Enzyme Activation in Blood-Perfused Artificial Organs

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T A B L E   O F   C O N T E N T S

PATHOBIOCHEMICAL MECHANISMS LEADING TO ACQUIRED COAGULATION DEFECTS

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SYSTEM-SPECIFIC AND LYSOSOMAL (UNSPECIFICALLY DEGRADING) PRO-
TEINASES

The reaction cascades of humoral systems like clotting,
fibrinolysis and complement are characterized by individual
and highly specific, chiefly enzyme-catalyzed reaction steps.
The cascades are triggered again by system-specific proteinases
with highly restricted substrate specificity, e.g. clotting by
thrombokinases and fibrinolysis by plasminogen activators (cf.
Fig. 1).Such cascades triggering proteinases are normally

Non - lysosomal Proteinases

(high cleavage specificity)	Activation of
o Tissue thrombokinases	➞ clotting
o Plasminogen activators*	➞ fibrinolysis (complement)

* Partly preformed only: synthesis stimulated

FIGURE 1. System-specific, non-lysosomal proteinases
present in the organism or its cells in relatively low concen-
tration but their synthesis and/or excretion may be enhanced
strongly by either an hormonal or inflammatory stimulus (1).
In contrast, lysosomal proteinases are preformed and stored

within the lysosomal granules in fully active form (2). The
lysosomal enzymes (cf. Fig. 2) represent an extremely potent

Lysosomal Proteinases (preformed)

neutral (pH 6–9)	acidic (pH 3–7)
• Elastases	• Cathepsin B
• Cathepsin G	• Cathepsins H, L
° Collagenases	• Cathepsins A, C
° Kininogenases (Kallikreins)	• Cathepsin D (Leukokininogenase)

cleavage specificity: • broad; ° high

FIGURE 2. Lysosomal proteinases of neutrophils

hydrolytic capacity which is responsible for the intracellu-
lar protein breakdown. In this respect lysosomal proteinases

Biological Substrates of Neutral Proteinases* from Neutrophils

Proteoglycans	Elastase, Cathepsin G
Elastin	Elastase, Cathepsin G
Collagen I	Collagenase
Collagen III	Elastase (Collagenase)
Clotting factors	Elastase, Cathepsin G
Fibrinolysis factors	Elastase, Cathepsin G
Complement factors	Elastase, Cathepsin G
Immunoglobulins	Elastase
Transport proteins	Elastase

* Per day approx. 1.5 g synthezid in men

FIGURE 3. Biological substrates of lysosomal pro-
teinases at physiological pH

are able to degrade and thus inactivate either individually or in combination even native proteins and glycoproteins (cf. Fig. 3). Pathobiochemical reactions of both lysosomal and system-specific proteinases are normally prevented by proteinase inhibitors ubiquitously present in the organism (cf. Fig. 4).

Abbr.	Inhibitor	M.W. x 1 000	Mean conc. mg/100 ml	Mean conc. µmol/l
$\alpha_2 M$	α_2-Macroglobulin	725	260	3.6
$\alpha_1 PI$	α_1-Protease·inhibitor	50	260	52
$\alpha_1 AC$	α_1-Antichymotrypsin	70	45	6.4
$\beta_1 CI$	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
$\alpha_2 PI$	α_2-Plasmin inhibitor	70	6	0.9
C1 INA	C1-Inactivator	100	24	2.4

FIGURE 4. Proteinase inhibitors in human plasma

MECHANISM OF ACTIVATION AND CONSUMPTION OF PLASMA FACTORS (cf. Fig. 5)

After cell damage, e.g. by endotoxins or trauma, blood system cascades are activated primarily by system-specific proteinases such as thrombokinases or plasminogen activators. Due to the high selectivity of all reaction steps involved, we would like to suggest for this reaction type the term s p e - c i f i c activation and, in consequence, for the consumption of factors due to specific activation processes the term s p e c i f i c c o n s u m p t i o n. In principal, these reactions are characterized by cleavage of one or only a few peptide bonds in a comparatively huge protein molecule resulting in the formation of active enzymes from proenzymes (e.g. thrombin from prothrombin) or accelerators from cofactors (e.g. factor VIIIa from factor VIII). The primary inhibitors present to prevent exceeding activation of the cascades are antithrom-

