

Evidence for the Nonmuscle Nature of the “Myofibroblast” of Granulation Tissue and Hypertrophic Scar

An Immunofluorescence Study

ROBERT J. EDDY, BSc, JANE A. PETRO, MD,
and JAMES J. TOMASEK, PhD

*From the Department of Anatomy, the Department of Surgery,
Division of Plastic and Reconstructive Surgery, and the
Department of Orthopaedic Surgery, New York Medical College,
Valhalla, New York*

Contraction is an important phenomenon in wound repair and hypertrophic scarring. Studies indicate that wound contraction involves a specialized cell known as the myofibroblast, which has morphologic characteristics of both smooth muscle and fibroblastic cells. In order to better characterize the myofibroblast, the authors have examined its cytoskeleton and surrounding extracellular matrix (ECM) in human burn granulation tissue, human hypertrophic scar, and rat granulation tissue by indirect immunofluorescence. Primary antibodies used in this study were directed against 1) smooth muscle myosin and 2) nonmuscle myosin, components of the cytoskeleton in smooth muscle and nonmuscle cells, respectively, and 3) laminin and 4) fibronectin, extracellular glycoproteins mediating

cell-matrix attachment in smooth muscle and nonmuscle cells, respectively. Myofibroblasts can be identified by their intense staining of actin bundles with either anti-actin antibody or NBD-phalloidin. Myofibroblasts in all tissues stained for nonmuscle but not smooth muscle myosin. In addition, nonmuscle myosin was localized as intracellular fibrils, which suggests their similarity to stress fibers in cultured fibroblasts. The ECM around myofibroblasts stains intensely for fibronectin but lacks laminin, which suggests that a true basal lamina is not present. The immunocytochemical findings suggest that the myofibroblast is a specialized nonmuscle type of cell, not a smooth muscle cell. (*Am J Pathol* 1988, 130:252-260)

TISSUE CONTRACTION is an important phenomenon in the repairing dermis of open wounds and in hypertrophic scarring. Initially, the shortening of collagen fibers in the extracellular matrix was thought to be responsible; however, this was challenged by the demonstration that wounds made in scorbutic animals contract independent of collagen production.¹ A cellular mechanism for contraction has been proposed by Gabbiani and co-workers^{2,3} after their discovery of a morphologically distinct cell found in actively granulating wounds. Upon ultrastructural examination, this cell type appeared to have characteristics of both fibroblasts and smooth muscle cells.^{2,3} In addition to having extensive rough endoplasmic reticulum and Golgi apparatus characteristic of fibroblasts, these cells contained large bundles of actin

microfilaments, which resemble the myofibrils of smooth muscle. The surface of these cells was covered by an interrupted amorphous layer of extracellular material similar to the basal lamina surrounding smooth muscle cells. Because these cells showed morphologic characteristics of both fibroblasts and smooth muscle cells, Gabbiani and associates² termed them “myofibroblasts.” The presence of these cells in

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Address reprint requests to Dr. James J. Tomasek, Department of Anatomy, Basic Sciences Building, New York Medical College, Valhalla, NY 10595.

actively contracting granulation tissue and hypertrophic scar,⁴ as well as other contractile tissues such as the palmar fascia during Dupuytren's disease,⁵ and their morphologic characteristics have led to the proposal that the myofibroblast is the cellular agent responsible for tissue contraction.³

After numerous morphologic studies, uncertainty still exists as to whether the myofibroblast more closely resembles the fibroblast or the smooth muscle cell. Therefore, we examined the cytoskeleton and extracellular matrix of myofibroblasts from human burn granulation tissue, rat granulation tissue, and human hypertrophic scar, with immunologic probes for proteins characteristic of either fibroblasts or smooth muscle cells. Two aspects of the myofibroblast were studied: 1) the type of myosin in the cytoskeleton and 2) the macromolecules in the extracellular matrix. The selection of these cellular components was based on their presumed role in the generation and transmission of contractile force.

Myosin is a cytoskeletal protein that interacts with actin to generate contractile force in both muscle and nonmuscle cells.⁶ The myosin associated with smooth muscle cells is distinct from that found in nonmuscle cells.⁷ To determine which species of myosin is associated with myofibroblasts of human burn granulation tissue, rat granulation tissue, and hypertrophic scar, we employed antibodies that can distinguish between smooth muscle myosin and nonmuscle myosin in conjunction with indirect immunofluorescence.⁸

