Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5982–5986, June 1995
Medical Sciences

On the mechanism of skin wound "contraction": A granulation tissue "knockout" with a normal phenotype

(wound repair/closure/surgical excision/pig)

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Contributed by Jerome Gross, April 4, 1995

ABSTRACT **This report explores the mechanism of spontaneous closure of full-thickness skin wounds. The domestic pig, often used as a human analogue for skin wound repair studies, closes these wounds with kinetics similar to those in the guinea pig (mobile skin), even though the porcine dermis on the back is thick and nearly immobile. In the domestic pig, as in the guinea pig, daily full-thickness excisions of the central granulation tissue up to but not including the wound edges in both back and flank wounds do not alter the rate or completeness of wound closure or the final pattern of the scar. A purse-string mechanism of closure was precluded by showing that surgical interruption of wound edge continuity does not alter closure kinetics or wound shape. We conclude that "tightness" of skin is not a key factor nor is the central granulation tissue required for normal wound closure. These data imply that *in vitro* models such as contraction of isolated granulation tissue or of the cell-populated collagen lattice may not be relevant for understanding the cell biology of *in vivo* wound closure. Implications for the mechanism for wound closure are discussed.**

Cellular mechanisms whereby open full-thickness excision wounds in adult mammalian skin are closed spontaneously are speculative (1–6). The currently prevailing hypothesis (6, 7), originally stated in 1956 (8, 9), proposes that the central granulation tissue generated shortly after wounding is a contractile machine that, through an undefined action of its fibroblasts, pulls the edges of the wound together. Recent papers on the subject (4, 7, 10, 11) promulgate the idea that a significant fraction of the mesenchymal cells of the granulation tissues, called myofibroblasts (12), have contractile powers that are exerted on collagen fibers, other matrix components, and each other. Observations by Harris and colleagues (13, 14) on the mechanical effects of fibroblast traction on the organization of fibrous collagen lattices led Ehrlich (15, 16) to an alternative hypothesis that proposes that "cells (fibroblasts) working as single units use cell locomotion forces to reorient the collagen fibrils associated with them" (15). The implication is that the reoriented matrix collagen of the granulation tissue transmits the contractile force (4). However, wounds close at a normal rate in scorbutic animals (8, 17) in which new collagen production is blocked, thus posing a serious hurdle for this concept unless one considers that other matrix components such as fibronectin (11, 18) have the necessary tensile properties.

The mechanism proposed by Abercrombie and colleagues (8, 9) was reexamined by Grillo and associates in 1958 (19) by using square full-thickness excision wounds in the guinea pig skin. Biochemical analyses of wound contents led the authors to question the proposed role of the central granulation tissues. Total removal of granulation tissues up to but not including the wound edge, periodically and frequently during the course of wound closure, did not alter the rate or extent of "contraction" compared with nonmanipulated control wounds in the same animal. In contrast, undercutting the wound edges between the dermis including panniculus carnosis and the deep fascia caused an immediate retraction, from which the edges soon resumed an inward movement. It was concluded (20) that the central granulation tissues were not required for contraction, but instead, a narrow 1- to 2-mm-wide rim of newly proliferated fibroblasts under the wound edge was responsible for closing the wound. This mass of fibroblasts, forming a subdermal "picture frame," tethers the dermal margins to the deep fascia below and, by directional mass migration, pulls the intact dermis inward.

It was stated as likely (2, 21–23) that full wound closure by "contraction" as studied in the guinea pig, rabbit, and rat may be limited to animals with very mobile skin but unlikely where the skin is more firmly attached as in many regions of the human and the domestic pig. The corollary question, then, is whether the granulation tissue excision experiments in the guinea pig (20) are relevant to wounds in pigs and humans. The experiments reported here in the domestic pig, a furless animal with thick nearly immobile back and flank skin considered to be an acceptable model for studies on human wound repair (24–26), answer both points. They raise other questions regarding the role of granulation tissue and the usefulness of some old and current (23) *in vitro* models for explaining the mechanism of wound "contraction."

