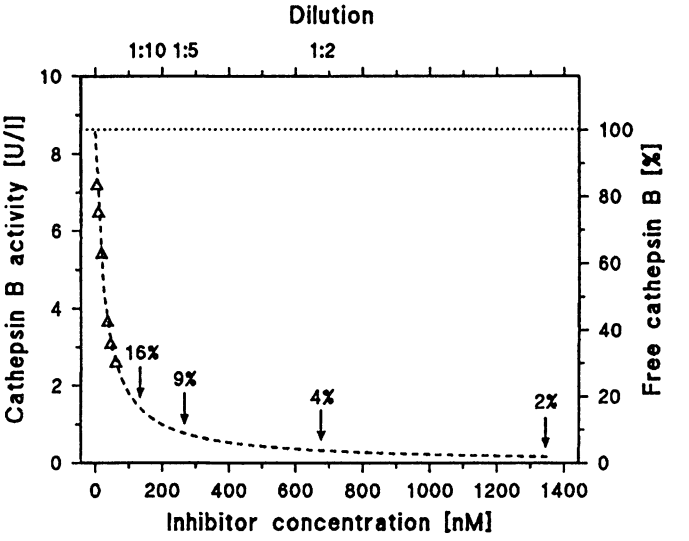

Fig. 3 Dissociation of cathepsin B activity on dilution of a peritonitis exudate. The active inhibitor concentration of the undiluted sample (1350 nM) was determined by titration of E-64 standardized papain. The dissociation curve was obtained by nonlinear regression analysis of the experimental data.

Extracellular Activity of Cysteine Proteinases

The pathobiochemical significance of released lysosomal cysteine proteinases in inflammation and tumor spread depends on their enzymatic activity outside the lysosome. We have found that, in contrast to earlier expectations, inactivation at neutral to alkaline pH does not preclude extracellular proteolytic activity. Half lifes at pH 7.40 (determined in continuous assays with small peptide substrates) were about 50–60 min for isolated cathepsin B and about 8 min for cathepsin L. Half-lifes at pH 7.40 of the cathepsin B activity of the breast cancer extracts (52–62 min) were identical with those of the isolated enzyme. The observed drastically increased inactivation at more alkaline pH may explain the low recovery of cathepsin B activity from the tumor extracts performed at pH 8.5 in this study. In a preliminary evaluation of 149 breast cancer tissue extracts we found a cathepsin B activity of 535 ± 119 mU/g (mean ± S.D.) compared to 45 ± 29 mU/g for 7 benign controls. About 4-fold higher activity of tumor homogenates has been reported by Dengler et al.[22] who extracted at pH 6.9.

Detailed kinetic studies revealed that the cysteine proteinase activity determined in the tumor extracts of our study was almost completely due to cathepsin B. This was confirmed by the determination of an inhibition constant of 3.2 nM for chicken cystatin which is close to that of isolated cathepsin B (2.9 nM).

Extracellular cysteine proteinase activity is controlled by a high potential of endogenous cysteine proteinase inhibitors (CPI), such as the kininogens and the 'low-Mr' cystatins, like cystatin C and the stefins A and B (*see* Barrett et al.[23] for review). Cathepsin L is very tightly bound by all of these inhibitors (Table 3) and should have almost no chance to remain active in the

Table 3 Inhibition of cathepsin B (Cath B) and cathepsin L (Cath L) by the plasma protein inhibitors. I_t, inhibitor concentration in blood plasma; K_i, inhibition constant

	I_t nM	K_i nM		I_t/K_i	
		Cath L	Cath B	Cath L	Cath B
Kininogens	5000[a]	0.012	390	4×10^5	13
Cystatin C	100[b]	< 0.005	0.8	2×10^4	125
Stefin A	< 20[b]	0.010	2.7	2×10^3	7
Stefin B	< 10[b]	0.003	74	3×10^3	0.1

[a]active concentration determined by titration
[b]antigen concentrations from Abrahamson et al.[25]

extracellular space unless the inhibitory capacity is drastically reduced. Therefore, concentrations and activity of CPI in and around tumor cells need to be investigated in more detail. Membrane-associated cathepsins that have been observed in tumor cells[24] may be able to escape inhibition by the extracellular CPI.

In contrast to cathepsin L, cathepsin B is only loosely bound by all CPI, especially by the kininogens which represent the major CPI of blood plasma.[13] We have shown that cathepsin B readily dissociates from its inhibitor complexes whenever the inhibitor concentration is reduced (Fig. 3). This dissociation may be enhanced by the presence of a good protein substrate. In tissues, the control of extracellular cysteine proteinase activity should depend critically on the active concentrations of stefins and cystatins which are not known. Moreover, altered 'tumor stefins' of reduced affinity for cathepsin B have been described.[2] The physiological significance of such findings remains to be established, however, as both stefins are 'slow-binding' inhibitors of cathepsin B requiring at least several minutes for complex formation.[16]

CONCLUSIONS

Extracellular control of the proteolytic activity of discharged lysosomal cysteine proteinases seems to be incomplete allowing these enzymes to play role in proteolysis-induced pathomechanisms of inflammation and tumor spread.

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