Actin Filaments in Normal Dermis and During Wound Healing

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During wound healing, it has been suggested, modified fibroblasts rich in actin filaments are responsible for wound contraction. With the use of specific fluorescent probe (NBD-phallacidin), the distribution of actin filaments are compared in normal dermis and in several wound contraction models, including open and burn wounds and full and thin-thickness skin autografts. Fibroblasts of normal dermis are slightly stained with NBD-phallacidin. Fibroblasts with actin filaments are increased in autografts, particularly at Days 15 and 21 after grafting, and are prominent in open and burn wounds. The wound contraction rate is not directly related to the presence of actin-staining fibroblasts. After stabilization of the contraction of open or burn wounds, fibroblasts rich in actin filaments remain. The superficial layer of full-thickness skin graft contains a similar actin distribution without concomitant contraction. It is concluded that the distribution of actin-rich fibroblasts corresponds morphologically to previous areas of necrosis or injury. (Am J Pathol 1987, 126:164-170)

WOUND HEALING and granulation tissue formation can lead to wound contraction. The contraction process involves the movement of intact dermis over the wound site, particularly in animals.[1] This repair is characterized by modifications of the cytoskeletal and contractile elements, particularly in the fibroblasts, which have been called myofibroblasts or modified fibroblasts.[2-6] These cells acquire the morphologic characteristics of smooth muscle cells. They have microfilaments bundles that are rich in actin filaments, as demonstrated by immunofluorescence with the use of autoantibodies obtained from persons with chronic active hepatitis.[3] Contracting wounds can be modified by smooth muscle contracting and relaxing substances.[3] Gabbiani et al[2] have suggested that these microfilaments are responsible for wound contraction, whereas Ehrlich et al[7] propose that the microtubules also play an important role. McGrath,[8] using the same autoantibodies with immunoperoxidase staining, showed a correlation between the rate of wound contraction and the number of myofibroblasts.

The goal of the present study is to compare the behavior of the actin filaments in different models of wound contraction using a small fluorescent probe, Nitrobenzooxadiole (NBD), conjugated to the phallacidin extracted from the mushroom *Amanita phalloides* (NBD-phallacidin). This probe has been shown to have a specific affinity for actin filaments.[9]

Materials and Methods

Wounding Procedure

Male Sprague-Dawley rats (250-300 g) (Charles River Breeding Laboratories, Wilmington, Mass) were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg) (Nembutal, Abbott Laboratories, North Chicago, Ill), and their backs shaved and depilated with sodium thioglycate cream (Nair, Carter-Wallace, Inc., Cranbury, NJ). A 2 × 2-cm square plastic template was placed on each side of the back of the animal, and a line was traced with eosin. Eight tatoo marks were made equidistant from each other, four on each quadrant and four between the others along the eosin outline.

Using tincture of iodine for cutaneous asepsis, we made two wounds on each rat. There were four groups

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for wound-healing studies. Each group contained 30 wounds. In one group, an open wound was made down to the level of the panniculus carnosus and left open, covered by a dressing as described below. In the second group, an identical open wound was made and then grafted with a full-thickness skin autograft using the skin that had been removed and then replaced with 8 interrupted sutures with 5/0 nylon. In the third group, an identical open wound was made and then grafted with a thin-thickness skin autograft using the abdominal skin of the same rat in the same condition as in the second group. In the fourth group, standard burn wounds were made by applying a lead cube equilibrated in boiling water for 30 seconds, which produced a third-degree burn injury. The depth of burn injury was confirmed histologically.

All wounds were covered with petroleum jelly and a gauze bandage wrapped around the trunk of the rats. The rats could move normally and retained the bandage for 5 days after injury. The animals were kept on their normal diet with water *ad libitum* and showed normal weight gain curves from Day 3 after surgery. Four normal cutaneous biopsy specimens were taken from normal animals under anesthesia and used as control specimens. The rats were sacrificed at Days 3, 7, 15, 21, and 42 after injury under anesthesia. Six wounds were analyzed at each time point. Skin grafts presenting complete necrosis at Days 7 and 15 were eliminated from the study (30%).