Smooth muscle cells are surrounded by a highly organized layer of extracellular material called the basal lamina.⁹ This structure functions in maintaining intercellular connections and transmitting changes in cell shape, such as contraction, across a tissue fabric. Basal laminae contain the glycoprotein laminin,¹⁰ which binds the cell to the surrounding Type IV collagen layer.¹¹ In contrast, fibroblasts have no basal lamina and therefore lack laminin.¹⁰ A different glycoprotein, fibronectin, attaches fibroblasts to their surrounding stromal (Types I and III) collagen.¹² Previous studies of myofibroblasts in human wound granulation tissue,¹³ experimental granulation tissue,¹⁴ and hypertrophic scar¹⁵ have demonstrated fibronectin at the surface of these cells. However, the distribution of laminin in these tissues is unknown. To determine the distribution of laminin with respect to fibronectin at the surface of myofibroblasts in granulation tissue and hypertrophic scar, we employed antibodies specific for laminin or fibronectin in conjunction with indirect immunofluorescence.

In this report, we demonstrate that myofibroblasts contain only nonmuscle myosin and are surrounded

by a matrix rich in fibronectin but lacking laminin. Therefore, our immunofluorescence studies of the cytoskeleton and extracellular matrix of myofibroblasts, in human and rat granulation tissue and hypertrophic scar, suggest that this cell has characteristics similar to fibroblasts, rather than smooth muscle cells.

Materials and Methods

Tissue

Granulation tissue was taken from 2 patients undergoing debridement of the burn wound. Hypertrophic scar tissue was taken from 1 patient undergoing scar contracture release. Granulation tissue was also taken from 4 female Sprague-Dawley rats each weighing approximately 250 g. Open wounds were made on rats which were anesthetized with an intraperitoneal injection of sodium pentobarbital in phosphate-buffered saline. Their backs were shaved and depilated (Nair, Carter Wallace, Inc., Cranbury, NJ), and an antiseptic solution (Podiodine, Larson Laboratories, Inc., Erie, Pa) was applied. A single 2 × 2-cm full-thickness excision was made on the dorsum of each animal and the wound left open. Two weeks after wounding, the animals were sacrificed and the granulation tissue excised. All the tissues were cut into 2–3-cm pieces and prepared for histologic and immunocytochemical staining by fixation in 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4, for 20 minutes at room temperature. The tissue was infiltrated with 30% sucrose, mounted in OCT compound (Lab-Tek Products, Miles Laboratories, Naperville, Ill), and rapidly frozen by immersion in liquid nitrogen. Cryostat sections (4–6 μ) were thaw-mounted onto gelatin-coated glass slides.

Antibodies

The anti-nonmuscle myosin antibody was a gift from Dr. Keigi Fujiwara (National Cardiovascular Research Institute, Suita-Shi, Osaka, Japan). It is a rabbit antiserum against human platelet myosin prepared as previously described.¹⁶ Previous studies with this antibody have shown it to react with the myosin of nonmuscle cells, including fibroblasts and vascular endothelial cells, but not with the myosin of smooth muscle cells.⁸

The anti-smooth muscle myosin antibody, also a gift from Dr. Keigi Fujiwara, is a rabbit antiserum against human uterine myosin prepared as previously described.⁸ By double immunodiffusion, this antibody formed a single precipitin line with extracts of

human uterus but not with extracts of human platelets or cardiac or skeletal muscle. Immunofluorescence microscopy shows this antibody to stain all visceral and vascular smooth muscle tested, but no other cell type, including epithelial, endothelial, and fibroblastic cells as well as cardiac and skeletal muscle.⁸

A monoclonal antibody raised against quail muscle actin was a gift from Dr. Michael Payne (Department of Anatomy, New York Medical College, Valhalla, NY). This antibody has been shown by immunoblot analysis and immunocytochemistry to react with both muscle and nonmuscle actins.¹⁷ Rabbit anti-serum against laminin was purchased from Collaborative Research, Inc. (Lexington, Mass). A monoclonal antibody prepared against human fibronectin was a gift from Dr. Albert J. T. Millis (Department of Biological Sciences, State University of New York, Albany, NY). The specificity of this antibody has been demonstrated by immunoblot analysis and immunofluorescence.¹⁸

The monoclonal antibodies against actin and fibronectin could not be used on rat granulation tissue because of the cross-reactivity of the goat anti-mouse secondary antibody with exogenous rat immunoglobulins in the tissue sections. In order to localize fibronectin, we used a rabbit anti-human fibronectin antibody. This antibody was an F(ab')₂ fragment generously provided by Dr. Henry P. Godfrey (Department of Pathology, New York Medical College, Valhalla, NY). It has been demonstrated to cross-react with rat fibronectin by immunoblot analysis and double immunodiffusion (personal communication, Dr. Henry P. Godfrey).

