Human Wound Contraction: Collagen Organization, Fibroblasts, and Myofibroblasts

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The closure of ungrafted sacrococcygeal pilonidal sinus excisional wounds was studied in 15 patients. Wound punch biopsies were taken on a regular basis, and histologic sections were made. To document changes, computer-assisted morphometric image analysis was employed. Initial average wound depth was 37.8 ± 4.6 mm, and complete closure (0 wound depth) was reached by 68 days. Wound contraction contributed 88 percent to wound closure, whereas the deposition of scar only contributed 12 percent. Maximum cells density within granulation tissue was reached by day 18. Myofibroblasts, identified by α-smooth muscle actin immunostaining, first appeared on day 11. Unlike those observed in laboratory animals, myofibroblasts were a minor cell population of granulation tissue, never exceeding 10 percent of the cells. The pattern of collagen fiber organization was documented by polarized light microscopy of Sirius red-stained sections. Early granulation tissue collagen fibers demonstrated a fine greenish birefringence, whereas more mature granulation tissue collagen fibers were thicker, displaying orange-yellowish birefringence. Myofibroblasts were associated exclusively with thicker collagen fibers, whereas fibroblasts were associated with both fine and thick collagen fibers. It is proposed that human wound contraction involves a volume change whereby normal dermal and adipose tissues are pulled into the defect by forces generated within fibroblasts. (Plast. Reconstr. Surg. 102: 124, 1998.)

Wound closure, resulting in the restoration of the skin's integrity, usually requires the de novo deposition of a dermis-like matrix within the defect, a scar. In addition to the deposition of new tissue in open wound closure, the "pulling in" of surrounding dermis and adipose tissue by wound contraction contributes to wound closure. The volume of scar deposited in a healed wound is influenced by the wound's initial size and by the role wound contraction plays in its closure. The greater the role of wound contraction, the less residual scar deposited.

Although wound healing by secondary intention has received much attention in recent years, almost all of the studies with human subjects have been limited to the prediction of healing time, wound infection, or the type of dressing to be used. In vivo studies on the basic pathophysiology of human wound contraction are few. Until recently, only two comprehensive histologic studies of human wound contraction had appeared. Both studies concluded that histologic differences may exist in human wound contraction compared with laboratory animals. The objectives of this study were to characterize human wound contraction in terms of a tissue volume change, the presence as well as location of myofibroblasts, and the pattern of collagen fiber bundles within maturing granulation tissue.

Cells within fibroblast-populated collagen lattices organize collagen fibers by compacting the fine collagen fibrils into thicker collagen fibers. In vivo, it is proposed that wound contraction occurs by compacting collagen fibrils within granulation tissue, which decreases its volume, leading to the pulling in of surrounding dermis and adipose tissue into the defect. By Sirius red staining and polarized light optics, the maturation of fine collagen fibers into thicker collagen fiber bundles within granulation tissue can be followed by changes in the birefringence pattern.
The maturation of granulation tissue parallels changes in the resident fibroblasts' expression of different isoforms of actin in their cytoskeleton. Normal dermal fibroblasts do not express alpha smooth muscle actin. Populations of fibroblasts having prominent stress fibers that contain α-smooth muscle actin are more differentiated fibroblasts, myofibroblasts. Myofibroblasts are readily identified in maturing granulation tissue and have been proposed as the cells responsible for generating the forces of wound contraction. In addition to their presence in contracting wounds, myofibroblasts have also been identified in contracting fibrotic lesions. Morphologically, myofibroblasts have thick bundles of microfilaments within stress fibers, indented nuclei, and cell-cell attachments as demonstrated by the electron microscope.

METHODS

The healing of sacrococcygeal pilonidal sinus excisional wounds was followed by periodic measurements of wound depth and by the histologic evaluation of invasive serial biopsies taken from day 3 postsurgery and continued until the wounds were completely closed. Morphometric image analysis was performed to document changes in the volume of tissue within the wound bed as wound closure proceeded.