Measurement of Wound Area

The wound edge was traced from the tatoo marks with the use of transparent plastic sheets. Wound areas were calculated with a microprocessor high-resolution Hipad digitizer (Houston Instrument, Bausch & Lomb, Austin, Tex).

Tissue Preparation

One portion of the wound was cut transversally, frozen directly in liquid nitrogen, and kept at −70 C. Cryostat sections (3–4 μ thick) were made with a Damon cryostat and collected on clean glass slides. Another portion of the wound was fixed in 10% formaldehyde and embedded in paraffin wax, and 6-μ-thick sections were cut and stained with hematoxylin and eosin (H&E).

Staining Procedures With NBD-Phallacidin

Cryostat sections were fixed and permeabilized by immersing in cold absolute acetone at −20 C for 10 minutes. Sections were air-dried and incubated with NBD-phallacidin (Molecular Probes Inc., Junction City, Ore) in phosphate-buffered saline (PBS) (33 ng for each tissue slide) for 35 minutes at room temperature in a moist atmosphere. They were then washed with PBS (4× 10 minutes) and mounted with Tris-glycerol under coverslips.

Control sections were fixed under the same conditions with acetone and incubated with PBS only. Other sections were fixed in 10% formaldehyde and stained with H&E. Each sample was examined and photographed using a Zeiss microscope (IM 35) equipped with filters for fluorescein isothiocyanate and phase-contrast optics. Kodak Tri-X film was used.

Results

Wound Contraction

The extent of wound contraction for each group is shown in Figure 1 in relation to the time after injury. After full- or thin-thickness skin autograft implantation, at Days 3 and 7, the wound size was similar to that at the zero time point. At Day 15, the small degree of wound contraction had reached its maximum extent. At Day 21, this small degree of contraction decreased; and at Day 42 reached the size at zero time. After an open wound, at Day 3, there was no change in the wound size from zero time. At Day 7, a decrease was significantly observed. At Day 15, an extensive contraction of the wound had occurred. At Days 21 and 42, the wound size became stable, without further change from that seen at Day 15. After a burn wound, at Days 3 and 7 after injury, the wound size was identical to that at zero time. At Day 15, the wound had

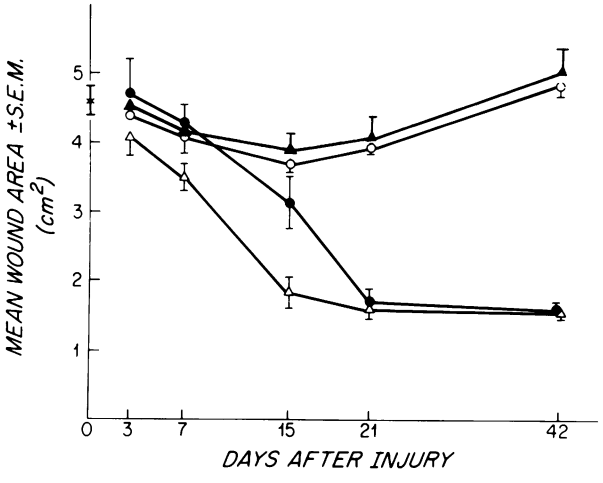


Figure 1 — Rate of wound contraction (mean I SEM) in thin-thickness (▲) and full-thickness skin (○) autografts with a transient contraction at Day 15, in open wounds with a strong contraction already present at Day 15 (△), and in burn wounds with a delayed contraction but identical at day 21 with open wounds (●). * Average of the injured areas at time zero.

begun to contract, but less than open wounds at the same time. At Days 21 and 42, the contracted wound had contracted maximally and become stable.

Microscopic Observations

In normal control dermis, the NBD-phallacidin strongly stained myocytes surrounding the sebaceous glands, hair follicles, and vessels (Figure 2A). It is only at high magnification (×900) that NBD-phallacidin-stained actin filaments were observed in the dermal fibroblasts (Figure 2B). The fluorescence was not uniform in all the cells, which indicated a heterogeneous population of fibroblasts. However, the fluorescence was found brighter than the autofluorescence of the control slides not stained with NBD-phallacidin.