METHODS

Domestic pigs of ≈50 pounds (1 pound = 453.6 g) starting weight were housed in appropriate and approved pens in the Massachusetts General Hospital Animal Resources Facility according to standards set by the American Association of Accreditation for Laboratory Animal Care. All surgical manipulations were performed after intramuscular ketamine/xylazine induction anesthesia, followed by halothane inhalation anesthesia. An antibiotic (1 g of cefazolin) was administered intramuscularly after each procedure. Hair in the surgical fields was removed with clippers and the fields were gently scrubbed with soap and water followed by betadine wash and rinsing with 70% isopropyl alcohol prior to surgery.

In each of the two animals used sequentially in these experiments, 16 full-thickness excision wounds, 2 × 2 cm squares, were cut in a pattern of eight on each side, with four located dorsally on the back several centimeters away from the midline and four located ventrally over the flank (Fig. 1*A*). All wounds were nearly identical in size and anatomical depth down to the deep fascia overlying the body wall muscle layer. Each wound area was first outlined with a template and eight tattoo markers were placed one at each corner and the others centered on each of the four sides by repeated punctures

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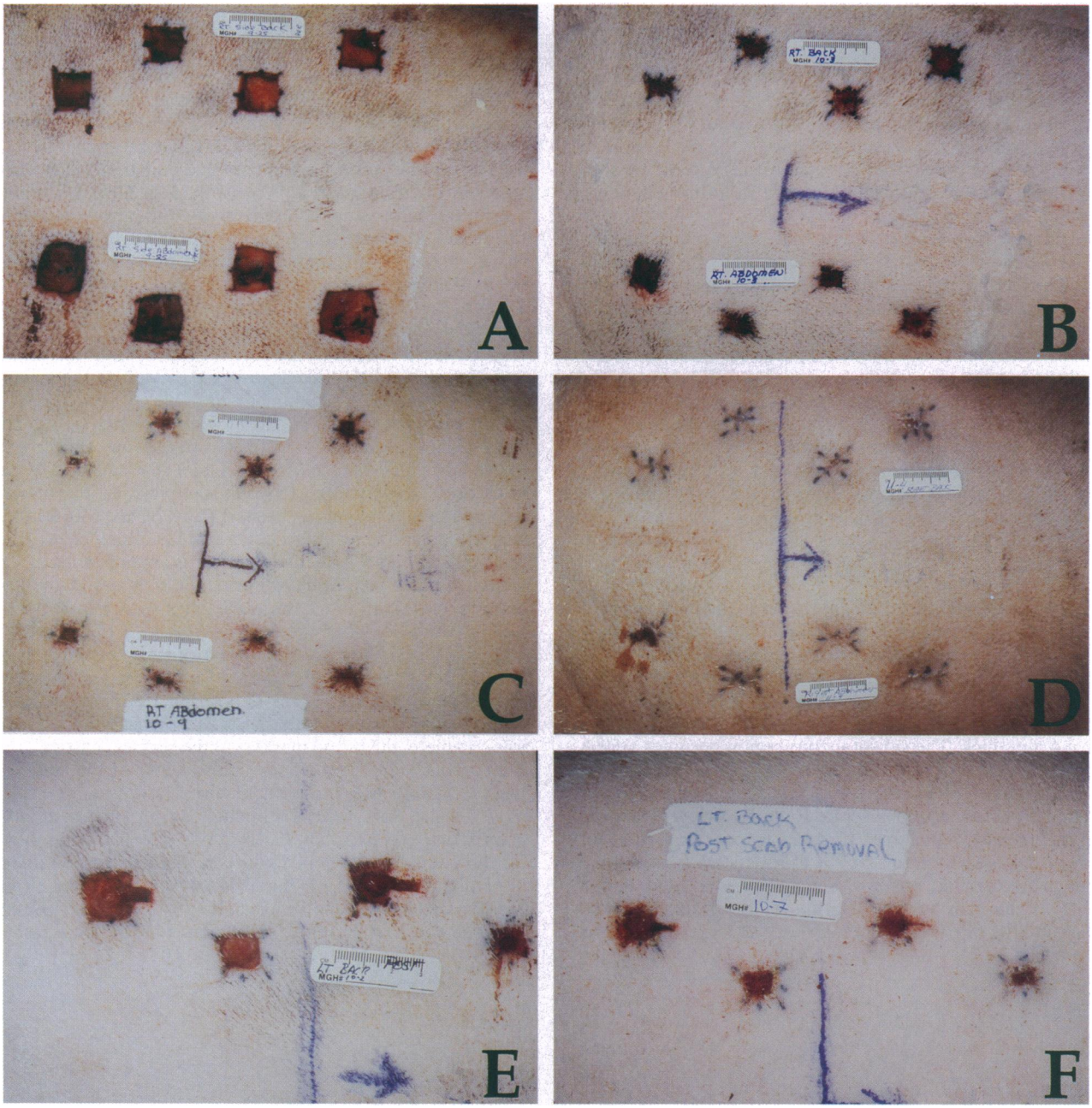

