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Onset and Localization of Collagen Synthesis during Wound Healing in Open Rat Skin Wounds[1] (40470)

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Early studies by Howes *et al.* (1) indicated that after wounding of skin there is a "lag phase" of about four to six days before significant fibroplasia and increased wound tensile strength could be detected. The existence of a "lag phase" was supported by Ross and Benditt's observation that there was no histological evidence of collagen fiber formation in open guinea pig wounds until the third day after wounding (2) and Madden and Peacock's observation that hydroxyproline synthesis did not increase until the fourth day in sutured rat wounds (3). In contrast, our studies have suggested that actual collagen synthesis in open rat skin wounds increases as early as 24 hr after wounding (4, 5). These observations raised the question as to what were the actual rates and kinetics of collagen synthesis early after wounding. In addition, because Ross noted that the peak number of collagen-synthesizing wound fibroblasts was not obtained in the wound until day 6 (2, 6), we sought to identify which wound area synthesized collagen as early as 24 hr after wounding. In the present study, collagen synthesis was found to be significantly increased in open rat skin wounds by 24 hr and the activity was localized in the panniculus carnosis.

Methods. Wounding procedure: Six to ten open wounds were made on shaved backs of male Sprague-Dawley rats (150–180 g) with either a 3 or 6 mm dermal punch through the level of the panniculus carnosis. The punched-out skin was discarded. On various days after wounding, the animals were sacrificed by decapitation and the entire dorsal skin, including the panniculus carnosis, was removed. Either a 4 or 8 mm dermal punch was then used to excise the entire wound and normal skin samples from the animal pelt.

Collagen synthesis. The excised wounds or normal skin samples were minced and incubated in 3 ml of Krebs-Ringer medium containing 10 μCi ^{3}H-5 proline (42 Ci/m*M*, Schwarz/Mann, Orangeburg, NY) as we have described previously (4). Duplicate analyses were made on all animals.

Following a 2-hr incubation at 37° under normal atmosphere, the samples were removed, frozen, homogenized and radioactive protein was precipitated at 4° with trichloroacetic acid (5%). The radioactive protein was then digested by purified bacterial collagenase to separate collagen from noncollagen protein (4). The radioactivity released by bacterial collagenase as well as the undigested noncollagen protein were each measured by liquid scintillation spectrometry. The *absolute* amount of collagen synthesized during this 2-hr period is reported as radioactivity per mg dry tissue weight. In addition, the *relative* amount of collagen synthesized was calculated by comparing absolute collagen synthesis to noncollagen protein synthesis (bacterial collagenase-resistant protein). A calculation was used to correct for the enriched imino acid content of collagen compared to other proteins (4). The student's *t* test was used for statistical analyses.

Groups for study of collagen synthesis in open rat wounds. Group I. Total wound (Fig. 1). Wounds made with a 3 mm punch and removed with a 4 mm punch were analyzed for relative collagen synthesis on days 1–5, 7, 11, 16, and 21 post-wounding. Three animals were studied on each day.

Group II. Skin-wound margin and granulating wound center (Fig. 1). Wounds were made with a 6 mm punch and then removed on days 1, 3 and 5 with a 6 mm punch. A 3 mm punch was then used to remove the granulating center of each specimen for separate analysis. Wounds from 12 animals were studied on days 1 and 3, and 10 animals on day 5.

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Group III. Skin-wound margin, granulating wound center above the panniculus (Fig. 1). Wounds were made with a 6 mm punch and removed on days 1, 3 and 5 with a 8 mm punch. The wounds were separated into granulating wound center minus the panniculus (6 mm punch), wound margin (remaining 2 mm) and panniculus carnosis underlying the wound. Collagen and total protein synthesis were measured in duplicate in each wound component. Four animals were studied on days 1 and 3, and two animals were studied on day 5.

Results. Group I. Relative collagen synthesis in complete full-thickness wounds was increased by 24–48 hr after wounding and was significantly greater than normal skin by 5 days ($p < 0.005$) (Fig. 2). Collagen synthesis continued to increase until day 7 and thereafter decreased until day 11 when the activity was the same as normal unwounded skin (Fig. 2). Absolute collagen synthesis ([^{3}H]proline incorporated into collagen per mg dry weight) followed the same pattern as relative collagen synthesis.