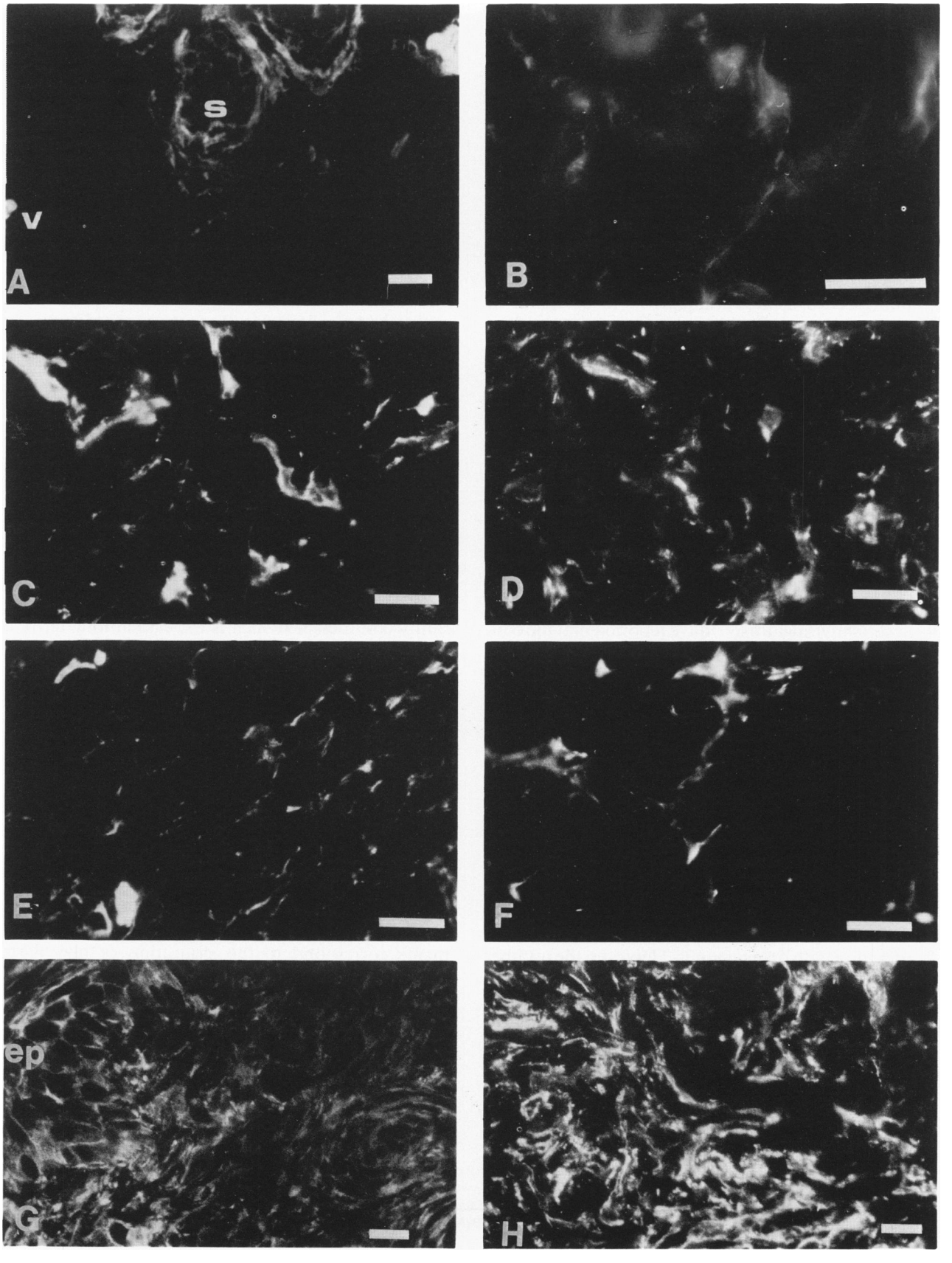
In thin- and full-thickness autografts, at Days 3 and 7, the intensity of NBD-stained actin filaments in the fibroblasts of the dermal grafts was either similar or stronger than that observed in normal dermis (Figure 2C). In the suture line, between graft and normal skin, a large number of actin-staining fibroblasts were observed. These staining fibroblasts were particularly evident around the inflammatory reactions induced by the suture. In the autograft bed, a narrow interrupted line of a large number of actin-staining fibroblasts were also found. No invasion of vessels was observed. At Day 15, the dermal autograft was richer in fluorescent-staining fibroblasts than normal dermis (Figure 2D). In the suture line, actin-staining fibroblasts were still present in half the cases; and in the autograft bed, no actin staining fibroblasts were observed. Vessels were identical to those found in normal dermis. At Day 21, the dermal autograft was similar to a normal dermis, both histologically and after NBD-phallacidin staining (Figure 2E). The suture line and autograft bed were not distinguishable from normal dermis. At Day 42, the fluorescent staining of the dermal autograft was as noted at Day 21 (Figure 2F).

