Wound Healing in Papain Treated Rats.* (29103)

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Crude papain is a proteolytic plant enzyme which appears to liberate the acid polysaccharides from cartilage following intravenous administration to rabbits(1,2). Spicer and Bryant(3) confirmed these observations and demonstrated that there was a diffusion of acid polysaccharides from the ground substance of rabbit ear cartilage into the surrounding tissues and blood vessels. Irving and Ronning(4) observed severe alterations of the epiphyseal cartilages in rats following intraperitoneal papain injections. There is as yet no evidence that papain acts upon "connective tissues" in general. The present investigation was designed to determine the influence of intraperitoneal papain administration upon the acid polysaccharides of normal skin and skin wounds in the rat.

Materials and methods. Eighteen albino rats weighing 150-200 g were used in this study. The skin from the back was cleaned and shaved. A punched circular wound ½ cm in diameter was made along the midline. Three days after surgery 12 rats were subjected to a single intraperitoneal injection of 0.5 ml (20 mg/kg) of activated crude papain in distilled water. The remaining 6 rats received equivalent volumes of distilled water and were used as controls. Two animals each were sacrificed at 1, 2, 4, 6, 8, and 10 days after administration of papain (*i.e.*, 4, 5, 7, 9, 11, and 13 days after injury). The controls were sacrificed at corresponding intervals. Specimens of normal skin were taken from the front dorsal region distant from the area of injury. The area of wound with the surrounding normal skin was also removed. Both the normal and injured skin were spread on a cork to prevent folding of the tissues. The specimens were fixed in 10% alcoholic formalin, processed by routine histological procedures, and embedded in paraffin. Serial sections were cut at 6 μ thickness, and stained initially with Hematoxylin and Eosin. To evaluate changes in specific constituents, additional sections were stained as follows: Van Gieson stain for connective tissue, Bielschowsky-Foot silver stain for reticular fibers, Periodic Acid Schiff for glycoproteins, and Mowry's colloidal iron procedure for acid mucopolysaccharides(8). Since the interpretation of microscopic findings in this study was based upon the intensity of staining reactions in various parts of the wound, the sections from experimental and control animals were stained simultaneously in a single batch of stain in the same staining rack and inserted and removed from the stains at the same time.

The principal of Mowry's Method for Mucopolysaccharides(8) is that the mucopolysaccharides present in tissue sections combine with iron from an acetic acid solution. The sites of iron binding in subsequent steps are colored as Prussian Blue, by the HCL-Potassium Ferro-Cyanide reaction. Mowry improved the selectivity of this staining reaction by use of a more dilute solution of colloidal iron reagent and a thorough rinsing in 12% acetic acid. The iron binding by this method is much more selective as compared to the previous modifications of this technique.

Results. Microscopic observations on the uninjured skin of experimental animals indicated that beginning at 2 days after treatment, there was a generalized decrease in the eosinophilic staining of the dermis. No significant difference was however observed in the PAS stained skin sections from control and papain treated rats. When these sections were stained by the colloidal iron method, there was a gradual but definite decrease in the concentration of acid polysaccharides in the ground substance of skin in the papain treated animals. The mast cells in the skin of these animals also showed a reduction in

* This investigation was supported by a U. S. Public Health Research Grant from Nat. Inst. of Dental Research.

their colloidal iron binding capacity (Fig. 1 & 2). Eight days after the papain treatment, the aggregates of acid polysaccharides began to reappear in the uninjured skin, and by 10 days the colloidal iron reaction appeared normal. This indicates that there is an initial decrease of the acid polysaccharide concentration in the uninjured skin following papain treatment and this lasts for approximately 6 days.

Since the most significant histochemical and histological differences between the experimental and control wounds appeared on the 5th and 7th days after injury, only these results will be reported.

The earliest signs of alteration in the histochemical characteristics of the healing process in wounds of papain treated animals was observed 5 days after injury (2 days following papain injection). While the inflammatory exudate appeared similar in both groups of animals, the wounds in experimental animals stained pale gray with Hematoxylin and Eosin, and many undifferentiated and immature fibroblasts were evident. In sections stained with colloidal iron for acid mucopolysaccharides, the connective tissue underneath the exudate demonstrated a decrease of the Prussian Blue reaction when compared with the control wounds (Fig. 3 & 4). This suggests that papain might have affected the concentration of acid polysaccharides in the ground substance.

Eight days after injury the wounds of papain treated animals still demonstrated a decreased Prussian Blue reaction (Fig. 5 & 6). This was in contrast to the wounds of control animals where aggregates of colloidal iron stained material were observed around fine fibrillar structures. These histochemical changes seemed to be delayed in the wounds of papain treated animals and appeared 11 days after injury (Fig. 7). Papain appeared to deplete the wound mucopolysaccharides after administration and the effect lasted for a short period.