bin III (AT III) for clotting, α_2-plasmin inhibitor (α_2PI) for
fibrinolysis and C1 inactivator (C1 INA) for complement and
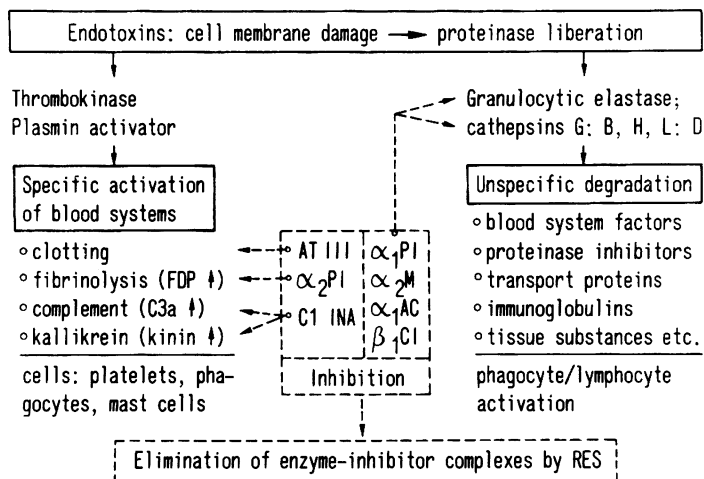kallikrein-kinin system proteinases (cf. Fig. 4 and 5).

FIGURE 5. Activation and consumption reactions:
specific activation of blood systems by system-
specific proteinases (left part); unspecific de-
gradation of plasma factors by lysosomal neutral
proteinases (right part); complex formation with
proteinase inhibitors and elimination of the en-
zyme-inhibitor complexes by phagocytes of the
reticulo endothelial system (RES).

Release of lysosomal proteinases from cells besides the
mentioned activators may lead to inactivation of blood system
factors by u n s p e c i f i c proteolytic degradation and
thus to u n s p e c i f i c c o n s u m p t i o n of plas-
ma factors etc. (cf. Fig. 4). Even proteinase inhibitors like
antithrobmin III (3) and the C1 inactivator might thus be inac-
tivated, for example, by elastase from polymorphonuclear granu-
locytes, but also transport proteins (M. Jochum and S. Lander,
unpublished observations) and immunoglobulins etc.. The primary
inhibitors of lysosomal enzymes are α_1-proteinase inhibitor
(α_1PI) (formerly α_1-antitrypsin, α_1AT), α_1-antichymotrypsin
(α_1AC) and especially α_2-macroglobulin (α_2M). The latter is
able to inhibit nearly all lysosomal proteinases but also sys-

tem-specific proteinases like plasmin, plasma kallikrein and thrombin.

Extensive pathobiochemical reactions by activated system-specific proteinases and/or liberated lysosomal proteinases are normally prevented by the proteinase inhibitors present in excess over the enzymes. Inhibition of the proteinases by the inhibitors occurs relatively rapidly and the stable enzyme-inhibitor complexes thus formed are eliminated within a short time by the cells of the reticulo endothelial system (4).

PATHOBIOCHEMISTRY OF LYSOSOMAL ENZYMES

Lysosomal proteinases are of special interest regarding their pathobiochemical potential because of their high amount present in the organism. Very rich in lysosomes are phagocytes like polymorphonuclear granulocytes (neutrophils) and macrophages but also endothelial cells, mast cells and fibroblasts (cf. Fig. 6). Remarkably, the last four cell types are especially
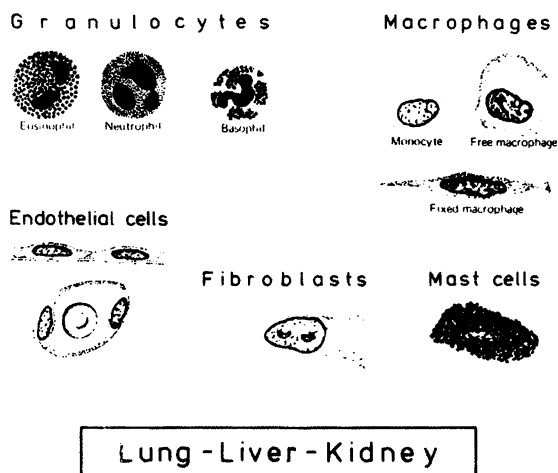

