A Comparative Study of Fibroblasts in Healing Freeze and Burn Injuries in Rats

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In rats, the healing process of a full-thickness dermal freeze injury differs from that of a burn wound. Whereas burn wounds heal by wound contraction, the movement of surrounding normal skin over the defect, freeze wounds heal without wound contraction. That absence of contraction may be due to the freeze wound's lack of myofibroblasts, the cells reportedly associated with wound contraction. Myofibroblasts can be demonstrated histologically by staining the F-actin filaments of the stress fibers with NBD-phallacidin, a fluorescent reagent specific to F-actin filaments. Fibroblasts in normal dermis have no staining stress fibers. However, staining myofibroblasts are uniformly distributed in the granulation tissue of the healing burn and in the islands of granulation tissue between residual connective tissue fibers in the healing freeze wound. These residual dermal fibers were identified by their patterns of birefringence. Residual connective tissue matrix persists following cold trauma and acts like an internal splint. Burn trauma destroys cells and the connective tissue matrix, which is completely replaced with granulation tissue which undergoes wound contraction. Freeze trauma kills the cellular components of dermis, while some residual connective tissue fibers endure. This study shows that the connective tissue matrix can play an important role in the control of wound contraction. (Am J Pathol 1984, 117:218–224)

WOUND CONTRACTION, the closure of full-thickness dermal injuries, is a cellularly mediated process. Abercrombie and his co-workers demonstrated a reduction in the synthesis and deposit of collagen in scorbutic guinea pigs whose wounds contracted normally.[1] Before that report, wound contraction was thought to be related to the contraction of collagen fibers. Subsequent to those observations, wound contraction was considered a cellularly mediated process, and the role of connective tissue matrix was thought to be less important.[2] A new, morphologically identifiable cell was discovered; and it became the cell reportedly responsible for connective tissue contraction (for review see Rungger-Brandle and Gabbiani[3]). When contractile connective tissue was examined with immunofluorescence-tagged anti-sera to actin filaments or with the electron microscope, morphologically altered fibroblasts were found. They were given the name "myofibroblasts."[4] The cells share morphologic features similar to those of smooth-muscle cells. When strips of granulation tissue rich in myofibroblasts are excised, they respond to some smooth-muscle pharmacologic agents.[5] It develops, therefore, that contracting wounds have myofibroblasts, whereas noncontracting wounds may not.

Full-thickness burn wounds in rats heal like open wounds, mainly by contraction; however, full-thickness freeze injuries do not. Ten days after burn trauma the healed area is reduced to about 40% of its original size; freeze injury wounds show no change in size.[6] Histologically, the depth of initial tissue destruction is similar in the two types of injury. However, there is subsequently less connective tissue matrix breakdown in the freeze injury.[6]

The inability of freeze injuries to heal by contraction may be related to an absence of myofibroblasts. Freeze trauma may impair the proliferation or migration of myofibroblasts into the site of repair. Therefore, we examined tissues from freeze and burn injuries in rats at 4, 7, 10, and 14 days after wounding for the presence and distribution of myofibroblasts. These cells were identified by their ability to stain with NBD-phallacidin

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(7-nitrobenz-2-oxa-1,3-diazole phallacidin). Phallacidin is an acid derivative of phalloidin, a toxin from the mushroom *Amanita phalloides*; it binds specifically to F-actin filaments.[7] The fluorescent derivative, NBD, is small (mol wt 1000 daltons) and provides a convenient fluorescent probe for the identification of F-actin filaments within cells in tissue sections.

Materials and Methods

Male Sprague–Dawley rats weighing between 300 and 400 g were anesthetized with ether, and their backs were shaved and washed with 70% ethanol. A brass probe with a surface of 2 × 2 cm was equilibrated in boiling water or in liquid nitrogen. A standardized burn wound was made by placing the probe equilibrated in boiling water on the skin surface for 20 seconds. A standardized freeze injury was made by placing an identical brass probe equilibrated in liquid nitrogen on the skin surface for 45 seconds. Four standard wounds were made on the back of each animal. The rats were returned to their cages, one rat per cage. Since pain receptors are destroyed in a full-thickness dermal injury, no analgesic medication was given to the rats. The animals were maintained on a standard laboratory diet, and none of the wounds appeared infected. Rats continued to gain weight and appeared active and normal throughout the study period. Wound specimens were taken with some surrounding normal tissue at days 4, 7, 10, and 14. The wound specimens were cut in half and placed in plastic tubes with 7% gelatin and were then immediately frozen in liquid nitrogen for 90 seconds in preparation for cryostat sectioning.