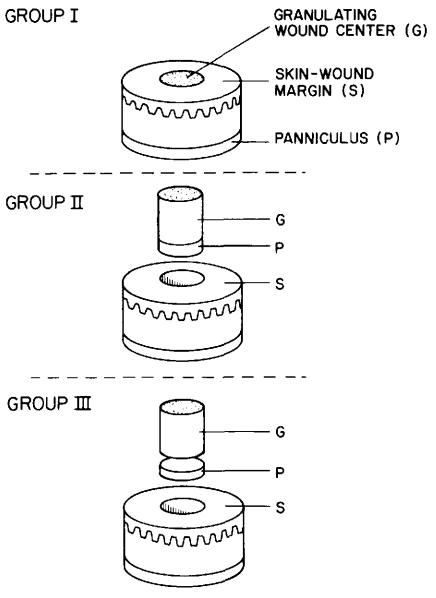

FIG. 1. Diagram of wounds and components that were analyzed. Group I wounds contained granulating wound center, skin-wound margin and underlying panniculus. The granulating wound center with panniculus attached was analyzed separately from the skin-wound margin in Group II animals. The wounds in Group III were subdivided into granulating wound center (minus panniculus), panniculus and skin-wound margin.

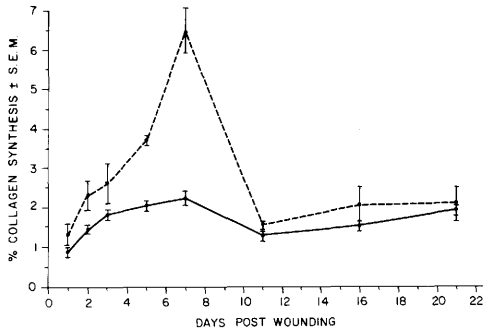

FIG. 2. Time course of relative collagen synthesis in full rat skin wounds (Group I). Solid line represents normal skin values and dashed line represents wound values. Duplicate samples from three animals were analyzed on each day. % Collagen Synthesis = cpm in collagenase digest/(cpm in residue × 5.4) + (cpm in collagenase digest) × 100 (4).

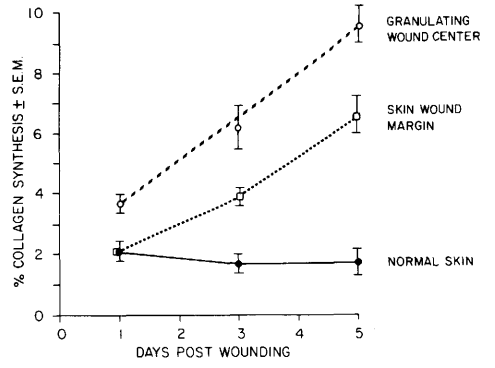

FIG. 3. Time course of relative collagen synthesis in granulating wound center and skin-wound margin (Group II). Wounds from twelve animals were studied on days 1 and 3 and 10 animals on day 5. Normal skin values were obtained from 8 animals. % Collagen Synthesis = cpm in collagenase digest/(cpm in residue × 5.4) + (cpm in collagenase digest) × 100 (4).

Group II. Dissection of the open wound into the granulating wound center (containing panniculus carnosis) and skin wound margin (Fig. 1), indicated that relative collagen synthesis was increased by 24 hr in the granulating wound center (Fig. 3). By days 3 and 5, relative collagen synthesis in the granulating wound center was significantly increased ($p < 0.005$) compared to normal skin and skin-wound margin ($p < 0.01$). Relative collagen synthesis in the skin-wound margin was significantly greater than normal skin on days 3 and 5 ($p < 0.005$). Normal skin collagen synthesis was less than either wound component and remained constant during the

5 days studied. Absolute collagen synthesis in the granulating wound center was significantly increased ($p < 0.025$) at 24 hr (141 ± 22 cpm/mg dry wt) compared to normal skin (40 ± 18 cpm/mg dry wt) and remained increased on days 3 and 5.

Group III. Further defining the observations of Group II, the central wound area was separated into panniculus carnosis and the granulating wound center above the panniculus and then these two wound components were analyzed separately (Fig. 1). Relative collagen synthesis in the wound panniculus was significantly elevated on days 3 and 5, whereas none of the other specimens was different from normal skin (Table I). In addition there was a significant increase in absolute collagen synthesis in the wound panniculus compared to all other areas as early as 24 hr post-wounding (Table II). Increased absolute collagen synthesis in the wound panniculus persisted throughout the five days of the study (Table II). Absolute collagen synthesis in the skin-wound margin was greater than normal skin at 24 hr and remained so for the 5 days of the study, whereas absolute collagen synthesis in the granulating wound center above the panniculus was elevated only at 24 hr (Table II).

