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Fibroblasts form a body-wide cellular network

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Abstract "Loose" connective tissue forms a network extending throughout the body including subcutaneous and interstitial connective tissues. The existence of a cellular network of fibroblasts within loose connective tissue may have considerable significance as it may support yet unknown body-wide cellular signaling systems. We used a combination of histochemistry, immunohistochemistry, confocal scanning laser microscopy (confocal microscopy), and electron microscopy to investigate the extent and nature of cell-to-cell connections within mouse subcutaneous connective tissue. We found that fibroblasts formed a reticular web throughout the tissue. With confocal microscopy, 30% of fibroblasts' processes could be followed continuously from one cell to another. Connexin 43 immunoreactivity was present at apparent points of cell-to-cell contact. Electron microscopy revealed that processes from adjacent cells were in close apposition to one another, but gap junctions were not observed. Our findings indicate that soft tissue fibroblasts form an extensively interconnected cellular network, suggesting they may have important and so far unsuspected integrative functions at the level of the whole body.

Keywords Connective tissue · Subcutaneous tissue · Connexin · Gap junctions · Cell signaling

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Introduction

Connective tissue fibroblasts typically are represented in histology textbooks as discrete fusiform or stellate cells with varying numbers of irregular cytoplasmic processes (Bevelander and Ramaley 1979; Ham 1980; Elias and Amenta 1986; Bloom and Faucett 1994; Kessel 1998; Zhang 1999; Gartner and Hiatt 2001). Perhaps because of the fibroblast's important relationship to the extracellular matrix, more attention has been paid to fibroblast–matrix interactions than to fibroblasts' interactions with each other. Several lines of evidence, however, suggest that fibroblasts are not separate cells, but rather are linked together in a reticular network extending throughout the whole body. Fibroblasts grown in cell culture can readily be seen to contact one another via cytoplasmic processes, and they are known to form gap junctions at points of contact between cells under certain conditions (Gilula et al. 1972; Ko et al. 2000). In whole tissue, however, the cytoplasmic processes of fibroblasts are more difficult to visualize with light microscopy. With electron microscopy, fibroblasts in load-bearing connective tissues, for example, tendon (Tanji et al. 1995) and periodontal ligament (Shore et al. 1980), have been shown to make various types of cell-to-cell contacts (adherens junctions, gap junctions, tight junctions, desmosomes, and simple close contacts). Furthermore, connexin 43 immunostaining at points of contact between fibroblasts in rat tendon suggests that these cells are linked by gap junctions (McNeilly et al. 1996). In these specialized tissues, gap junctions between fibroblasts are thought to play a role in detecting and coordinating responses to mechanical loads. Gap junctions between myofibroblasts have also been described in healing wounds (Gabbiani et al. 1978) where formation of a cellular network is thought to be important for wound contraction (Jester et al. 1995). The findings of Novotny et al. further suggest that fibroblasts throughout connective tissue may be connected with each other even in the absence of a wound (Novotny and Gommert-Novotny 1990; Novotny and Gnoth 1991). Using silver impregnation of paraffin tissue sections, these authors

showed that human skin and subcutaneous tissue fibroblasts possess long, branching processes that appeared to contact each other. However, the light microscopy imaging techniques that were used did not allow confirmation and precise visualization of points of cell-to-cell contact.

In this study, we have used a combination of histochemistry, confocal microscopy, and electron microscopy to demonstrate that mouse subcutaneous connective tissue fibroblasts do indeed form a cellular network. We also performed immunohistochemistry for connexin 43 (the principal connexin found in connective tissue) to investigate the nature of the cellular connections within this network.

Materials and methods

Subcutaneous tissue dissection

Subcutaneous connective tissue was harvested immediately after death from the abdomen of C57Black6 male mice weighing 20–25 g. An 8×3-cm flap containing dermis, subcutaneous muscle, and subcutaneous tissue was dissected from the abdominal wall musculature. Subcutaneous tissue samples (20–30 mm in width, 60–80 mm in length, and 20–50 μm in thickness) were then dissected from the subcutaneous muscle, while applying minimal traction on the tissue.

Fibroblast cell culture

Human dermal fibroblasts (Cell Applications) were maintained in T-25 flasks in fibroblast growth medium (Cell Applications) at 37°C, 5% CO_2 in a humidified incubator unless otherwise indicated. Cells were subcultured according to directions. Briefly, human dermal fibroblasts at ~90% confluence were treated with a trypsin/EDTA solution (Cell Applications). Trypsinized cells were incubated in trypsin neutralizing solution (Cell Applications), centrifuged at 200 *g* for 5 min to pellet the cells, and plated at high density (~10,000 cells per cm^2) on 22-mm Thermanox plastic coverslips (Nalge Nunc, Naperville, IL). Cultured cells were fixed and processed for microscopy 5–7 days after plating.

