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Histochemical Studies of Wound Healing in the Hair-loss Mouse

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Summary. Adult female hair-loss (hl) mice (Mus musculus) were given incised wounds through the full thickness of the dorsal skin. At specified intervals thereafter (i. e., 1, 2, 4, 6, 12, and 20 hours), the animals were sacrificed and the wound sites were excised, processed, and sectioned. Enzyme and carbohydrate histochemical procedures were carried out on frozen and paraffin sections respectively. Nonspecific esterase was demonstrated in one hour wounds and a slight increase was observed through six hours. No other enzyme histochemical procedure gave a satisfactory reaction in the wound site. The Periodic Acid-Schiff reaction for carbohydrates revealed a steadily increasing reaction from one to six hours and a decrease in intensity by twelve hours. The causative agent for the PAS reaction may be serum glycoproteins transported to the wound site as part of the inflammatory process.

Key words: Wound healing, histochemical studies, wound healing.

Zusammenfassung. Erwachsenen weiblichen Mäusen (Mus musculus) wurde die dorsale Haut durchgeschnitten. Nach gegebenen Zeiten (1, 2, 4, 6, 12 und 20 Stunden) wurden die Tiere getötet und die Wunden exzidiert, vorbereitet und geschnitten. Die Fermenthistochemie wurde an Gefrierschnitten durchgeführt, während die Histochemie der Kohlehydrate an Paraffinschnitte ausgeführt wurde. Die Esterase konnte bei einem Wundalter von einer Stunde dargestellt werden; sie war ein bißchen stärker in 6 Stunden alten Wunden. Keine andere Fermenthistochemie, die an den Präparaten durchgeführt wurde, ergab befriedigende Resultate. Die sogenannte "Periodic acid-Schiff"-Reaktion für Kohlehydrate zeigte eine regelhafte Verstärkung in den 1 bis 6 Stunden alten Wunden; dann nahm die Intensität in den 12 Stunden alten Wunden ab. Die „PAS"-Reaktion könnte dadurch entstanden sein, daß die Serum-glykoproteine durch Entzündungsprozesse in die Wunden gelangten.

Schlüsselwörter: Wundheilung, histochemische Untersuchung – Histochemie, Wundheilung.

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The problem of age estimation of wounds is a significant one in forensic science. One of the most important considerations in the investigation of a violent death is whether or not a given wound was inflicted before death. Since 1960 histochemical methods have been applied to the study of wound healing in order to arrive at a set of criteria that provide a reliable basis for wound age estimation. Raekallio [1] demonstrated that several enzymes appeared in a sequence during the first eight hours following vital wounding in guinea pigs. Leucine aminopeptidase was demonstrated as early as two hours after a vital wound was sustained. Acid phosphatase and alkaline phosphatase were demonstrated at four and eight hours, respectively, in the site of vital wounds.

Further research by Raekallio [2] and Fatteh [3] confirmed the earlier results by Raekallio and confirmed the vital nature of the reactions. Fatteh carrier out studies on human autopsy material and surgical specimens in order to provide medicolegally significant data. The surgical material obtained by Fatteh was up to three hours old and was removed from antiseptically cleansed skin of patients under general anesthesia during routinely scheduled operations. Wounds older than three hours were obtained at autopsy and no control over type and number of specimens was possible. The sequence of enzyme appearance was different in humans and guinea pigs. Acid phosphatase, for example, appeared in the guinea pig by one hour but was not evident in the human until six hours.

Raekallio [4] reviewed the literature to date concerning wound age estimation. Various histochemical findings were presented along with results of biochemical experimentation showing a sharp rise in histamine and serotonin during the first few minutes following vital wounding in guinea pigs. Raekallio concluded that the estimation of wound age is much more reliable when several independent methods are used, especially with respect to enzymes.

It appears from the reports in the literature that the ability to estimate the age of a vital wound is complicated by individual physiological differences, the age of the victim, the type and severity of the wound, and the type of treatment a wound receives. More data may cast light on the magnitude of the effect of these variables, but as Raekallio [4] suggests, as much independent data as possible must be accumulated on a particular vital wound before a reliable estimation of wound age is to be determined. The purpose of the present study was to investigate enzyme and carbohydrate histochemical changes following incised wounding in the hair-loss mouse.