In the case of the full-thickness skin grafts, most of the superficial cutaneous glands were largely necrotic, as well as the epidermal layer. In addition, at Day 15, necrosis of one-quarter thickness of the upper dermal autograft was observed on H&E-stained histologic sections. Below this necrosis, a new epidermal layer had totally covered the underlying connective tissue, which was rich in actin filament-staining fibroblasts, similar to that seen in granulation tissue (Figures 2G and H). At Day 21, in the superficial layer of the autograft, actin-staining fibroblasts were present as described at Day 15. These actin-staining filaments were generally oriented parallel to the epidermal layer. The necrosis observed was attributed to the initial and transient ischemia of the autograft. At Day 42, the superficial rich actin area was only found in a few cases and reduced in size. The epidermal layer became normal.

In open wounds at Day 3, vessels and a few inflammatory cells invaded the clot, and within the subcutaneous muscle bundles a few fibroblasts were visible. In these areas, NBD-phallacidin stained mainly the new vessels. It was difficult to distinguish inflammatory cells and fibroblasts at this time, but few cells with round or oval nuclei showed a diffuse staining without identifiable filaments; they were probably inflammatory cells. At Day 7, the granulation tissue was more apparently organized. The cells of this new tissue were rich in actin filaments present throughout fibroblasts without filamentous organization (Figure 3A). By Day 15, the scar tissue comprised the thickness of the new tissue and was well organized. The scar tissue was totally covered by an epidermal layer in many cases. Actin filaments of fibroblasts were spread in all directions, but occasionally actin filaments appeared parallel to the surface (Figure 3B). The vessels of the scar tissue had a single layer of cells, but these endothelial cells were strongly stained. At Day 21, the morphologic features were identical to those on Day 15 (Figure 3C). At Day 42, after NBD-phallacidin staining, the upper two-thirds of repair tissue showed actin filaments aligned parallel to the surface (Figure 3D). The lower third of the dermis near the subcutaneous muscle was rich in large vessels and poor in NBD-phallacidin-stained fibroblasts.

In burn wounds, at Day 3, histology showed an invasion of inflammatory cells, and vessels were only present at the level of the subcutaneous muscle. NBD-phallacidin staining was obscured by extensive autofluorescence of the necrotic tissue. At Day 7, inflammatory cells had completely replaced the edematous tissue between necrotic tissue and subcutaneous muscle. The inflammatory reaction was greater than in open wounds. After NBD-phallacidin staining, cells had a diffuse fluorescence in the cytoplasm surrounding oval or round dark nuclei (Figure 3E). Autofluorescence of necrotic tissue still obscured the observation of the upper layers. At Day 15, granulation tissue was the major tissue present. With NBD-phallacidin staining, the area of granulation tissue was similar to that found in open wounds, but the fibro-

