The Application of Histochemical Methods to the Age Evaluation of Skin Wounds
Experimental Study in Rabbits

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Enzyme histochemical methods allow determination of wound age, especially in the range of a few hours, and are used to distinguish between postmortem and antemortem skin wounds. The methods are based on the determination of the presence and changes of the enzyme reaction in the wound area. Increased activity of nonspecific esterases was observed approximately 1 hour after wounding and was followed by an increase in adenosine triphosphatase at approximately 2 hours and alkaline phosphatase at approximately 3.5 hours. Maximum enzyme activity was reached for nonspecific esterases at 24 hours, for adenosine triphosphatase at 20 hours, and for alkaline phosphatase at 32 hours after wounding.

Key Words: Wound age evaluation—Enzyme histochemistry—Alkaline phosphatase—Adenosine triphosphatase—Nonspecific esterases—Rabbits.

The determination of wound age as well as the distinction between antemortem and postmortem wounds, both in humans and in animals, has been one of the most important medicolegal problems (1, 2). Cohnheim in 1867 was the first to discuss this subject, basing his research on observations on the evolution of wound healing (1). During wound healing, histologic and enzyme processes take place that are visible microscopically and histochemically. Researchers who have dealt with wound age estimation (1–5) have used several histochemical, biochemical, histologic, and immunohistochemical methods.

Histochemical methods define wound age according to the changes in enzyme activity in the wound area, biochemical methods determine the values of histamine and serotonin, and histologic methods determine the age of the wound according to the chronological order of appearance of the cellular elements (neutrophils, granulocytes, macrophages, lymphocytes, etc.) (1, 2, 6, 7). The histologic methods are simple to apply but have the disadvantage of giving clear results 8–16 hours after the injury occurs. Immunohistochemical methods (determination of fibronectin, tenascin, and collagen types I, III, VI, and V) may also be used to determine the age of the human skin wound (8–10).

MATERIALS AND METHODS

Treatment of Experimental Animals

Seventy-four New Zealand white rabbits (50 female and 24 male, 1 to 42 months old) were used (Fig. 1). Incisions 0.5 to 2.0 cm long were inflicted on the skin of rabbits after local anesthesia. The specimens were taken after the rabbits were killed or while they were under general anesthesia. A total of 356 antemortem skin wounds were examined, in which the after infliction intervals ranged from 0.5 to 144 hours (0.5, 1, 1.5, 2, 2.5,
In 46 of the antemortem wound cases, the specimens were removed between 6 and 72 hours after the death of the rabbits. Additionally, 86 postmortem skin wounds were examined. The specimens were fixed in liquid nitrogen immediately after they were taken, and were cut in 10-μm sections in cryostat at −20°C and immediately after the histochemical examination was performed (11,12). The enzymatic activity of nonspecific esterases, adenosine triphosphatase (ATPase), and alkaline phosphatase was investigated. Additionally, each specimen was stained by hematoxylin and eosin to detect the typical morphology of the lesions in the wound area.

The control specimens were tissues from injuries incubated without substrate.

Alkaline Phosphatase
A total of 256 antemortem skin wounds were examined. The azo dye coupling method was used for the histochemical determination of alkaline phosphatase activity (13,14). The reagents were sodium a-naphthyl phosphate 10 mg, Tris buffer 0.1 mol/l (stock solution) pH 10.0 10 ml, diazonium salt fast red TR 10 mg. The pH of the incubating medium was 9.2. The specimens were incubated at room temperature for 10 to 60 minutes, washed in distilled water, counterstained in 2% methyl green solution (chloroform extracted), washed with running tap water, and mounted in glycerin jelly. Alkaline phosphatase activity appeared as a reddish brown stain.

Nonspecific Esterases
A total of 221 antemortem skin wounds were examined. The Nachlas and Seligman method modified by Pearse (2, 5) was used for the histochemical determination of nonspecific esterase activity. The reagents were sodium a-naphthyl acetate 10 mg, acetone 0.25 ml, phosphate buffer 0.1 mol/l (stock solution) pH 7.4 10 ml, and diazonium salt fast blue B salt 50 mg. The specimens were incubated at room temperature for approximately 5 minutes, washed in distilled water for 5 minutes, and mounted in glycerin jelly. The nonspecific esterase activity appeared as a reddish brown stain.

Adenosine Triphosphatase
A total of 136 antemortem skin wounds were examined. The Wachstein and Meisel method modified by Pearse (13) was used for the histochemical determination of ATPase activity. The reagents were ATP disodium salt 2 mg, Tris buffer 0.2 mol/l pH 7.2 0.4 ml, lead nitrate 2% 0.1 ml, magnesium sulfate 2% 0.1 ml, distilled water 0.5 ml, and 2,4-dinitrophenol 1.5 mg. The specimens were incubated at 37°C for approximately 60 minutes, washed in distilled water, immersed in 1% ammonium sulfide for 1 minute, rinsed under running tap water, and mounted in glycerin jelly. ATPase activity appeared as a brownish black deposit.

The enzyme activity of alkaline phosphatase, of nonspecific esterases, and of ATPase was determined in the postmortem skin wounds by the histochemical methods used for the determination of antemortem skin wounds.

RESULTS

Antemortem Wounds
A total of 356 antemortem wounds were examined.

Alkaline Phosphatase
In normal skin, the epidermal layer showed no staining. In the skin appendages, vessel walls, and dermal fibroblasts, positive reactions were observed. In the vital skin wounds, the increase in alkaline phosphatase activity appeared approximately 3.5 hours after wounding. Maximum enzyme activity was reached for alkaline
phosphatase approximately 32 hours after wounding, whereas 1.6% of the examined specimens gave a negative result (Table 1, Fig. 2).