Staining for stress fibers containing F-actin was done on 4-μ-frozen sections. These sections were fixed in 4% paraformaldehyde, permeabilized in cold acetone (−20 C for 5 minutes), and air-dried. NBD-phallacidin (Molecular Probes Inc., Ore) was used according to the manufacturer's directions. Each section was stained by applying 10 μl of NBD-phallacidin solution (100:1 dilution) in 100 μl of phosphate-buffered saline (PBS) for 20 minutes at room temperature. Control sections were incubated in PBS alone. The slides were then washed in PBS and mounted in PBS/glycerol (1:9 vol/vol) containing *p*-phenylenediamine, which inhibited bleaching of fluorescent staining.[8] The slides were viewed with an inverted Zeiss IM 35 microscope fitted with epifluorescence and standard FITC filters. The slides were photographed with Kodak Ektachrome film, ASA 400, uprated in processing to 1600 ASA. After the fluorescence studies, coverslips were stained with hematoxylin and eosin. These histologic sections were examined by brightfield microscopy and photographed on Kodak Panatomic X film. Collagen fiber birefringence was viewed by polarized light optics and recorded on Kodak Tri-X film.

To confirm the specificity of the NBD-phallacidin, fixed and permeabilized sections were incubated for 20 minutes with unlabeled phalloidin (Sigma Chemical Co., St. Louis, Mo). These slides were washed in PBS and then incubated with NBD-phallacidin as described above. The concentration of the phalloidin was 20 μg per section, 300 times greater than the NBD-phallacidin, whose concentration was 66 ng per section. Pretreatment of the sections with unlabeled phalloidin prevented fluorescent staining of the granulation tissue myofibroblasts and epidermal cells. However, deep dermal muscle fibers with added unlabeled phalloidin stained very weakly.

Results

Gross Appearance

Full-thickness burn injuries heal mostly by contraction, whereas freeze injuries do not. By 10 days, healing burn injuries are reduced in area to approximately 40% of the size of the original wound. In standardized freeze injuries no reduction in the size of the healing wound is apparent. Each type of injury has a scab, but the scab area is smaller in the burn injury at 14 days, while the associated scab area of the freeze injury remains unchanged in size during the first 2 weeks of the healing process.

Histologic Findings

Histologic sections of the tissue taken 4 days after burn injury showed considerable destruction of both epidermis and dermis. No viable cells were present in the center of the wound, but inflammatory cells and fibroblasts were visible at the interface between the surviving tissue and area of necrosis (Figure 1A). The inflammatory cells, mostly polymorphonuclear leukocytes, stained diffusely with NBD-phallacidin, (Figure 1B). At the lateral edge of the wound adjacent to viable tissue an occasional fibroblast was stained with NBD-phallacidin. Above were newly formed epidermal cells, which also stained with a pattern characteristic of these cells (Figure 1C). No staining cells were visible in the central necrotic tissue.

The tissue taken 4 days after freeze injury was edematous and had inflammatory cells at the interface between dead and surviving dermis. A few viable fibroblasts were visible within the central wound area and appeared to be associated with the residual connective tissue matrix (Figure 2A). These cells stained with NBD-phallacidin and were judged to be myofibroblasts (Figure 2B).