Materials and Methods

Adult female mice exhibiting the hair-loss (hl) phenotype [5] were given two full thickness wounds of the dorsal skin with a pair of fine-tipped scissors. The two wounds were of different ages. No anaesthesia was used and no treatment of the wounds was performed. After a predetermined time (0, 1, 2, 4, 6, 12, and 20 hours), the mice were killed and the wound sites excised and fixed in 4 % glutaraldehyde in a 0.1 M phosphate buffer (pH = 7.2) [6]. After an overnight buffer rinse, half of the tissues were frozen and sectioned in a cryostat at eight micra, and half were processed through paraffin embedding and sectioned at six micra. The tissues were oriented in such a way that the plane of sectioning was perpendicular to the body surface with one edge showing the vital wound and the other edge showing a wound made immediately after death. Each skin section thus served as its own control. Other skin sections showed postmortem wounds of similar age to the vital wounds in order to evaluate the effect, if any, of postmortem activity that might mimic a vital wound. Routine hematoxlin and eosin stains were carried out on each of the wounds.

Enzyme Histochemistry

Cryostat sections were employed for all enzyme histochemical procedures because destruction of enzyme activity was observed in nearly all paraffin sections stained with specific enzyme techniques. Controls performed were of two types: positive and negative. The negative controls were all reagent blanks, and the positive controls consisted of tissues known to contain the enzyme in question and processed with the wound sections. The possibility that a reaction in the experimental medium, but not in the reagent control, might be a false positive necessitates the use of positive controls [7]. A positive control consists of a tissue known to contain the enzyme in question. No enzyme histochemical procedure lacked a negative control although positive controls were not run during each experiment.

Carbohydrate Histochemistry

Identical tissues to those used for enzyme histochemistry were embedded in paraffin and sectioned at six micra. The Periodic Acid-Schiff (PAS) method for carbohydrates [8], the Alcian Blue method for acid mucopolysaccharides [9], and a combined PAS-Alcian Blue technique for mucosubstances were performed on wound sections of differing ages [10]. Metachromatic staining was also performed, but did not constitute a major postion of the research. The specificity of the carbohydrate reactions was controlled through enzyme digestion and chemical reactions.

Grading of Reactions

Reactions were graded on a semi-quantitative scale of zero to three at the time of first observation. A zero rating was applied to a reaction where no discernible increase was evident; a plus one rating was assigned to a barely visible reaction; a plus two rating was assigned where a clear, distinct reaction was visible; and a plus three rating was given to all reactions that exhibited a much higher intensity than a plus two reaction. Where a background stain was present, such as a normal tissue enzyme level, the relative intensity of the reaction provided the basis for grading.

Results and Discussion

Enzyme histochemical reactions showed variable results. Reactions were not always consistent from animal to animal and from section to section from the same tissue block. Of the four enzymes studied, only nonspecific esterase showed what might be called a consistent reaction. Acid phosphatase alkaline phosphatase, and adenosine triphosphatase exhibited reactions that were not clear enough to make a valid judgment concerning wound age. For example, adenosine triphosphatase showed a positive reaction in the bile canalculi of the liver where one might expect it, and a positive reaction in the basal parts of the sebaceous glands, but little or no reaction in the wound sites. Alkaline phosphatase showed a positive reaction in six hour wounds, but the localization was not clear in any of the cases. Acid phosphatase localizations were a little better: a positive reaction was evident by two hours, but negative controls often exhibited false positive reactions. Because of these considerations, it appears that the histochemistry of the phosphatases as performed in our laboratory is not a good indicator of wound age in the mouse.