The fluorescent probe 7-nitrobenz-2-oxa-1,3-diazole (NBD) phalloidin was used to identify myofibroblasts in rat granulation tissue. Phalloidin is an acidic derivative of phalloidin, a toxin from the poisonous green mushroom *Amanita phalloides*, which binds specifically to filamentous or F-actin of the cytoskeleton.¹⁹ NBD-phalloidin has been reported to stain the F-actin of stress fibers in isolated living cells and fixed tissues,²⁰ as well as to stain myofibroblasts in granulation tissue.²¹

Immunofluorescence Staining

Cryosections of human granulation tissue and hypertrophic scar were washed in phosphate-buffered saline (PBS), pH 7.4, for 5 minutes. Sections to be stained with anti-cytoskeletal antibodies were permeabilized with 0.2% Triton X-100 in PBS for 3 minutes and washed with PBS three times for 5 minutes each. Sections to be stained with anti-extracellular

matrix antibodies were blocked with normal goat serum diluted 1:10 in PBS for 30 minutes at room temperature. Sections were then incubated with the appropriate primary antibody for 30 minutes at room temperature, except for the anti-myosin antibodies, which were incubated overnight at 4 C. Antibodies were diluted in PBS (anti-actin antibody, 1:500; anti-nonmuscle myosin and smooth muscle myosin antibodies, 1:1000; anti-laminin antibody, 1:50; mouse anti-fibronectin antibody, 1:100; and rabbit anti-fibronectin antibody, 1:50). The sections were washed in PBS three times for 5 minutes each and incubated for 30 minutes with the appropriate secondary antibody (goat anti-rabbit IgG-rhodamine or fluorescein or goat anti-mouse IgA + IgG + IgM-rhodamine [Cappel Laboratories, Malvern, Pa] diluted 1:100 in PBS). The slides were washed with PBS three times for 5 minutes each, mounted in a 2:8 solution of PBS and glycerol, and coverslipped. Cryosections of rat granulation tissue were washed in PBS for 5 minutes and permeabilized in cold acetone (-20 C) for 4 minutes and allowed to air-dry. NBD-phalloidin (Molecular Probes, Inc., Junction City, Ore) was prepared according to manufacturer's instructions. Each section was incubated with 10 μ l NBD-phalloidin stock solution diluted to 200 μ l with PBS for 20 minutes at room temperature. Sections were washed twice rapidly in PBS, mounted in a 1:1 solution of PBS and glycerol, and coverslipped.

Double fluorescent staining of the same sections with anti-actin and anti-laminin antibodies was accomplished by separate incubations of the primary antibodies, followed by simultaneous incubation with the appropriate contrasting fluorescent secondary antibodies. Double fluorescent staining of the same sections with NBD-phalloidin and anti-laminin was accomplished by incubation with anti-laminin antibody and the contrasting secondary antibody, followed by incubation with NBD-phalloidin.

Immunofluorescence staining controls for both anti-myosin and anti-laminin polyclonal antibodies were performed by incubating tissue sections with rabbit preimmune serum at the same concentration as the antibody, followed by the appropriate fluorochrome-conjugated goat anti-rabbit IgG. Immunofluorescence controls for anti-actin and anti-fibronectin monoclonal antibodies were performed by replacing the antibody with PBS followed by incubation of the sections with the appropriate fluorochrome conjugated goat anti-mouse IgG. In both controls, no fluorescent staining was detected. All slides were examined with a Leitz orthoplan microscope equipped with epifluorescence optics and both rhodamine and fluorescein filters for viewing double-

stained sections. Photographs were taken with Kodak TRI-X Pan Film push-processed to 1000 ASA.

Results

Actin Staining

Human burn granulation tissue and hypertrophic scar were examined by indirect immunofluorescence for the presence of myofibroblasts. Previous studies have demonstrated that the cytoplasm of these cells stains intensely with anti-actin antibody.²² Myofibroblasts were found to be the dominant cell type in granulation tissue (Figure 1A), whereas the pattern of actin staining in hypertrophic scar showed myofibroblasts to be organized into nodular structures (Figure 2A), confirming previous ultrastructural observations.⁴ In contrast, normal dermal fibroblasts do not stain with anti-actin antibody (Figure 1A). The inability of anti-actin antibody to stain normal fibroblasts is not due to the absence of actin in these cells, but is most likely due to their lack of large bundles of actin microfilaments. Vascular smooth muscle cells are the only other cell type in human granulation tissue and hypertrophic scar that stains with anti-actin antibody (Figures 1A and 2A).

The fluorescent probe NBD-phalloidin was used to stain actin in myofibroblasts of rat granulation tissue. Myofibroblasts were found to be the dominant cell type, conforming to the staining pattern observed in human granulation tissue (Figure 3A).

