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Morphological and Immunochemical Differences Between Keloid and Hypertrophic Scar

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There are two types of excessive scarring, keloid and hypertrophic scar. Contrary to hypertrophic scars, keloids do not regress with time, are difficult to revise surgically, and do not provoke scar contractures. These two lesions require different therapeutic approaches but are often confused because of an apparent lack of morphological differences. We have investigated the collagen organization and the possible presence of α-smooth muscle (SM) actin-expressing myofibroblasts in these conditions. Keloids contain large, thick collagen fibers composed of numerous fibrils closely packed together. In contrast hypertrophic scars exhibit nodular structures in which fibroblastic cells, small vessels, and fine, randomly organized collagen fibers are present. We confirm that such nodular structures are always present in hypertrophic scar and rarely in keloid. Furthermore, only nodules of hypertrophic scars contain α-SM actin-expressing myofibroblasts. Electron microscopic examination supports the above-mentioned differences in collagen organization and in fibroblastic features and shows the presence of an amorphous extracellular material surrounding fibroblastic cells in keloid. The presence in hypertrophic scar myofibroblasts of α-SM actin, the actin isoform typical of vascular SM cells, may represent an important element in the pathogenesis of contraction. Interestingly, when placed in culture fibroblasts from hypertrophic scars and keloids express similar amounts of α-SM actin, suggesting that local microenvironmental factors influence in vivo the expression of this protein. Thus several morphological and immunohistochemical differences exist between hypertrophic scar and keloid that are useful for the biological and pathological characterization of the two lesions. (Am J Pathol 1994, 145:105–113)

Trauma that creates tissue loss gives rise to the repair process and eventually ends with scar tissue. In some situations (eg, second- and third-degree burn injuries) healing may be complicated by the development of hypertrophic scars, which are characterized by elevation above the skin surface, redness, and itching. They are limited to the initial boundaries of the injury, tend to regress with time, can be revised by plastic surgery, and may produce scar contractures, eg, when located over joints. It is accepted that excessive scarring is related to the depth of initial tissue loss.[1] Another excessive scarring condition is the keloid. This type of lesion differs from hypertrophic scar by developing from either a deep or a superficial injury. Keloids are also red and itchy but they exceed the boundaries of the initial injury, do not regress with time, are difficult to revise surgically, and do not provoke contractures.[2–5] These two types of lesion are often confused and the titles keloid and hypertrophic scar are often used interchangeably in describing excessive scarring. To the clinician they are distinct lesions which require different approaches to resolve them.

Histological differences between keloid and hypertrophic scar have been reported using hematoxylin

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and eosin, periodic acid-Schiff and Masson's trichrome staining.[6] Abnormally large collagen bundle complexes were identified in keloids but were absent from hypertrophic scars.[6] These complex collagen bundles were shown to be associated with important amounts of "ground substance" mucopolysaccharides.[6] However, these techniques were unable to distinguish any unique morphological differences between the fibroblasts present in keloid or hypertrophic scar.

A histological characteristic of hypertrophic scar is the presence of nodules containing a high density of cells and collagen.[6–8] These nodules have a similar appearance to the nodules described in Dupuytren's contracture.[9] They are cigar-shaped and run parallel to the surface of the skin, are located in the middle or deeper layer of the scar, and are oriented along the tension lines of the scar.[10] Fibroblasts in nodules have been reported to have long processes which are intimately attached to collagen fibers.[11] The absence of such nodules is a characteristic of keloid.

Myofibroblasts are differentiated fibroblasts found in granulation tissue and fibrotic lesions.[12–15] They have been originally identified by means of electron microscopy. They differ from normal fibroblasts by their characteristic cytoplasmic bundles of microfilaments, nuclear indentations and cell-to-cell or cell-to-stroma connections.[13] Moreover, a large proportion of myofibroblasts expresses smooth muscle proteins such as α-smooth muscle (α-SM) actin and desmin.[16,17] A monoclonal antibody against α-SM actin has been used to differentiate fibroblasts and myofibroblasts in histological and electron microscopical sections.[16,18] It is well accepted that myofibroblasts appear temporarily in granulation tissue during wound healing,[12,17,19,20] but are present permanently in hypertrophic scars[21] and other fibrotic settings.[9,16]

