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III. Sepsis and Shock

Pathobiochemistry of Sepsis: Role of Proteinases, Proteinase Inhibitors and Oxidizing Agents

M. JOCHUM, J. WITTE*, K.-H. DUSWALD*, D. INTHORN**, H. WELTER*, H. FRITZ

Abteilung für Klinische Chemie und Klinische Biochemie in der *Chirurgischen Klinik Innenstadt der Universität München, Nußbaumstraße 20, und **Chirurgische Klinik Großhadern, D-8000 München, W.-Germany

Summary

Degradation of structural elements and excessive consumption of humoral factors, especially of plasma proteinase inhibitors, by proteolysis and/or oxidation is a major cause of multiple organ failure in sepsis or septic shock. Such pathobiochemical reactions seem to be induced primarily by extracellularly liberated lysosomal proteins from PMN granulocytes (e.g. elastase, cathepsin G, myeloperoxidase, lactoferrin) as well as oxygen radicals produced during extensive phagocytosis. In clinical studies on septicemia and septic shock the consumption of plasma proteins including proteinase inhibitors was inversely correlated to the liberation of lysosomal factors, especially the granulocytic elastase. Administration of relatively specific elastase-cathepsin G-inhibitors (Bowman-Birk inhibitor, eglin) in experimental septicemia proved to be a promising therapeutic approach to reduce consumption of plasma proteinase inhibitors and development of interstitial lung edema in severe inflammation.

Zusammenfassung

Die Zerstörung von Gewebesubstanzen und der übermäßige Verbrauch humoraler Faktoren, insbesondere von Plasma-Proteinaseinhibitoren durch Proteolyse und/oder Oxidation wird als eine wichtige Ursache für das multiple Organversagen in der Sepsis bzw. dem septischen Schock angesehen. Derartige pathobiochemische Prozesse dürften vorwiegend durch extrazellulär freigesetzte lysosomale Proteine (z. B. Elastase, Cathepsin G, Myeloperoxidase, Laktoferrin etc.) und oxidierende Produkte hervorgerufen werden, die während einer verstärkten Phagozytosetätigkeit gebildet werden. In klinischen Studien zur Septikämie bzw. zum septischen Schock war der Verbrauch von Plasmaproteinen einschließlich der Proteinaseinhibitoren invers korreliert zur Freisetzung lysosomaler Faktoren, insbesondere der PMN Elastase. Die Anwendung relativ spezifischer Elastase/Cathepsin G-Inhibitoren (Bowman-Birk-Inhibitor, Eglin) in experimentellen Sepsisstudien erwies sich als vielversprechender therapeutischer Ansatz, dem übermäßigen Verbrauch von Proteinaseinhibitoren und der Ausbildung eines interstitiellen Lungenödems bei schweren entzündlichen Erkrankungen entgegen zu wirken.

Key Words: Antithrombin III, Bowman-Birk inhibitor, cathepsin G, eglin, elastase, factor XIII, inhibitor imbalance, inhibitor therapy, inflammation, lactoferrin, lysosomal factors, α_2-macroglobulin, myeloperoxidase, α_1-proteinase inhibitor, PMN granulocytes, septicemia, septic shock, unspecific proteolysis.

Introduction

Despite of numerous studies concerned with sepsis or septic shock, the pathogenesis of this disease is still discussed controversially. However, evidence accumulates that leukocytes, particularly the polymorphonuclear (PMN) granulocytes, may play a central role in the development of multiple organ failure in severe septicemia. Invasion of PMN granulocytes into the inflamed area is triggered by production of chemoattractants such as leukotrienes, fibrin/ogen degradation products, complement-derived anaphylatoxins (C5a, C3a) etc. as well as by increased vascular permeability due to e.g. liberation of kinins from kininogens. During the attachment to and engulfment of invasive organisms and cell debris in the infectious focus, the granulocytes release various aggressive substances extracellularly (e.g. oxygen radicals, hydrolytic and proteolytic enzymes) which in turn may impair structural elements (basal membranes, elastin, collagen, fibronectin, proteolycans etc.) as well as humoral factors (especially proteins of the "blood systems": clotting, fibrinolysis, complement, and kallikrein-kinin system) [for review of literature, see[1]].