Nonspecific esterase showed good localization in the wound sites and no reaction at all in the negative controls. The positive controls (kidney) showed the expected positive reaction in the tubules. The time of the earliest observable reaction in the skin wounds of the mouse was one hour. The reaction was strong (plus two) and discrete for two, four, and six hour wounds. By twenty hours, the reaction had decreased in intensity and become quite diffuse. According to Oya [11], esterase reactions were demonstrated in human skin wounds made shortly after death and allowed to remain at room temperature for a period of time. In the current research involving the hair-

loss mouse, no such increase in esterase activity was observed in wounds made after death and allowed to remain for several hours. Postmortem autolysis was very much in evidence, though. The most logical source of the esterase in the wound site is the leucocytes, and it would be difficult, at best, for leucocytes to transport esterase to the site of a postmortem wound.

The conflict between data obtained in the present research and data reported by Oya (1970) relative to postmortem esterase activity in wounds may be due, in part, to differences in human and mouse response to wounding. It is unlikely that the cause of the postmortem esterase reaction observed by Oya is the same as the cause of the vital reaction observed in regenerating wounds. Since esterase histochemical procedures are usually nonspecific, and because there are a number of enzymes capable of hydrolyzing ester bonds, it may be necessary to use alternate substrates to identify the particular esterase present. Through the use of more specific histochemical or biochemical techniques, it may be shown that different esterases are involved with antemortem and postmortem reactions.

Nevelös and Gee [12] reported an increase in periodic Acid-Schiff (PAS) reactivity in the sites of some human wounds but did not discuss its nature or origin. The results reported by Robertson and Hodge [13] suggest that carbohydrate staining is not conclusive proof of vital wounding since carbohydrates were demonstrated in human wounds made shortly after death and allowed to sit for a period of time. In the hair-loss mouse, no postmortem PAS reaction was observed in wounds made at the point of death and allowed to "incubate" at 37°C for four and six hours, respectively. Antemortem appearance of PAS positive substances in the wound site is a vital reaction to wounding. If, in fact, a postmortem process causes an increase in carbohydrate at the wound site, it would be a simple matter to perform other confirming tests to establish the vital nature of a wound. It seems unlikely, however, that a postmortem process would affect only a wound site. Other areas of the skin could be expected to show a similar reaction. In any case, as Raekallio [1] has pointed out, it is not conducive to accurate wound age estimation to use just one test.

A steadily increasing PAS reaction was observed in incised skin wounds of hair-loss mice, first visible in a one-hour wound and increasing in intensity through six hours (Table 1). This increase in reactivity was associated with the migration of leucocytes to the wound site with the exception of the one-hour wound where little or no leucocytic infiltration was observed. By twelve hours, the intensity of the PAS reaction had faded somewhat while the degree of cellular infiltration had remained the same. Extensive tissue destruction was observed at this time. The decrease in reactivity was also observed in the twenty hour wounds.

Gersh and Catchpole [14] induced inflammatory reactions in mouse skin by intradermal injections of turpentine. After one hour, PAS-positive fibroblasts appeared in the wound site. Gersh and Catchpole held that the PAS reaction of the fibroblasts revealed the precursors of the ground substance of connective tissue.

No PAS-positive fibroblasts were seen in hair-loss mouse wounds one hour of age, but the ground substance showed a positive reaction at that time. Gersh and Catchpole did not report a ground substance reaction until six hours had elapsed.

The PAS reaction was confined to the connective tissue layer and was diffuse rather than granular in appearance. This description was constant for all wounds observed.

Table 1. Period acid-schiff reaction in wounds of known age

Time after wounding in hours	Number of test animals	PAS reaction intensity	Control intensity
1	2	+1	0
		+1	0
2	2	+1	0
		+1	0
4	2	+2	0
		+2	0
6	2	+3	0
		+3	
12	2	+2	0
		+2	
20	3	+2	0
		+2	0
		+2	0
4 hour postmortem	1	0	0
6 hour postmortem	1	0	0

Control Results

An acetylation-deacetylation reaction was performed on tissue sections. The results of these confirm the carbohydrate nature of the causative agent for the positive PAS reaction in the wound site.

A no-oxidation control was performed on representative sections; there was no staining, ruling out the possibility of contamination by free aldehyde groups.