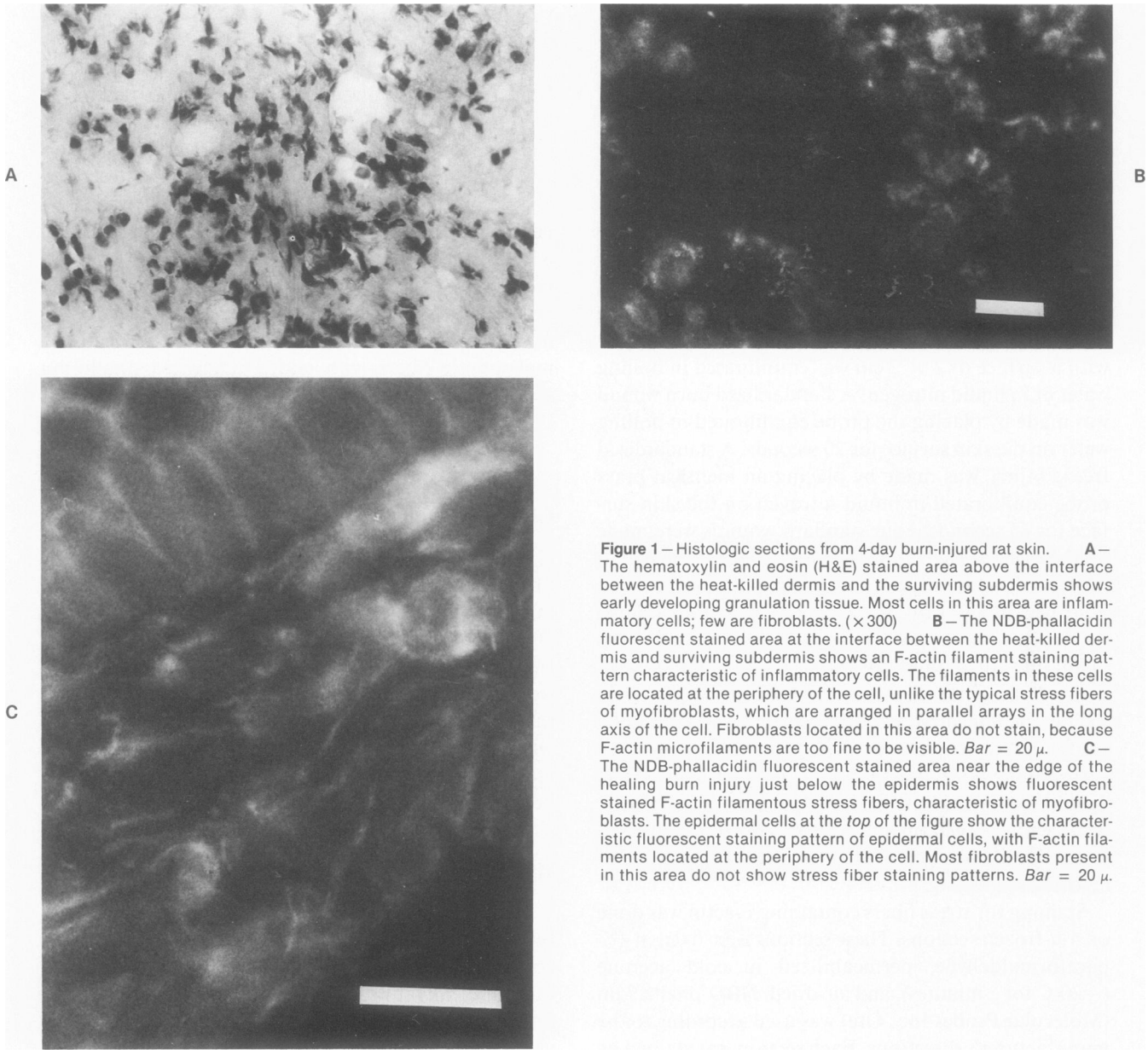

Figure 1 — Histologic sections from 4-day burn-injured rat skin. **A** — The hematoxylin and eosin (H&E) stained area above the interface between the heat-killed dermis and the surviving subdermis shows early developing granulation tissue. Most cells in this area are inflammatory cells; few are fibroblasts. (×300) **B** — The NDB-phallacidin fluorescent stained area at the interface between the heat-killed dermis and surviving subdermis shows an F-actin filament staining pattern characteristic of inflammatory cells. The filaments in these cells are located at the periphery of the cell, unlike the typical stress fibers of myofibroblasts, which are arranged in parallel arrays in the long axis of the cell. Fibroblasts located in this area do not stain, because F-actin microfilaments are too fine to be visible. *Bar* = 20 μ. **C** — The NDB-phallacidin fluorescent stained area near the edge of the healing burn injury just below the epidermis shows fluorescent stained F-actin filamentous stress fibers, characteristic of myofibroblasts. The epidermal cells at the *top* of the figure show the characteristic fluorescent staining pattern of epidermal cells, with F-actin filaments located at the periphery of the cell. Most fibroblasts present in this area do not show stress fiber staining patterns. *Bar* = 20 μ.