Myosin Immunofluorescence Staining

The type of myosin present in human burn granulation tissue, hypertrophic scar, and rat granulation tissue was determined by indirect immunofluorescence. Myofibroblasts were found to stain intensely with anti-nonmuscle myosin antibody but not with anti-smooth muscle myosin antibody (human granulation tissue, Figures 4A and B; hypertrophic scar, Figures 5A and B; rat granulation tissue, Figures 6A and B). In addition, normal dermal fibroblasts and vascular endothelial cells stain positively with anti-nonmuscle myosin antibody (Figures 4A, 5A and 6A). Individual fibers within myofibroblasts in human burn granulation tissue and rat granulation tissue were observed to stain with anti-nonmuscle myosin antibody (Figures 7A and B). Anti-smooth muscle myosin antibody staining was seen only in vascular smooth muscle cells around blood vessels (Figures 4B, 5B, and 6B).

Laminin Immunofluorescence Staining

The extracellular matrix surrounding myofibroblasts in human granulation tissue, hypertrophic scar, and rat granulation tissue was examined for laminin by indirect immunofluorescence. The sections were double-stained with anti-actin antibody or NBD-phalloidin for colocalization of actin and laminin. No staining with anti-laminin antibody was observed at the surface of these myofibroblasts or in the surrounding matrix (human granulation tissue, Figure 1B; hypertrophic scar, Figure 2B, rat granulation tissue, Figure 3B). Anti-laminin antibody staining was observed in the basal lamina of vascular smooth muscle cells (Figures 1B and 3B).

Fibronectin Immunofluorescence Staining

The extracellular matrix surrounding myofibroblasts in human granulation tissue, hypertrophic scar, and rat granulation tissue was examined for fibronectin by indirect immunofluorescence. Positive staining with anti-fibronectin antibody was observed throughout the matrix of human granulation tissue (Figure 8) and rat granulation tissue (Figure 10). In hypertrophic scar, fibronectin staining was concentrated in the nodular structures (Figure 9), which confirmed previous observations.¹⁵

Discussion

In this study, we have shown that the cytoskeleton of myofibroblasts from human burn granulation tissue, hypertrophic scar, and rat granulation tissue contain nonmuscle myosin but not smooth muscle myosin. The extracellular matrix of these myofibroblasts contains abundant amounts of fibronectin as previously reported.¹³⁻¹⁵ In addition, this study has demonstrated that these cells lack the basal lamina-specific glycoprotein laminin, associated with smooth muscle cells. Previous studies demonstrating the morphologic similarity of myofibroblasts to smooth muscle suggest that they may exert and transmit contractile forces by a smooth muscle type mechanism. However, the absence of smooth muscle myosin and the basal lamina-specific attachment protein laminin in these cells strongly argues against this hypothesis. The presence of nonmuscle myosin in the cytoskeleton and fibronectin in the extracellular matrix of the myofibroblast suggests that they more closely resemble a nonmuscle type of cell.

Normal fibroblasts do not display large bundles of actin microfilaments *in situ*, such as those seen in myofibroblasts, although they can be made to do so by culture on a planar substratum, such as a plastic