Discussion. The initial phase of wound repair (day 0 to 4) has been described by Howes *et al.* (1) as a "lag phase" during which time there is a delay in fibroplasia and no gain in tensile strength. More recently, Ross' (2, 6) electron and light microscopy studies have demonstrated intense inflammatory cell activity, neovascularization, and the onset of fibroblast migration during the "lag" period. Therefore, the more appropriate term "inflammatory or substrate phase" was adopted. However, Ross' electron microscopy studies did not detect new collagen fibrils until day four (6). Similarly, Madden and Peacock (3) observed no significant change in the specific activity of hydroxyproline until the third day after wounding.

In contrast, the present studies demonstrate that collagen synthesis begins in the wound center as early as 24 hr after wounding (Fig. 3). When the wound center is further dis-

TABLE I. RELATIVE COLLAGEN SYNTHESIS IN NORMAL SKIN AND VARIOUS WOUND AREAS (GROUP III).[a]

	% Collagen synthesis ± SEM[b]				
Day	Normal skin	Normal skin panniculus	Skin wound margin	Granulating wound center minus panniculus	Wound panniculus
1	3.2 ± .6	2.8 ± .4	2.4 ± .3	1.8 ± .1	3.1 ± .2
3	3.3 ± .6	2.3 ± .2	3.1 ± .8	2.3 ± .5	7.3 ± .4[c]
5	3.8 ± .4	2.4 ± .1	4.0 ± .9	2.7 ± .3	10.3 ± .2[c]
Mean	3.4 ± .3	2.5 ± .3			

[a] The values for days 1 and 3 are from four observations and values for day 5 are from two observations. The mean values for normal skin and normal skin panniculus represent ten observations.

[b] %Collagen Synthesis = cpm in collagenase digest/(cpm in residue × 5.4) + (cpm in collagenase digest) × 100 (4).

[c] $p < 0.001$ compared to normal skin panniculus and normal skin.

TABLE II. ABSOLUTE COLLAGEN SYNTHESIS IN NORMAL SKIN AND VARIOUS WOUND AREAS (GROUP III).[a]

	Collagen synthesis, cpm/mg dry wt. ± SEM				
Day	Normal skin	Normal skin panniculus	Skin-wound margin	Granulating wound center minus panniculus	Wound panniculus
1	66 ± 3	136 ± 25	119 ± 21[c]	817 ± 91[c]	2237 ± 379[b]
3	60 ± 12	181 ± 40	143 ± 45[c]	22 ± 3	3045 ± 342[b]
5	19 ± 1	345 ± 37	176 ± 36[c]	21 ± 1	3085 ± 209[b]
Mean	54 ± 7	196 ± 31			

[a] The values for days 1 and 3 are from four observations and values for day 5 are from two observations. The mean values for normal skin and normal skin panniculus represent ten observations.

[b] $p < 0.001$ compared to normal panniculus.

[c] $p < 0.05$ compared to normal skin.

sected, there is significant absolute collagen synthesis at 24 hr localized in the panniculus carnosis (Table II). It is not surprising that relative collagen synthesis in wound panniculus is not different than normal skin at 24 hr (Table I) while absolute collagen synthesis in panniculus is significantly increased (Table II). This is because all protein synthesis in the wound panniculus carnosis is significantly increased at 24 hr. However, by day 3 the increase in absolute collagen synthesis is greater than all other protein synthesis. Therefore, both relative and absolute collagen synthesis in the wound panniculus are significantly greater than normal skin (Tables I and II).

These present obervations are not in disagreement with other studies. For example, in Ross' electron microscopy studies of guinea pig open wounds, the panniculus carnosis was not analyzed (6). If Ross had examined the panniculus, he may have observed collagen fiber formation prior to day 4. Madden and Peacock's inability to observe increased collagen synthesis until day three in sutured wounds which contained a 1 mm margin of normal skin may have been due to normal skin diluting the small amount of panniculus carnosis in these wounds (3). In contrast, the open wound model used in our studies provides sufficient wound tissue for analysis without contaminating normal skin. In addition, only by dissecting the open wound into components, could the increased absolute collagen synthesis be detected in the panniculus carnosis at 24 hr (Table II). Furthermore, Madden and Peacock's studies relied on changes in the specific activity of tissue hydroxyproline during wound healing (3). This technique may be inaccurate because alterations in the amino acid cell pool size would alter radioactive hydroxyproline specific activity and therefore alter collagen synthesis data. Moreover, as their data is calculated on the basis of collagen content in the wound at the time of sampling, increased wound collagen degradation could yield a spurious increase in collagen synthesis. The present studies overcome these problems because collagen synthesis is determined relative to total protein synthesis. This method assumes that the same cell pool amino acids are used for both collagen and noncollagen protein synthesis.