Application of up-to-date biochemical techniques in clinical research revealed very recently that extracellular release of granulocytic products is substantially correlated with the outcome of patients suffering from sepsis or septic shock[2]. Hence, elucidation of the underlying pathomechanisms may provide more successful tools for prophylaxis and therapy.

This report mainly deals with two topics: (i) the considerable consumption of plasma proteinase inhibitors due to augmented liberation of lysosomal proteins and/or enhanced activation of blood system proteinases, (ii) a therapeutic approach to overcome the often *lethal consequences of the proteinase inhibitor deficit* in the organism.

Lysosomal Factors of PMN Granulocytes

Lysosomal factors are liberated from various body cells under pathological conditions. In this respect PMN granulocytes, which are attracted during severe inflammatory processes in high amounts in the primary shock organs (lungs, liver and kidneys), are of special interest. These cells are equipped with a powerful proteolytic, hydrolytic and oxidizing potential in their lysosomes, enabling physiologically the intracellular protein catabolism as well as degradation of phagocytized extracellular material in the phagolysosomes[3].

Of the lysosomal proteinases known so far, *elastase and cathepsin G*, the neutral proteinases from the azurophilic granules of PMN granulocytes are most interesting because of their occurrence in high quantities within the granules and their very low substrate specificity[4,5]. When liberated extracellularly, these proteinases degrade and inactivate various plasma proteins as, for example, the proteinase inhibitors antithrombin III, α_2-plasmin inhibitor and C1-inactivator[6,7], before the enzymes are inhibited by their major antagonists, α_1-proteinase inhibitor, α_1-antichymotrypsin and α_2-macroglobulin. Moreover, an amplification of the elastolytic activity due to the combined action of both elastase and cathepsin G has been reported recently[8].

Myeloperoxidase – also localized in the azurophilic granules – catalyzes the reaction of hydrogen peroxide (H_2O_2) with chloride ions (Cl^-) in the phagolysosomes, thus forming various oxidants which are strongly bactericidal[9].

The antibacterial effect of *lactoferrin*, which is primarily produced in the specific granules of granulocytes (but also in body cells such as glandular epithelial cells), is well established. Patients with recurrent infections as a consequence of lack of specific granules and lactoferrin as well have been described only re-

cently. Moreover, several promoting and inhibiting effects of lactoferrin have been proposed. However, most of the studies could not be confirmed by others and even contradictory results were reported. Hence, at present the functional role of lactoferrin in inflammation is still an open question[10].

Activation and Consumption of Blood Proteins

If the forementioned proteins – especially the proteinases – are released extracellularly, the inflammatory response may be enhanced via two major routes[1]:

Selective or limited proteolysis leads to proenzyme and/or cofactor activation of the blood systems (clotting, fibrinolysis, complement, kallikrein-kinin system) and to the formation of biologically highly potent peptides such as kinins, anaphylatoxins and fibrin/ogen degradation products. *Unspecific proteolysis* especially due to the action of elastase and cathepsin G destroys not only blood system factors, immunoglobulins and many other proteins but also proteinase inhibitors simply by proteolytic digestion.

Normally, selective activation of blood systems and unspecific proteolysis are kept under a well-balanced control by potent and specific plasma proteinase inhibitors[11]:

Antithrombin III (AT III) regulates clotting, *α_2-plasmin inhibitor (α_2PI)* fibrinolysis, and *C1-inactivator (C1-INA)* both, the classical complement pathway and the intrinsic coagulation cascade. The latter is achieved by inhibition of plasma kallikrein and Hageman factor respectively its low molecular weight fragment.

The occurrence of complexes between *α_2-macroglobulin (α_2M)* and plasma kallikrein or plasmin in plasma under certain pathological conditions indicates that this multifunctional glycoprotein is also involved in the regulation of the blood system cascades. However, present evidence suggests a predominant protective role of α_2-macroglobulin in prevention of unspecific proteolysis by inhibition of all types of liberated lysosomal proteinases (serine, thiol, aspartate, metallo proteinases). Due to its high molecular weight, the inhibitory function of α_2M is normally restricted to the vascular bed. *α_1-Proteinase inhibitor (α_1PI)*, the major antagonist of the lysosomal neutrophil elastase, is present in remarkable high concentration in blood, but occurs also in interstitial fluid and mucous secretions. *α_1-Antichymotrypsin (α_1AC)*, a rapidly responding acute phase reactant, is a potent inhibitor of lysosomal neutrophil cathepsin G and mast cell chymase. Compared to α_1PI and α_1AC, the plasma concentrations of the other given inhibitors are clearly lower. Nevertheless, the proteinase inhibitors represent approximately 60% of the plasma proteins other than albumin and the immunoglobulins. This is an indirect indication upon the *significance of proteinase inhibitors as regulatory proteins* of the organism.