Here we have investigated the possibility that biologically and possibly diagnostically relevant differences between keloid and hypertrophic scar could be determined at both the light and electron microscopic levels. The organization of collagen fibers was determined by polarized light microscopy of Sirius red-stained sections. The presence or absence of myofibroblasts was demonstrated by α-SM actin immunostaining in dermis, normal scar, keloid and hypertrophic scar. These findings were verified by electron microscopy. Our results indicate that hypertrophic scars and keloids have distinct pathological features. Such features may help in the interpretation of the clinical behavior and the pathogenesis of these lesions.

Materials and Methods

Specimens

Specimens for study were obtained from four medical centers. A total of 13 normal skin, 7 normal scar, 17 keloid, and 22 hypertrophic scar biopsies from patients 5 to 42 years of age were examined. Fresh tissue biopsies were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin wax. Sections (5 μ thick) were prepared. For the electron microscope studies, samples of normal skin (3), normal scar (5), keloid (7) and hypertrophic scar (6) were fixed in 2.5% cacodylate-buffered glutaraldehyde (Merck, Darmstadt, FRG).

Sirius Red Staining

Sirius red staining of paraffin sections was used according to Constantine and Mowry.[22] Briefly, sections were postfixed in Bouin's solution for 24 hours and incubated for 20 minutes in a solution containing 1 mg picrosirius red in 1 ml of a saturated picric acid solution. The sections were washed in water, mounted, and observed using a Zeiss Axiophot microscope (Carl Zeiss Inc., Oberkochen, FRG) equipped for light polarization. Photographs were taken with Ektachrome EPY-64X film (Kodak, Rochester, NY).

Immunochemical Staining of Tissue Section

Paraffin-embedded cut sections were pretreated with 7% H_2O_2 in distilled water and subsequently with 0.1 mol/L periodic acid, 0.005 mol/L $NaBH_4$, and normal serum. The sections were incubated for 2 hours with anti-αSM-1, a mouse immunoglobulin-G-2a (IgG-2a) monoclonal antibody against α-SM actin[18] diluted 1:200. The presence of α-SM actin was examined by means of the streptavidin-biotin complex peroxidase method (Dako A/S, Glostrup, Denmark). The revelation of peroxidase activity was done with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St Louis, MO). Slides were counterstained with hematoxylin. The sections were washed in water, mounted, and observed using a Zeiss Axiophot microscope (Carl Zeiss Inc.). Evaluation of the degree of nodular fibroblastic staining was made by two independent observers using the following arbitrary scale: – = not seen, +/– = focal positivity, and + = diffuse positivity. The significance was evaluated by means of the χ^2 test. Photographs were taken with Ektachrome EPY-64T film (Kodak).

Electron Microscopy

Glutaraldehyde-fixed biopsies were postfixed in 2% OsO_4 (Merck), dehydrated in graded ethanols, and embedded in Epon 812 (Fluka Chemie AG, Buchs, Switzerland). Semithin sections were cut and stained with methylene-blue. Representative areas were selected for thin sections. These were collected on copper grids, double-stained with uranyl acetate and lead acetate (Merck), and examined with a Philips 400 electron microscope (Philips SA, Zurich, Switzerland).

Cells, Culture Conditions, and Immunofluorescence Staining

Cell culture experiments were made using fibroblasts grown out from explants of normal dermis, keloid, and hypertrophic scar biopsies. The cells were grown in Eagle's Essential Medium (GIBCO AG, Basel, Switzerland) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L glutamine and containing 10% fetal bovine serum (GIBCO AG). Cultures were incubated at 37 C in a humidified atmosphere of 5% CO_2 and 95% air with medium changes three times per week.

Immunofluorescence was performed on methanol-fixed cells in plastic tissue culture dishes (60 mm). For double indirect immunofluorescence, we used anti-αSM-1 and a rabbit polyclonal anti-actin antibody recognizing all actin isoforms.[18] Tetramethylrhodamine isothiocyanate-labeled goat anti-mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) were used for the second step. Preparations were mounted and viewed with a Zeiss Axiophot microscope equipped with epi-illumination and specific filters for rhodamine and fluorescein (Carl Zeiss Inc.). Photographs were taken on T-MAX black-and-white film (Kodak).