Following ethic committee approval and having obtained informed consent, 15 patients [12 males, 3 females with a mean age of 30.1 years (range 21 to 40 years)] were studied. They all had standard, elective, sacrococcygeal pilonidal sinus excision wounds (Fig. 1). All patients were well, and none had any concomitant disease that might adversely affect their wound healing. Weekly measurements of wound depth and 6-mm punch biopsies were taken from day 3 postsurgery and terminated when wound closure was complete.

Histologic Evaluation

All biopsies were formalin fixed and paraffin embedded, and 5-μm-thick sections were cut. Sections were stained with hematoxylin and cosin and quantitatively examined using the Kontron IBAS image analyzer. The system used a low-power image of the whole biopsy that was projected onto a video screen, where four tissue layers could be clearly identified (Fig. 2). The area of each layer was measured by projecting the microscopic image onto a computer screen and circumscribing the area with a drawing tablet. The mean depth of the layers was then calculated by dividing the area by the width of the biopsy. The mean width of each biopsy was calculated by making five serial width measurements (Fig. 2).

As shown in Figure 2, four distinct layers were identified: superficial slough, granulation tissue (as evidenced by patent blood vessels with surrounding edema), a collagenous layer, and a layer of fat tissue. Because the wound bed originally consisted of fat, only biopsies demonstrating the basal fat layer were included in the study as evidence that the biopsy represented the full thickness of de novo healing tissue. The de novo healing tissue included both the granulation tissue layer and the more mature collagenous tissue layer.

Sirius Red Staining

Fixed embedded sections were stained with Sirius red by the technique of Constantine and Mowry. Sections were postfixed in Bouin's solution for 24 hours and then incubated for 20 minutes in saturated picric acid solution containing picrosirius red at 1 mg/ml. The sections were rinsed in water, mounted, and viewed with an Olympus BH-2 microscope equipped with polarized light optics. Photographs were taken with Ektachrome Elite 200 film (Kodak, Rochester, N.Y.).

Immunostaining of Tissue Section

Paraffin-embedded sections were pretreated with 7% hydrogen peroxide in distilled water and subsequently with 0.1 M periodic acid, 5 mM NaBH₄, and normal serum. The sections
were incubated for 2 hours with anti-α smooth muscle actin antibody, a mouse IgG₂₄ monoclonal antibody against α-smooth muscle actin diluted 1:200.11,17 The presence of α-smooth muscle actin was demonstrated by means of the streptavidin-biotin complex peroxidase technique (Dako A/S, Glostrup, Denmark). The substrate for peroxidase activity was 3-amino-9-ethylcarbazole (Sigma, St. Louis, Mo.). Slides were counter stained with hematoxylin. The sections were washed in water, mounted, and viewed. Evaluation of the number of myofibroblasts staining in a 20-power objective field was made by two independent observers. The count of α-smooth muscle actin positive cells was performed on three randomized areas within the granulation tissue section, and the cell type was determined using the following classification. Vascular smooth muscle cells of the blood vessels were positive for α-smooth muscle actin and functioned as a positive internal control for immunostaining specificity and intensity. The absence of α-smooth muscle actin staining in spindle-shaped cells defined fibroblasts. Similarly, the presence of α-smooth muscle actin staining in spindle-shaped cells, established not to be vascular smooth muscle cells, defined myofibroblasts.

RESULTS

In total, 107 biopsies were analyzed from the 15 patients studied. The mean time for closure of these excisional wounds was 68.3 days, range 38 to 83 days (Fig. 3). The mean early wound depth (day 3) was 37.6 ± 4.6 mm, which represented the depth of the surgical excision. By quantitative morphologic analysis of the biopsy sections at day 3, the mean depth for the staging area of granulation tissue deposition, the region that separated the scab on its top surface and viable deep dermis on its bottom sur-

Fig. 2. A schematic representation of the four tissue layers within the 6-mm punch biopsy. The procedure involved in calculating the areas of the four layers and measuring the overall mean width of the four tissue layers is presented (left). (Right) the calculation of the mean depth of each layer is presented.

