PROTEASES
Potential Role in Health and Disease

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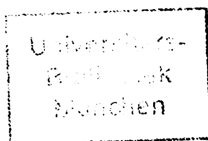

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PROTEINASES AND THEIR INHIBITORS IN SEPTICEMIA - BASIC CONCEPTS AND CLINICAL IMPLICATIONS

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INTRODUCTION

The Role of Lysosomal Enzymes in Inflammation

During the inflammatory response various systemic or local tissue cells are activated thereby releasing internal, mostly lysosomal enzymes. They trigger the activation of the clotting, fibrinolysis and complement cascades, the disrupture of cell membranes and tissue structures, and the release of toxic peptides (Fig. 1).

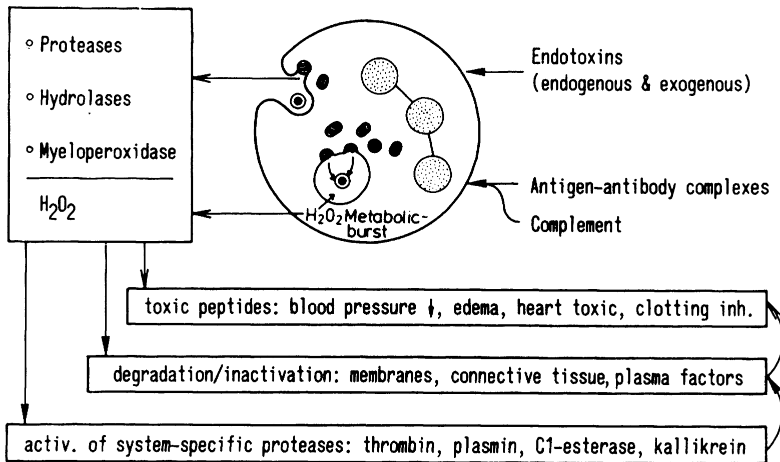

Fig. 1: Liberation and effects of lysosomal factors. For details see text.

Phagocytes, especially the granulocytes and monocytes or macrophages, but also fibroblasts, endothelial cells and mast cells are known to be very rich in such internal or lysosomal enzymes.

So far, only the properties and pathobiochemical effects of enzymes of the azurophilic and specific lysosomes of polymorphonuclear granulocytes (neutrophils) have been investigated in more detail. Such enzymes, for example the neutral elastase and cathepsin G as well as the acidic cathepsins are preformed and stored in the lysosomes in fully active form[1,2]. In this way, they can respond immediately to perform their biological function, namely the degradation of extracellular and intracellular material after phagocytosis.

Outside the phagocytes, proteolytic action of the lysosomal enzymes is normally prevented or balanced by proteinase inhibitors present in plasma, interstitial fluid and body secretions (Fig. 2). These inhibitors

Abbr.	Inhibitor	M.W. x 1 000	Mean conc. mg/100 ml	Mean conc. µmol/l
α_2M	α_2-Macroglobulin	725	260	3.6
α_1PI	α_1-Protease inhibitor	50	260	52
α_1AC	α_1-Antichymotrypsin	70	45	6.4
β_1CI	β_1-Collagenase inh.	40	1.5	0.4
ITI	Inter-α-trypsin inh.	160	45	2.8
AT III	Antithrombin III	65	26	4.0
α_2PI	α_2-Plasmin inhibitor	70	6	0.9
C1 INA	C1-Inactivator	100	24	2.4

Fig. 2: Plasma inhibitors directed against lysosomal proteinases of various body cells or plasma-derived proteinases.

comprise together with inhibitors of clotting, fibrinolysis and complement proteinases more than 10 % of the plasma proteins. Non-lysosomal proteinases like, for example, thrombokinases and plasminogen activators are

faced only with a very low inhibitory potential in body fluids. They are, therefore, privileged candidates for the activation of clotting and fibrinolysis if released into the circulation after increased production due to an inflammatory stimulus.