FIGURE 6. Blood and tissue cells with high numbers of lysosomes

concentrated in lungs, liver and kidneys, i.e. in organs known as primary shock organs; above all, the neutrophils accumulate rapidly in the lungs during an inflammatory response. In view of the fact that approximately 1.3 grams of neutral lysosomal

proteinases (primarily elastase and cathepsin G) have to be
inhibited and eliminated per day only from disintegrating neu-
trophils under physiological conditions, it is not surprising
that more than 10 % of the plasma proteins are represented by
proteinase inhibitors. This high inhibitory potential - further
inhibitors are present in cytosol and mucous secretions - is a
clear additional indication for the pathobiochemical effecti-
veness of lysosomal proteinases if released extracellularly
from their membrane-coated organelles, the lysosomes. On the
other hand, a high proteolytic activity in the lysosomes is
necessary in order to fulfil their biological functions:
(1) degradation of all wasted or potentially dangerous endo-
genous substances (intracellular catabolism) and (2) degrada-
tion of invasive organisms after uptake into the phagosomes of
phagocytes (cf. Fig. 7) (5,6,7).
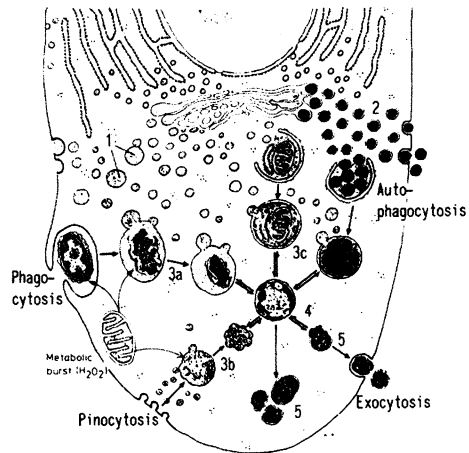
1, primary lysosome
3a. phagolysosome
3b. phagosome
3c, autophagosome

2, secretory granules
4, secundary lysosome
5, residual body

FIGURE 7. Functional significance of lysosomes
within the cell. Substances taken up in phagosomes
by phagocytosis are degraded subsequently by lyso-
somal enzymes. (from R. V. Krstić (ed) Ultrastruk-
tur der Säugetierzelle (Springer-Verlag Berlin 1976),
modified by the authors of this paper)

SIGNIFICANCE OF PROTEINASE INHIBITORS

During a massive inflammatory process both system-specific and lysosomal proteinases are liberated or accumulate extra-cellularly in higher concentration leading to a massive consumption or inactivation of the corresponding proteinase inhibitors (8,9,10). Hence, the inhibitory potential of the organism is diminished and thus in turn the effectiveness of the proteinase-induced pathobiochemical processes is enhanced leading to a progressive consumption of factors of the blood systems. Consequences of these processes are severe clinical symptoms including dissiminated intravascular coagulation. The justification of this assumption is supported by results of a clinical trial described in the following sections.

LYSOSOMAL ELASTASE AS A DIAGNOSTIC MARKER
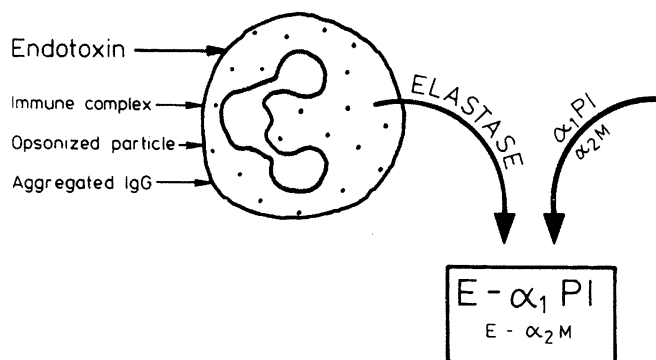
FIGURE 8. Complex formation between liberated lysosomal elastase (E) and α_1-proteinase inhibitor (α_1PI) as well as (to a much less extent) α_2-macroglobulin (α_2M). Whereas the E-α_1PI complex is enzymatically inactive, α_2M-bound elastase is still able to hydrolyse low molecular weight (synthetic) substrates.