A scab of connective tissue components and cellular debris covered the wound 10 days after burning. Beneath the scab was a large distinct area of granulation tissue (Figure 3A). Burn-destroyed tissue appeared to have accumulated within the scab, and the granulation tissue area was made up of newly synthesized tissue. When examined under polarized light optics, the granulation tissue showed no birefringence (not illustrated). The granulation tissue contained large numbers of myofibroblasts, which stained intensely with NBD-phallacidin (Figure 3B).

Ten-day freeze wounds showed islands of granulation tissue within the area of healing, intermingled with residual connective tissue matrix (Figure 4A). Under examination by polarized light optics, this connective tissue matrix matrix showed a distinct dermal-like pattern of birefringence (Figure 4B). The pattern of birefringence was identical to that found in the normal, undamaged skin adjacent to the wound. It was concluded that this tissue was made of residual dermal connective tissue fibers. Few cells were evident within this residual matrix, but the islands of granulation tissue contained densely packed myofibroblasts, which stained intensely with NBD-phallacidin (Figure 4C). Cell density and NBD-phallacidin staining was identical to healing burn wound granulation tissue.

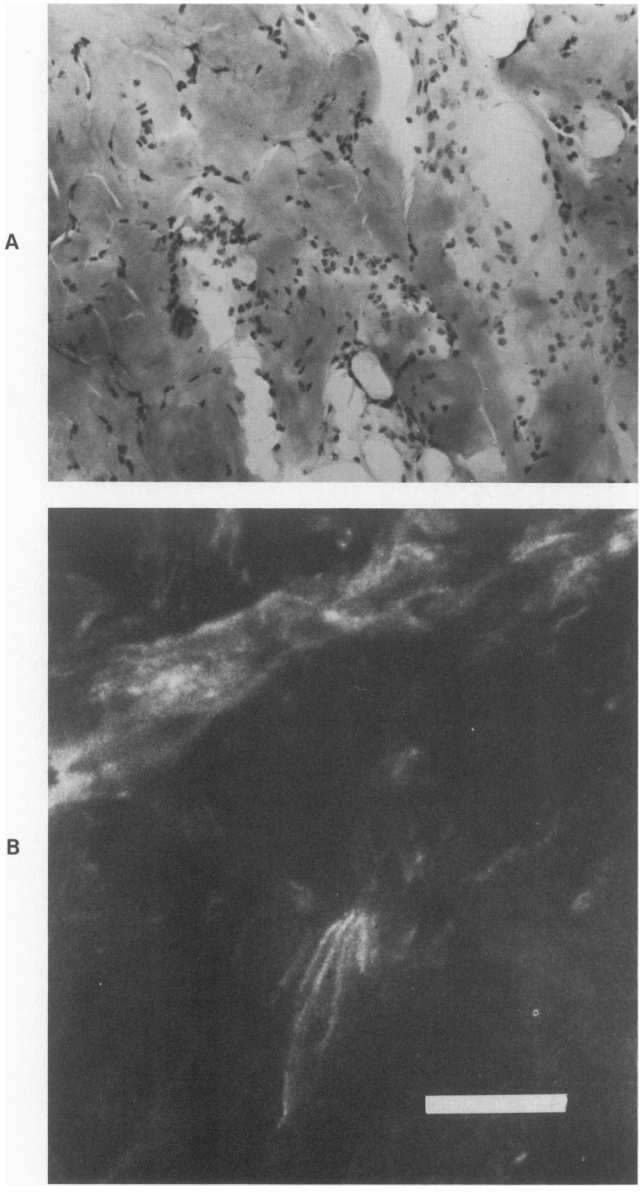

Figure 2—Histologic sections from 4-day freeze-injured rat skin. **A**—The H&E-stained section shows the presence of inflammatory cells and fibroblasts in the area above the interface between the freeze-killed and surviving dermis. The cells are clustered very densely between surviving connective tissue dermal fibers. There is low cell density when they reside within connective tissue fibers. (×160) **B**—The NDB-phallacidin fluorescent stained section from an area similar to that in the H&E-stained section shows fluorescent stained F-actin filament, stress fibers, within the cytoplasm. By staining criteria, these cells are myofibroblasts, and they appear in greater density in healing freeze-injured granulation tissue. *Bar* = 20 μ.

