LOCAL AND SYSTEMIC CHANGES OF ACID PROTEINASES DURING ADVANCED STAGES OF BURN INJURIES

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Abstract. Rats were subjected to burns extending to 15% of their body surface. Enzyme activities were measured by a slightly modified Barrett technique between the 12th and 18th day after the burn injury. A statistically significant activation of acid proteolytic enzymes, in relation to mg protein, was seen in liver, spleen, kidney and stomach. In relation to grams fresh weight, an activation was seen in the kidney. Necrotic skin subsequent to burn injury showed a marked rise in activation compared with the skin of the control group, as well as with the skin of the burned animals. Increased enzyme contents were also detected in the intact skin of burned animals, compared with the skin of the control group.

Thanks to therapeutical progress made in recent decades, cases of death occurring during the early stages of burn injuries are rare. Nevertheless a declining of lethality rate in severe burns could not be observed by Ramirez, Tamondong, DelCastillo & Dino (1970). In most cases death occurs at an advanced stage, between the 10th and 30th day (Schoenenberger, Stödtler, Allgöwer, Burkhart, Müller & Zellner, 1974). Therefore it seemed worth investigating the behaviour of acid proteolytic enzymes during this period. Not only are the local changes in the skin of interest, but changes in important organs of the body are also of consequence.

MATERIALS AND METHODS

We used female Sprague-Dawley rats having an overall weight of 220 g. A third-degree burn was inflicted, extending over 15% of body surface, by contact with hot water (90°C) for a period of 15 sec. On the 10th day we performed a necrectomy. The burned skin areas were kept deep-frozen until biochemical examination. For the treatment of the burn wound we used lyophilized pig skin.

The rats were subsequently kept in individual cages under aseptic conditions. Between the 12th and the 18th day the animals were exsanguinated, the organs taken away and deep-frozen until required for examination.

First the skin was minced with scissors, after which the tissue was homogenized in an Ultra-Turrax apparatus for a total period of 30 sec, cooled with crushed ice. The tissue suspension was centrifugated at 30000*g* for 30 min at 4°C. For the assay the supernatant was used. In order to measure the acid proteolytic activity we modified the method of Barrett (Barrett, 1967), which is based on Anson's procedure (Anson, 1939). As basic reaction, haemoglobin is degraded by the enzyme, with the liberation of peptides soluble in the presence of trichloroacetic acid. For the incubation mixture we used as substrate pure lyophilized bovine haemoglobin, obtained from Serva; we produced an 8% (w/v) solution. A 1 M sodium formate buffer with a pH of 3.0 was used. The assay, which had a final pH of 3.2, contained 0.25 ml sodium formate buffer; 0.25 ml haemoglobin solution; and 0.5 ml enzyme sample. The solution was incubated at 45°C for 90 min and was stopped by the addition of 5 ml of 3% (w/v) trichloroacetic acid. The contents of the tubes were mixed thoroughly and incubated for a further10 min. The solution was filtered with a Selecta filter paper (no. 595 1/2). The filtrate was measured at 280 nm. Reagent blank values were obtained by adding the trichloroacetic acid immediately after the addition of the enzyme sample. Assays were carried out in duplicate. Each test filtrate was accompanied by a blank.

One unit of enzyme activity was the quantity that would have produced an increase in extinction (ΔE_{280}) of 1.0 unit in the assay.

The estimation of the protein content was performed by the Biuret method according to Weichselbaum (Weichselbaum, 1946). The results were evaluated with Student's *t*-test.

Table I. *Alterations in the activity of acid proteinases during late stages of burn injuries, related to mg protein (mU/mg protein)*

	Controls			Burned			
	n	$\bar{x}$	*s*	*n*	$\bar{x}$	*s*	*p*
Liver	13	81	35	10	106	27	≤0.05
Spleen	13	145	36	10	232	88	≤0.01
Kidney	13	174	48	10	255	89	≤0.0125
Stomach	8	426	141	10	898	365	≤0.0025
Skin	13	224	105	10	199	82	≤0.3