Inhibition of activated or liberated proteinases by the given inhibitors means that formation of vasoactive or toxic peptides such as kinins or anaphylatoxins is also depressed. The same is true for proteolysis-induced stimulation of cellular systems like thrombin-induced platelet aggregation or the anaphylatoxin-induced chemotactic response to granulocytes. Therefore, *inactivation of the regulatory proteinase inhibitors of the blood systems by unspecific proteolysis represents one of the most striking pathological effects* caused by lysosomal proteinases.

Even α_1-proteinase inhibitor is proteolytically inactivated by a lysosomal metallo enzyme from macrophages[12], the lysosomal thiol proteinase cathepsin B and a bacterial elastase[11]. Moreover, *oxidation of the methionine residue in the enzyme-reactive site of α_1-proteinase inhibitor* leads to a significant reduction of the affinity of this inhibitor to neutrophil elas-

tase[13]. Such oxidizing agents, for example, superoxide anion, hydroxyl radicals and hydrogen peroxide, are produced in high amounts in the phagolysosomes to facilitate together with myeloperoxidase intracellular protein breakdown. If they are released simultaneously with the lysosomal enzymes under pathological conditions, they may impair locally the inhibition of extracellularly liberated elastase, because the complex formed with oxidized α_1PI is readily dissociated by substrates exhibiting high affinity for elastase (e.g. elastin). This might lead to tissue injury following rapid accumulation of polymorphonuclear granulocytes in the lungs during the inflammatory response.

In view of the pathogenesis of multiple organ failure the imbalance of the physiological equilibrium between proteinases and their inhibitors should be seriously considered as a major reason of the underlying pathological mechanism.

Clinical Studies

a) Hyperdynamic septic shock

Our first clinical study on septicemia was performed on patients (n = 18) suffering from strictly defined hyperdynamic septic shock[14]. Hemodynamic and biochemical parameters were evaluated in suitable time intervals till 96 hours after taking the initial values at diagnosis of hyperdynamic shock ($\bar{x}_0 - \bar{x}_{96}$). Within the observation period 4 patients died, whereas the others survived the shock event. However, all these patients except 1 succumbed within 6 to 84 days after the end of the investigation, either due to direct sequels of shock (respiratory insufficiency, oliguria or anuria) or to toxic heart failure and malignant disease.

Besides various other plasma proteins the concentrations of α_2-macroglobulin were determined by radial immunodiffusion with standardized immunodiffusion plates (Behringwerke, Marburg). Estimation of antithrombin III concentration in plasma was carried out by electro-immunodiffusion according to *Laurell*[15]. The functional activity of the fibrin stabilizing factor XIII was checked by the "Clotting Factor XIII Rapid Reagent" (Behringwerke, Marburg). Native inter-α-trypsin inhibitor (ITI 160000) and its acid stable degradation product (ITI 30000) in serum were detected as described recently[16].

Although the hyperdynamic shock phase is commonly considered to be the initial stage of the disorder, the present results demonstrate that the plasma levels of the measured parameters were already abnormal when the first clinical and hemodynamic signs of septic shock appeared (Tab. 1).

There is good evidence to assume that the *substantial consumption or turnover of plasma proteinase inhibitors* during the observation period is an essential though indirect indication of the considerable liberation of proteinases in septic shock:

Consumption of antithrombin III, the most important inhibitor for maintaining homeostasis of the clotting system[17], normally reflects the liberation of thrombin and factor Xa; their inhibition and elimination is primarily ef-

Table 1 Plasma or serum levels of various proteins in septic shock

Parameters	$\bar{x}_0$ (± SEM)	$\bar{x}_{96}$ (± SEM)
factor XIII (% of normal)	46.1 ± 4.9	52.9 ± 4.8
antithrombin III (% of normal)	47.4 ± 2.8	58.9 ± 5.6
α_2-macroglobulin (% of normal)	48.8 ± 5.2	46.8 ± 4.5
inter-α-trypsin inhibitor (mIU/ml)		
native, acid-labile	42.7 ± 3.6	46.4 ± 3.4
acid-stable	15.7 ± 1.7	21.7 ± 2.5

Values are given as mean $\bar{x}$ (± SEM) for the beginning of the shock phase ($\bar{x}_0$) and after 96 h ($\bar{x}_{96}$).