Figure 2 — NBD-phallacidin-stained tissue sections observed under fluorescent light. **A** and **B** — Normal rat dermis with sebaceous glands *(s)* and vessel *(v)* rich in stress fibers (**A**) and at fibroblasts at high magnification (**B**) containing actin filaments. **C–F** — Thin-thickness skin autograft at Days 7 (**C**), 15 (**D**), 21 (**E**), and 42 (**F**). **G** and **H** — Full-thickness skin autograft. Only the upper layer of the dermal portion of the graft is presented and shows a typical granulation tissue at Day 15 after grafting below the epidermis *(ep)*. (Bar = 20μ)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blasts were still mixed with inflammatory cells, which were easily distinguishable at this time (Figure 3F). At Day 21, the histologic and NBD-phallacidin observations were similar to the 15th and 21st day after open wound injury (Figure 3G). At Day 42, in most cases, actin filaments were oriented parallel to the surface (Figure 3H).

Discussion

NBD-phallacidin is a useful means of detecting actin filaments in the "modified" fibroblasts present in granulation and scar tissue of open and burn wounds. Actin has been shown previously by immunofluorescence using antibodies[3,5,6] to be found in these tissues. In addition, in this study we show that fibroblasts of skin grafts exhibit a temporary increase in actin filament content. Furthermore, actin filaments have been identified in the fibroblasts of the normal dermis, which has not been observed previously with the use of immunofluorescence[3] and immunoperoxidase.[8] The latter difference may be due to the small molecular size of the NBD-phallacidin (molecular weight, 1000 daltons; approximate diameter, 1.2 nm) and its high specificity for actin filaments, not soluble G-actin monomers. The use of NBD-phallacidin may be a better probe for the study of the role of actin filaments in tissue in various biologic situations.

Several investigators[3,5,6] have reported that the contractile property of wounds is effected by the actin filaments found in the myofibroblasts of granulation tissue and scars. However, this study suggests that actin filament-rich fibroblasts are not involved directly in wound contraction. Indeed, the time course of the actin filaments in the wound cells does not coordinate with wound contraction. At 15 days after wounding, as well as after full-thickness skin grafting, the contraction process is stable. Nonetheless, at these times, there are still actin-rich fibroblasts present in the wound or in the graft. McGrath[8] has previously shown a parallel behavior between the increased number of myofibroblasts and the increase in wound contraction with open wounds. However, the number of myofibroblasts then decreased; but they were still present by 20 weeks after wounding.[4] In a full-thickness skin graft model, Rudolph[10] has observed a large number of myofibroblasts maximal from Day 14 to Day 21 and then decreasing rapidly during the expanding phase of autograft healing after 3 weeks, as shown in the present study.

