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Cathepsin D: A New Marker of the vitality of the Wound

Comparative Study with Histamine and Serotonin

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Summary. We have studied the variation of specific activity of the most important lysosomal enzyme: Cathepsin D. This study has been developed in pig skin incision wounds for its application to the differential diagnosis between vital and postmortem wounds. At the same time, we have determined histamine and serotonine levels to make a comparative study with Cathepsin results.

Our results show the utility of this new marker to determine the vitality of wounds, even when the time elapsed until death has been very short.

Nevertheless, there is no correlation between Cathepsin D results and those of histamine or serotonin, a fact that induces us to think that this new marker is not useful to determine wound age.

Key words: Vitality of wounds, Cathepsin D – Cathepsin D, marker of the vitality of wounds

Zusammenfassung. Wir haben die Variation der spezifischen Aktivität des wichtigsten lysosomalen Enzyms untersucht: Cathepsin D. Diese Untersuchung ist in Schnittwunden in Schweinehaut entwickelt worden im Hinblick auf die Anwendung der Methode zur Differentialdiagnose zwischen vitalen und postmortalen Wunden. Gleichzeitig wurden die Histamin- und Serotonin-Konzentrationen bestimmt, um einen Vergleich mit den Cathepsin-Ergebnissen zu ermöglichen. Unsere Ergebnisse zeigen die Brauchbarkeit dieses neuen Markers, um die Vitalität von Wunden zu bestimmen, auch bei sehr kurzen Überlebenszeiten. Trotzdem besteht keine Korrelation zwischen den Cathepsin-D-Ergebnissen und den Bestimmungen der Histamin- und Serotonin-Gehalte. Das ist ein Befund, der uns daran denken läßt, daß dieser neue Marker nicht zur Wundaltersbestimmung geeignet ist.

Schlüssenwörter: Vitale Reaktion, Cathepsin D – Cathepsin D, ein Marker zur Vitalitätsbestimmung

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Introduction

The use of enzymes as vitality markers is relatively recent. Its validity has been largely demonstrated, especially by Raekallio [1–4], who showed variations of the enzymatic activity at a wounds edge, which make possible the determination of the vitality of the wounds even when the time elapsed between wounding and death has been very short.

In a former work [5], we have studied the behavior of lysosomal enzymes [including Cathepsin D (EC 3.4.23.5.)], based on its participation in processes of protein interchange and destruction of necrotic elements to prepare the tissue reparation that follows the traumatic damage represented by wounds.

In this work we have repeated the same experiments but with different times of evolution. Besides thus, we have determined histamine and serotonin levels in the same pieces of skin where Cathepsin D was studied. Both amines are related with the initial phases of the reaction of the living tissue to the aggression, and are well-known as vitality markers [3, 4, 6]. This is the reason why we have used them as controls and references for Cathepsin D.

Materials and Methods

We have employed ten domestic pigs (average age: 5 months, approximate weight: 120 kg). We made six wounds in the back of every pig; every wound had a different time of evolution (time elapsed between the moment of wounding and the moment of death).

These times were of 0, 10, 20, 30, 60, and 120 min. All the animals were killed by dislocation and, once washed and shaved, we made another wound (postmortem wound) for reference; this wound was placed in a homolateral place of the back.

Wounds were gathered with 5-6 cm of skin around the incision and frozen at −30° C until the moment of study.

Before determinations were performed each wound we divided into the following samples:

1) Vital Wounds
   A Control zone. Undamaged skin.
   B_1 Vital wound edge, until 3 mm.
   B_2 Vital wound edge, from 3 mm.
2) Postmortem wounds
   C_1 Postmortem wound edge, until 3 mm.
   C_2 Postmortem wound edge, from 3 mm.

The determination of Cathepsin D was made according to Turk et al. [7] who modified the original method of Anson (1939). This method is based on the degradative action of Cathepsin D over the hemoglobin in an acid medium.