Phalloidin staining

Three different fixation methods were used: (1) 1% reagent-grade paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4, (2) 3% PFA in PBS, and (3) 95% ethanol. Tissue samples were fixed for 30 min then rinsed in PBS. Samples fixed in PFA were incubated with Texas red-conjugated phalloidin (4 U/ml; Molecular Probes, Eugene OR) with 0.1% Triton X-100 for 1 h at 4°C and counterstained for 5 min with Sytox green (Molecular Probes) nucleic acid stain. For samples fixed with ethanol, the same method was used except that Triton X-100 was omitted. We found that Triton did not significantly improve staining quality with ethanol fixation. Samples were mounted on glass slides using 50% glycerol in PBS with 1% *N*-propyl gallate as a mounting medium.

Confocal scanning laser microscopy

Tissue samples stained with Texas red-conjugated phalloidin were imaged with a BioRad MRC 1024 confocal microscope (BioRad Microsciences, Hercules, CA) using a 60× oil immersion lens (N.A. 1.4) and 568 nm laser excitation. Stacks of images with a total thickness of 20 μm were acquired at a 1-μm interimage interval with an iris aperture of 2.5. Stacks of individual images were used for morphometric analysis. Stacks were also made into projections to yield the images shown in Figs. 1, 4, and 6.

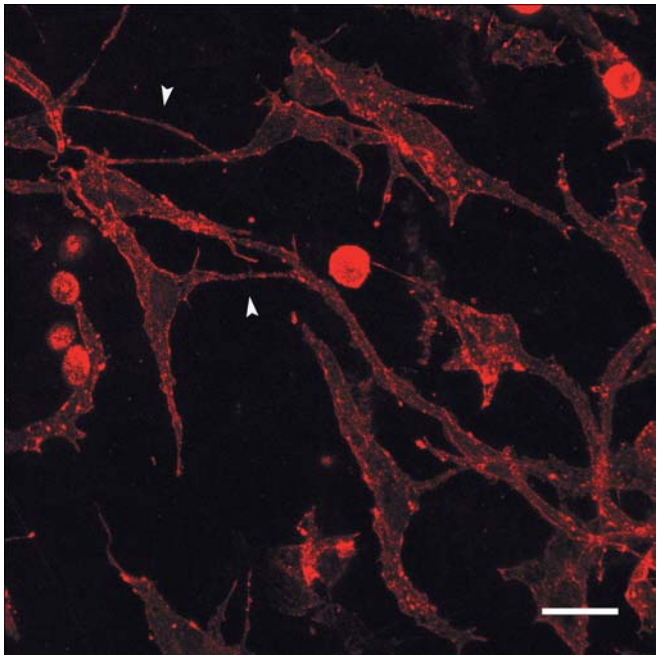

Fig. 1 Mouse subcutaneous tissue fibroblast network. Tissue was fixed in 95% ethanol, stained with Texas red-conjugated phalloidin and imaged with confocal microscopy. Note the thin cellular processes present between individual cells (*arrowheads*). *Scale bar* 40 μm

Morphometric analysis

Two samples, each from a different mouse, were used for morphometric analysis. Each sample was stained with Texas red-conjugated phalloidin and imaged by confocal microscopy. Four 60× fields (313×313 μm) were imaged per sample, one field at the center of each sample quadrant. For each field, a stack of images was acquired as described above. The image stacks were imported into the analysis software package MetaMorph (version 6.0; Universal Imaging, Downingtown, PA) for morphometric analysis. In each set of images, all visible cell processes were identified by following each process through successive images within the stack. A cell process was defined as an extension of a cell's cytoplasm that was greater than 2 μm in length and less than 2 μm in width. In each stack, we counted: (1) "blind-ended" processes and (2) "joining" processes, defined as processes that appeared continuous with confocal microscopy (i.e., without a visible break from one cell to another). The lateral (x, y) resolution of our 60× oil objective (1.4 N.A.) was 0.22 μm. In the z (depth) direction, our ability to differentiate between continuous versus discontinuous overlapping cell processes depended on the thickness of our optical sections relative to our step size. Based upon our 60× objective and iris aperture of 2.5, the theoretical thickness of our optical sections was approximately 1 μm. Since we matched the stepper motor step size to this section thickness in acquiring our stacks, we expect to have missed very little information between optical sections.