**Nonspecific Esterases**

In normal skin, there was a site of intense esterase activity between the stratum granulosum and the stratum corneum. The root sheaths, the upper bulbs of the active hair follicles, and the dermal fibroblasts showed strong reactions. In the vital skin wounds, the increased activity of nonspecific esterases appeared approximately 1 hour after wounding. Maximum enzyme activity was reached for nonspecific esterases approximately 24 hours after wounding, whereas 1.4% of the examined specimens gave a negative result (Table 1, Fig. 2, Fig. 3, and Fig. 4).

**Adenosine triphosphatase**

In the normal skin, the skin appendages, vessel walls, musculi arrectores pilorum, and dermal fibroblasts gave positive reactions. A moderate reaction was detectable in the stratum granulosum and stratum basale. In the vital skin wounds, the increased ATPase activity appeared approximately 2 hours after wounding. Maximum enzyme activity was reached for ATPase approximately 20 hours after wounding, whereas 0.8% of the examined specimens gave a negative result (Table 1, Fig. 2).

Forty-six sections of the 356 total antemortem skin wounds were sampled for the enzyme histochemical examination 6 to 72 hours after the rabbits were killed. They also showed changes in the activity of alkaline phosphatase, nonspecific esterases, and ATPase similar to those in the 310 antemortem skin wounds.

**Postmortem Wounds**

Sections from 86 postmortem wounds were examined and showed no increase in enzyme activity.

Our results clearly showed that the intensity and the appearance of enzyme activity did not depend on the sex and the age of rabbits.

**DISCUSSION**

Two zones may be histologically distinguished around the antemortem wound.

In the central zone, a decrease in the vitality of the cells of the connective tissue (negative vital reaction) appears in the immediate vicinity of the wound edge. This comes as a consequence of the mechanical damage caused by the injury and the reduction of the blood supply caused by the local destruction of the blood vessels and inflammation. The cells of that area show a progressive loss of enzyme activity 1 to 4 hours after the injury occurs. All the above changes are considered to be early signs of the forthcoming necrosis in the central zone of the trauma.

In the peripheral zone, a significant increase in enzyme activity occurs (positive vital reaction). Both the activity and the quantity of the enzymes increase, as is shown by quantitative measurements of aminopeptidases and phosphatases. This increase comes from the cells in the peripheral zone of the traumatized area, from the plasma outside the blood vessels, and from the lym-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Wounds examined</th>
<th>Earliest appearance</th>
<th>Maximum enzyme activity</th>
<th>% negative reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>256 (72%)</td>
<td>3.5 ± 0.1 h</td>
<td>32 ± 0.2 h</td>
<td>1.6</td>
</tr>
<tr>
<td>Adenosine triphosphatase</td>
<td>136 (39%)</td>
<td>2.0 ± 0.1 h</td>
<td>20 ± 0.2 h</td>
<td>0.8</td>
</tr>
<tr>
<td>Nonspecific esterases</td>
<td>221 (63%)</td>
<td>1.0 ± 0.1 h</td>
<td>24 ± 0.2 h</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**FIG. 2.** Time of appearance and of maximum intensity of enzyme reactions in antemortem skin wounds in rabbits.

**FIG. 3.** Activity of nonspecific esterases 24 hours after wounding (Nachlas and Seligman method, modified by Pearse) (×10).
yses were repeated, the number of negative results was nonspecific esterases, and 2 for ATPase. When the analyses were originally performed, we obtained 6 negative results for alkaline phosphatase, 4 for nonspecific esterases, and 0.8%, or 1 specimen of 136 for alkaline phosphatase; 1.4%, or 3 specimens of 221 for nonspecific esterases; and 0.8%, or 1 specimen of 136 for alkaline phosphatase. It should be mentioned that in all negative result cases the experiment was repeated. In our study, ATPase gave a positive result. The advantage of the methods used in our experiments is that the changes in enzyme activity were reliable up to 72 hours after death (2).

In our experiments, no enzymatic reactions were given by the postmortem wounds, although some researchers maintain that there may be a small increase of the nonspecific esterases in postmortem wounds (1,2,6,7).

Some authors accept that young human skin wounds give more pronounced enzymatic reactions because the healing time is shorter (1,2). That was not observed in our experiments in rabbits. Bleeding, cachexia, cold environment, and cranial fractures do not delay the inflammatory reactions (1,2,4). Some other investigators have studied burns using enzymatic histochemistry (16–18).

Because the above methods are basically qualitative, it is important to use the appropriate method so that the coloring is as intense as possible and the result is easily detectable (19). Comparison of our results given by the azo dye coupling and naphthol AS-B1 methods, in the case of alkaline phosphatase, confirms the above statement. In a time interval of 3.5 hours after wound infliction, the first method gave a clearly positive result, and the second method gave results that were difficult to distinguish. A similar result was observed by the comparison of the Nachlas-Seligman and Pearse methods for the nonspecific esterases. In our experiment, the renewal of the diazonium salts and the buffers used every 6 months was essential because it had an immediate result on the intensity of the coloring of the enzyme reaction (7,13).

The above methods of enzymatic histochemistry are considered to be reliable, and their contribution to the medicolegal determination of wound age and the differentiation between antemortem and postmortem skin wounds in combination with the rest of the necropsy findings is crucial.

REFERENCES