For this purpose, 1 g dried skin without fat was homogenized in 2 ml buffered NaCl 1% − 1-Buthanol 2%-Triton-X-100 0.1%, keeping a temperature of 4° C. Of this homogenate 100 µl was incubate for 40 min at 37° C with 0.5 ml buffered hemoglobin 2.5% (w/v) in 0.1 *M* sodium acetate (pH: 3.8) − 0.25 ml of buffered 0.1 *M* sodium acetate (pH: 3.8) and distilled water to complete 1 ml. When the time had elapsed, we stopped the incubation with 1 ml buffered trichloroacetic acid 5% keeping this mix at 4° C for 10 min. Then it was centrifuged for 20 min at 5,000 rpm, and the supernatant was filtered with Whatman paper no. 4. The resultant was measured at 280 nm with a Beckman model 25 spectrophotometer.

These results were compared with those obtained after measuring a tyrosine curve activity as Turk [7] explained.

Specific activity (SA) was finally calculated in relation to the amount of proteins, which was made using the classical method of Lowry [8].

Histamine was determined according to Shore et al. [9] who developed a method based on the butanolic extraction of histamine; this butanolic layer is further mixed with NaOH and heptane to eliminate contaminants and similar products; finally, histamine is extracted with HCl 0.1 *N* and condensed with Ortho-phtalal-aldehyde (OPT) in acid medium, resulting in a strong fluorescence that was measured with a Perkin-Elmer model MPF 43 A Spectrofluorimeter (Exc. 360 nm; Em. 450 nm). For their calculation, results were compared with a standard curve made with histamine (Sigma R).

To determine serotonin, we have followed the method used by Raekallio and Mäkinen [4] who made an slight modification on to the original method of Udenfriend et al. [10]. This method is based on the use of the native fluorescence of serotonin in acid medium (pH: 2.5) which can be measured with a Spectrofluorimeter. We have used a Perkin-Elmer type, model MPF 43 A. Results were compared, for their calculation, with a standard curve made with serotonin (Sigma R).

The statistical study of the results was carried out with Student's *t*-test for coupled samples to compare differences between diverse zones. Additionally we made tests of lineal regression and correlation to compare the evolution of histamine and serotonin levels with those of Cathepsin D.

The anatomopathologic study was carried out in samples conserved in formalin adjusted to pH: 7.5 with calcium carbonate. Subsequently, samples were included in paraplast and stained with HE to be observed with an optic microscope model HM-LUX.

Results and Discussion

Results are expressed in Table 1, and the graphic representations are in Fig. 1–3.

Results present an excellent statistical signification in all the series studied (Table 2) except in the one of 0 min of evolution; this confirms the value of this enzyme as vitality marker.

This evident statistical signification, even in the series of short evolution, has a logic explanation according to a wound's physiopathology: after tissue damage appears necrosis, with an accumulation of lactic acid that causes a great acidification of the medium; from this acidification arises a lysosomal instability with final liberation of the enzymes that they contain [11]. In the early phases, Cathepsin D degrades proteins and helps the intracellular digestion [12]. This intervention can be easily detected because there is a lot of this protease in most of the tissues, including skin [13].

Results of histamine and serotonin are as expected. Their evolution with time are also according to the studies of some authors [4, 6] (Fig. 2, 3).

Concentration increase of the markers employed (histamine, serotonin, and Cathepsin D) have appeared in the two zones close to the wound that we have studied: B_1 and B_2 (Fig. 1–3). Nevertheless, we consider that zone B_1 (vital wound edge, until 3 mm) is the best one to study for this kind of markers for two main reasons: highest increases of all the markers have taken place in this zone, and the anatomopathologic phenomena (polynuclear cell infiltration, diapedesis, and exudation) are also more important in this zone.

Correlation studies (Table 3) show significant results between histamine and serotonin in all the series, which has an easy explanation because both are amines with a similar physiopathologic role.

Table 1. Mean values ($n = 10$) and SD for Cathepsin D, histamine, and serotonin in all the different times of evolution. (A = control zone; B_1 and B_2 = vital wound; C_1 and C_2 = postmortem wound)