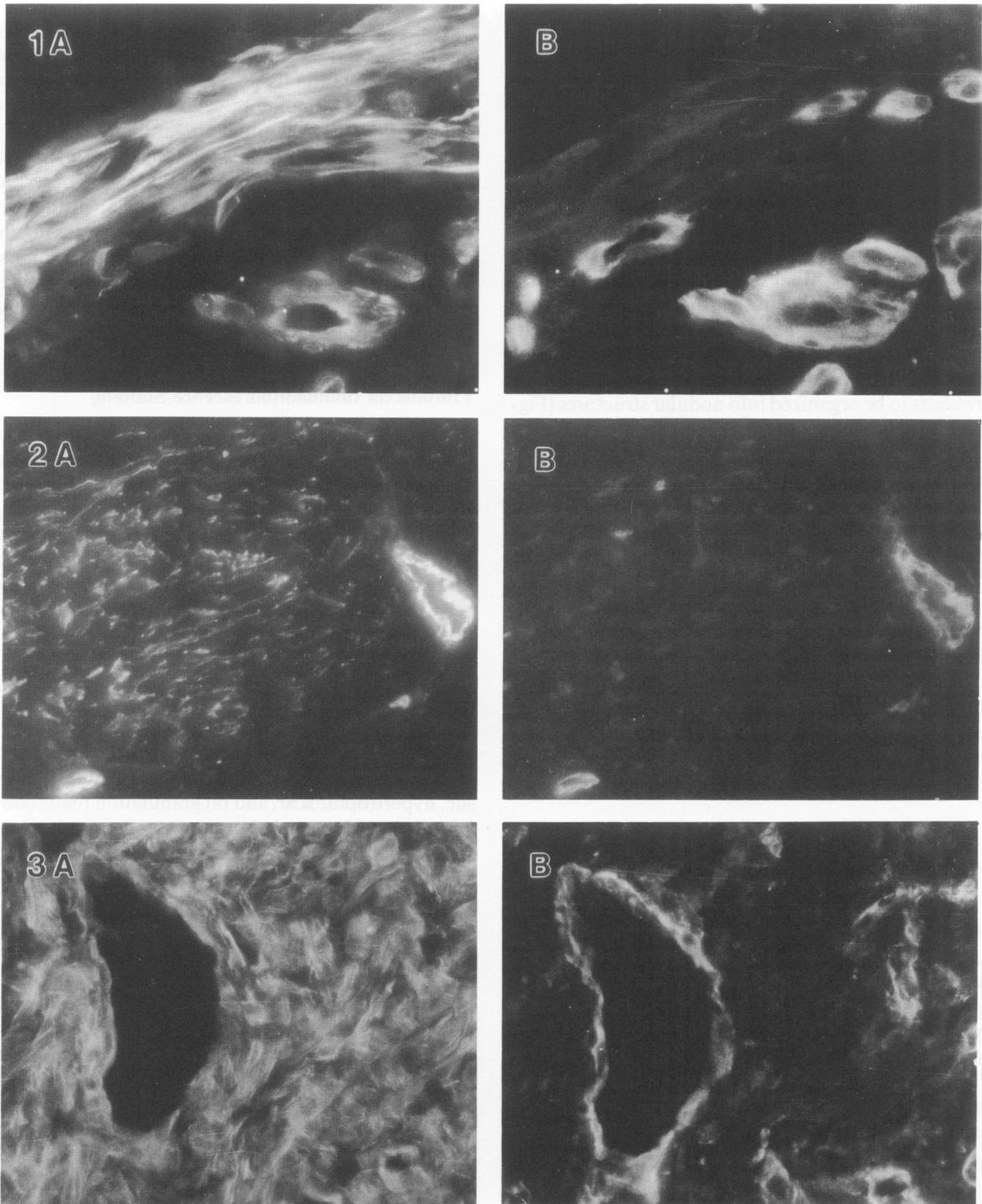


Figure 1-3—Fluorescent micrographs of cryosections of human burn granulation tissue (**Figure 1**), human hypertrophic scar (**Figure 2**), and rat granulation tissue (**Figure 3**) double-stained with anti-actin antibody (**1A, 2A**) or NBD-phalloidin (**3A**) and anti-laminin antibody (**1B, 2B, 3B**). **Figures 1A, 2A, and 3A**—Myofibroblasts are identified by intense fluorescent staining of the cytoplasm for actin. Fibroblasts in the surrounding stroma do not stain. Vascular smooth muscle cells around blood vessels stain brightly for actin. **Figure 2A**—An aggregation of myofibroblasts characteristic of a hypertrophic scar nodule. **Figure 3A**—Myofibroblasts containing stress fibers display a filamentous staining pattern. **Figures 1B, 2B, and 3B**—No staining for laminin is observed around myofibroblasts. As expected, there is no staining for laminin around stromal fibroblasts. The basal lamina around vascular smooth muscle cells of arterioles stains positively. ($\times 375$)