FIG. 1. Photographs of the skin wound fields on the right side of the body of pig 2. The four daily-excised wounds are on right side of blue vertical line, the four controls are to the left of the line, and the back wounds are the upper set. Calibration stickers on skin in each field permit normalization of magnification variations. Selected days after wounding are as follows: (*A*) Day 1. (*B*) Day 10. (*C*) Day 16. (*D*) Day 41. The effect of a thin strip excision perpendicular to wound edge to check the "purse string" theory. Left back. (*E*) Immediately after strip excision on day 9. (*F*) Same wounds on day 14.

perpendicular to the skin of a 27-gauge needle administering india ink from a tuberculin syringe. These vertical tattoo marks left continuous, clearly visible trails from the skin surface to the deep muscle. Skin excisions along the template were made through the tattoo marks down to the deep fascia overlying body wall muscle.

In pig 1, the central granulation tissues were completely excised under full anesthesia every 3 days to within 1 mm or less from the dermal walls of the wound bed, visibly down to the deep fascia. Bleeding was easily controlled without sutures or electrocautery. These excisions were performed in four of the eight wounds on each side, two each from the anterior dorsal and ventral fields on the left and the same number in the posterior dorsal and ventral fields on the right side, thus controlling for anatomical position. The remaining wounds were left undisturbed as controls. Self-adhesive sterile 4 × 14 inch dressings (Coverlet O. R. Adhesive Surgical Dressing 2249; 1 inch = 2.54 cm) were applied and covered with a webbing body sleeve sutured in place. Granulation tissue excisions were stopped on day 16 and wounds were allowed to heal undisturbed for another 41 days, after which the animal was killed with intravenous anesthetic and the wounds were widely excised for histologic study. Area measurements were made as reported below.

Due to significant regrowth of central granulation tissues in the 3-day interval between excision of wound contents, a second experiment (pig 2) was modified only in that excisions were performed daily (also under full anesthesia) for 15 days. This experiment is reported below in detail.

Wound fields were photographed with size markers. Tracings of wound edges with marks locating the tattoos were made

on transparent plastic sheets prior to and immediately after excisions of the wound content. Area measurements were made from photocopies of the transparencies and also from the photographs, cutting out the enclosed areas and weighing the paper cutouts. Direct area measurements were also calculated from wounds in the second pig by computerized tracing from the transparencies. Results comparing the data obtained via each method were essentially identical. Percent of area change from day 1 as a function of days after initial wounding was tabulated and charted for each wound.

The possible involvement of a purse-string mechanism closing the wound was tested at ≈50% closure by excising a 1-mm-wide full-thickness strip of tissue spanning the wound edge on one side in a nonmanipulated control wound and in one wound repeatedly excised. In another pair of wounds, a single incision was made in each of the four sides, similarly spanning the edge.

Tissues taken for histologic study of the wound edges, central granulation tissues, and final scars at selected time points were fixed in paraformaldehyde, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin/eosin, Mallory's trichrome, and Sirius red stains (histology not shown).

RESULTS

The backskin excision sites, ≈5 cm lateral to the midline, were nearly immobile upon simple palpation in the anesthetized pig with somewhat greater mobility displayed over the flanks. The full thickness of the back skin in the freshly cut wounds on day 1, measured ≈1 cm in thickness compared with ≈0.5 cm over the flank. The freshly cut back skin dermis is a dense fibrous layer ≈4 mm thick. The subcutaneous region occupies >50% of the full thickness of the skin. Histologically, it is a dense firm white fatty tissue with scattered lipocytes and fibroblasts, laden with numerous collagenous septa bound to the deep fascia covering the body wall muscle. In the intact back skin, two thin sheets of collagenous fascia and a thin striated muscle layer (panniculus carnosis) traverse the subcutaneous fat layer parallel to the skin surface. Dermis of the flank skin was thinner and the fatty subcutaneous tissue was greatly reduced in thickness and missing the panniculus carnosis and thin fascial sheets present in the back skin. The walls of the excised areas remained vertical during the early phases of closure. Very little solid material other than blood clot was removed with each wound excision. Care was taken to visualize the full extent of the white fascial floor, indicating grossly complete granulation tissue removal each day. There appeared to be very little granulation tissue regrowth within the 24-h intervals.