Time of evolution								
Zone	0 min	10 min	20 min	30 min	60 min	120 min	Zone	P.M.
Cathepsin D (SA)								
B_1	0.40 ± 0.06	0.56 ± 0.08	1.02 ± 0.12	0.73 ± 0.08	0.78 ± 0.008	0.74 ± 0.07		
B_2	0.38 ± 0.05	0.61 ± 0.07	0.98 ± 0.08	0.70 ± 0.08	0.73 ± 0.07	0.71 ± 0.07	C_1	0.35 ± 0.03
A	0.35 ± 0.03	0.37 ± 0.03	0.38 ± 0.03	0.37 ± 0.03	0.37 ± 0.03	0.37 ± 0.03	C_2	0.34 ± 0.03
Histamine (μg/g)								
B_1	0.75 ± 0.23	0.98 ± 0.19	1.33 ± 0.34	1.14 ± 0.31	0.78 ± 0.22	0.72 ± 0.19		
B_2	0.74 ± 0.20	0.92 ± 0.16	1.28 ± 0.29	1.06 ± 0.25	0.76 ± 0.23	0.69 ± 0.18	C_1	0.41 ± 0.09
A	0.57 ± 0.11	0.62 ± 0.15	0.69 ± 0.17	0.57 ± 0.13	0.52 ± 0.11	0.51 ± 0.10	C_2	0.38 ± 0.09
Serotonin (μg/g)								
B_1	5.32 ± 1.16	12.3 ± 2.84	9.16 ± 2.18	5.22 ± 1.36	6.16 ± 1.88	6.62 ± 1.31		
B_2	5.13 ± 1.12	8.57 ± 2.32	8.31 ± 1.58	4.71 ± 1.28	5.58 ± 1.91	6.18 ± 1.20	C_1	4.12 ± 0.83
A	4.52 ± 0.83	4.62 ± 0.98	4.61 ± 1.11	3.82 ± 0.83	4.16 ± 0.94	4.12 ± 0.87	C_2	4.06 ± 0.77

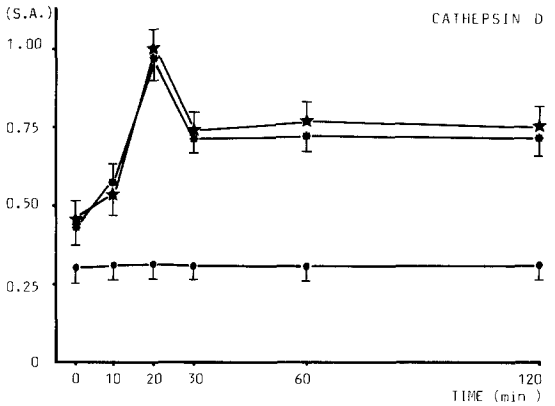

Fig. 1. Evolution of Cathepsin D levels with time in all the zones (B_1, B_2, and A) of the vital wounds B_1: ★; B_2: ❋; A: ●

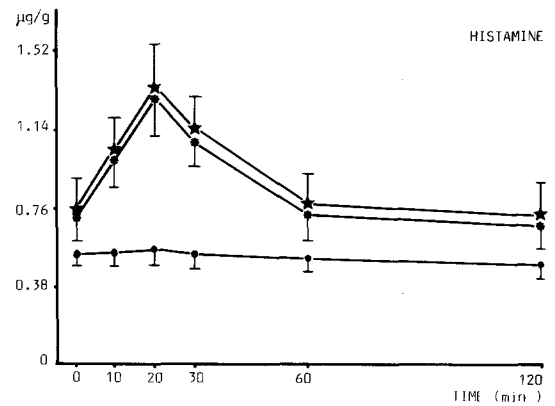

Fig. 2. Evolution of histamine levels along the time in all the zones (B_1, B_2, and A) of the vital wounds B_1: ★; B_2: ❋; A: ●

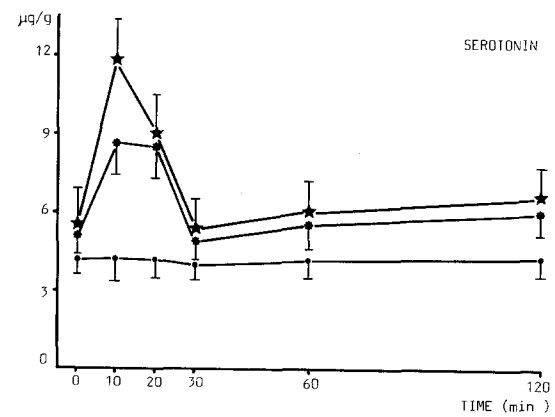

Fig. 3. Evolution of serotonin levels along the time in all the zones (B_1, B_2, and A) of the vital wounds B_1: ★; B_2: ❋; A: ●