The present study demonstrates for the first time that significant collagen synthesis begins as early as 24 hr after wounding and that most of the activity is in the panniculus carnosis (Table II). The cellular source of this increased collagen synthesis is not clear. Ross' observation that a significant quantity of fibroblasts do not migrate into the wound until day six (6) suggests that the early collagen synthesizing cells must be local. Perhaps local dormant fibroblasts are stimulated at the time of wounding to increase collagen synthesis. The stimulus for this very early collagen synthesis may be reduced oxygen tension and increased lactate production as suggested by Hunt *et al.* (7) or released tissue amines as suggested by Sandberg (8). In addition, Postlethwaite and Kang have observed that collagen and collagen degradation products are chemotactic for blood monocytes (9). As these macrophages accumulate in the wound, they may in turn stimulate local dormant fibroblasts to synthesize collagen. Several laboratories have evidence which suggests that inflammatory cells stimulate fibroblasts and collagen synthesis (10–13). Leibowich and Ross demonstrated that wound fibroplasia was diminished by anti-macrophage serum (10) and they have identified a macrophage factor which stimulates fibroblasts *in vitro* (11). Likewise, Casey and coworkers (12) noted that collagen synthesis was increased in rat wounds after instillation of allogenic macrophages while Clark and his colleagues (13) noted that activated macrophages stimulated neovascularization and fibroplasia in rabbit cornea.

The collagen produced immediately following wounding may be vital in establishing initial wound integrity and may provide a "scaffold" for new vessel ingrowth and inflammatory cell migration. Therefore, as the inflammatory cells are attracted and guided into the wound by the newly synthesized collagen lattice, the macrophages in turn release chemotactic factors to stimulate fibroblasts in the wound margin to migrate into the wound and synthesize additional collagen.

In addition, this early synthesized wound collagen may have a different composition than normal dermal collagen. Epstein has found that normal human adult dermis con-

tains about 85% Type I collagen and about 15% of Type III collagen (14). In early granuloma pouch wounds Bailey and co-workers (15) noted an increase in Type III collagen in the early stages of granuloma formation which decreased with time until a normal ratio of Type I to III collagen was re-established. However, this wound model is based on a foreign body reaction to turpentine and may not accurately reflect normal wound healing. Gay *et al.* (16) recently reported early increased Type III collagen in human dermal wounds which returned to a normal distribution of Type I and III after 72 hr. Collagen Typing in healing dermal wounds and its significance remains to be thoroughly investigated.

Although the present studies demonstrate that relative collagen synthesis in the wound returns to a normal level by day 11 (Fig. 2), previous studies by Madden and Peacock (3) noted significant increases in primary wound collagen synthesis until at least 70 days after wounding. Once again, the technique used in their studies relied on hydroxyproline specific activities and alterations in collagen content which can be inaccurate as discussed above.

The present studies demonstrate that the initial phase after wounding certainly is not a "lag" phase and is more than merely an "inflammatory" or "substrate" phase. Rather, this initial period of wound repair is characterized by significant collagen synthesis.

Summary. Previous studies suggest that there is an initial "lag" or "latent" phase following wounding. During this 4- to 6-day period, there is very little collagen synthesis as judged by tensile strength measurements, light and electron microscopy, and changes in the specific activity of hydroxyproline. In the present study a sensitive biochemical method was used to measure collagen synthesis during the initial phase of open wound repair in rat skin and a significant increase in collagen synthesis was detected as early as 24 hr. Analysis of various wound components demonstrated that the early increased collagen synthesis was localized in the panniculus carnosis. Collagen synthesis continued to increase with the greatest activity occuring on day 7 and by day 11 had returned to normal skin levels. We conclude from these studies that collagen synthesis begins by 24 hr after wounding and that the initial phase of wound repair is not preceded by a "lag phase".

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