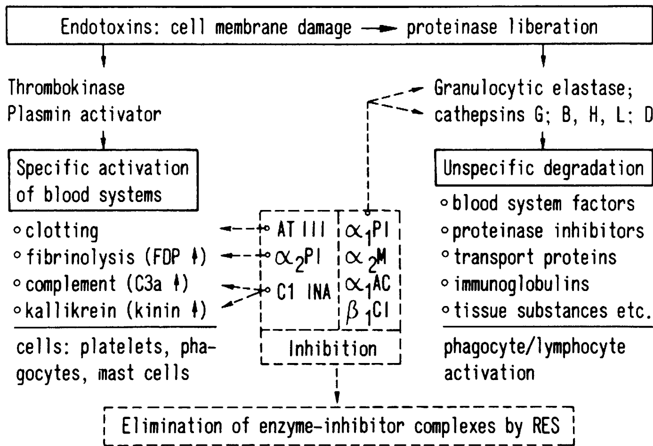

Fig. 3: Consumption of plasma factors during inflammation. For details see text.
Abbreviations: antithrombin III (AT III), α_2-plasmin inhibitor (α_2PI), C1 inactivator (C1 INA), α_1-proteinase inhibitor (α_1PI, formerly α_1-antitrypsin), α_2-macroglobulin (α_2M), α_1-antichymotrypsin (α_1AC), β_1-collagenase inhibitor (β_1CI), fibrin (-ogen) degradation products (FDP), complement factor (C 3a), reticuloendothelial system (RES).

Lysosomal enzymes liberated during severe inflammation like septicemia or septic shock can enhance, together with thrombokinases and plasminogen activators, the inflammatory response via two major routes characterized by either substrate-specific or substrate-unspecific proteolysis (Fig. 3).

The system-specific proteinases, thrombokinases and plasminogen activators, trigger the activation of the clotting, fibrinolysis and complement cascades (summarized as 'blood systems') by substrate-specific proteolysis of proenzymes and cofactors. The activated enzymes are subsequently inhibited by their natural inhibitors; the enzyme-inhibitor complexes thus formed are rapidly eliminated from the circulation by the reticuloendothelial system (RES). Hence, in a series of steps based on highly specific interactions not only the proteinases respectively their zymogens are consumed but also their natural antagonists, the system-specific inhibitors. Until recently, the given sequence of reactions was assumed to be exclusively responsible for the development of disseminated intravascular coagulation (DIC).

Results obtained very recently indicate that an additional reaction path may contribute considerably to the consumption of plasma factors during severe inflammations. This implies inactivation of plasma factors by substrate-unspecific proteolysis due to liberated lysosomal proteinases. Egbring and coworkers observed in animals a significant decrease in several clotting factors after infusion of endotoxin or human neutrophil elastase[3]. Similar results were obtained by Ohlsson and coworkers[4,5] in canine endotoxemia. Moreover, in patients suffering from sepsis or septic shock a striking consumption of blood system factors, immunoglobulins and proteinase inhibitors has been found by the teams of Egbring[6], Aasen[7], Gallimore[8] and Witte[9].

The potency of lysosomal enzymes for the degradation of native plasma protein inhibitors was also demonstrated in vitro by the inactivation of antithrombin III due to catalytic amounts of neutrophil elastase[10]. α_1-Protease inhibitor (formerly α_1-antitrypsin) is inactivated in a similar way by the macrophage elastase[11] and, in addition, by oxidation of its reactive-site methionine residue via the lysosomal myeloperoxidase/hydrogen peroxide system[12].

RESULTS

Neutrophil Elastase and Plasma Factors in Sepsis

Assay of liberated elastase. Due to the presence of an excess of the endogenous inhibitors α_1-protease inhibitor (α_1PI) and α_2-macroglobulin (α_2M), direct measurement of the neutrophil-derived proteinase activities in plasma or other body fluids is not feasible. However, increased levels of the elastase-α_1PI complex would be already a clear indication for elastase liberation. Quantitative estimation of the plasma levels of the elastase-α_1-protease inhibitor complex was carried out with a highly sensitive enzyme-linked immunoassay[13]. Briefly, the E-α_1PI complex of the plasma sample was bound to surface-fixed antibodies directed against neutrophil elastase. After washing, a second alkaline phosphatase-labelled antibody directed against α_1PI was fixed to the complex. Under suitable conditions, the activity of fixed alkaline phosphatase towards p-nitrophenylphosphate is proportional to the concentration of the E-α_1PI complex in the sample.