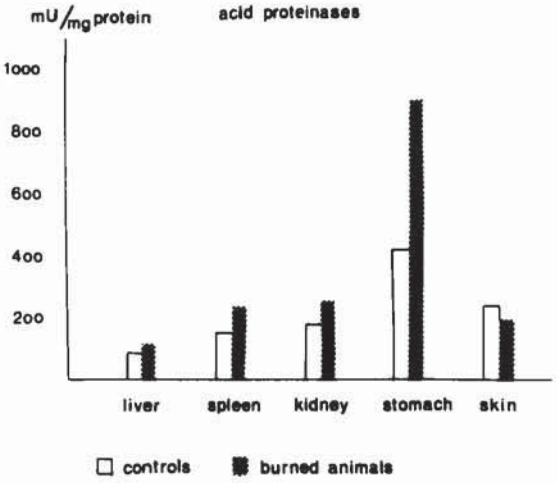

Fig. 1. Changes in acid proteolytic activity late after the burn injury (mU/mg protein).

RESULTS

The method of Barrett is best suited to the estimation of Cathepsin D. Haemoglobin is an excellent substrate for this enzyme. As is known, this is located in the lysosomes. It is a glycoprotein having a molecular weight of 24 000 and belongs systematically to the carboxyl endopeptidases. It digests proteins and peptides of at least five residues. The pH optimum is between 2.8–5.0. The presence of cathepsin D in lysosomes of many cell types and in many species has been amply demonstrated by the methods of subcellular fractionation. The acidic pH optimum of cathepsin D—in common with the lysosomal enzymes—suggests that it is best adapted for a role in the intracellular digestion of proteins, in the acidified environment provided by the lysosomal system (Barrett, 1977). Recently, the availability of antisera against cathepsin D has permitted the microscopical localization of the enzyme (Poole, Hembry & Dingle, 1973; Weston & Poole, 1973). Not only was the enzyme localized intracellularly in the lysosomes, but it could even be detected extracellularly around cells engaged in secreting it (Poole et al.) It has been suggested that the living leukocytes may be able to create a local acidic environment for the extracellular action of the acid proteinases on the vessel wall, as investigations with rabbits showed (Cochrane & Aiken, 1966). An unexpected additional role for cathepsin D in phagocytes is suggested by the recent finding that human enzyme has a bactericidal effect at pH 6.4 which is blocked by pepstatin (Thorne, Oliver & Barrett, 1976). In the analysis, the other enzymes are not of any great importance in comparison with the cathepsin D, as regards the pH optimum, the occurrence and the substrate specificity.

Table II. *Alterations in the activity of acid proteinases during late stages of burn injuries, related to grams fresh weight (U/g fresh weight)*

	Controls			Burned			
	n	$\bar{x}$	s	n	$\bar{x}$	s	p
Liver	13	10.3	1.4	10	11.2	2.1	≤ 0.15
Spleen	13	16.4	2.0	10	16.3	1.7	≤ 0.45
Kidney	13	13.9	1.7	10	16.1	2.0	≤ 0.01
Stomach	8	23.9	2.1	10	23.3	1.0	≤ 0.25
Skin	13	6.8	1.4	10	8.3	1.1	≤ 0.005

Cathepsin B, which belongs to the thiolendopeptidases, has a pH optimum of between 3.5 and 6.0, depending on the substrate and the origin.

With haemoglobin as substrate, the pH optimum ranges from 4.0 to 4.5. Cathepsin B is certainly widely distributed among species. Hitherto, cathepsin H and cathepsin L, both thiolendopeptidases, have been demonstrated in rat liver lysosomes; their pH optima are 6.0 and 5.0 respectively. The analysis of enzyme activities in the stomach presents a particularity, as pepsin exists here with a pH optimum between 1.0 and 2.0.

We used the method mentioned above to examine the enzyme activities in the following organs: liver, spleen, kidney and stomach. Significant changes as

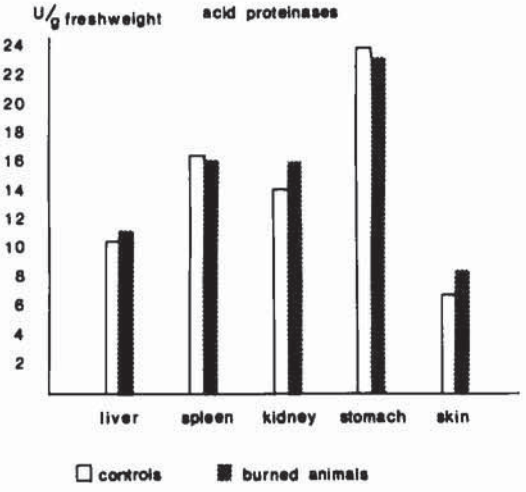

Fig. 2. Changes in acid proteolytic activity late after the burn injury (U/g fresh weight).