There was a similarity in the staining for reticular fibers of 5-day-old wounds of control and experimental animals. The reticular fibers which were produced as fine strands in wounds of papain treated animals persisted as late as 8 days (Fig. 8 & 9) while the fibers in wounds of control animals showed marked thickening. This suggests that the formation of collagen fibers in the control wounds progressed more rapidly than in the wounds of the papain treated animals.

The delay in rate of collagen fiber formation in experimental wounds was confirmed when sections of 11-day-old wounds were stained with Van Gieson's connective tissue stain (Fig. 10 & 11). In the control wounds the collagen fiber formation had progressed and the bundles stained bright red. The fibroblastic activity was normal for that stage of wound healing. The wounds from papain treated animals demonstrated pale staining thin strands of collagen and an abundance of fibroblasts. This indicated that the rate of collagen fiber formation or maturation was retarded in spite of the large number of fibroblasts. With further time after injury, there was no significant difference in the histochemical patterns of healing wounds between experimental and control animals.

Discussion. Our results suggest that intraperitoneal administration of papain to rats decreased the concentration of acid polysaccharides in the ground substance of normal skin and skin wounds within 48 hours. This effect lasted only for a short period. Although similar effects have been demonstrated in the cartilage(1,2,3,4), following administration of papain to rabbits and rats, we have not found any studies in the literature concerning the connective tissue of rat skin. The influence of papain upon skin appeared to be slower and lasted for a longer period than upon cartilage. This may be explained on the basis of observations by Haulth and Westerborn (5) and Merkow and Lalich(6) who pointed out that the rat is more resistant to papain action than is the rabbit. The dose level used in the present study was less than that employed by other investigators. It is further possible that the passage of this enzyme from the peritoneal cavity into the skin may be slower than from the serum into the cartilage.

Observations on skin wounds suggest that there was an initial delay in the various stages of wound healing in the rat skin fol-