Results

Light Microscopy and Immunohistochemistry on Paraffin-Embedded Tissues

Both keloid and hypertrophic scar showed increases in the deposition of connective tissue, the density of blood vessels, and the number of cells compared with normal scar and dermis. The epidermal layer in some cases was thicker in keloids and hypertrophic scars compared with that found in normal situations, but this was not a consistent finding. As expected, subepidermal appendages and rete pegs were absent in keloid and hypertrophic scar as well as in normal scar. The organization of the connective tissue as well as the orientation of cells differed between hypertrophic scar and keloid. A major histological difference was the presence of distinct nodules in hypertrophic scar and their absence in keloid and normal scar.

The organization of collagen fibers in dermis, keloid, and hypertrophic scar was examined by viewing Sirius red-stained histological sections with polarized light. As shown in Figure 1a, the birefringent collagen fibers in normal skin were red and composed of fine fibers arranged in a basket-like weave pattern. In keloid (Figure 1b), the birefringent collagen fibers were yellow-green and composed of abnormally thick fibers arranged in parallel arrays. In hypertrophic scar (Figure 1c), the birefringent collagen fiber pattern was distinct for fibers within and outside of nodules. The birefringent collagen pattern within nodules (Figure 1c, top left) showed fine, green-colored fibers. The birefringent collagen fibers surrounding the nodules (Figure 1c, lower right) were yellow-green and thicker. The birefringent banding pattern of these surrounding fibers showed a stripe design perpendicular to the long axis of the fibers. Thus collagen is organized differently in these two types of lesions.

Explicit differences between keloid and hypertrophic scar were also found in the cellular features of the two lesions. With α-SM actin-immune staining normal dermis, normal scar, keloid, and hypertrophic scar exhibited positive smooth muscle cells (SMCs) in the walls of blood vessels which served as an internal control. In dermis (Figure 1d), normal scar (data not shown), and keloid (Figure 1, e and g), the α-SM actin staining was also restricted to the blood vessel wall. In contrast, α-SM actin was present in vascular SMCs as well as in cells located within the nodular structures unique to hypertrophic scar (Figure 1, f, h, and i). These α-SM actin-positive cells present in the nodules of hypertrophic scar are myofibroblasts.[16] Table 1 shows the summary of positive-staining myofibroblasts in dermis, normal scar, keloid, and hypertrophic scar. No α-SM actin-positive myofibroblasts were found in dermis or normal scar, and they were also absent in 15 of 17 keloids examined. Thus in general, myofibroblasts were present in hypertrophic scars, but absent in keloids ($P < 10^{-5}$). In the two cases where myofibroblasts were demonstrated in keloids there was weak α-SM actin staining located in