Normal value for factor XIII, antithrombin III and α_2-macroglobulin: 100% pool plasma

Normal range for inter-α-trypsin inhibitor:
native, acid-labile: 50–80 mIU/ml;
acid-stable: 6– 9 mIU/ml.

mIU = milli inhibitor units

fected by AT III. During heparin medication to prevent thrombosis or disseminated intravascular coagulation, the consumption of AT III is even further intensified, since the heparin-AT III complex also reacts with other activated clotting enzymes or plasma kallikrein[18]. Most remarkably, in patients surviving the shock phase (n = 15), the AT III level increased continuously, whereas in those who died (n = 3) close to the end of the observation period AT III levels decreased dramatically (Fig. 1). Although *in vitro* effects should be transferred to *in vivo* conditions only with reservation, we suppose that at least part of AT III consumption measured in the present trial may be due to proteolysis by granulocytic proteinases[6], especially in the patients who died during the septic shock phase.

The very low level of α_2-macroglobulin throughout the septic shock phase is especially striking. Obviously, considerable amounts of various proteinases were permanently released into the circulation and eliminated by α_2M. Considering this finding, an important function can be ascribed to α_2M in protecting the organism against non-specific proteolysis during septic shock.

In vitro investigations showed that especially granulocytic elastase was able to split off most rapidly an acid-stable inhibitor (ITI 30000) from native inter-α-trypsin inhibitor (ITI 160000)[19]. Since in the present study the level of native ITI was significantly reduced whereas the concentrations of the acid-stable ITI-derived inhibitor was considerably elevated, it may be deduced that granulocytic proteinases are responsible for the prominent turnover of ITI in septic shock and septicemia. Measurement of the ITI turnover could be an indirect marker of leukocytic proteinase activity although the biological function of ITI 160000 or ITI 30000 is not yet clear.

Besides turnover or consumption of several plasma proteinase inhibitors, the significantly low level of the fibrin stabilizing clotting factor XIII may be also indicative of enhanced proteolytic activity due to degranulation of leukocytes. F XIII seems to be a preferred substrate of granulocytic proteinases, since in experimental septicemia the high consumption of this clotting factor could be prevented by previous systemic application of a specific inhibitor of granulocytic elastase and cathepsin G[20]. Moreover, *Egbring* et al.[21] de-

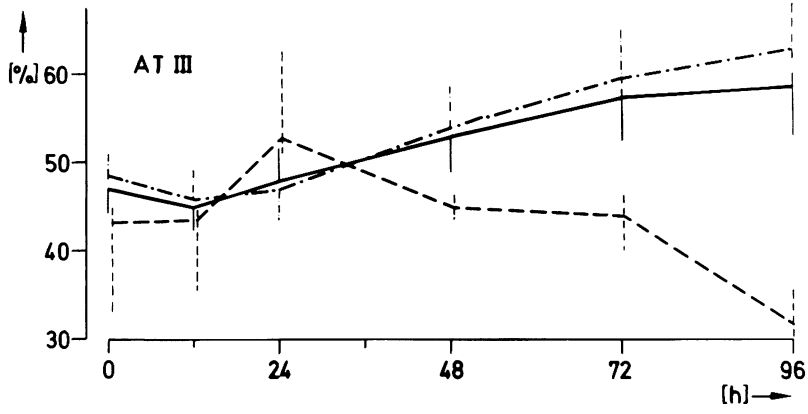

Fig. 1: Plasma levels of antithrombin III (AT III) during 96 h of hyperdynamic septic shock. Indicated are mean values $\bar{x}$ (± SEM) of patients (n = 15) surviving the shock phase (– · – · –), of patients (n = 3) who died close to the end of the observation period (– – – –) and of the whole group (n = 18, ——).
Normal range: 75–100% of pool plasma.