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\overline{W} = \frac{W_1 + W_2 + W_3 + W_4 + W_5}{5}
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\overline{D}_{GT} = \frac{\text{AREA OF GT LAYER}}{\overline{W}}
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face, was 0.56 ± 0.14 mm. As the repair process progressed, the mean depth of the granulation tissue increased for 28 days, after which time no further increase was measured (Fig. 3). At complete wound closure, the mean depth of the scar was 4.83 ± 0.75 mm, demonstrating that de novo tissue deposition only accounts for 12.5 percent (4.83 mm/37.6 mm) of the healed wound. The closure of this defect was mostly by pulling in surrounding dermal and adipose tissues, wound contraction, and not de novo tissue deposition.

The histologic evaluation of these healing wounds showed an acute inflammatory response in evidence at day 3. At that time, there was no granulation tissue, and the dead space contained mostly fibrin populated with neutrophils. The tissue was void of both α-smooth muscle actin-positive cells and connective tissue birefringence. By day 6, early granulation tissue was in evidence, and neutrophils and a few fibroblasts were identifiable in the hematoxylin and eosin-stained section (Fig. 4, above). By α-smooth muscle actin staining, positive vascular smooth muscle cells were identified within the medial wall of blood vessels in the adjacent dermis and adipose tissue, but no myofibroblasts were resident in this early granulation tissue. The organization of the newly deposited collagen in the early granulation tissue was minimal, as demonstrated by insignificant Sirius red-stained polarized light birefringence (Fig. 4, center). In contrast, the collagen fibers in normal dermis produced intense birefringence (Fig. 4, below).

Granulation tissue was well established at the base of the healing wound by 11 to 14 days, where new blood vessels were identified (Fig. 5, above, left) and modest populations of myofibroblasts were evident within the deeper granulation tissue (Fig. 5, above, right). The earliest time point when myofibroblasts were identified in these contracting wounds was day 11. By Sirius red staining and polarized light optics, a pattern of fine birefringence of the newly deposited collagen matrix was evident (Fig. 5, center, left). The birefringence was green, indicative of fine, minimally organized, immature collagen fibers, typical of young granulation tissue.

Myofibroblasts were established in granulation tissue at 18 days (Fig. 5, center, right). However, fibroblasts (spindle-shaped cells negative for α-smooth muscle actin) were the most abundant cell type in that granulation tissue.

The density of fibroblasts and myofibroblasts had reached a maximum by 18 days, where both fibroblasts and myofibroblasts had increased equally from day 11. The percentage of myofibroblasts to fibroblasts was only 10 percent. The collagen fibers showed more intensive birefringence as the collagen fibers became longer and thicker (Fig. 5, below), and
these myofibroblasts were limited to these areas.

At 39 to 40 days, the overall density of spindle-shaped cells (both myofibroblasts and fibroblasts) in granulation tissue had declined compared with the density seen at 18 days (hematoxylin and eosin staining as shown in Fig. 6, above). The proportion of myofibroblasts was unchanged (Fig. 6, center), and they were located in areas where more organized collagen fibers as demonstrated by intense birefringence were present (Fig. 6, below). Based on these observations, the remodeling phase of repair was well underway at this time.

**Fig. 5.** Histologies of healing wounds at days 11 and 18 are presented. (Above, left) An 11-day-old wound stained with hematoxylin and eosin shows prominent blood vessels; see arrows (magnification 100×). (Above, right) A 14-day-old wound stained for α-smooth muscle actin shows myofibroblast populations; see arrow. For vascular smooth muscle cell, see arrowhead (magnification 200×). (Center, left) An 11-day-old wound stained with Sirius red and viewed by polarized light is shown (magnification 100×). (Center, right) An 18-day-old wound immunostained for α-smooth muscle actin myofibroblasts at the arrow and unstained fibroblasts at the arrowheads (magnification 200×). (Below) An 18-day-old wound stained with Sirius red and viewed by polarized light shows the increase in birefringence as a consequence of increasing collagen organization (magnification 100×).

**Discussion**

The anatomic location of an open wound dictates the role wound contraction will play in its closure.17 Open wounds on the extremities demonstrate minimal wound contraction. An open wound on the sacrococcygeal region is a site where wound contraction plays a major role in wound closure, and the need for a skin graft is unnecessary. Though wound contraction seems slower in people than in animal models, such as rats, it is an effective way to restore lost tissue. Repair by wound contraction produces a healed defect filled with der-
The filling in of the defect with scar tissue plays a modest role in the closure of sacrococcygeal pilonidal sinus excisional wounds.