Table III. *Comparison of acid proteolytic activity between skin of controls, unburned and burned skin during late stages of burn injuries (mU/mg protein)*

	n	$\bar{x}$	s		n	$\bar{x}$	s	p
Skin of controls	13	224	105	Unburned skin	10	199	82	≤0.30
Skin of controls	13	224	105	Burned skin	10	182	170	≤0.30
Unburned skin	10	199	82	Burned skin	10	182	170	≤0.40

compared with mg protein were found in the liver, the kidney and the stomach. In all organs the activity was clearly elevated in the animals with third-degree burns over 15% of the body surface, as compared with the control animals. The activity in the stomach showed a particularly distinct rise (Fig. 1; Table I). Measured in relation to grams fresh weight, there was also a significant rise in the kidney. The increase in the skin was also evident. The results in the liver showed at least a tendency to an increase, while there were no changes vis-á-vis g fresh weight in the spleen and the stomach (Fig. 2; Table II).

The conditions in the skin are especially interesting. Therefore we compared three groups: (1) the normal skin of control animals, (2) non-burned skin, removed far from the burn wound, and (3) burned skin that was removed during the necrectomy on the 10th day post-burn.

In relation to g fresh weight, both the unburned skin and the burned skin showed a highly significant rise in activity. In the burned skin the activity found was almost twice as high as that in the control animals. On comparing the intact skin of the burned animals with the burned skin, we could discern a significant increase in activity in the burned area. No significant changes were found in relation to mg protein (Tables III; IV).

DISCUSSION

In so far as these results are significant, they all show the same elevated enzyme activity in the burned animals. On a scale representing the percentage rise in activity in relation to g fresh weight, the burned skin occupies top place, with almost 100%. The kidney was the only organ in which the activity in relation to g fresh weight as well as to mg protein was significantly raised.

The highest increases in activity related to mg protein were found in the stomach: viz. about 100%. We must remember that when using this method on this organ, the activity in pepsin is also included. By and large, one can note an increased number of harmful factors to the gastric wall. The spleen, with an increase of 70%, takes second place in the table of activity increase. Considering the abundance of granulocytes with their abundance of lysosomes, this increase can be easily explained. The kidney has a rise in activity of 50% and finally the liver follows with a 25% increase.

As the experimental results show, a raised proteolytic potential exists intralysosomaly and possibly extracellularly. The extracellular increase occurs when there is no local possibility of an inactivation by inhibitors such as α_2-macroglobulin. Supposing that the protein digestion as was shown with rabbit macrophages (Dingle, Poole, Lazarus et al., 1973) were to take place in the area of the burn wound as well, the increase of the proteolytic potential would be a very useful reaction. A possible bactericidal effect of cathepsin D in phagocytes, as was found in the human body, would fit in reasonably with this reaction (Thorne et al.).

According to the results of Dean (1975), cathepsin D plays a major part in the degradation of cel-

Table IV. *Comparison of acid proteolytic activity between skin of controls, unburned and burned skin during late stages of burn injuries (U/g fresh weight)*

	n	$\bar{x}$	s		n	$\bar{x}$	s	p
Skin of controls	13	6.8	1.4	Unburned skin	10	8.3	1.1	≤0.005
Skin of controls	13	6.8	1.4	Burned skin	10	12.2	1.0	≤0.005
Unburned skin	10	8.3	1.1	Burned skin	10	12.2	1.0	≤0.005

lular proteins. The recorded increases in activity at a distance from the wound may be responsible for a raised catabolism.

The significance of cathepsin D in this context is controversial (Huismann, Lanting, Doodema et al., 1974). Huismann is of the opinion that it is mainly thiolendopeptidases (chiefly cathepsin B) which are involved in the intracellular proteolysis.

The observation that polymorphonuclear leukocytes accumulated in the vessel walls of rabbits, causing a destruction of the basement membrane by means of cathepsin D and E is particularly interesting. A rise in permeability to carbon particles and red cells follows the damage. A similar mechanism might account for the damage to glomerular basement membrane (Cochrane et al.). This could be rather important for the earlier stages of burn injury. A no less interesting aspect is the raised activity of the intact skin of burned animals. This means that split thickness skin grafts also have a modified enzyme reaction.

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