Fourteen days after burn injury, the granulation tissue contained large numbers of myofibroblasts, which stained uniformly with NBD-phallacidin. Fourteen-day-old freeze wounds appeared identical to 10-day freeze wounds in all respects, including the fact that no wound contraction had occurred. The islands of granulation tissue were densely populated with myofibroblasts identical to those at 10 days shown in Figure 4C.

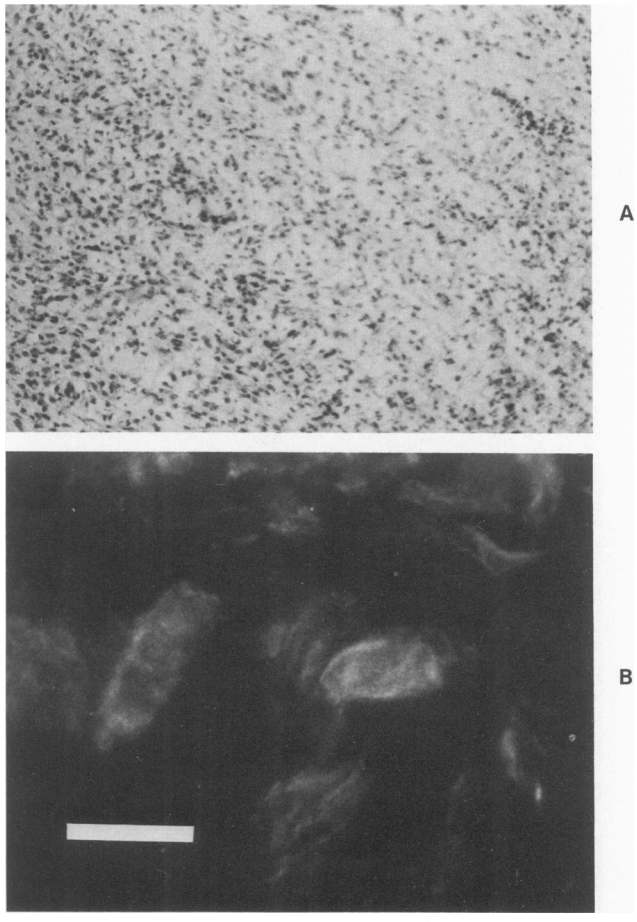

Figure 3—Histologic section from 10-day burn injury. **A**—The H&E-stained area of granulation tissue within the center of the healing contracting burn wound shows high cell density and newly synthesized connective tissue. (×16) **B**—The NDB-phallacidin fluorescent stained granulation tissue in an area similar to that in **A** shows F-actin fluorescent stress fibers characteristic of myofibroblasts. Most cells in the areas of highest cell density are myofibroblasts. *Bar* = 20 μ.

Discussion

These findings indicate that there is a role for the connective tissue matrix in controlling wound contraction. There appears to be cooperation between fibroblasts or myofibroblasts and their surrounding connective tissue matrix. Burn injury trauma immediately destroys cells and connective tissue matrix. By contrast, freeze injury trauma kills cells, but it does not destroy connective tissue matrix immediately or completely. The cell population of the freeze injury is replaced by migrating fibroblasts, which populate this dead space. Freeze trauma allows a relationship to develop between the migrating mesenchymal cells and the residual connective tissue matrix, while burn injury does not permit the development of such a relationship. The heat-damaged connective tissue matrix becomes included in the scab and is sloughed off. No residual connective tis-