Table 2. Series with Statistical Signification

Marker	Series (time in min)	Statistical signification
Cathepsin D	10, 20, 30, 60, 120	$P < 0.01$
Histamine	0, 10, 20, 30, 60, 120	$P < 0.01$
Serotonin	0, 10, 20, 30, 60, 120	$P < 0.01$

Table 3. Correlations with statistical signification among the different markers used

Histamine/Serotonin			
0 min	$r = 0.903$	$t_{exp} = 5.952$	$P < 0.001$
10 min	$r = 0.657$	$t_{exp} = 2.469$	$P < 0.050$
20 min	$r = 0.756$	$t_{exp} = 3.272$	$P < 0.020$
30 min	$r = 0.837$	$t_{exp} = 4.342$	$P < 0.010$
60 min	$r = 0.513$	$t_{exp} = 2.146$	$P < 0.100$
120 min	$r = 0.604$	$t_{exp} = 2.732$	$P < 0.100$
Histamine/Cathepsin D			
60 min	$r = 0.550$	$t_{exp} = 1.866$	$P < 0.050$
120 min	$r = 0.557$	$t_{exp} = 1.899$	$P < 0.050$
Serotonin/Cathepsin D			
30 min	$r = 0.693$	$t_{exp} = 2.724$	$P < 0.050$

We have also found correlation between histamine and Cathepsin D in the series of 60 and 120 min, and between serotonin and Cathepsin D in the serie of 30 min. We think we should not try to find an exact correlation among these markers; thus, we think that they are all elements that have a parallel intervention in the same process, the inflammatory reaction, but without a direct relation among themselves.

In conclusion, we can confirm the great utility of Cathepsin D as wound vitality marker, even in those of very short evolution. These results need confirmation on human skin, studies that will be performed as soon as possible.

On the other hand, the negative correlation between Cathepsin D and histamine or serotonin in the series that we have studied can permit us to insinuate that this enzyme is not useful to determine a wound's time of origin.

The use of histamine and serotonin, as we all know mainly from Raekallio [4], is useful to make differential diagnoses between vital and postmortem wounds and also to determine wound age.

References

1. Raekallio J (1961) Histochemical studies in vital and postmortem skin wounds. Merkatorin Kirjapaino, Helsinki

2. Raekallio J (1965) Die Altersbestimmung Mechanismus bedingter Hautwunden mit enzymhistochemischen Methoden. Max-Schmidt-Römhild, Lübeck
3. Raekallio J (1971) Enzyme histochemistry of wound healing. Gustav Fischer, Stuttgart
4. Raekallio J, Mäkinen PL (1969) Serotonin and histamine – content as vital reaction. Zacchia 46:80–87
5. Hernandez-Cueto C (1984) Study of Cathepsin A and D and Ca, Mg, Cu, Zn, Fe, Na and K concentrations in wound edges. Its application to differential diagnosis between vital and postmortem injuries. J Forensic Sci Soc 24:301–302
6. Berg S (1972) Die Alterbestimmung von Hautverletzungen. Z Rechtsmed 70:121–135
7. Turk V, Tamara L, Kregar I (1984) Cathepsin D, Cathepsin E. Methods of enzymatic analysis, 3rd edn, vol 5. Verlag Chemie, Weinheim
8. Lowry OH, Rossbrough NJ, Farr AL, Randall RS (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
9. Shore PA, Burkhalter A, Cohn VH (1959) A method for the fluorimetric assay of histamine in tissues. J Pharmacol Exp Ther 127:182–186
10. Udenfriend S, Weissbach H, Clark CT (1955) The determination of 5-hydroxytryptamine (serotonin) in biologic tissues. J Biol Chem 215:337–344
11. Dingle JT, Barret AJ, Weston PD (1971) Cathepsin D characteristics of immunoinhibition and the confirmation of a role in cartilage breakdown. Biochem J 123:1–13
12. Ricciutti MA (1972) Myocardial lysosomes stability in the early stages of acute ischemic injury. Am J Cardiol 30:492–497
13. Lazarus GS, Poole AR (1975) Immunocytochemical localization of Cathepsin D in rabbit skin. Arch Dermatol 111:1150–1153

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