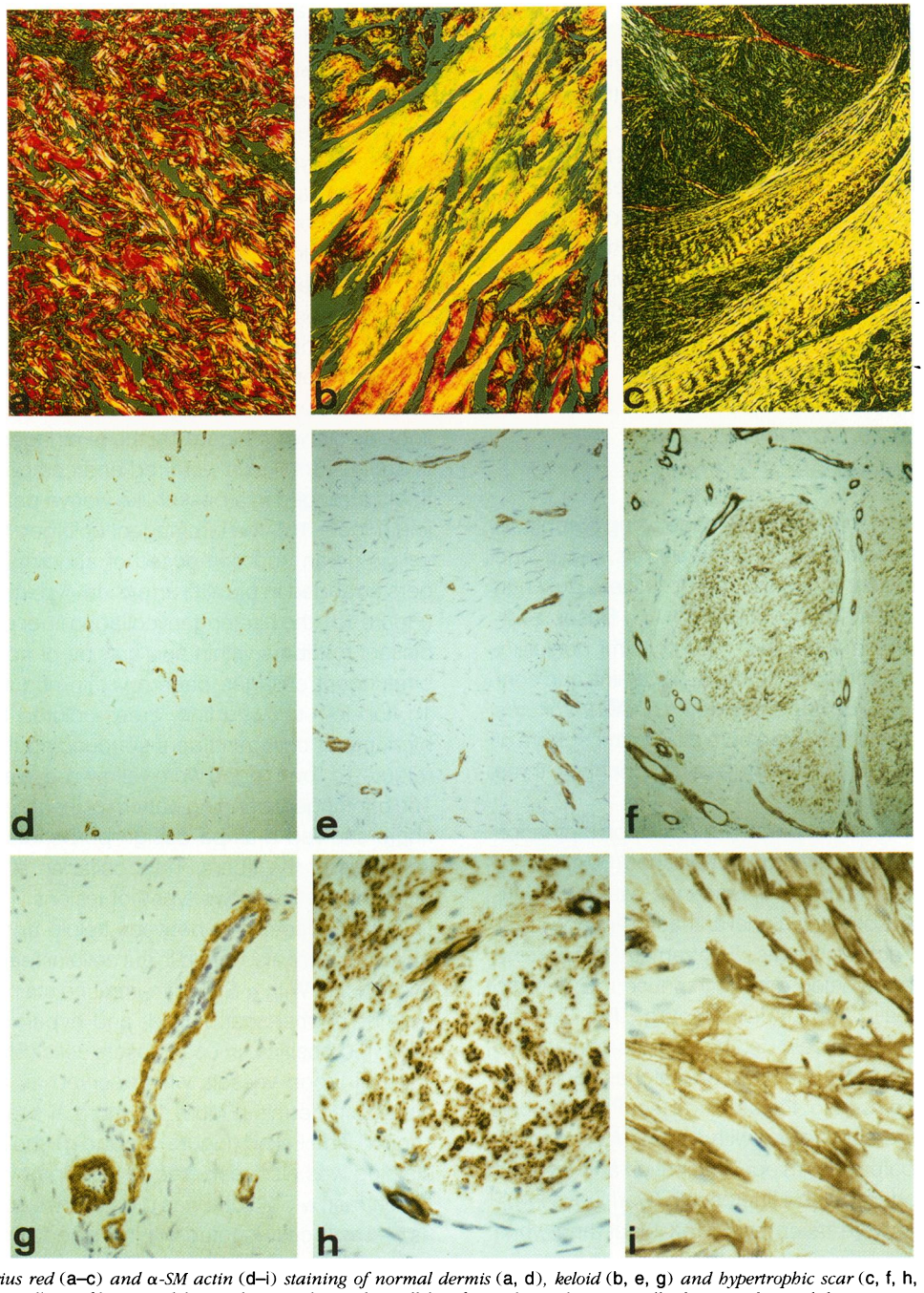

Figure 1. *Sirius red* (a–c) *and α-SM actin* (d–i) *staining of normal dermis* (a, d), *keloid* (b, e, g) *and hypertrophic scar* (c, f, h, i). *In normal dermis* (a) *the collagen fibers are delicate; they are disposed parallel in fascicules and occasionally show regular undulation as in tendon sheets. In keloids* (b) *the collagen fibers appear much thicker and more irregular than in normal dermis; they show a very coarse arrangement without any nodular or fascicular disposition. In hypertrophic scar* (c) *the collagen fibers appear as delicate filaments that are of fairly regular thickness and are arranged in definite nodular structures resembling those characteristics of fibromatosis such as in Dupuytren's or Ledderhose's diseases. α-SM actin expression is observed in the vascular wall of normal dermis* (d), *keloid* (e, g) *and hypertrophic scar* (f, h, i). *In hypertrophic scar* (f, h, i), *myofibroblasts present in the nodules are identified by α-SM actin-positive staining. In cross-section, α-SM actin is mainly localized at the cell periphery* (h). *Tangential section shows α-SM actin-containing stress fibers running parallel to the long axis of the cell* (i). a–f: ×*100;* g–i: ×*400.*

cells within nodular structures deep in the subcutaneous layer of the lesion. The presence of nodules in keloid was a rare finding and coincided with the presence of α-SM actin-positive cells. Hence in a human scar situation (with the exception of typical granulation tissue), myofibroblasts appear to reside exclusively in nodules and to be absent from non-nodular locations.