Immunohistochemistry

Immunohistochemistry was performed for the detection of connexin 43 using an indirect immunofluorescence technique. Tissue samples and cells grown on coverslips were fixed in 95% ethanol

for 30 min, blocked with 10% normal donkey serum in PBS with 1% bovine serum albumin for 30 min, and incubated in primary rabbit anti-connexin 43 antibody (Sigma, St. Louis, MO) at 1:2,000 dilution overnight at 4°C. They were then washed three times in PBS with 1% bovine serum albumin for 10 min and finally incubated in Alexa 488-conjugated donkey anti-rabbit antiserum (Molecular Probes) at 1:800 dilution for 1 h at room temperature. The samples were then counterstained with phalloidin as described above. Negative controls were performed by omitting the primary antibody, and positive controls by staining heart tissue for intercalated discs.

Electron microscopy

Subcutaneous tissue samples were fixed for 1 h at 4°C in a mixture of 2.5% glutaraldehyde and 1.0% PFA in Millonig's phosphate buffer. Following three rinses in buffer, the tissue pieces were encapsulated in 2% agarose (Sea Prep ultra low gelling temperature; Biowhitaker Molecular Applications, Rockland, ME) to maintain stability and orientation. The agarose was solidified by soaking for 15 min in the same fixative as above. Human dermal fibroblasts were fixed in a mixture of 2.5% glutaraldehyde and 1% PFA in Millonig's buffer for 45 min at 4°C. Following fixation, both tissue pieces (in agarose) and cells were postfixed in 1% OsO_4, dehydrated through a graded series of alcohols, and embedded in Spurr's hydrophobic plastic resin. Ultrathin sections were cut with diamond knives, retrieved onto copper grids, post-stained with uranyl acetate and lead citrate, and finally examined in a Jeol 1210 transmission electron microscope operating at 60 kV.

Results

Histochemical staining of subcutaneous tissue

Confocal microscopy of Texas red-conjugated phalloidin-stained subcutaneous tissue revealed the presence of an extensively interconnected cellular network (Fig. 1). The cells forming this network were identified as fibroblasts on the basis of the following observations: (1) the interconnected cells were the overwhelmingly predominant cell within the tissue; (2) occasional more globular cells without processes, presumably lymphocytes and phagocytes, were also seen; and (3) the density of the cellular network was consistent with the density of the dominant cell type seen in unstained subcutaneous tissue with phase contrast microscopy.

Individual fibroblasts were bipolar or multipolar, with cytoplasmic processes up to 200 μm in length. We found that ethanol fixation preserved these fibroblast processes better than fixation in 1% or 3% PFA (Figs. 1, 2). The morphology of the cells was consistent with that of the cells described by Novotny using silver impregnation (Novotny and Gnoth 1991). When fibroblast processes were followed through successive images within a stack, some processes ended blindly (Fig. 3 *closed arrows*) while others could be followed continuously from one cell to another without a visible break (joining process; Fig. 3 *open arrows*). In ethanol-fixed tissue, mean (±SD) numbers of blind-ended versus joining processes per stack were 26.4±8.6 and 10.8±6.8, respectively. Thus, approximately 30% of visible processes appeared continuous with confocal microscopy.

Connexin 43 immunolabeling showed punctate staining associated with fibroblasts. Most staining was at or near the cell surface and at apparent points of cell-to-cell contact (Fig. 4). In some cases, a fibroblast process appeared to extend all the way to another cell with connexin present at the apparent point of contact with that cell's body (Fig. 4A, D). In other cases, connexin was present along a process joining two cells (Fig. 4B). Occasionally, two adjacent cells had positive connexin staining at multiple points of contact (Fig. 4C).

Histochemical staining of cultured fibroblasts

As with tissue, fibroblasts fixed with ethanol had more visible cellular processes compared with cells fixed with PFA (Fig. 5). Immunostaining for connexin 43 was more extensive in the cultured cells than in subcutaneous tissue. However, as in the whole tissue, staining was most prominent at points of cell-to-cell contact (Fig. 6). Intracytoplasmic connexin staining was also seen.