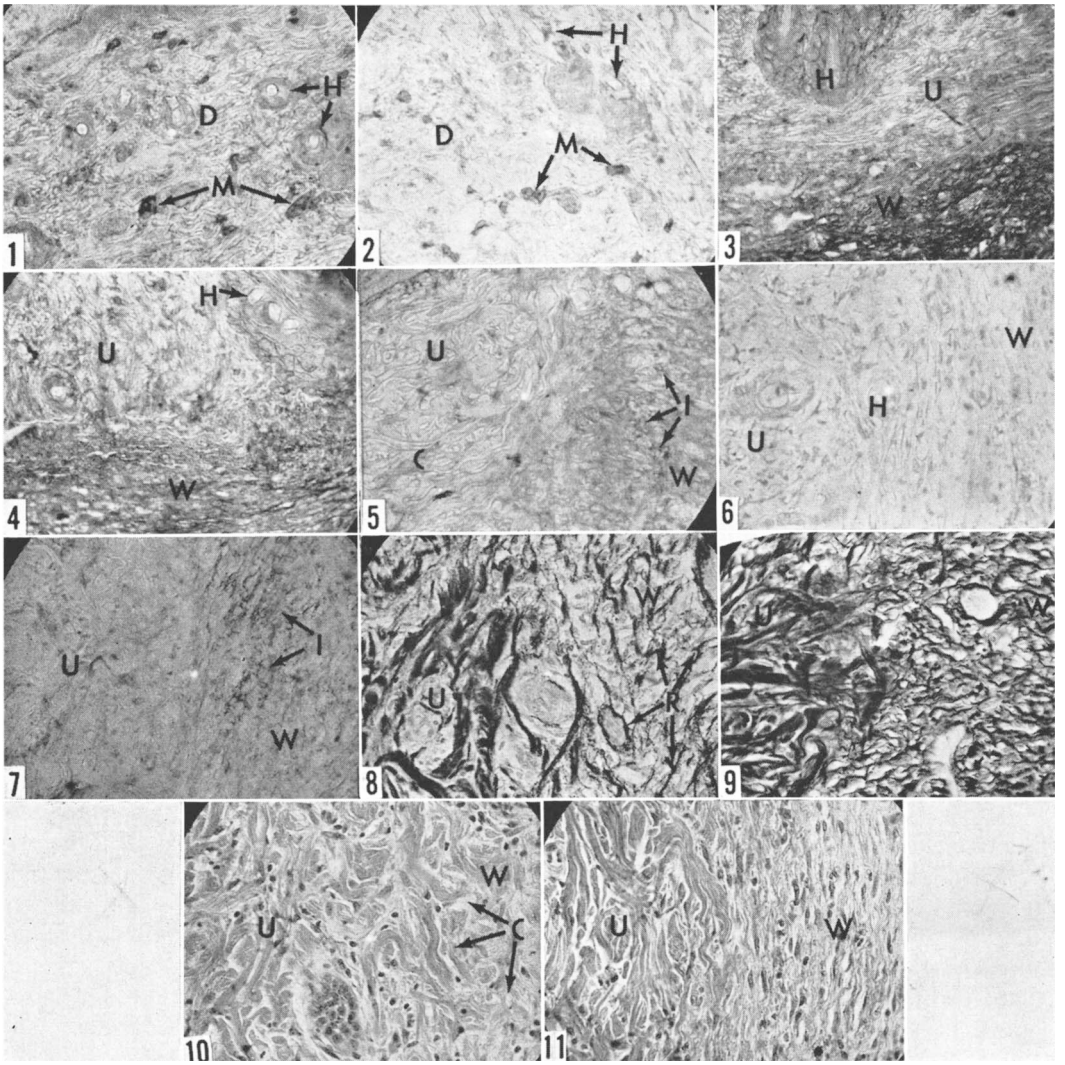

FIG. 1. Section of uninjured skin from control animal stained by colloidal iron method. Note the diffuse staining reaction of intercellular ground substance in dermis (D). Mast cells (M) show intense Prussian Blue staining. (Orig. mag. ×400)

FIG. 2. Uninjured skin from an experimental animal 24 hr after papain treatment. Note loss of Prussian Blue color in dermis and mast cells following colloidal iron staining. (Orig. mag. ×400)

FIG. 3. Section through a 5-day-old skin wound from a control rat stained with colloidal iron method. Area of the wound (W) shows an intense Prussian Blue reaction. U—uninjured skin adjacent to wound. H—hair follicle. (Orig. mag. ×400)

FIG. 4. Section of 5-day-old skin wound from the rat, 24 hr after papain treatment. Note a decrease in staining reaction of injured area (W), following colloidal iron staining. (Orig. mag. ×400)

FIG. 5. Eight-day-old skin wound in control animal demonstrates granular aggregates of colloidal iron stained material (I) associated with fibrillar structures. Collagen fiber bundles (C) in uninjured area appear distinct. (Orig. mag. ×400)

FIG. 6. Wound of the same age as in Fig. 5, 3 days following papain treatment. Area of injury (W) does not show distinct fibrillar arrangement and note absence of colloidal iron stained material. Collagen fiber bundles in uninjured region (U) are not distinct. (Orig. mag. ×400)

FIG. 7. Six days following papain treatment, the area of skin wound (11 days) demonstrates fibrillar arrangement with granular material intensely stained with colloidal iron (I). This is comparable to the 8-day-old wound in control animals. Collagen fiber bundles in un-

injured skin (U) appear to be distinct. (Orig. mag. ×400)

FIG. 8. Eight-day-old wound of control animal stained with Bielschowsky silver stain for reticular fibers. The newly formed fibers demonstrate dense granular aggregates (R) of silver. (Orig. mag. ×400)

FIG. 9. The 8-day-old wound in papain treated animals shows a fine network of reticular fibers. (Orig. mag. ×400)

FIG. 10. Section from 11-day-old healing wound of a control animal stained with Van Gieson connective tissue stain. Note formation of new collagen fiber bundles (C), with intense fibroblastic activity. (Orig. mag. ×400)

FIG. 11. Compare with Fig. 10. In the wound of papain treated animal, formation of collagen fiber bundles is not well advanced, and fibrillar structure shows a pale staining reaction. Fibroblastic activity appears normal.

lowing systemic administration of papain. This was based upon the presence of fine immature reticular fibers as late as 8 days after surgery and the delay in formation of collagen fibers in the wounds of experimental animals. The retardation of the rate of fibroblastic differentiation in these wounds may partially explain this observation.

The delayed rate of collagen fiber formation in the experimental animals may also have been due to the effect of papain in depleting(1,2,3) the connective tissue ground substance of acid polysaccharides which are presumed to play an important role in the conversion of reticular fibers into collagen bundles(7). That papain depletes the ground substance of its acid polysaccharides was observed in this investigation as a decrease in the Prussian Blue reaction in the connective tissue of 5-day-old experimental wounds following colloidal iron staining. With the progress of healing, *i.e.*, 11 days after injury, these reactions were at a maximum intensity while a considerable decrease in the staining reaction of control wounds had begun by this time. Therefore, it appears that the effect of papain upon wound healing lasted approximately 5-6 days, and the accumulation of acid polysaccharides in the connective tissue ground substance began after the influence of papain had in large part worn off. Paralleling this accumulation of acid polysaccharides was the formation of discernible collagen bundles.

Summary and conclusions. Intraperitoneal injections of 20 mg/kg body weight of activated crude papain to rats resulted in a decrease in the concentration of acid polysaccharides in the normal skin. There was an initial delay in the various stages of wound healing in the skin. Retardation in differentiation of fibroblasts and maturation of reticular fibers into collagen bundles was observed. The depletion of acid polysaccharides from the connective tissue ground substance in wounds of papain treated animals was associated with a delay in the maturation of collagen.

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Received July 23, 1963. P.S.E.B.M., 1964, v115.