Table 1. *α-SM Actin Expression in Fibroblastic Cells of Connective Tissues*

Tissue	α-SM actin expression*		
	–	+/–	+
Dermis n = 13	13	0	0
Normal scar n = 7	7	0	0
Keloid n = 17	15	2	0
Hypertrophic scar n = 22	0	4	18

Scale: –, not seen; +/–, focal positivity; +, diffuse positivity.
* Vascular walls are always labeled for α-SM actin.

Myofibroblasts located in the nodules of hypertrophic scar were generally orientated parallel to one another. Within the myofibroblasts α-SM actin was organized in stress fibers running parallel to the long axis of the cell as demonstrated in Figure 1i. Figure 1h shows the stress fibers cut in cross-section where α-SM actin-positive material is mainly localized at the cell periphery.

The density of blood vessels within keloid and hypertrophic scar appeared higher compared with dermis and normal scar. We did not attempt to quantify their densities because of previous work specifically suggesting this point.[23] By α-SM actin staining, the SMCs of blood vessels in hypertrophic scar were found in both nodules and the connective tissue surrounding nodules (Figure 1, f and h). As shown in Figure 1g, the endothelial cells within blood vessels in keloid were somewhat rounded and projected into the lumen of the vessel. Likewise, in hypertrophic scar, rounded endothelial cells projected into the lumen of blood vessels. This was found in blood vessels located both within and outside of nodules. In contrast, endothelial cells lining the blood vessels of dermis were flattened and adherent to the vessel wall.

Electron Microscopy

To confirm these differences found by light microscopy between keloid and hypertrophic scar transmission electron microscopy was done (Figure 2, A and B). Fibroblasts in keloid (Figure 2A) did not show cytoplasmic microfilaments. A well-developed rough endoplasmic reticulum was present. The banded collagen fibrils of keloid were organized into thick fibers that were separated from the membrane surface of the fibroblast by a diffuse amorphous substance surrounding the surface of the cell (Figure 2A). This was readily evident in all keloid specimens examined and not found in hypertrophic scar or normal dermis specimens. Hypertrophic scar nodules showed typical myofibroblasts (Figure 2B) rich in peripheral microfilament bundles usually oriented parallel to the long axis of the cell. These myofibroblasts presented numerous and long cytoplasmic extensions connecting different cells through gap- and adherens-junctions (data not shown). Their plasma membrane showed focal deposition of basal lamina, plasmalemmal attachment plaques, was associated with fine, banded collagen fibers (Figure 3A), and presented typical fibronexus (Figure 3B).[24] The fine collagen fibers associated with the cell membrane were randomly arranged.

Cell Culture and Immunofluorescence

The possibility that keloid fibroblasts are defective in producing α-SM actin was examined in tissue culture. Fibroblasts grown out from keloid explants and maintained in monolayer cultures for five passages were stained for α-SM actin. As shown in Figure 4, keloid-derived fibroblasts have the capacity in culture to synthesize α-SM actin and assemble it in microfilaments making up stress fiber structures. Not all keloid-cultured fibroblasts demonstrated α-SM actin-positive stress fibers. Like dermal and hypertrophic scar-derived fibroblasts,[25] about 20% of the keloid-derived fibroblasts were positive for α-SM actin.

Discussion

Differences between hypertrophic scar and keloid are often considered to be insignificant,[1] and there are conflicting reports as to whether there are histological distinctions between these two scars.[2,6,26] However, there is little disagreement about distinctions concerning the gross appearance of these lesions. Our results confirm and extend the reports of histological differences between keloid and hypertrophic scar including the presence of nodules containing a high density of cells and fine fibrillar collagen in hypertrophic scar and their exceptional appearance in keloid.[6] Moreover, we show that such nodules contain α-SM actin-expressing myofibroblasts, which are generally absent in keloids. It is proposed that these nodules represent a characteristic feature of hypertrophic scars. Small nodules containing few α-SM actin-positive myofibroblasts were found in two cases of keloid. The possibility exists that these keloids with nodules correspond to a mixed scar comprising both keloid and hypertrophic scar regions. Possible reasons for the heterogeneous composition of those two