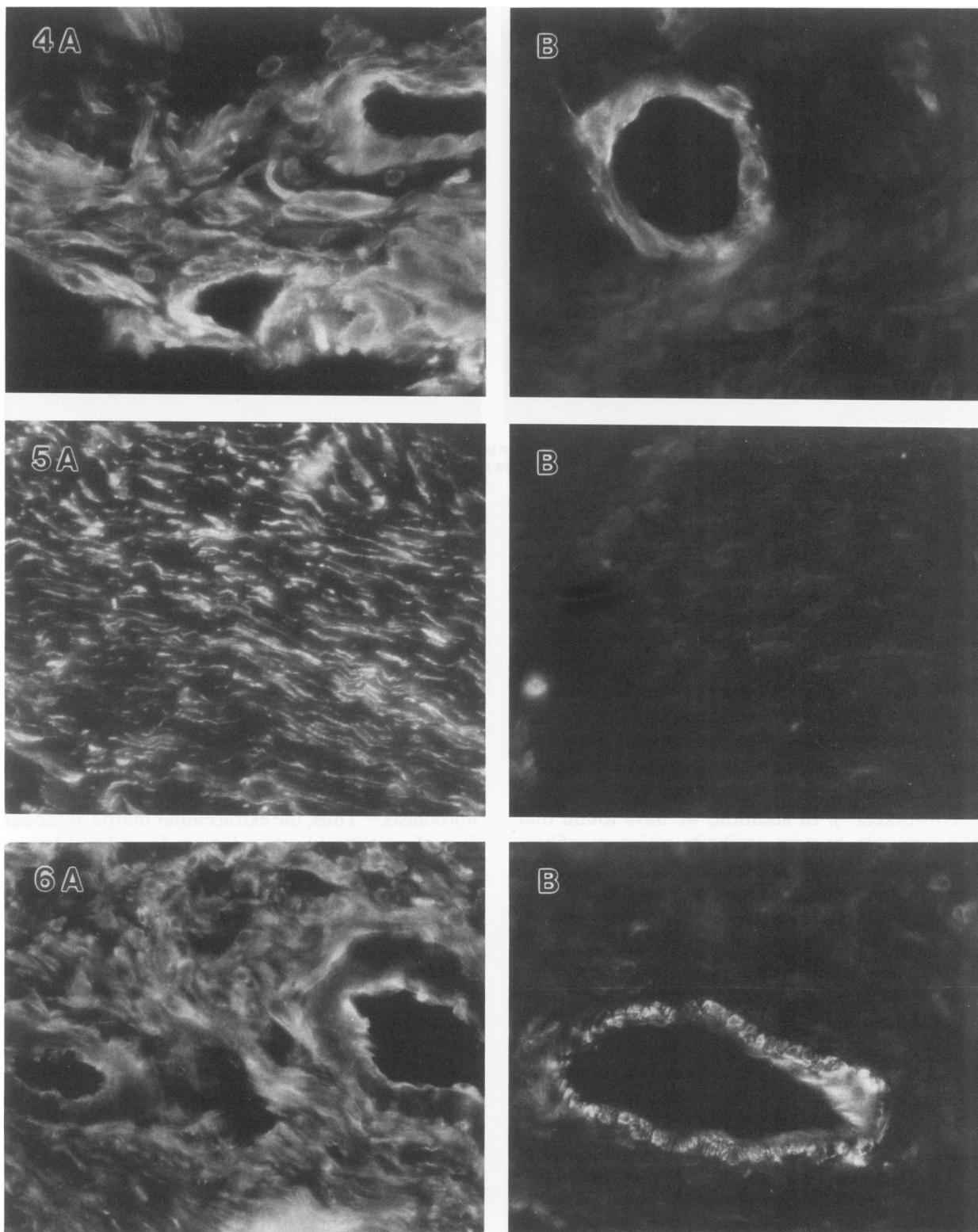


Figure 4-6—Indirect immunofluorescent micrographs of cryosections of human burn granulation tissue (Figures 4), human hypertrophic scar (Figure 5), and rat granulation tissue (Figure 6) stained with either anti-nonmuscle myosin (4A, 5A, 6A) or anti-smooth muscle myosin (4B, 5B, 6B) antibodies. Figures 4A, 5A, and 6A—Myofibroblasts stain intensely for nonmuscle myosin. Vascular smooth muscle does not stain with anti-nonmuscle myosin antibody, whereas vascular endothelium does stain. Figures 4B, 5B, and 6B—Myofibroblasts do not stain with anti-smooth muscle myosin antibody. As expected, vascular smooth muscle stains intensely with this antibody. ($\times 375$)