The proposed mechanism of wound contraction is that granulation tissue collagen fibers are compacted by cellular forces. The compaction of granulation tissue produces a force that pulls in the surrounding tissues. The wound contraction process does not involve the contraction of cells that directly pull on the surrounding tissues.

It is advanced that wound contraction closure of sacrococcygeal pilonidal sinus excisional wounds is a three-dimensional process. Wound contraction at the surface is viewed as a decrease in area, a two-dimensional change. However, within the wound site there is a three-dimensional change occurring, where the volume of dermis occupying the wound site is increasing while the volume of granulation tissue is not contributing to filling in the defect. In the sacrococcygeal pilonidal sinus excisional wound model, the initial defect had a much greater volume compared with the experimental excisional wound made in a rat. Full excision rat wounds are much shallower because the region where the sacrococcygeal pilonidal sinus excisional wound is made, the skin is much thicker. The closure of such a deep wound requires more time.

The suggested mechanism for wound contraction is cellular generated forces reorganizing collagen through the mechanical translocation of the collagen fibrils, where fine collagen fibrils become thicker and longer fibers. That degree of organization of collagen fibrils is demonstrated by the intensity and pattern of polarized light induced birefringence. The contracting rat wound granulation tissue shows minimal birefringence and a high density of resident myofibroblasts at 7 days. On the other hand, human contracting wounds do not show myofibroblasts before day 11. The myofibroblasts within 14-day rat granulation tissue are associated with collagen fibrils demonstrating minor birefringence (unpublished observation). In contrast, myofibroblasts of human granulation tissue are associated with collagen fibers with established birefringence. The myofibroblasts associated with fine collagen fibrils in rat granulation tissue compared with their association with thick collagen fibers in human granulation tissue cannot be attributed to differences in species specificity. In the nodules of human hypertrophic scar, myofi-
broblasts are only associated with fine collagen fibrils. Here, in human contracting wounds, myofibroblasts are only associated with thicker collagen fibers. These differences imply that hypertrophic scar myofibroblasts are associated with immature collagen fibers, whereas in wound contraction they are associated with mature collagen fibers. These contrasts imply that the processes of wound contraction and scar contracture involve different cellular mechanisms.

In the tight-skin mouse, the contraction of full excision wounds was delayed for 3 weeks, but the granulation tissue from those noncontracting wounds displayed a high density of myofibroblasts. At the end of week 4, wound contraction proceeded at a normal rate, when cells demonstrating myofibroblast morphology had disappeared. At week 5, cells having morphologic characteristics of myofibroblasts had reappeared when the rate of wound contraction had slowed. In tight-skin mouse healing, myofibroblasts appeared between 1 and 3 weeks, when no wound contraction occurred; disappeared between weeks 3 and 4, when wounds contracted by 50 percent; and reappeared between weeks 4 and 6. These results do not support a role for myofibroblasts in wound contraction.

Normal mature scar and keloids, which display no scar contracture, do not have myofibroblasts. The birefringence patterns of collagen in those tissues show variations, but generally the collagen is organized as thick collagen fibers that demonstrate yellowish-orange birefringence. Both myofibroblasts and fibroblasts have been identified within tissues undergoing either wound contraction or scar contracture. The granulation tissue of laboratory animals shows high densities of myofibroblasts between 1 and 3 weeks, whereas the proportions of myofibroblasts in wound contraction in these model wounds were low. Wound contraction was effective in closing these large wounds in the absence of a high density of myofibroblasts.

This implies that fibroblasts generate the cellular forces responsible for wound contraction, whereby fibroblasts organize the fine collagen fibrils into thick collagen fibers. A proportion of these fibroblasts differentiate into myofibroblasts, which occurs after the collagen fibrils have been organized. The contracting unit responsible for wound contraction seems to be the compaction of the connective tissue within granulation tissue, which tugs on the surrounding dermis and adipose tissues. There is no evidence to support the concept that cells pull directly on the surrounding tissues to cause wound contraction.

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