monstrated that in the course of septicemia in man the active transglutaminase (subunit A) and the carrier protein (subunit S) of F XIII were consumed to a comparable degree. The reduction of both subunits, however, is not consistent with the usual consumption of F XIII during blood clotting, since subunit A and F XIII activity disappear completely after coagulation of plasma, whereas subunit S remains unchanged. As the elastase-like granulocytic proteinase (ELP) caused a similar reduction of the activity of both F XIII subunits in plasma[21], it is very likely that proteolysis by ELP is involved in patients with septicemia or septic shock.

b) Major surgery and septicemia

To verify the release of granulocytic proteinases into patient's plasma in severe inflammation, we used the PMN granulocytic elastase as a marker enzyme. The liberated proteinase is present in the circulation primarily in form of the elastase-α_1-proteinase inhibitor (E-α_1PI) complex. A small amount of neutrophil elastase may be bound also to α_2-macroglobulin, however, compared to the E-α_1PI complex the E-α_2M complex is much more rapidly eliminated from the circulation[22].

In the prospective clinical study, plasma was drawn in suitable time intervals from patients subjected to major abdominal surgery, followed either by uncomplicated recovery (group A, n = 11) or by septicemia (groups B, n = 14, and C, n = 16). Patients of group B survived the infection, whereas patients of group C died due to severe septicemia or septic shock[2].

Evaluation of the amount of complexed elastase was performed with an enzyme-linked immunoassay[23] now commercially available (PMN Elastase, E. Merck, Darmstadt). The inhibitory activity of AT III against thrombin was determined using the chromogenic peptide substrate S-2238 (Deutsche Kabi, München). The biological activity of F XIII was assayed with the "Clotting Factor XIII Rapid Reagent" (Behringwerke, Marburg). The inhibitory activity of α_2M was detected with an α_2-Macroglobulin Test Combination (Boehringer, Mannheim) according to *Ganroth*[24].

The changes in the plasma levels of *complexed elastase (E-α_1PI)* are summarized for each group in Figure 2 (lower part):

Patients without postoperative infection showed only a moderate (up to threefold) increase of the preoperative value (60–120 ng/ml)* following surgery, whereas the septic patients exhibited multiple elevated levels in the septic phase. Therefore, blood specimens should be taken at least every 6 to 12 hours during the acute phase of an inflammatory process.

At the time of diagnosis of septicemia highly significantly elevated E-α_1PI levels were measured corresponding to an up to sixfold or even tenfold mean increase in groups B and C. Individual peak levels were found to be as high as 2500 ng/ml in both groups. In patients with persisting septicemia the E-α_1PI levels remained high until lethal outcome (group C), whereas recovery from septicemia was reflected by a simultaneous decrease of the E-α_1PI levels to the normal range (group B).

Concomitant to the increase of complexed elastase in plasma a significant *decrease of the inhibitory activity of AT III and α_2M as well as of the fibrin stabilizing activity of F XIII* could be demonstrated (Fig. 2). The diminished activities of these factors at onset of septicemia were normalized in all patients overcoming the infection (group B), whereas a further significant decrease was found in those who died (group C).

c) Multiple trauma and infection

More recently, we were able to measure the liberation of granulocytic myeloperoxidase

* The preoperative mean value of group C was already elevated: of these patients 6 individuals suffered from defined infections before operation. Surgical removal of the infection focus may have caused the slight mean decrease of the E-α_1PI complex following operation.