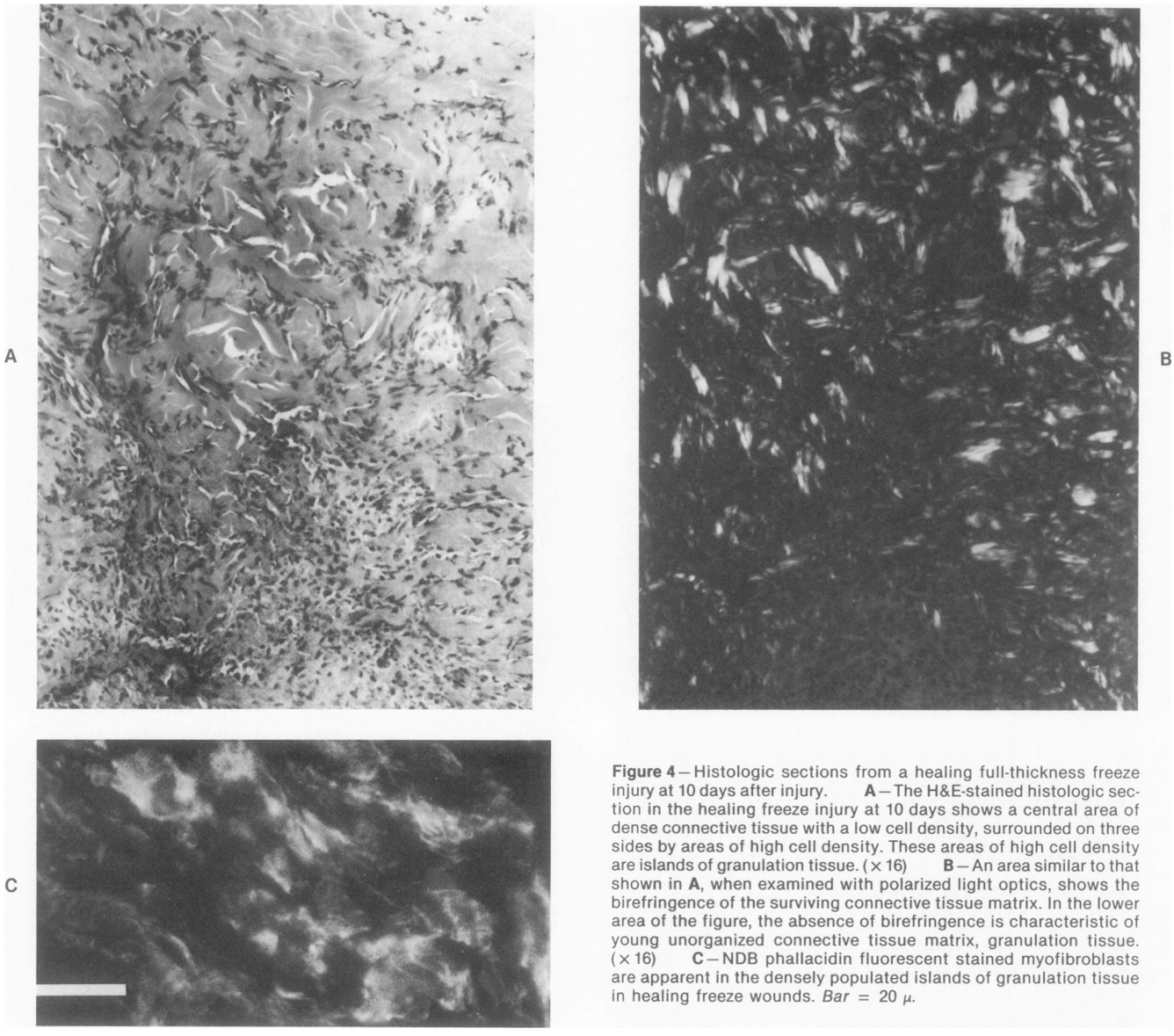

Figure 4—Histologic sections from a healing full-thickness freeze injury at 10 days after injury. **A**—The H&E-stained histologic section in the healing freeze injury at 10 days shows a central area of dense connective tissue with a low cell density, surrounded on three sides by areas of high cell density. These areas of high cell density are islands of granulation tissue. (×16) **B**—An area similar to that shown in **A**, when examined with polarized light optics, shows the birefringence of the surviving connective tissue matrix. In the lower area of the figure, the absence of birefringence is characteristic of young unorganized connective tissue matrix, granulation tissue. (×16) **C**—NDB phallacidin fluorescent stained myofibroblasts are apparent in the densely populated islands of granulation tissue in healing freeze wounds. *Bar* = 20 μ.

sue fibers remain associated with newly synthesized granulation tissue. During the repair process in freeze injuries, the turnover of connective tissue matrix is retarded.[6] The residual connective tissue matrix retains much of its structural integrity, as evidenced by its pattern of birefringence. The inability of mesenchymal cells to rapidly alter this residual matrix appears to be related to the structural integrity of that matrix. The morphologic appearance of the resident cellular population is identical to that of contracting healing burn wounds.

It appears that when fibroblasts become sedentary, and cell–cell contacts become prominent; then stress fibers develop.[9] Myofibroblasts may be sedentary cells that readily form cell–cell contacts.