Direct observation of all wound fields before and immediately after daily complete excision of granulation tissues over the entire experimental sequence revealed little change in shape or size as an immediate result of excision with little obvious difference in areas between the control and excised wounds. Fig. 1 *A–D* is a selected set of photographs of the right side of the wound field of pig 2 at days 1, 10, 16, and 41, respectively. The area of the four excised wounds in each field was identified by the inked arrows sketched on the animal's body after day 1. The animal's left-side wound fields (not shown except for Fig. 1 *E* and *F*) looked similar to the right even though the anterior–posterior orientation of control and excised wounds was reversed. Fig. 2 plots the daily time course of closure for each of the 16 wounds in pig 2, in 50% of which the granulation tissue was excised daily. There was no initial wound expansion or delay period. Measurements of wound areas prior to and immediately after granulation tissue excisions were identical. Both excised experimental and unexcised controls closed at about the same rates with minor fluctuations. At day 34, when the wounds had been fully closed for ≈16 days, a stellate pattern of tattoo markers reveals the familiar asymmetry of the thin closure scars and their geographic differences (Fig. 1*D*). This final pattern was not altered by daily excisions of granulation tissue. When wound closure was complete, the tattoo markers at the surface, particularly at the corners, were significantly apart. Those at the centers of the edges were nearly approximated. There was little movement in the dorsoventral direction as previously noted in guinea pig (19) and rabbit (27) wounds. The data (not shown) for time-dependent wound closure for pig 1 (excisions at 3-day intervals) varied only in details from those of pig 2 (daily excisions), and the kinetics of closure of the excised wounds followed those of the unexcised wounds in the same fashion.

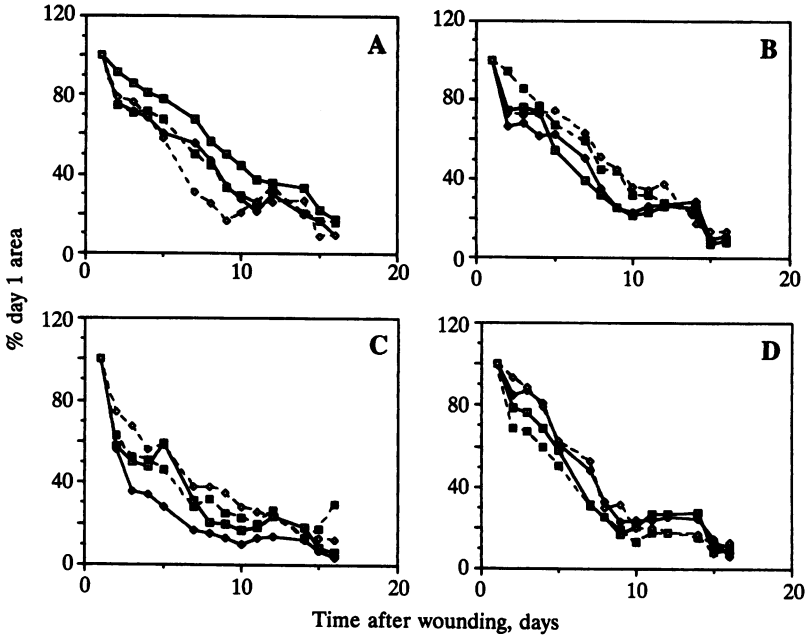

FIG. 2. Time dependance of wound area closure of the 16 skin wounds on pig 2, including left and right sides, backs, and flanks. Data are normalized to day 1 areas. Data from each of the wound areas are shown. (*A*) Left back. (*B*) Right back. (*C*) Left flank. (*D*) Right flank. Continuous lines represent the control wounds and dashed lines represent the daily-excised wounds.