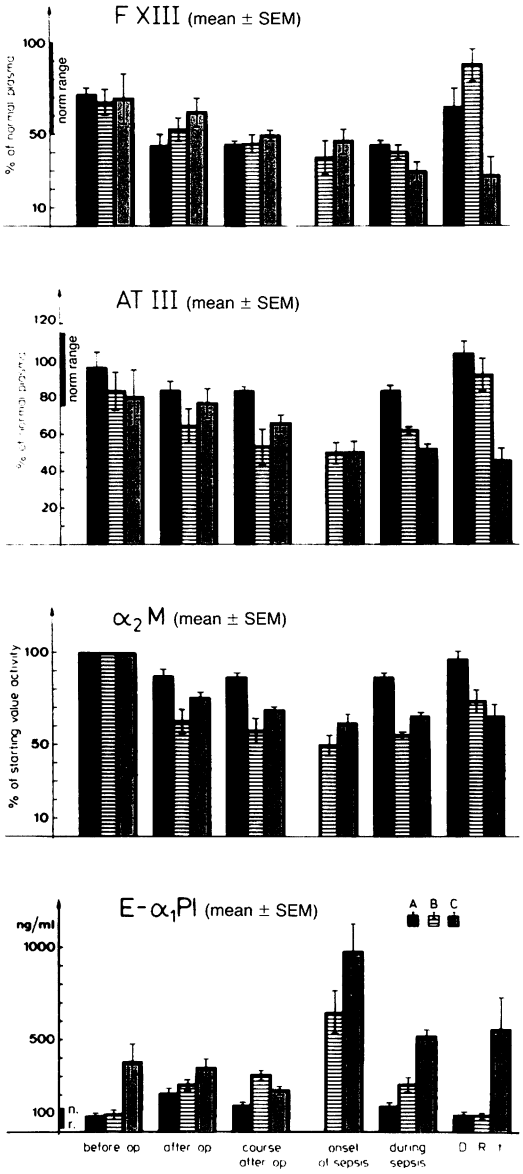

Fig. 2: Plasma levels (mean ± SEM) of elastase-α_1-proteinase inhibitor complex (E-α_1PI), antithrombin III (AT III), α_2-macroglobulin (α_2M) and factor XIII (F XIII) in patients subjected to major abdominal surgery:
A = patients without postoperative infection (n = 11)
B = patients surviving postoperative septicemia (n = 14)
C = patients dying due to septicemia or septic shock (n = 16)

Values are given for the day before operation, the day after operation, the early postoperative phase, at onset of sepsis and during septicemia. Last determinations were performed on day of discharge (D) for group A, on day of recovery (R) for group B and before death (†) for group C. nr = normal range.

and lactoferrin from PMN granulocytes into plasma by specific enzyme-linked immunosorbent assays. These assays have been developed by S. Neumann and W. Rautenberg (Biochemical Research Department, E. Merck, Darmstadt) and are not yet commercially available.

Normal plasma concentrations of myeloperoxidase and lactoferrin were found to be 20–60 ng/ml and 100–300 ng/ml, respectively.

First data of a patient suffering from multiple trauma with lung contusion showed a concomitant release of elastase, myeloperoxidase and lactoferrin (Fig. 3). The severity of the trauma was indicated by the highly elevated levels of all three granulocytic proteins at the time of the first measurement (6 hours post trauma). The subsequent pattern of the plasma levels of all three lysosomal proteins reflected quite perfectly infectious complications (pneumonia) at days 2–3 and 4–5 after trauma as well as the amelioration of the clinical situation of the patient in the posttraumatic course.

Remarkably, a perfect correlation existed in the patient's plasma not only between the azurophilic elastase and myeloperoxidase levels but also between these enzymes and lactoferrin from the specific granules. This suggests, that both types of granules have been equally involved in phagocytosis and extracellular liberation of the lysosomal proteins in the underlying kind of inflammation. Whether the same holds true for other inflammatory processes is currently under investigation.

In general, *elevated plasma levels of granulocytic lysosomal proteins indicate participation of polymorphonuclear granulocytes in an inflammatory event* taking place elsewhere in the organism. The amount of the E-α_1PI complex as well as of myeloperoxidase and lactoferrin seems to reflect the intensity of