If myofibroblasts are important for wound contraction, then a cooperation between cells and the surrounding connective tissue matrix must occur. In the case of granulation tissue, where the connective tissue matrix shows less integrity, the mesenchymal cells populating it are capable of contracting that tissue. In the case of freeze injury, where much of the connective tissue matrix retains its integrity, the mesenchymal cells within it are unable to contract that fibrous tissue. It appears, then, that the forces of wound contraction reside in the mesenchymal cells in the fibrous tissue. At the same time, it seems that a controlling force for contraction is retained by the connective tissue matrix surrounding those cells. When, as in a freeze injury, the connective tissue matrix surrounding the mesenchymal cells has great structural integrity, contraction does not occur.

Another question that is raised is whether myofibroblasts are responsible for the contractile force of wound contraction. In the burn injury undergoing contraction, much wound contraction occurred in the absence of myofibroblasts. Burn wounds had contracted by 40% at 7 days, but few myofibroblasts could be identified by NDB-phallacidin staining stress fibers within the granulation tissue. There is the possibility that migrating fibroblasts eventually form cell–cell contacts and that their cytoplasmic microfilaments aggregate into stress fibers. These myofibroblasts may not be involved in the contraction process. Instead, those cells that have not attained the myofibroblast morphology, that is, migrating fibroblasts with no stress fibers, surrounded by connective tissue rather than by cell–cell contacts, might be responsible for the contraction process. We realize that this is only a hypothesis; studies for investigation of this concept with other models of wound concentration continue in the laboratory.

These results show clear differences in the healing processes following burn and freeze injury. Following a burn, the heat-killed tissue is incorporated into a scab and is totally replaced by granulation tissue. This concurs with the findings of Li et al,[6] who showed that burn wounds have a great initial loss of collagen, followed by increased collagen deposition. The early loss of collagen and the subsequent collagen deposition are much less pronounced in the freeze wound. Healing freeze wounds retained the characteristic birefringence of the original dermal matrix collagen fibers. This residual matrix apparently prevents wound contraction in the same way that skin grafted into an open wound prevents wound contraction.[10] If the center of a healing freeze wound is excised 1 week after injury, the rate of contraction of the wound is the same as that of an open wound.[6]

Myofibroblasts were found in healing burn and freeze wounds. They were present in healing freeze wounds as early as 4 days after injury. Vascular patency is not destroyed in a freeze wound,[11] and this may give rise to an environment which promotes early cell migration into the damaged tissue. Because there is no evidence of contraction in a freeze wound, the question is why the mesenchymal cells stain as myofibroblasts. NBD-phallacidin stains the actin-rich stress fibers, and recent observations *in vivo* suggest that stress fibers occur in cells as a result of increased tension.[12–15] *In vitro* studies demonstrated that the artificial application of tension to cytoplasmic gels causes the microfilaments, normally randomly oriented, to line up parallel to one another.[16,17] The microfilaments in the cortex of *Physarum* become oriented only when contraction is resisted and they are under increased tension.[18,19] The myofibroblasts in the islands of granulation tissue in the healing freeze wounds pull against the resistance developed by the residual connective tissue matrix.[20]

Microfilaments have been shown to aggregate into bundles when cell–cell contacts are made.[9] This is a rapid process which can occur within 20 seconds after cell–cell contact. It appears to occur in healing burn and freeze wounds. Granulation tissue from both types of healing wounds show high cell density. Numerous cell–cell contacts are present and should permit the development of microfilament bundles, or stress fibers. Islands of granulation tissue in healing freeze wounds exhibit the greatest filament staining where cell density is greatest. Mycfibroblasts may develop there because of the great density of fibroblasts. Conversely, myofibroblasts may disappear from healing wounds when cell density decreases and more cell–connective tissue matrix contacts develop.

The process of wound contraction may be controlled by the connective tissue matrix of the wound. Where little native connective tissue matrix remains, as in a burn or open wound, the wound heals by contraction. Fibroblasts in granulation tissue have little difficulty moving and distorting this matrix. Where the structural integrity of the connective tissue matrix remains intact, as in a freeze wound, the wound does not contract because the resident fibroblasts are unable to move or distort it. The contractile process as observed in wound healing is a cooperation between mesenchymal cells and their surrounding connective tissue matrix.

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