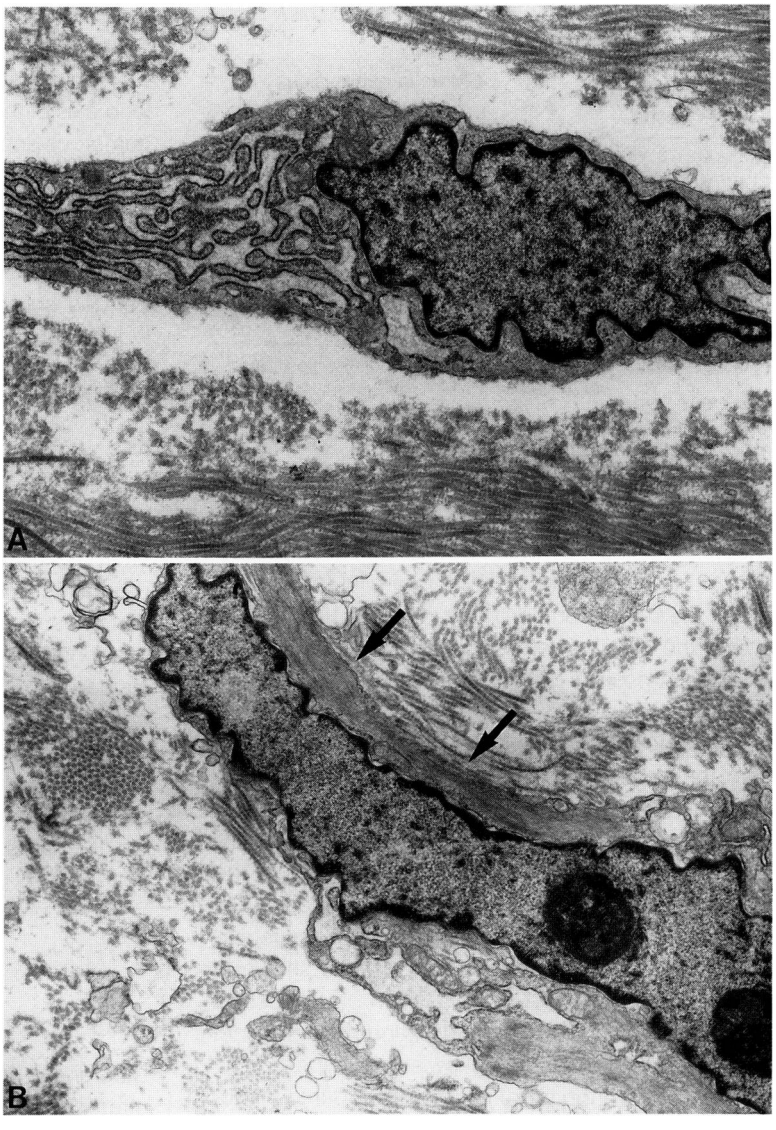

Figure 2. *Electron microscopic appearance of fibroblastic cells in keloid* (**A**) *and hypertrophic scar* (**B**). *In keloid* (**A**), *the fibroblast has the classical appearance with cisternae of rough endoplasmic reticulum; collagen fibrils are separated from the cell membrane of the fibroblast by an electron transparent substance surrounding the entire cell. In hypertrophic scar nodules* (**B**), *typical myofibroblasts rich in peripheral microfilament bundles* (**arrows**) *are seen; they are associated with banded collagen fibers immediately adjacent to the cell surface.* ×*12000.*

scars may be related to the origin of the fibroblasts that participated in the repair process or a deviation in the local inflammatory response during early repair.