In a first approach, we were interested to see whether a relationship exists between the plasma levels of E-α_1PI and the severity of postoperative infections. To achieve this purpose, besides other factors the levels of factor XIII (Faktor XIII-Schnelltest, Behringwerke AG Marburg) and antithrombin III (S-2238, Deutsche Kabi Munich) were continuously monitored because both clotting factors are known to be easily degraded by neutrophil elastase in vitro.

Patients. In the clinical trial, patients subjected to major abdominal surgery were included if the operation time exceeded 120 min. Diagnosis of septicemia in the postoperative course was confirmed by prospectively established septic criteria. In the prospective study more than 120 patients were included. Thirty of them fulfilled the defined septic criteria during the postoperative course. Of these patients, fourteen survived the infection (group B) whereas sixteen died as a direct result of septicemia (group B). Eleven patients being without infection after abdominal surgery served as controls (group A) (Fig. 4).

- Defined infection site & pos. bact. culture
- Body temperature > $38.5^{0}C$
- Leukocytosis with > 15 000 cells/mm^3 or
  Leukocytopenia with < 5 000 cells/mm^3
- Platelets < 100 000/mm^3 or drop > 30 %
- (Positive blood culture)

Fig. 4: Prospectively defined sepsis criteria. Diagnosis of septicemia in the postoperative course was confirmed in patients fulfilling all these conditions.

Elastase-α_1PI levels. With the enzyme-linked immunoassay elastase levels between 60 and 110 ng/ml were found in 153 healthy individuals. In patients without preoperative infection (group A and B), the operative trauma was followed by an increase of the E-α_1PI level up to 3-fold of the normal value. Patients suffering from preoperative infections (6 out of 16 in group C) showed already clearly elevated preoperative E-α_1PI levels. Immediately after surgery a slight decrease was observed, probably due to elimination of the infection focus. Before onset of sepsis, the E-α_1PI concentrations of group B and C showed a moderate elevation but no significant changes compared to the postoperative levels. However, at the beginning of septicemia a highly significant increase of the E-α_1PI levels could be detected: up to 6-fold in group B and up to 10-fold in group C. Peak levels were found above 2 500 ng/ml in both groups. The E-α_1PI levels of septic patients who recovered showed a clear tendency towards normal values. In patients with persisting septicemia, high levels of E-α_1PI were measured until death. (Fig. 5).

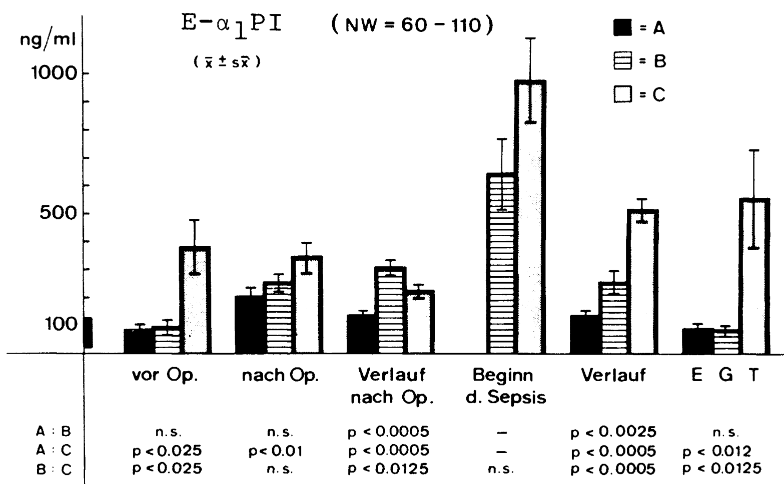

Fig. 5: Plasma levels of elastase-α_1-proteinase inhibitor complex (E-α_1PI) in patients subjected to major abdominal surgery.