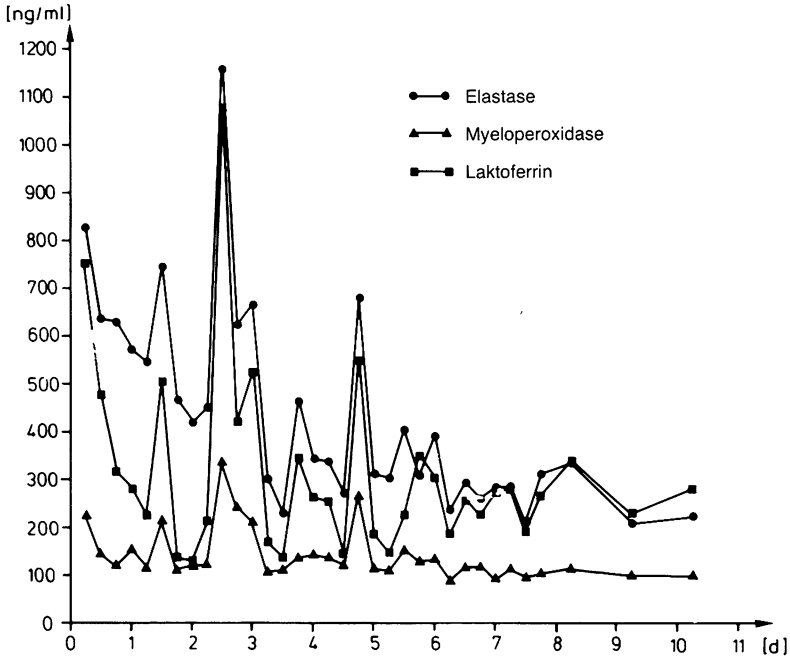

Fig. 3: Plasma levels of complexed elastase (E-α_1PI), myeloperoxidase and lactoferrin in a patient suffering from severe multiple trauma.
Normal range: complexed elastase = 60–120 ng/ml,
myeloperoxidase = 20–60 ng/ml,
lactoferrin = 100–300 ng/ml.

both the inflammatory stimulus and the response thereto of the neutrophils.

In our opinion, lysosomal and other cell-derived proteinases are preferential candidates for establishing a diagnostic pattern of an inflammatory response for mainly two reasons: (i) Due to their high potential for inactivation of plasma proteins and structural elements by degradation or production of inflammatory mediators proteinases represent most interesting tools for elucidation of pathomechanisms and (ii) they offer means for therapeutic approaches with suitable exogenous proteinase inhibitors.

Experimental Animal Studies

Administration of a potent inhibitor of granulocytic elastase and cathepsin G (Bowman-Birk inhibitor from soybeans) diminished considerably the endotoxin-induced consumption of various plasma factors including antithrombin III and factor XIII in dogs[25].

With regard to the role of granulocytic proteinases in inflammatory diseases like septic shock and multiple trauma further therapeutic approaches with generally available proteinase inhibitors seemed to be desirable. Eglin, a potent inhibitor of PMN elastase and cathepsin G[26] from the leech Hirudo medicinalis, is now available by genetic engineering[27] in quantities sufficient for experimental studies (Ciba Geigy, Basel). In order to elucidate the potential therapeutic effectiveness of this miniprotein in severe inflammation we have developed a septicemia model in pigs[28].

Septicemia was induced by i.v. infusion of 3 x 10^{10} E. coli for 2 hours. In another group eglin was simultaneously applied for 4 hours in a dose of 3.85 mg x kg^{-1} x h^{-1} giving rise to plasma levels up to 6 µmol/l.

Compared to the untreated septicemia group with a mean survival time of 6 hours, most of the eglin-treated animals survived the experimental period of 30 hours.

The therapeutic effectiveness of eglin was corroborated by improvement of many of the parameters measured, especially by (a) only modest morphological alterations of the lungs (i.e. less interstitial edema formation), (b) a minor rise in extravascular lung water, (c) *diminished consumption of antithrombin III, factor XIII and α_2-macroglobulin,* (d) and only a retarded and weak increase in body temperature.

In animals with normal kidney function, eglin was excreted in urine to 75–95% within 12 hours. Accumulation in plasma, however, occured in pigs with renal insufficiency due to local microthrombi formation which clearly was not abolished by eglin treatment. Therefore, thrombin inhibitors, e.g. hirudin from the leech, have to be administered in addition to diminish activation of the coagulation cascade under these circumstances[29]. Details of studies in which aprotinin, C1-inactivator and superoxide-dismutase (as a radical scavanger) also showed discrete therapeutic effects will be presented elsewhere.

Conclusion

Summarizing the results of our clinical and experimental animal studies, it is obvious that *in sepsis and septic shock the natural defence mechanisms against augmented proteolysis are considerably overstressed* thus leading to fatal consumption of many vital plasma proteins and to development of multiple organ failure. *Administration of suitable proteinase inhibitors should primarily prevent the exhaustion of endogenous plasma inhibitors.* Until such inhibitors are available for use in humans, substitution with fresh frozen plasma or antithrombin III concentrates[30] seems to be the method of choice.

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