The nodules present in hypertrophic scar are similar to those seen in Dupuytren's disease.[9] In both lesions they are surrounded by bands of thick collagen fibers. The presence of hypertrophic scar over a joint often leads to scar contracture, which impairs motion; in Dupuytren's disease the contraction of the scar-like tissue in the palmar fascia leads to impaired movement of the fingers. Keloids are not associated with scar contracture and lack nodules as well as α-SM actin-positive myofibroblasts. Thus it appears that the formation of nodules containing typical myofibroblasts may be related to the pathogenesis of scar contracture. Further studies on the formation of these nodular structures are needed to understand the mechanism for establishment of scar contracture; however, some considerations can already be drawn at this point. Myofibroblasts are a common feature of experimental and human granulation tissue, and they appear related to wound contraction establishment irrespective of their mechanism of action. Generally myofibroblasts disappear during normal scar formation probably through apoptotic changes,[17] and nodular structures are not produced. It is conceivable that an alteration of the process of myofibroblast disappearance in distinct locations results in the pathological formation of nodules.

The major component of stress fibers in myofibroblasts is α-SM actin, which is the actin isoform characteristic of SMC in general and of vascular SMC in particular.[27] Although the role of different actin isoforms is not presently well established, their phylogenetic conservation and their association with specific contractile tissues (such as skeletal, cardiac, or

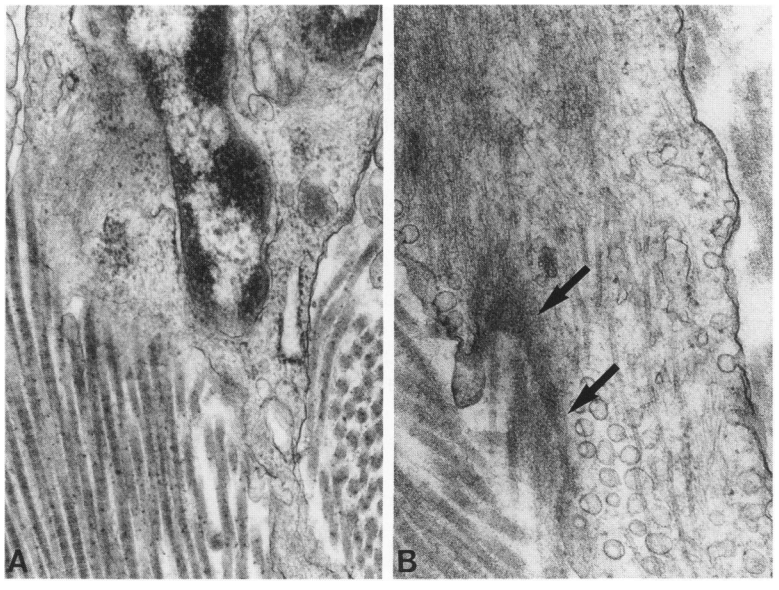

Figure 3. *Details of myofibroblast interactions with extracellular matrix in hypertrophic scar. Myofibroblasts are associated with fine banded collagen fibers on their cell surface* (**A**)*; typical fibronexus* (**arrows**) *are also observed* (**B**). ×*40000.*

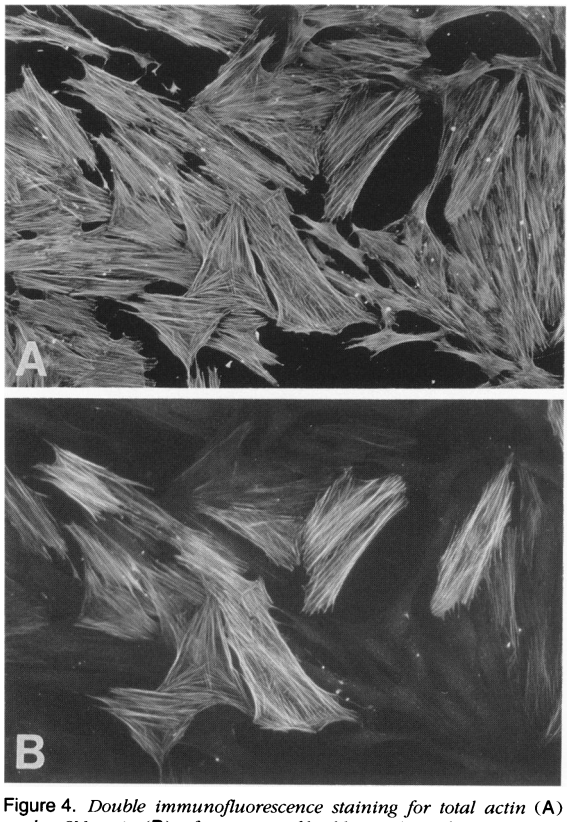

Figure 4. *Double immunofluorescence staining for total actin* (**A**) *and α-SM actin* (**B**) *of passage 5 fibroblast culture from keloid. A proportion of cells expresses α-SM actin.* ×*400.*