A = patients (n=11) being without postoperative infection
B = patients (n=14) surviving postoperative septicemia
C = patients (n=16) dying as a result of septicemia

The E-α_1PI levels are given as mean values ($\pm$ SEM) for the day before operation, the day after operation as well as for the postoperative phase before sepsis, at onset of sepsis and during septicemia. Last determinations were done on day of discharge (D) for group A, on day of recovery (R) for group B, and before death (D or †) for group C.

nr = normal range.

Antithrombin III activity. In non-infected patients the activity of AT III, the most important inhibitor of the clotting system, was in the normal range during the whole observation period. In infected patients, however, the AT III activity was found already below the clinically critical concentration of about 75 % of the standard mean value before onset of septicemia. This low value normalized in all patients overcoming the infection,

whereas a further significant decrease (up to 45 % of the norm) was found in group C patients with lethal outcome. Probably, the extremely low AT III activity in the latter patients, having permanently elevated E-α_1PI levels, may be due to a significant degree to degradation by lysosomal enzymes and especially by elastase.

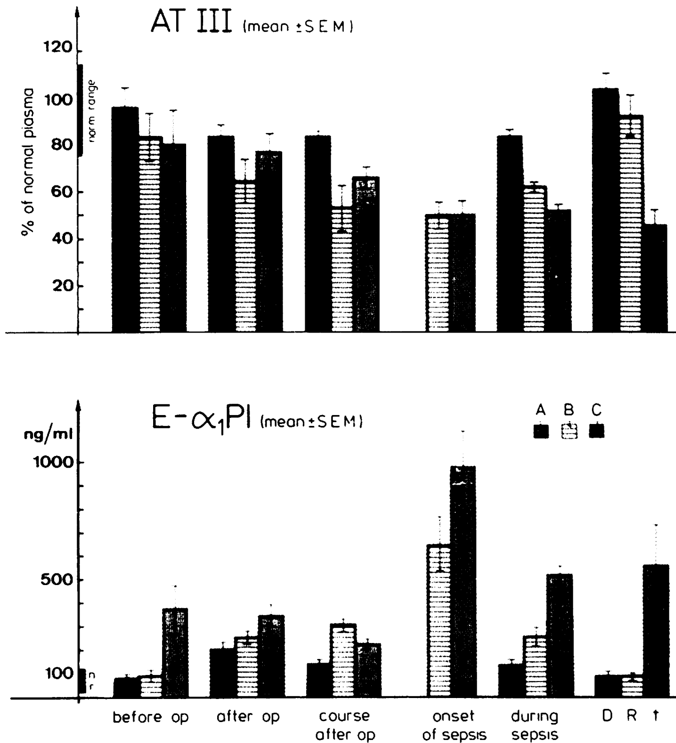

Fig. 6: Plasma levels of the inhibitory activity of antithrombin III (AT III) compared to the amount of the elastase-α_1-proteinase inhibitor complex (E-α_1PI) in patients subjected to major abdominal surgery.
For details, see legend to Fig. 5

<u>Factor XIII activity and A & S subunit levels</u>. Similar results were obtained for F XIII, the fibrin stabilizing coagulation factor. In plasma of patients who did not survive septicemia, the F XIII activity decreased

up to 28 % of the standard mean value. As measured by immunoelectrophoresis, these patients also had very low concentrations of both subunit A, comprising the active enzyme, and subunit S, representing the carrier protein (data not shown). In contrast, group A patients with uncomplicated postoperative course showed normal or only slightly decreased concentrations of subunit S, although subunit A and fibrin stabilizing activities were often significantly reduced.