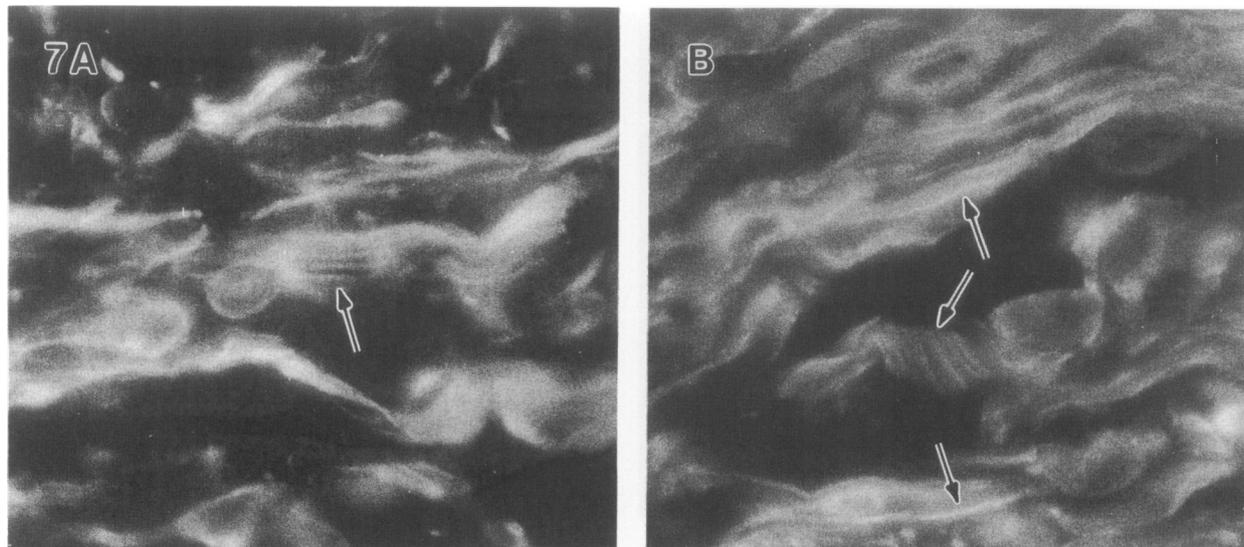


Figure 7—Indirect immunofluorescent micrographs of cryosections of human burn granulation tissue (A) and rat granulation tissue (B) stained with anti-non-muscle myosin antibody. Individual stress fibers within myofibroblasts are stained (arrow). ($\times 925$)

culture dish.²³ These bundles of microfilaments are referred to as stress fibers. In addition to actin, stress fibers are composed of α -actinin, nonmuscle myosin, and tropomyosin, which are organized in a periodic manner along their length.²³ Ultrastructural studies have shown that stress fibers in cultured cells resemble the bundles of actin microfilaments in myofibroblasts.³ Our study has demonstrated that myofibroblasts contain nonmuscle myosin, not smooth muscle myosin (see also the preliminary report by Benzonana and co-workers²⁴). In addition, we have found that anti-nonmuscle myosin antibody stains myofibroblasts in a filamentous pattern, similar to the staining of stress fibers.²⁵ These results suggest that the bundles of microfilaments present in myofibroblasts are similar to stress fibers.

Fibroblasts and smooth muscle contain different types of actin.²⁶ Recently, a monoclonal antibody has been prepared against α -actin,²⁷ a type of actin specific for smooth muscle.²⁶ Immunocytochemical studies of human wound granulation tissue using this antibody have demonstrated the lack of α -actin in myofibroblasts.²⁷ These results are consistent with the hypothesis that the microfilament bundles present in myofibroblasts in granulation tissue are similar to stress fibers present in cultured fibroblasts.

The extracellular matrix at the surface of myofibroblasts in granulation tissue and hypertrophic scar was found to lack laminin. Previous ultrastructural studies have described the extracellular material at the surface of myofibroblasts as basal lamina-like.²⁻⁵ The absence of laminin demonstrates that this extracellu-

lar material is biochemically distinct from basal lamina. Rather, there is a large amount of fibronectin present at the myofibroblast's surface in granulation tissue and hypertrophic scar, as shown here as well as in other studies.¹³⁻¹⁵ The fibronectin-rich extracellular fibrils located at the myofibroblast's surface have been found to be in close transmembrane association with intracellular actin microfilaments.²⁸ This transmembrane association, termed the fibronexus, has previously been observed at the surface of cultured fibroblasts.²⁹ Thus, the extracellular matrix at the surface of myofibroblasts is distinct from basal lamina and resembles that observed around cultured fibroblasts.

The cytoskeleton and extracellular matrix of myofibroblasts in Dupuytren's disease, a human disease characterized by a progressive contraction of the palmar fascia, have recently been examined.³⁰ This immunocytochemical study has demonstrated that Dupuytren's myofibroblasts contain nonmuscle myosin and fibronectin and lack smooth muscle myosin and laminin, similar to that observed here for granulation tissue and hypertrophic scar. Myofibroblasts from Dupuytren's disease as well as those from infiltrating ductal breast carcinomas have also been shown to contain the intermediate filament protein vimentin but to lack desmin,³¹ a protein characteristic of most smooth muscle cells.³² Therefore, myofibroblasts that form in a variety of contractile tissues appear to be a nonmuscle cell type. However, it should be stressed that although our results have demonstrated the nonmuscle nature of the myofibroblast, they do not indi-