smooth muscles) suggest that they are connected with the production of contractile forces. Thus the appearance of α-SM actin in myofibroblasts may be related to the forces important in the retraction of connective tissue during pathological scarring. In agreement with this possibility electron microscopic examination of keloid and hypertrophic scar has confirmed the presence of stress fibers in myofibroblasts of this last condition and their absence in keloid fibroblasts. There is a report describing bundles of microfilaments in keloid fibroblasts but only around small vessels[28]; these cells however may represent pericytes. These cells failed to show α-SM actin staining in our study.

The electron microscopic observations revealed an unusual pericellular structure surrounding keloid fibroblasts. The chemical makeup of this pericellular structure is unknown. A similar pericellular structure was reported in noncontracting tight skin mouse wounds.[29] In both situations, the collagen fibrils were separated from the fibroblasts by this matrix. In tight skin mice, this pericellular material disappeared 3 weeks after wounding when the wounds began to contract. In keloids, it can remain for as long as 2 years, which was the age of one of the lesions examined. More work on the characterization and function of this pericellular material will be important for the understanding of the biological behavior of keloid fibroblasts *in vivo*.

Blood vessels were easy to identify by the α-SM actin staining because α-SM actin is a major component of the contractile apparatus of vascular SMC.[27] We confirm the observation that the number of blood vessels in hypertrophic scar and keloid was increased compared with normal scar and dermis.[23] It has been reported that nodules lack blood vessels and that they are dependent on the internodular vascular supply.[10] Rounded endothelial cells within the vessels of hypertrophic scar and keloid[28] were also

present. The importance of these rounded endothelial cells projecting into the lumen of vessels has been speculated to be critical for the development and maintenance of hypertrophic scar and keloid[28] and may be a common pathway in the development of fibrotic lesions.

Our *in vitro* data, showing that fibroblasts cultured from normal dermis, keloid, and hypertrophic scar biopsies express α-SM actin suggest that culture conditions are able to abolish the difference in α-SM actin expression by fibroblasts observed *in vivo*. We can assume that cultured fibroblasts from normal dermis and keloids that do not express α-SM actin *in vivo* are stimulated to produce α-SM actin by serum factors. Recently we have shown that transforming growth factor-β1 (TGF-β1) induces the expression of α-SM actin in cultured fibroblasts.[30] Furthermore, preincubation of culture medium containing whole blood serum with neutralizing antibodies to TGF-β1 resulted in a decrease of α-SM actin expression by fibroblasts in replicative and nonreplicative conditions. TGF-β1 could represent one of the main regulators of α-SM actin expression *in vivo* and *in vitro*. However, we cannot exclude the possibility that *in vivo*, factors inhibiting α-SM actin expression are present in keloids. Further studies will be necessary to establish in proliferating fibroblasts during keloid and hypertrophic scar evolution the presence of factors possibly secreted by inflammatory cells[31] able to inhibit or induce the expression of α-SM actin.

There are clear differences between the organization of collagen in dermis, keloid, and hypertrophic scar. Large, thick collagen fibers in keloids were demonstrated by polarized light microscopy and by electron microscopy. These collagen fibers were composed of numerous fibrils closely packed together. In contrast, the collagen fibers in the nodules of hypertrophic scar as demonstrated by polarized light birefringent patterns and electron microscopy were fine and randomly organized. In general the collagen structures of keloid were organized in thicker fibers than those of the nodules of hypertrophic scar. This organization difference implies that the collagen fibers of keloid lack the appropriate orientation to participate in scar contracture.

In conclusion, our work reports several morphological and immunohistochemical differences between hypertrophic scar and keloid. These differences are useful for the pathological distinction between these two lesions and support different mechanisms for their development. Further work on these mechanisms may be important for understanding the pathogenesis and for influencing the evolution of both conditions.

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