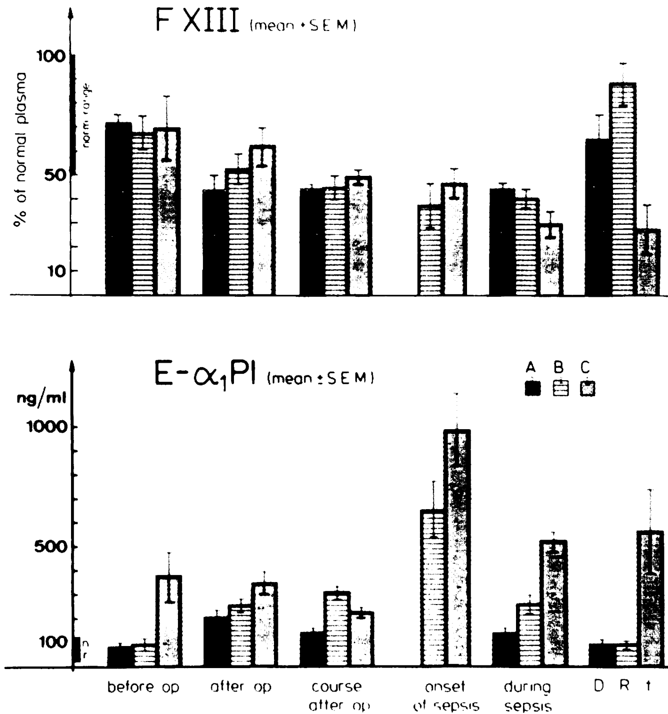

Fig. 7: Plasma levels of the fibrin stabilizing activity of factor XIII (F XIII) compared to the amount of the elastase-α_1-proteinase inhibitor complex (E-α_1PI) in patients subjected to major abdominal surgery.
For details, see legend to Fig. 5

As demonstrated formerly by Egbring and coworkers[6] and Ikematsu and coworkers[14], reduction of both subunits of

F XIII cannot be due to activation of the clotting cascade alone. During clotting, that means by the action of thrombin only subunit A is consumed simultaneously with the F XIII activity but not subunit S. Elastase, however, is able to degrade both subunits to a similar degree. These data and the results presented in our clinical trail suggest that in the patients suffering from septicemia, unspecific proteolytic degradation by granulocytic elastase and/or other lysosomal proteinases is involved to a significant degree in the depletion of F XIII.

Conclusions from the Clinical Studies. The results of the clinical studies show that in inflammatory diseases a correlation exists between the release of a lysosomal enzyme marker, the neutrophil elastase, and the clinical situation of the patient respectively the consumption of selected plasma factors. We take this as a clear indication that liberated lysosomal factors and especially neutrophil proteinases contribute significantly to the inflammatory response of the organism by substrate-u n s p e c i f i c degradation of plasma and other factors. Early application of suitable and potent exogenous proteinase inhibitors should prevent or at least diminish, therefore, such destructive proteolytic processes.

Inhibitor therapy in experimental endotoxemia

To confirm this assumption, we established an endotoxemia model in dogs by intravenous infusion of E. coli endotoxin for 2 hours. Thereby, a significant decrease was observed in the plasma levels of the clotting factors antithrombin III, prothrombin and factor XIII, of the fibrinolysis factors plasminogen and α_2-antiplasmin, and of the complement factor C3. The levels were followed up over an experimental period of fourteen hours and their alterations checked for statistical significance[15] (Fig. 8).

Simultaneous intravenous administration of a relatively specific inhibitor of neutrophil elastase and cathepsin G, the Bowman-Birk inhibitor from soybeans, clearly reduced the endotoxin-induced decline of the tested plasma factors. The 7 000 dalton inhibitory miniprotein was effective in dosages ranging from 3 - 8 mg (i.e. 10 - 25 trypsin inhibiting units) per kilogram body weight. A reasonable assumption would be, therefore, that the endogenous inhibitor was able to prevent or reduce the neutrophil proteinase-induced consumption reactions very effectively.