Amylase digestion failed to remove the wound site PAS reactivity, but did remove the reactivity of the muscle layer and isolated structures near the degenerated hair follicles. The results of the amylase digestion indicate that glycogen is not a major causative factor in the wound site reaction, although a slight decrease in PAS positivity was observed in a six-hour wound section following amylase digestion.

Hyaluronidase digestions were carried out on representative sections. It was borne in mind that the PAS reaction of acid mucopolysaccharide-containing structures is not a result of hyaluronic acid or any of the chondroitin sulphates, but rather a result of contaminating polysaccharides occurring naturally along with these substances [15, 16]. The hyaluronidase digestion failed to remove the wound site reactivity. A PAS-Alcian Blue method for acid mucopolysaccharides was performed before and after hyaluronidase digestion. No Alcianophilia was observable at the wound site in either case, ruling out acid mucopolysaccharides as the causative factor in the carbohydrate reaction at the wound site. The type of reaction observed following PAS-Alcian Blue staining is consistent with the type of reaction expected for neutral mucosubstances [10]. The lack of acid mucopolysaccharides in the wound site is in agreement with the results reported by Nevelös and Gee [12] of experimentation on human connective tissue wounds.

Several sialidase digestions were performed, using a number of tissue sections. Sialidase digestions failed to remove any of the PAS reaction at the wound site. The results indicate that sialic acid and its derivatives play a negligible role in the PAS reaction of vital wounds.

According to Leblond et al. [17], when glycogen is eliminated as a causative agent in a PAS reaction in paraffin sections, the only other reactive polysaccharides remaining are the glycoproteins. The carbohydrate-protein complexes contain hexoses, methylpentoses, and hexosamines (Glegg et al., [18]). Biochemical assays reported by Leblond correlated with the PAS stainability. The glycoproteins include collagen and fractions of serum albumen and immunoglobulins. Robertson and Hodge [13] suggest that the collagen fibers swell and undergo a change that results in a higher PAS reactivity. This contention has merit. Another possibility for the increase in PAS reactivity is the transport of the plasma elements of the blood to the wound site. The transport of PAS positive fractions of serum would explain the increase noted with time of the PAS reaction in vital wounds. Van Winkle [19] suggests that fibroblasts may synthesize glycoproteins, although evidence for this is circumstantial.

If the causative factor at the wound site for the PAS reaction is a glycoprotein and if the latter is carried in with the initial exudate, a true vital reaction exists, for no inflammatory response can occur after death. A difficulty would exist, though, with accurate wound age estimation if factors that affect the inflammatory process are not taken into consideration. Since the current research was performed using standardized incisions, no data are available concerning the effect of varied kinds of wounding (i. e. puncture, abrasion, contusion, etc.).

If the origins of the wound site reactivity are glycoproteins synthesized by fibroblasts, the vital nature of the reaction is also assured, for vital processes are involved. It is unlikely, however, that the initial reaction of the wound involves fibroblast activity since the PAS reaction peak occurred at six hours when no new fibroblasts were evident. The later increase in PAS reactivity recorded by Dunphy and Udupa [20] quite possibly could result from fibroblast synthesis of glycoproteins.

The decrease in PAS reaction intensity with time apparently is not in accord with the results reported by Dunphy and Udupa. They record an increasing PAS reaction during the several days following vital wounding in man and report a decrease in reaction intensity after about ten days. They did not report any data for the first few hours. Therefore, it is possible that a decrease is observable and that the PAS reaction in the first few hours may have a different cause than the reaction later on.

From the evidence obtained in the current research, it appears that the PAS reaction in the wound site during the first few hours results from serum glycoproteins that are transported as part of the inflammatory process. This contention is supported by Schilling [21] who held that an early permeability to plasma proteins takes place in the healing wounds. White et al. [22], using dogs as experimental animals, observed glycoproteins early in the healing wounds and acid mucopolysaccharides later in the inflammatory process. Further support for the vascular origin of PAS positive agents at the wound site is provided by Jackson et al. [23], whose biochemical studies of healing connective tissue in rats revealed that the increase in glycoproteins was due to extravascular serum and was probably unrelated to collagen synthesis.

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