Connective Tissue XI. Chemical Pathology of Necrotic Wounds.* (29444)

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The 18th-century English surgeon John Hunter was the first to express the belief that the reaction of a tissue to injury was essentially the same despite the character of the injurious agent(1). Lewis some 160 years later was able to demonstrate that a very large variety of injurious agents all invoked a similar "triple response" from the connective tissue(2).

Using a sequential extraction technique to effect the chemical dissection of the connective tissue into its various morphological and chemical components(3,4) we have characterized chemically the process of croton oil produced necrosis(4,5). In this paper we have compared the chemical and histochemical characteristics of 3 different kinds of model necrotic injury (alkaline burns, thermal burns and histamine produced wounds) with the results obtained from studying croton oil induced wounds. These results are described below, and tend to support the claims of Hunter that all injuries produce similar responses not only during the immediate and initial period of inflammation, but also during the subsequent necrotic phase of injury.

Materials and methods. Four groups of 72 male 250-270 g Sprague-Dawley rats each were anesthetized and subjected to one of the following forms of injury to the abdominal skin on the left side of each animal.

a) Intradermal injection of 0.5 ml of 1 N NaOH

b) Intradermal injection of 0.5 ml of 50% croton oil in peanut oil

c) Topical burning of a thin cotton gauze disk (3 cm in diameter) containing 0.2 ml of ethanol

d) Intradermal injection of 0.5 ml of an isotonic saline solution containing 50 mg/ml of histamine HCl

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At 0, 4, 7, 10, 15 and 23 days after injury, groups of 12 animals each which had been injured by each of these 4 techniques were sacrificed and the injured tissue shaven, dissected free of adhering fat, fascia and muscle and excised. About half of the injured tissue from each wound from each animal was hydrolyzed in 10 ml/g of 4 N HCl for 8½ hours at 100°C. Most of the remaining tissue from the 12 animals representing each particular day of sacrifice from each group of wounded rats was pooled into 3 pools each representing all 12 animals. These triplicate samples were extracted sequentially in the cold with 0.15 M NaCl, 0.5 M NaCl and 0.5 M citrate buffer (pH 3.6) as has been described previously (3-6). These solvents have been shown to extract quantitatively the "ground-substance," salt soluble and acid soluble collagens respectively(3,4). Aliquots of each of the extracts were also hydrolyzed in 4 N HCl as described above. The hydrolysates from both the whole tissue and the extracts were analyzed for nitrogen (3), hydroxyproline(7) and hexosamine(8) as has been described previously(5,6).

A small sample of injured tissue at various days after injury from each animal with each of the 4 types of wound described above was collected and placed in buffered formalin, imbedded in paraffin and sectioned in the usual manner. These tissue sections were stained with Masson trichrome stain for collagen, Rheinhold colloidal iron for acid polysaccharides and periodic acid-Schiff stain for glycoproteins and glycogen(1).

The results of the chemical analyses were all expressed in terms of μmoles per mmole of total dermal nitrogen to obviate variations in tissue water and fat content. The total hexosamine and hydroxyproline to nitrogen ratios were expressed as the mean of 12 animals analyzed in triplicate. Standard deviations of these means never exceeded 7% of

TABLE I. Effects of Time After Injury upon Total Hexosamine (Hex) and Hydroxyproline (Hypro) Content of the Wound in μmoles per mmole of Total Dermal Nitrogen (Obviating Variations in Tissue Water and Fat).

Days after injury	Types of injury: NaOH Hex	NaOH Hypro	Croton oil Hex	Croton oil Hypro	Burns Hex	Burns Hypro	Histamine Hex	Histamine Hypro
0	1.9 ± .10	43 ± 2	1.9 ± .10	43 ± 2	1.9 ± .10	43 ± 2	1.9 ± .10	43 ± 2
4	*3.5 ± .15	*20 ± 1	*5.1 ± .25	*13 ± 6	*3.8 ± .14	*21 ± 1	*2.6 ± .13	*18 ± 1
7	*3.4 ± .16	*20 ± 1	*7.8 ± .30	*18 ± 8	*4.4 ± .20	*27 ± 1	*3.3 ± .16	*21 ± 1
10	*3.4 ± .14	*21 ± 1	*6.4 ± .30	*17 ± 8	*4.8 ± .21	*27 ± 1	*3.3 ± .15	*19 ± 1
15	*2.9 ± .13	*26 ± 1	*6.9 ± .32	*21 ± 1	*4.5 ± .22	*29 ± 1	*3.5 ± .15	*28 ± 1
23	*2.4 ± .11	*26 ± 1	*2.3 ± .11	*28 ± 1	2.1 ± .10	*38 ± 2	*2.3 ± .11	*36 ± 2

* Significantly different from day 0 ($p < .05$).

the mean. Standard deviations of the means of the triplicate extractions were about 8% of the mean. If these latter means differed from the control "day 0" mean by more than 2 standard deviations then the mean was significantly different from that of the control ($p < .05$).