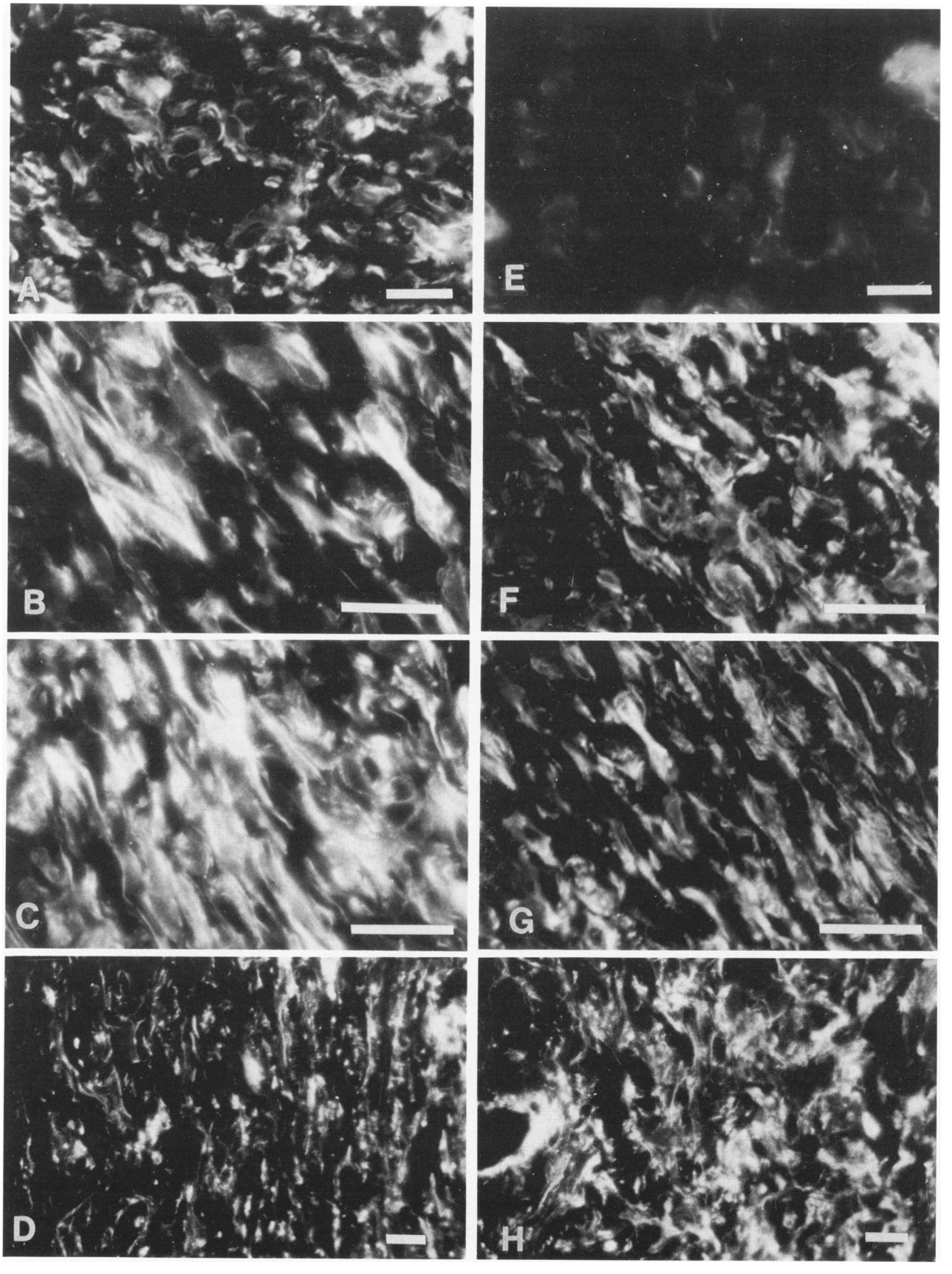
In culture, fibroblasts are rich in stress fibers when they are adherent.[11] Stress fibers are composed of actin filaments,[12] and NBD-phallacidin specifically binds to actin.[9] From cell culture studies, it appears that cell adhesion may be more involved than cells with contractility during wound healing. *In vivo,* Singer et al[13] have demonstrated that the formation of fibronexus by myofibroblasts corresponds to an adhesion site to the extracellular matrix (ECM) in the granulation tissue. In addition, it has been hypothesized that adhering fibroblasts are pulling the substrate to which they are attached.[14] Thus increased adhesivity may allow the pulling of the ECM during wound healing. Thus, fibroblasts rich in actin would be in the wound to rearrange the ECM.

Another observation that argues against a direct role of the actin-rich cells in contraction is that they correspond morphologically to the areas of injury or necrosis. Indeed, a partial necrosis as observed in the superficial layer of full-thickness skin graft, a direct necrosis of the skin by burn injury, or the absence of tissue as in open wounds results in an increase of actin filaments in fibroblasts. A subepidermal edema by ischemia at Day 7 after-grafting has been also noticed by Hinshaw et al,[15] who concluded that this observation was comparable to a second-degree burn. In second-degree burn wound, contraction is temporary and not largely involved during wound healing. On the other hand, Klein and Rudolph[16] have shown that only at Day 120 the full-thickness skin graft is significantly contracted. If such is the case at long term, a nonconcomitant contraction is observed in our model, and the superficial area of the skin graft rich in actin is progressively reduced and remains in only very few cases at Day 42 after-grafting.