Neutrophils are stimulated by endotoxins or other inflammatory stimuli thereby releasing an elastase besides other lyso-

somal constituents. Elastase (E) reacts with various susceptible proteins and finally with native α_1-proteinase inhibitor (α_1PI); the stable enzyme-inhibitor complex (E-α_1PI) thus formed (cf. Fig. 8) is eliminated from the circulation with a half-life time of approximately 1 hour. Using a recently developed enzyme-linked immunoadsorbent assay, it is now possible to estimate the concentration of this complex in blood plasma and other body fluids (11,12). α_1PI is normally present in body fluids in such a high concentration that liberated elastase is inhibited within a short time, i.e. free active elastase is normally not detectable in plasma samples etc. (A small amount of active elastase may be found; this is due to the presence of elastase in the complex with α_2-macroglobulin, where it is still able to catalyse the hydrolysis of synthetic substrates).

LIBERATION OF LYSOSOMAL ELASTASE DURING SEPSIS

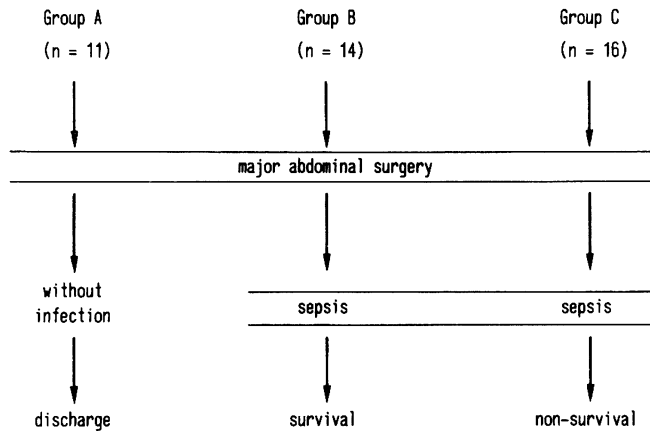| Group A (n = 11)	Group B (n = 14)	Group C (n = 16)
↓	↓	↓
major abdominal surgery		
↓	↓	↓
without infection	sepsis	sepsis
↓	↓	↓
discharge	survival	non-survival

FIGURE 9. Patient groups of the prospective clinical trial and number of patients per group.

In a prospective clinical trial we studied the course of E-α_1PI plasma levels in 3 different groups of patients (cf. Fig. 9) subjected to major abdominal surgery (cf. Fig. 10) (13,14,15):

group A with uncomplicated postoperative course,

group B with postoperative sepsis and recovery,

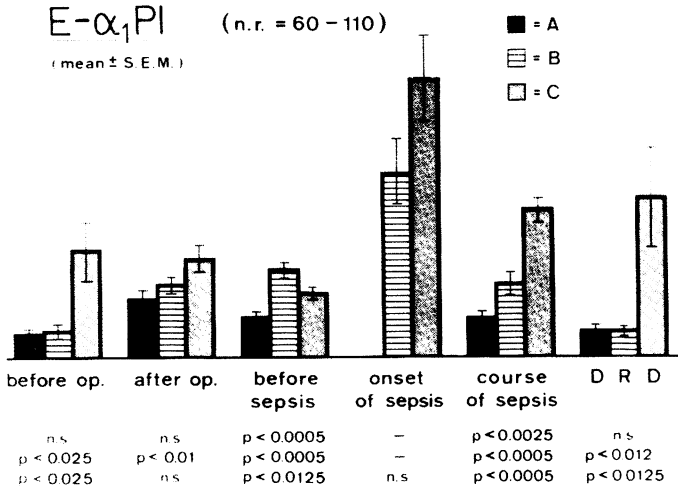group C with postoperative sepsis and lethal outcome.

	before op.	after op.	before sepsis	onset of sepsis	course of sepsis	D R D
	n.s	n.s	$p < 0.0005$	–	$p < 0.0025$	n.s
	$p < 0.025$	$p < 0.01$	$p < 0.0005$	–	$p < 0.0005$	$p < 0.012$
	$p < 0.025$	n.s	$p < 0.0125$	n.s	$p < 0.0005$	$p < 0.0125$

FIGURE 10. Plasma levels of the elastase-$\alpha_1$protein-ase inhibitor complex (E-α_1PI) in patients subjected to major abdominal surgery:
A: patients without postoperative infections (n=11)
B: patients who survived postoperative sepsis (n=14)
C: patients who died due to postoperative sepsis (n=16)
The E-α_1PI levels are given as mean values for the day before operation, the day after operation, the time before sepsis, at onset of sepsis, and for the course of septicemia. Last determinations were done on day of discharge (D) in group A, on day of recovery (R) in group B, and shortly before death (D) in group C.