Results. The ratios of total hexosamine and hydroxyproline to total dermal nitrogen of 4 different kinds of wounds at various days after injury are shown in Table I. The data indicate that the tissue from all 4 types of wound contained more hexosamine and less hydroxyproline (collagen) than normal with time after injury. After eschar sloughing (about 15 days after injury), healing had begun and by 23 days after injury 3 of the 4 types of wound gave some evidence of healing. That is, with 3 types of lesions there were increases in collagen content of the granulation tissue and losses of hexosamine from the wound on a nitrogen basis by the 23rd day after injury. All 4 injuries resulted in essentially the same chemical changes within the wound both quantitatively and qualitatively during the pre-eschar sloughing necrotic phase of the lesion, however. The nitrogen content of all wounds remained constant (*i.e.* 4.6 ± 0.2 mmoles/g).

The histochemistry of all 4 types of injury demonstrated essentially the same staining characteristics from 2 to 15 days after injury. The morphology of all injuries was that of coagulation necrosis creating a sterile, full-thickness ulcer throughout the whole area of injury as has been described previously(5). A comparison of the staining characteristics of the various wounds at 7 days after injury with each other and with normal control skin is shown in Table II. The data in this Table indicate that histochemically all 4 types of injury demonstrated a loss of both collagen and mucopolysaccharides. Once again, both morphologically and histochemically, all 4 types of injury resulted in essentially similar changes within the wounded tissue.

These wounds differed considerably in numbers of leucocytes and macrophages within the injured area. The croton-oil produced lesions contained few, if any, of these cellular elements, whereas the histamine produced injuries contained large numbers, while the concentration of these cells within the alkali and thermal burns was less than that found in the histamine wound but more than that of the croton-oil lesion.

The hexosamine and nitrogen content of the isotonic saline extracts of each type of wound at various days after injury is shown in Table III. These results indicate an essen-

TABLE II. Histochemistry of Various Necrotic Wounds One Week After Injury.*

Stains	None	NaOH	Croton oil	Burn	Histamine
1) Periodic acid-Schiff	±/0	+++	+++	+++	+++
2) Colloidal iron	++	0	0	0	0
3) Masson trichrome (collagen)	+++	0	0	0	0

* 0 to +++ representing the spectrum of staining intensity.

TABLE III. Effect of Injury upon Amount of Hexosamine (Hex) and Nitrogen (N) Soluble in Isotonic Saline from the Wound in μmoles per mmole of Total Nitrogen.

Days after injury	Types of injury: NaOH Hex	NaOH N	Croton oil Hex	Croton oil N	Burns Hex	Burns N	Histamine Hex	Histamine N
0	1.0 ±.07	.17 ±.01	1.0 ±.07	.17 ±.01	1.0 ±.07	.17 ±.01	1.0 ±.07	.17 ±.01
4	*1.9 ±.13	*3.30 ±.02	*2.1 ±.15	*.44 ±.03	*1.7 ±.10	*.26 ±.02	*2.3 ±.14	*.30 ±.02
7	*2.1 ±.14	* .30 ±.02	*4.0 ±.28	*.60 ±.04	*2.7 ±.18	*.43 ±.03	*3.0 ±.20	*.40 ±.03
10	*2.3 ±.14	* .35 ±.02	*5.1 ±.35	*.68 ±.04	*3.7 ±.26	*.51 ±.03	*3.3 ±.21	*.44 ±.03
15	*2.4 ±.14	* .40 ±.03	*6.0 ±.40	*.71 ±.05	*4.0 ±.26	*.60 ±.04	*3.5 ±.22	*.44 ±.03
23	1.2 ±.08	.15 ±.01	1.1 ±.07	.20 ±.01	1.2 ±.07	.20 ±.01	1.0 ±.07	.19 ±.01

* Significantly different from day 0 ($p < .05$).

tially parallel increase in the amounts of both wound hexosamine and nitrogen soluble in 0.15 M NaCl. These parallel increases with time after injury are consistent with the previously described accumulation of glycoproteins within the wound (4,5,9,10,11). Although the apparent amount of glycoprotein contained within the croton oil-produced wound was greater than that found within the other 3 kinds of injury, all 4 types of lesion showed qualitatively the same accumulation of glycoprotein within time after injury.

The loss of salt soluble collagen from the site of necrotic injury was similar both quantitatively and qualitatively for all 4 kinds of wound (Table IV). These data also indicated that the necrotic tissue from all 4 types of injury contained essentially normal amounts of citrate soluble collagen until healing (day 23) when quantitatively similar increases in the wound concentrations of acid soluble collagen were recorded.

