PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 161, 337–340 (1979)

Quantitation of Collagen Types I and III during Wound Healing in Rat Skin (40548)

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The four major Types of collagen in the body each have unique structural and functional relationships. Mature dermis is composed of approximately 80% Type I collagen and 20% Type III, whereas Type III collagen is increased in the fetus and newborn (1). Alteration of the normal distribution of the various collagen Types is usually associated with pathologic states. For example, the fragile, extensible skin of Ehlers–Danlos IV patients is associated with a lack of Type III collagen (2) and atherosclerotic plaques contain excessive Type I collagen (3).

We have reported collagen synthesis increases significantly by 24 hr in open rat skin wounds (4); however, studies to date have not determined directly the Types of collagen synthesized during this early phase of wound healing. Bailey *et al.* (5) noted that rat dermal granulation tissue, in response to foreign substances, contains a higher proportion of Type III collagen than mature skin. In addition, Gay *et al.* (6) reported that Type III collagen was the first immunologically detected species in Cellstic implants in children. In the present studies, collagen was separated by chemical Type and increased Type III collagen was observed at 10 hr after wounding.

Materials and methods. Full-thickness, 4-mm punch wounds were made on the shaved backs of male Sprague–Dawley rats (100–150 g). Each animal received 24 wounds and four rats were sacrificed by decapitation at each time point for collagen-Type analysis. The complete wound, including the underlying panniculus carnosis, was excised allowing approximately a 1-mm margin of normal skin. Punch biopsies of normal tissue were obtained from areas at least 1 cm distant from wound sites.

The biopsies of either normal skin or wound tissue were minced and suspended in 12 ml of Krebs–Ringer medium containing 100 μg/ml β-aminopropionitrile (BAPN) (Aldrich Chemical Co., Metuchin, N.J.) and 40 μCi [5-^{3}H]proline (49 Ci/mM, Schwarz/Mann, Orangeburg, N.Y.). BAPN was added to prevent collagen crosslink formation. After incubation for 2 hr at 37°, the specimens were homogenized with a Tissuemizer (Tekmar, Co., Cincinnati, Ohio) and adjusted to 0.5 M with glacial acetic acid. Pepsin (Boehringer–Mannheim Biochemicals, Indianapolis, Ind.) was added to give 1:50 (w/w) and collagen was extracted at 4° for 48 hr. Sodium chloride was added to the supernatant (12,000 g) to give a final concentration of 12% and the collagen was removed by centrifugation (12,000 g). The precipitate was resuspended in 0.5 M acetic acid and dialyzed against 0.02 μ phosphate buffer (pH 7.4). The resulting precipitate was dialyzed against 0.1 M acetic acid and then lyophilized.

The dried collagen specimens were weighed and dissolved in 1 M $CaCl_2$–0.05 M Tris (pH 6.5) at 55°. The solubilized collagen preparation (5 mg/ml) was chromatographed on a 1.5 × 150-cm column of BioGel A5-M (200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 1 M $CaCl_2$–0.05 M Tris (pH 6.5) at room temperature with a flow rate of 10 ml/hr (7). Fractions (2.5 ml) were monitored for collagen at 230 nm. Aliquots (1 ml) were dissolved in 10 ml Biocount (Research Products International Corp., Elk Grove Village, Ill.) and counted with a liquid scintillation spectrometer. Type III collagen elutes in Region A, whereas Type I alpha chains are in Region C and crosslinked Type I alpha chains are in Region B (Fig. 1). Confirmation of the identity of Type III collagen in Region A was achieved by demonstration of cysteine and reduction and alkylation of Region A that resulted in a product which cochromatographed with Type I alpha chains (Region C) (8). Radioactivity in Region A (Type III collagen) was

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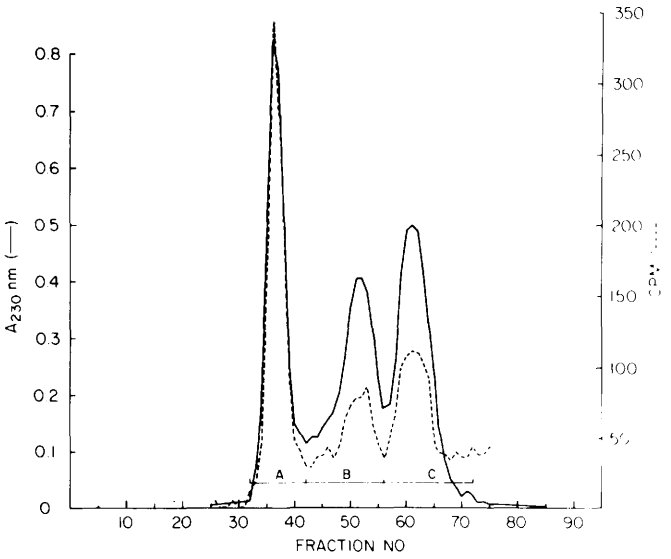

FIG. 1. Elution profile of collagen Types I and III separated on an A-5 M column (1 × 150 cm). Approximately 15 mg of purified collagen was applied to the column and the percentage Type III (Region A) was determined by dividing the radioactivity in Region A by the total radioactivity in Regions A, B, and C.

divided by the sum of the radioactivity in Regions A, B, and C × 100 to determine the percentage of Type III collagen. Procollagen Types I and III are not observed using this procedure because the amino and carboxy terminal extensions are removed during the solubilization procedure using pepsin (9).