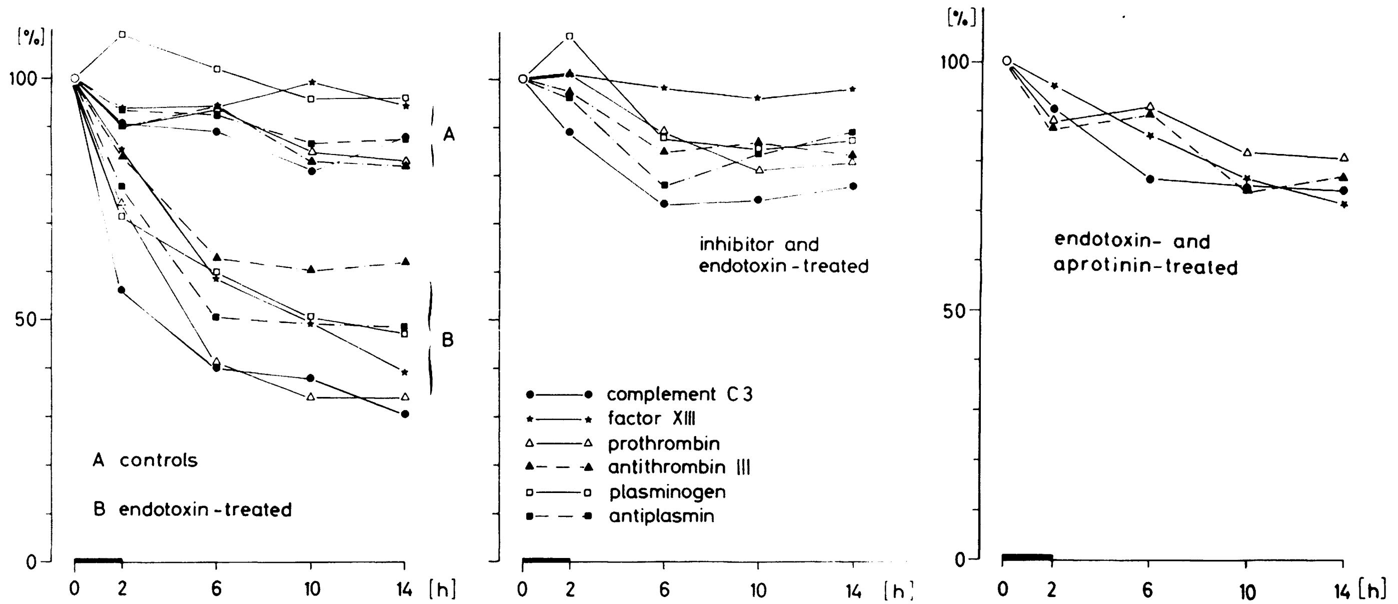

Fig. 8: Changes in the plasma levels of various plasma factors during the acute phase of experimental endotoxemia in dogs (n=6 for each group) without or with inhibitor treatment. Applied inhibitor dosages: 10 - 25 trypsin inhibiting units (Bowman Birk inhibitor) or 80 000 kallikrein inhibitor units (aprotinin) per kg body weight over the observation period of 14 hours. Data are given as mean values in percentage of the individual starting values. Thick line at the abscissa = endotoxin infusion period. For further details see text and ref. 15.

Surprisingly, intravenous administration of the broad-spectrum proteinase inhibitor aprotinin (Trasylol) reduced the endotoxin-induced reduction of the plasma factors to a similar degree (Fig. 8). We must emphasize, however, that the administered aprotinin dose of 80 000 kallikrein inhibitor units (i.e. 9.6 mg of the basic 6 500 dalton miniprotein from bovine organs) per kilogram body weight over the observation period of 14 hours would correspond to 5.6 Mill. kallikrein inhibiting units in the case of a 70 kg patient or to 9.6 Mill KIU/24 h. Although we do not know at present the lowest dosage of aprotinin effective in our animal model, we speculate that a relatively high dose is necessary to inhibit effectively endotoxin-induced activation of the intrinsic clotting cascade and thus also systemic fibrinolysis as well as complement reactions.

GENERAL CONCLUSIONS

In severe inflammatory processes, multiple trauma or shock various cells like neutrophils, macrophages, endothelial cells, and mast cells are stimulated or desintegrated. In this way a high potential of lysosomal enzymes is released of which the proteinases are of special pathogenetic effectiveness. Recent studies in our laboratory and by others indicate strongly that substrate-unspecific proteolysis by lysosomal proteinases and especially by the neutrophil elastase contributes to a significant degree to the consumption and/or degradation of extracellular substances in such diseases. On the other hand, early administration of convenient exogenous inhibitors directed against the lysosomal enzymes and/or plasmin should have a positive therapeutic effect also in humans.

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