Subtraction of the sum of the various soluble concentrations of hydroxyproline from total concentration of this imino acid permits the calculation of the amount of insoluble collagen found in each of the 4 kinds of wounds at various days after injury. These results (Table V) indicate that all 4 types of injury demonstrated extremely similar losses

TABLE IV. Hydroxyproline Content of 0.50 M NaCl and 0.5 M Citrate Buffer (pH 3.6) Extracts of Various Wounds at Various Days After Injury (in μmoles/mmole *Total* Wound Nitrogen).

Days after injury	Types of injury: NaOH NaCl	NaOH Citrate	Croton oil NaCl	Croton oil Citrate	Burns NaCl	Burns Citrate	Histamine NaCl	Histamine Citrate
0	4.0 ±.28	1.6 ±.09	4.0 ±.26	1.6 ±.09	4.0 ±.28	1.6 ±.09	4.0 ±.28	1.6 ±.09
4	*1.8 ±.11	1.6 ±.10	*1.2 ±.09	1.7 ±.11	*2.0 ±.14	1.5 ±.10	*2.1 ±.14	1.7 ±.11
7	*1.0 ±.07	1.4 ±.10	*1.1 ±.07	1.4 ±.10	* .9 ±.06	1.5 ±.10	*1.1 ±.07	1.4 ±.09
10	1.0 ±.06	1.3 ±.07	*1.0 ±.07	1.8 ±.12	* .9 ±.06	1.7 ±.12	* .9 ±.06	1.5 ±.10
15	*2.0 ±.04	2.0 ±.14	*2.0 ±.13	1.9 ±.12	*2.1 ±.12	1.7 ±.12	* .9 ±.06	2.1 ±.14
23	*1.1 ±.07	*5.3 ±.35	*1.3 ±.08	*5.4 ±.30	*1.0 ±.07	*5.6 ±.38	*2.1 ±.14	*6.3 ±.42

* Significantly different from day 0 ($p < .05$).

TABLE V. Insoluble Collagen Content of Various Kinds of Necrotic Wounds at Various Days After Injury (μmoles of Hydroxyproline per mmole of Total Wound Nitrogen).

Days after injury	Types of injury: NaOH	Croton oil	Burns	Histamine
0	36.4 ± 2.5	36.4 ± 2.5	36.4 ± 2.5	36.4 ± 2.5
4	*15.6 ± 1.1	* 9.1 ± .6	*15.5 ± .8	*13.2 ± .8
7	*16.6 ± 1.1	*14.5 ± .9	*23.6 ± 1.5	*17.5 ± 1.1
10	*17.7 ± 1.2	*13.2 ± .8	*23.4 ± 1.6	*15.6 ± 1.0
15	*21.0 ± 1.4	*16.1 ± 1.0	*25.2 ± 1.7	*24.0 ± 1.7
23	*18.6 ± 1.2	*19.3 ± 1.3	*30.4 ± 2.1	25.6 ± 1.8

* Significantly different from day 0 ($p < .05$).

of collagen from the wound. Despite the complete loss of collagen staining with any of the 4 types of injury described above, all wounds still contained about ⅓ of the normal amounts of tissue collagen *vis a vis* total wound nitrogen.

Discussion and conclusions. These results indicate that both chemically and histochemically, the reaction of the connective tissue to injury was essentially independent of the type of injury employed. Further, these results also suggest that croton oil-produced wounds are a reasonable and reliable model system for the study of the chemical pathology of inflammation, and therefore such a study of croton oil-induced injuries does not result in information unique to this irritant alone. The essential similarity of the response of the connective tissue to a variety of injuries seems reasonable biologically since it should be obvious that while the integument is subject to an infinite variety of insult, the tissue could not possibly maintain an equally infinite variety of response. Therefore, it would appear that the inflammatory response is essentially non-specific with respect to the nature of the irritant employed to elicit the response.

These results also suggest that this general inflammatory response involved two major changes in the nature of the connective tissue: 1) A loss of insoluble collagen from the wound as well as a decrease in amount of soluble collagen within the wound and; 2) accumulation of large amounts of glycoprotein within the necrotic wound. Healing of these necrotic wounds involved the loss of this glycoprotein from the wound, and accumulation of increasing amounts of collagen and acid mucopolysaccharides within the wound tissue. Repair of these wounds also seems to be associated with increased amounts of acid soluble collagen.

Finally, these chemical changes with tissue necrosis do not seem to be associated with changes in the number or nature of the formed circulating cellular elements invading the injured area, since despite the marked difference in concentration of these cells in the various types of wound, the chemical analyses of these wounds did not differ remarkably.

Summary. Four representative forms of necrotic injury were studied both chemically and histochemically. The results indicate that the inflammatory response was independent of the nature of the irritant employed to elicit this response. Therefore results of the study of croton oil-induced wounds are not unique to this irritant but are of general applicability to the inflammatory process itself. The general inflammatory response appears to involve primarily a loss of both soluble and insoluble collagen from the necrotic wound and accumulation of a glycoprotein within the wound.

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