Statistical analysis of data. Either the Student's *t* test for independent samples or paired *t* test was used for statistical analyses.

Results. Open rat skin wounds synthesized significantly more Type III collagen at 10 hr compared to wounds studied at all other times (Table I). In addition, the ratio of wound Type III collagen to normal skin Type III from the same animal was significantly increased at 10 hr ($P < 0.01$), whereas there were no significant differences at later time points (Table II). Furthermore, the percentage Type III collagen synthesized by normal skin at 10 hr (24.4 ± 2%) was increased signficiantly ($P < 0.03$) compared to normal skin at all other times (20.4 ± 0.6%).

Discussion. The period immediately following wounding has been termed a "lag" phase (10, 11), but recent studies reported by this laboratory have demonstrated significant collagen synthesis by 24 hr (4). The present quantitative studies demonstrate that there is a significant increase in the percentage Type III collagen synthesized at 10 hr (Tables I and II). However, by 24 hr the percentage Type III collagen synthesis in the wound has returned to that of normal skin (approximately 20%). This is the first study to directly quantitate changes in collagen Types during wound healing where the analysis was based on chemical characterization.

TABLE I. PERCENTAGE TYPE III COLLAGEN AFTER WOUNDING.

Time after wounding	Number of observations	Percentage Type III collagen ± SE
10 hr	4	31.1 ± 2.8[a]
24 hr	4	19.8 ± 3.0
48 hr	3	18.9 ± 1.8
72 hr	3	20.0 ± 1.1
5 days	2	18.1 ± 0.3
7 days	4	20.9 ± 1.4
9 days	3	17.4 ± 0.8
12 days	3	15.5 ± 0.7

[a] $P < 0.05$ compared to all other times.

Bailey *et al.* (5) reported that a "a high proportion of Type III collagen" was produced in response to turpentine injection or to sponge implantation. However, these foreign-body wound models do not accurately represent a normal wound and the increased Type III collagen may be the result of the increased vascularity (12) of the foreign-body granuloma. Furthermore, in their studies, collagen concentrations were approximated by weighing selective salt precipitates (5). Gay *et*

TABLE II. RATIO OF WOUND TO NORMAL SKIN TYPE III COLLAGEN AFTER WOUNDING.

Time after wounding	Type III collagen wound/normal skin
10 hr	1.28[a]
24 hr	0.99
48 hr	0.99
72 hr	0.99
5 days	0.95
7 days	1.14
9 days	0.82
12 days	0.64

[a] $P < 0.01$ compared to all other times.

al. (6) also suggest that Type III collagen is synthesized during the initial phase of wound repair in humans. But again, their studies utilized foreign-body Cellstic implants with cellulose sponge to collect cells and wound fluid. Collagen Types I and III were then estimated by visual inspection of Cellstic slices based on indirect immunofluorescence (6). It is of interest to note that Gay did not detect Type I collagen until 72 hr after wounding using the Cellstic model (6). Nevertheless, these qualitative studies are in agreement with our present quantitative studies demonstrating increased Type III collagen synthesis in the early phase of open wound repair (Tables I and II).

The early increase in Type III collagen synthesis observed in the present studies is probably derived from local fibroblasts which are activated by the wounding process. The early Type III collagen (Tables I and II) may be important in establishing the initial wound structure and provide a basic lattice for subsequent healing events. For example, Type III collagen may guide inflammatory cells and fibroblasts into the wound site and also provide a matrix for reestablishment of blood supply.

However, it is unlikely that Type III collagen contributes significantly to wound tensile strength because the greatest increase in tensile strength is not observed until later phases of wound repair (10). Moreover, Type III collagen is composed of fine "reticulin" fibers (13) compared to the stronger fibers of Type I collagen (14). The "reticulin" fiber form of Type III collagen has been demonstrated by histologic methods to be present during the initial phases of wound repair (15).

Our observation that normal skin distant (1 cm) from the wounded area also had increased Type III collagen synthesis at 10 hr compared to normal skin at all other times suggests systemic changes in collagen Types following wounding. Alternatively, there may be local changes in the "normal" skin due to neovascularization.

The present studies coupled with our previous findings of increased collagen synthesis during the onset of wound repair (4) suggest that these initial events after wounding are important factors for subsequent wound healing. Although we have reported a significant increase in the percentage of Type III collagen synthesized at 10 hr, further *in vivo* studies are needed to fully characterize collagen metabolism during wound healing.

Summary. The collagen Types synthesized during early wound healing have not been quantitatively analyzed. Therefore, open wounds were made on the backs of Sprague–Dawley rats and analyzed at various times for newly synthesized collagen Types I and III. Biopsies were incubated in tissue culture medium with [^{3}H]proline and collagen was extracted and purified prior to separation on an A5-M column. Type III collagen was increased significantly at 10 hr compared to all other times through Day 12. By 24 hr, the percentage Type III collagen returned to a normal skin value of 20%. The early appearance of Type III collagen is associated with an early increase in collagen synthesis and may function in providing initial wound structure and support for subsequent healing events.

This work was supported by NIH Grant GM-20298. The authors are grateful for the fine technical assistance of Ms. Mary Anna Link and Ms. Benita Atiyeh and the typing assistance of Ms. Rae Spivey and Ms. Jean Usry.

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Received February 22, 1979. P.S.E.B.M. 1979, Vol. 161.