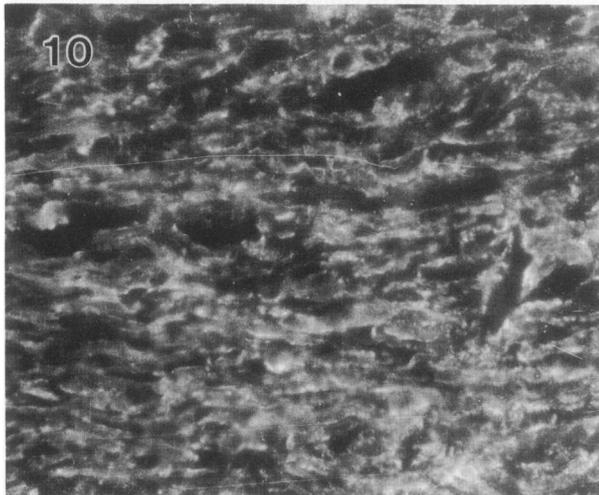
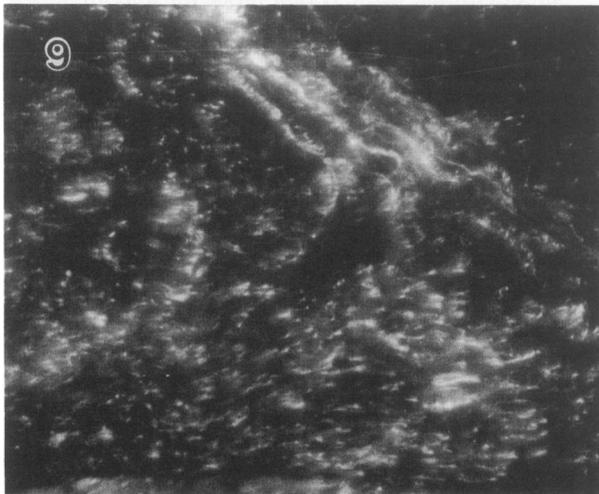
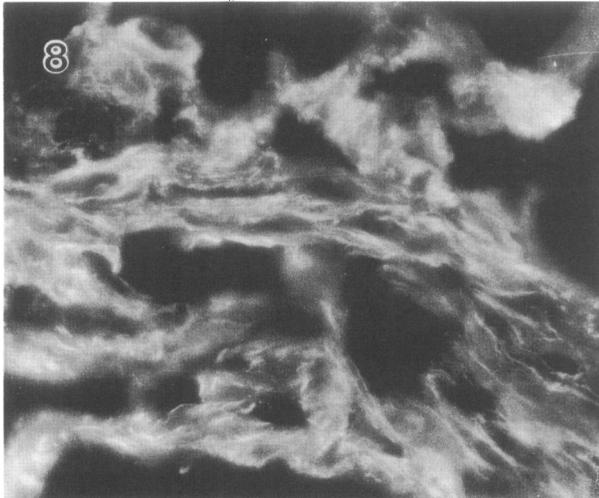


Figure 8–10—Indirect immunofluorescent micrographs of cyrosections of human burn granulation tissue (**Figure 8**), human hypertrophic scar (**Figure 9**), and rat granulation tissue (**Figure 10**) stained with anti-fibronectin antibody. **Figures 8 and 10**—Bright fibrillar staining for fibronectin is seen in the extracellular matrix around myofibroblasts. **Figure 9**—The extracellular matrix around myofibroblasts of the hypertrophic scar nodule stains intensely for fibronectin. Stromal fibroblasts show very little staining. ($\times 375$)

cate the origin of this cell type. Recent studies have convincingly shown that smooth muscle myosin disappears, while nonmuscle myosin appears, in rapidly proliferating smooth muscle cells during culture *in vitro*.³⁵ Therefore, the lack of smooth muscle myosin in myofibroblasts does not rule out the possibility that these cells are derived from smooth muscle.

If the myofibroblast is responsible for the contraction in granulation tissue and hypertrophic scarring, it must be capable of generating an intracellular contractile force and possess a means of transmitting this force to the surrounding tissue. Nonmuscle cells contain a cytoplasmic actomyosin system that is capable of generating contractile forces⁶ and a collagen- and fibronectin-rich extracellular matrix that is capable of transmitting such a force across a tissue fabric. Stress fibers present in cultured fibroblasts have been demonstrated to contract under appropriate conditions.^{33,34} Thus, there exists in the myofibroblast a nonmuscle contractile mechanism with the potential to generate the intracellular forces necessary for tissue contraction. Fibronectin, which is present at the surface of myofibroblasts and extends into the collagen-rich matrix,^{13,28} could function in the formation of cell-to-cell and cell-to-matrix connections. These connections would then serve to transmit the contractile forces generated by the myofibroblast across the tissue as a whole. Fibronectin is ideally suited for such a role, because it is divided into functional domains that can bind to both the surface of cells and the surrounding collagen.¹²

In conclusion, we suggest that the myofibroblast is a nonmuscle cell that has formed stress fibers *in vivo* and accumulated large amounts of fibronectin at its surface. Our findings indicate that the resemblance of this cell to smooth muscle is strictly morphologic and does not extend to characteristic cytoskeletal or extracellular components. Future studies of tissue contraction in wound healing, its possible control in hypertrophic scarring, and the origin and fate of this interesting cell type should take into account these findings on the properties of its cytoskeleton and extracellular matrix.

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