There was no significant difference in the closure rates and lack of response to granulation tissue excision between wounds high on the back and those on the flanks or between those located caudally and those ventrally on the body walls. Apparently the presence or absence of the panniculus muscle was of little consequence.

The possible role of a purse-string effect involving some structural or functional entity operating circumferentially in the wound edges was eliminated by interrupting continuity at ≈50% closure by excising a thin strip of tissue perpendicular to and spanning the wound edge of two left back wounds, one control and one excised (Fig. 1*E*). The new cross-wound edges remained immobile and in several days were almost completely sealed (Fig. 1*F*) without causing a significant change in rate of closure. In another experiment on two other similarly paired wounds on the left side, single full-thickness incisions perpendicular to each of the four wound margins extending into both the granulation tissues and the surrounding normal skin were made through the center of each of the four sides of the wound, also at ≈50% closure. Within a few hours, the new incision edges spread apart but the intervening old adjacent wound edges did not change their position with regard to the overall dimensions of the wound nor was there any change in their subsequent rate of closure.

In some wounds, both control and experimental, usually late in closure, there was considerable excavation under one or both anteroposterior edges, the cavities being filled with serous fluid. This phenomenon, suggesting resorption of underlying granulation tissue, observed in both control and repeatedly excised wounds, left a depressed area around the thin stellate scar when closure was complete.

DISCUSSION

This study confirms the original hypothesis (20) that the central granulation tissue is not a principle contributor to the mechanism of wound closure. We also conclude that the degree of adhesion of the dermis to the underlying body wall, often referred to as "looseness" or "tightness," does not seem to influence the kinetics of wound closure nor does the mechanism of closure of full-thickness excision wounds in various locations on the trunk require the presence of the central granulation tissue. Similar experiments (J.G., M. August, and R.B., unpublished data) have also been repeated in the tight skin mutant (*tsk*) mouse (28, 29) in which the skin is more firmly adherent to the body wall, with essentially the same results. We (unpublished data) have also repeated the earlier experiments of Watts *et al.* (20) in the guinea pig, confirming and expanding the basic data.

The initial delay of 10 days in the time course of full-thickness domestic pig skin wound closure, reported by Welch *et al.* (11), was not seen in the studies reported here. It may reflect experimental differences in their study including location, wound shape (oval vs. square), and mode of measurement by extrapolation from histological sections as compared with direct fresh whole-wound measurements used here.

When wound closure is complete, the tattoo markers at the surface, particularly at the corners, are significantly apart. Those at the centers of the edges are nearly approximated. Because adjacent orthogonal edges near the corners are closer to each other than are opposing parallel sides, they will make contact and stop moving sooner, thus creating the final stellate closure pattern.

The possibility of closure by a purse-string mechanism had been studied earlier by Phillips and Peacock (30) by using multiple repeated full-thickness incisions across each side of square excision wounds in the backs of rats. Although the rate of closure was slowed, the kinetics were similar with a delay of 23% (26 vs. 20 days) for complete closure compared with controls. More recently, Martin and Lewis (31) have invoked this sphincter mechanism based on the appearance of a continuous ring of actin cables within epithelial cells at the rim of round skin wounds in 11-day chicken embryos. A direct experiment on the effect of wound-edge interruption was not reported. The simple observation in the adult rodent and pig that the shape of the wound changes with time from square to sharply stellate rather than to round makes this explanation unlikely for the adult mammalian skin wound. The lack of significant effect of surgical interruption of the wound edge on closure kinetics in our experiments confirms this point.

Mechanical participation of the epidermis growing over the granulation tissue is unlikely since it was removed daily with the latter. However, the adjacent intact epidermal cells, and perhaps those of near-by hair follicles or glands responding locally to the injury and its consequences, may via paracrine activity induce local subdermal fibroblast proliferation and migration.