A constant alignment of actin filaments is another observation of interest. These aligned filaments are parallel to the surface in both cases after third-degree injury and in the upper layer of full-thickness skin autografts. These lines are seen regularly associated with a complete epidermal covering, and they are more related to a stabilization of the contraction as observed at Day 42 than to a concomitant contraction as seen at Days 7 and 15. These aligned actin filaments correspond to the elongation of fibroblasts which follow the newly formed collagen bundles as observed on H&E-stained tissue sections. This suggests a strong adhesion of fibroblasts to the ECM and an absence of contraction.

The increased number of fibroblasts rich in actin filaments at Days 15 and 21 in autografts may also be explained by an invasion of host cells from the donor site into the graft, as it has been shown by Hinshaw et al[15] and Rudolph et al.[17] However, cellular invasion can be observed in the suture line and the autograft

Figure 3— NBD-phallacidin-stained tissue sections of open wounds at Days 7 (A), 15 (B), 21 (C), and 42 (D) and burn wounds at Days 7 (E), 15 (F), 21 (G), and 42 (H) after injury. (Bar = 20μ)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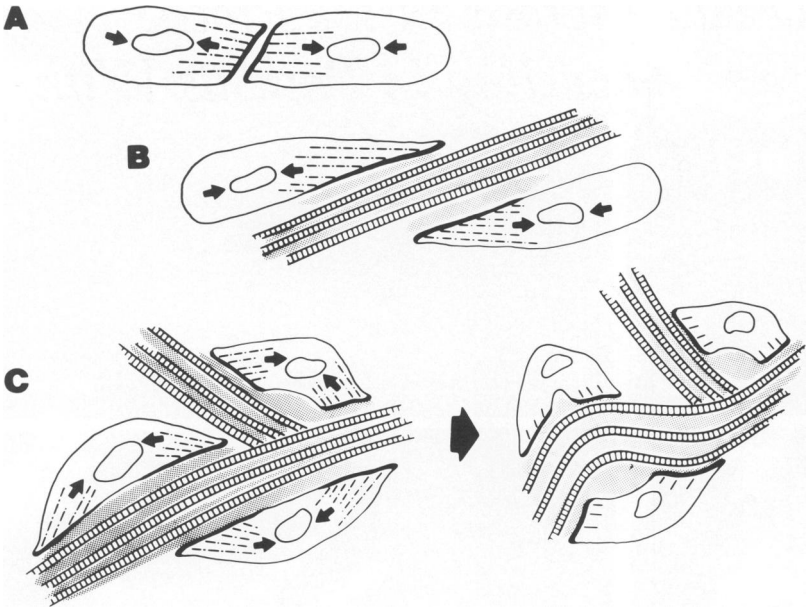

Figure 4—Different mechanisms involved during wound healing. ⊸⊸⊸, actin filaments; ▥▥▥, collagen; ▒▒▒, fibronectin and glycosaminoglycans. **A**—Cell–cell contact as described by Gabbiani et al[2] induces wound contraction. **B**—Cell–ECM contact induces wound contraction[2] and corresponds to strong adhesion sites, as described by Singer et al.[13] **C**—Both adhesion and pulling can induce remodeling of the ECM.

bed, whereas it is not observed in the skin graft. On the other hand, in culture, migrating cells have a diffuse and weak stress fibers,[11] as seen at Days 3 and 7 of open and burn wounds, whereas mobile or migrating fibroblasts lack or have diffuse stress fibers. We believe that fibroblasts of the skin graft have been modified *in situ* and do not represent a population of cells which have migrated into the ECM of the graft, because oriented actin filaments are well observed in these fibroblasts.

In conclusion, rich actin-modified fibroblasts, with contact specialization such as gap junctions[2] and fibronexus,[13] are involved in wound contraction and cell adhesion by cell–cell and cell–ECM contacts. In addition to these two mechanisms, we suggest that the adhesion, and its "pulling" property, of the modified fibroblasts in part of the remodeling of the newly formed ECM during wound healing (Figure 4).

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