In patients without infection signs before operation (group A) normal E-α_1PI levels were found (<100 ng/ml). The operative trauma caused a 2 - 3 fold increase in the E-α_1PI level which decreased relatively rapidly towards normal levels during recovery (cf. Fig. 10). Postoperative sepsis (cf. Fig. 11), however, was accompanied with highly significantly elevated E-α_1PI plasma levels which in turn normalized during recovery (group B, Fig. 10).

Some of the patients (n = 6) of group C showed signs of infection already before operation which is reflected in the preoperatively elevated mean level of E-α_1PI (Fig. 10). Post-

Sepsis Criteria

- Defined infection site & pos. bact. culture
- Body temperature > 38.5°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)

FIGURE 11. Criteria used for diagnosis of sepsis

operatively, a slight decrease of the complex concentration was observed which is probably due to elimination of the infection focus in those patients suffering from an infection already before operation. Onset of sepsis is again accompanied with a highly significant increase of the E-α_1PI level and this high level was maintained until lethal outcome. Remarkably, in those patients recovering from sepsis (group B) the E-α_1PI level dropped already significantly during sepsis. This behaviour of the E-α_1PI plasma level might become a prognostic marker for septic patients. However, the validity of this concept has still to be proven in clinical trials with higher numbers of patients.

CORRELATION BETWEEN ELASTASE RELEASE AND CONSUMPTION OF PLASMA FACTORS

From the clinical trial it is evident that the degree of liberation of a lysosomal marker substance from neutrophils may reflect the severity of the inflammatory response of the organism. Therefore, the search for a possible correlation between the plasma level of E-α_1PI and factors of the blood systems as postulated above was of major interest. Indeed, a reverse correlation could be established for the following parameters (cf. Fig. 12): antithrombin III, clotting factor XIII and α_2-macro-

globulin. That means, in the given clinical trial high levels of E-α$_1$PI were accompanied with low levels of these factors and vice versa. Obviously, liberation of high amounts of neutrophil elastase is correlated with a high degree of consumption of plasma factors susceptible to elastase cleavage.

Parameters with High Significance
↑↑ highly significant; ↑ significant; n, not significant

Parameter	Sepsis	Survival	Non-survival
E-α$_1$ PI complex	↑↑	n	↑↑
Antithrombin III	↓↓	n	↓↓
Factor XIII	↓↓	n	↓↓
α$_2$ M concent.	↓↓	n	↓↓
activity	↓↓	n – ↓	↓↓
C-reactive protein	↓↓	n	↓

E: Elastase; α$_2$M: α$_2$-Macroglobulin

FIGURE 12. Correlation between plasma levels of E-α$_1$PI and other plasma factors in the given patients after major abdominal surgery.

The C-reactive protein (CRP), an acute phase reactant, showed a concentration pattern similar to that of E-α$_1$PI; however, CRP proved to be a less specific sepsis indicator compared to E-α$_1$PI (14,15).

RESUMÉ AND FUTURE PROSPECTS

The results of the given and ongoing clinical trials indicate that clotting disorders during sepsis are not due exclusively to the activation of blood systems by or via system-specific proteinases (e.g. thrombokinases, plasminogen activator, intrinsic clotting cascade, etc.). Obviously, lysosomal proteinases released from stimulated inflammatory cells are involved to an appreciable degree in the development of consumption coagulopathy or disseminated intravascular coagulation. The underlying mechanism is unspecific proteolytic degradation leading

to inactivation of various blood system factors (3,4,8,16,17).

The given $E-\alpha_1 PI$ assay is a valuable tool for studying pathological processes in which neutrophils are involved; this concerns besides sepsis and septic shock primarily disorders of the rheumatic type (18) as well as myelogic leukemia (14). In addition, this assay may be used to prove the effectiveness of a proteinase inhibitor therapy (e.g. with aprotinin, the effective ingredient of Trayslol^R) on a broader basis by objectively measurable plasma factors.

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