The contractility of excised fresh granulation tissue influenced by various drugs active on smooth muscle (32, 33) has been used to justify the conclusion that granulation tissue functions *in vivo* to close the excision wound by contraction. However, this correlation is purely circumstantial. If, in an intact wound, there was a significant additive contribution to the process by contraction of the central granulation tissue, it is unlikely that the closure rate as shown here in the absence of granulation tissue would remain unaffected. From earlier studies in the guinea pig (20) showing that removal of the wound edge or simply undercutting it distracts the edges whereas removal of central granulation tissues in both guinea pig and domestic pig wounds does not alter closure kinetics, it would appear that the pulling action of the picture frame is necessary and sufficient to close the wound with normal kinetics. Although the mass of fibroblasts generated under the wound edges may be structurally contiguous with those of the central granulation tissue, their functional behavior may differ significantly. The granulation tissue may function by adding increased stability to the open wound and by providing cells and materials for scar formation since there seems to be inadequate instructions for true regeneration. We conclude that the term "contraction" is a misleading descriptor of spontaneous wound closure.

The proposed mechanism based on polarized coordinated migration of a rim of densely packed freshly proliferated fibroblasts, the "picture frame," underlying and pulling inward the dermal edges, is not dissimilar to morphogenetic mass cell migrations well described in other contexts in the embryo (34–36). This idea differs significantly from the widely held concepts of wound closure by contraction of central granulation tissue either by myofibroblasts pulling on matrix collagen (12) or by fibroblasts altering the organization of the new matrix by migrating through it (16).

These observations also bear on relevance to the mechanism of wound closure of the numerous experiments on contraction *in vitro* of fibroblast-populated collagen lattices, summarized by Grinnell (23), since central granulations appear not to be required for the process. This implication does not detract from the value of this *in vitro* model for the study of cell–matrix interactions; it simply means that the model probably does not contribute directly to our understanding of the *in vivo* mechanism of wound closure.

The mode of attachment of the picture-frame cells to the overlying dermis, to the underlying deep fascia, and to each other probably involves a complex of intra- and extracellular structural molecules such as fibronectin, proteoglycans (11, 18), integrins, other binding proteins, and direct cell–cell contacts. The mechanism whereby synchronized attachment and release of the picture-frame cells from the deep fascia accomplishes coordinated inward movement of the wound edges may involve the programmed action of specific proteases and other lyases. The specific stimuli for local cell proliferation

and directional mass migration are yet to be disentangled from the welter of cytokines, integrins, matrix molecules, and their fragments known to be present in the wound (37), any one or combination of which may have such capacities.

Quantitative compositional measurement of the granulation tissue of excision skin wounds in guinea pigs (19), rats (38), and rabbits (27) as a function of closure rate, indicating considerable resorption, was observed morphologically in the pig wounds studied here. The loss of granulation tissue fibroblasts underlying the advancing dermal edges in closing wounds by apoptosis has been described (39). Simultaneous removal of associated extracellular matrix, no doubt by local enzymatic activity, contributes to the minimal nature of the ultimate superficial scar. (Of interest is the fact that a scar nearly the area of the original wound persists below the dermis.) As yet unknown factors, possibly secreted by the overlying dermal cells or epidermis at the wound edge, may induce these resorptive processes. The long delay in closure of such wounds in large areas of human skin probably results in scarring by conversion of granulation tissue to dense collagenous matrix.

The source of the wound fibroblasts is of much interest. Histologic examination of the dermis adjacent to the wound edge does not indicate cell proliferation or change in cell density, morphology, or the collagenous matrix structure. These observations (not presented here) confirm earlier histologic (22, 40, 41) and autoradiographic (42, 43) reports of nonproliferation of dermal fibrocytes surrounding excision wounds or morphologic alterations in the dermal collagenous matrix. In contrast, there is active proliferation of fibroblasts in subcutaneous tissue underlying the wound edge and continuing into the central granulation tissue (43). Thus, there is little evidence that wound fibroblasts are derived from the adjacent dermis. They may prove to be a population different from the dermal cells, probably hypodermal fibroblasts, lacking the potential for regenerating the normal dermal architecture or inducing epidermal differentiation. The observations reported here, and accompanying speculations, may bear on significant mechanistic differences between wound repair and regeneration.

We thank Drs. Mark W. J. Ferguson, James W. May, Jr., Paul F. Goetinck, and Hermes Grillo for their critical evaluations of the manuscript and helpful suggestions and Ms. Barbara Dewey for her excellent assistance in preparing the manuscript. This work was funded by the Cutaneous Biology Research Center through the Massachusetts General Hospital/Shiseido Co. Ltd. Agreement and a gift from the J. R. Proctor Memorial Research Fund to J.G.

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