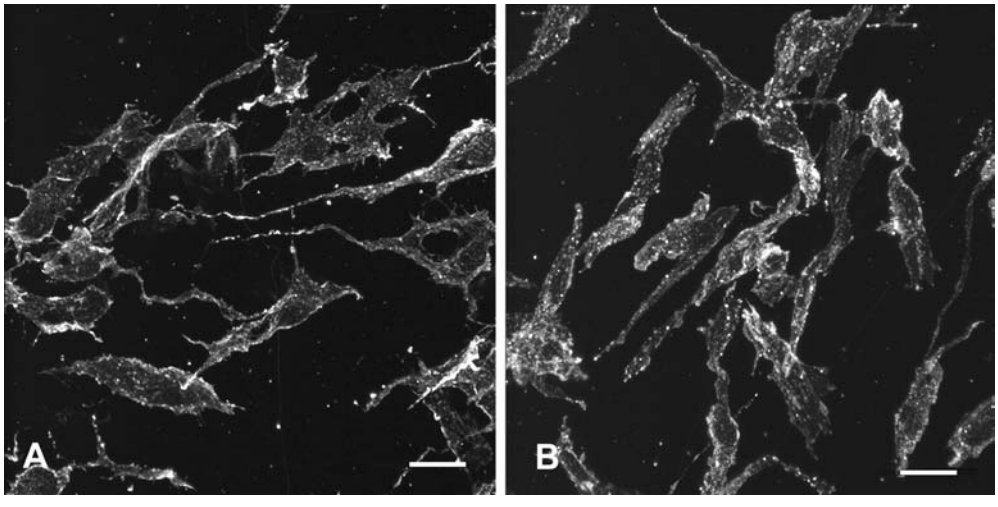

Fig. 2A, B Texas red-conjugated phalloidin-stained mouse subcutaneous tissue fixed with 1% paraformaldehyde (PFA; **A**) or 3% PFA (**B**). Note that the 1% PFA preserved fibroblast processes to a better extent than 3% PFA, but not as well as 95% ethanol (see Fig. 1). *Scale bars* 40 μm

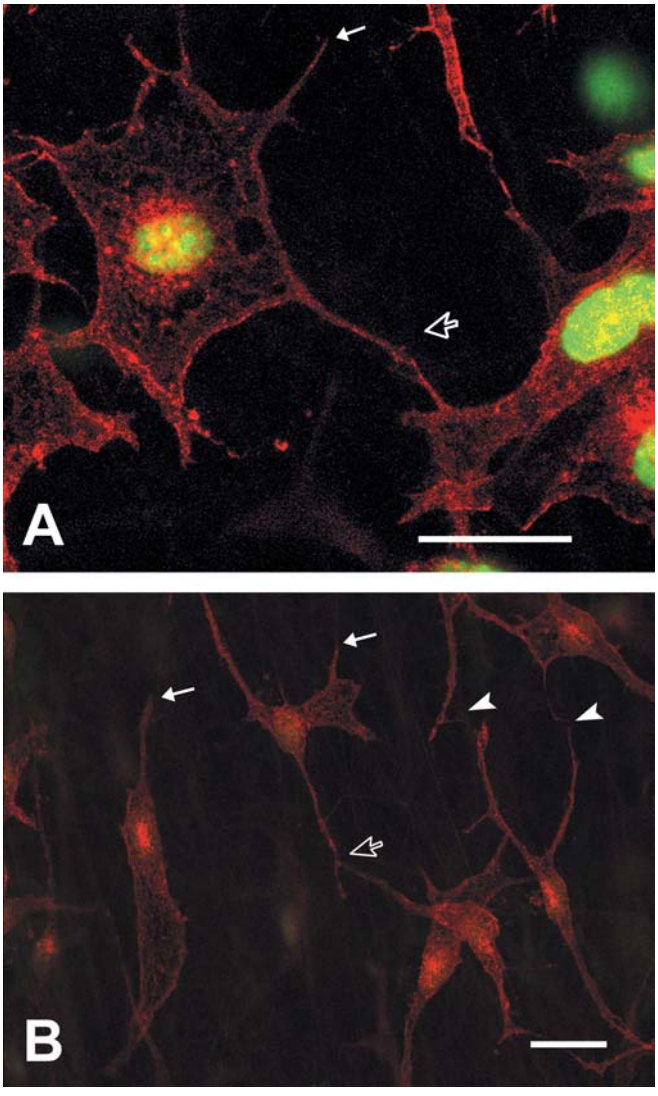

Fig. 3A, B Subcutaneous tissue fixed with 95% ethanol, stained with Texas red-conjugated phalloidin and Sytox green, and imaged by confocal microscopy. Examples of fibroblast processes joining two cells (**A**, **B**; *open arrows*) and ending blindly (**A**, **B**; *closed arrows*). In cases where processes approached each other but a visible gap was present (**B**; *arrowheads*), processes were counted as ending blindly. *Scale bars* 40 μm

Electron microscopy

Cytoplasmic processes from different cells could be seen to contact each other (Fig. 7). However, no specialized junctions were identified. In subcutaneous tissue, elongated thin cellular processes emerged primarily from the tapered ends of fibroblasts. These processes were observed to course with an undulating appearance through the extracellular matrix for long distances, often terminating by abutting against another cell or cell process (Fig. 7A). At high magnification, the region of contact between cell processes did not display any membrane junctional specialization (Fig. 7A *inset*). In cultured fibroblasts, points of contact were observed mostly between individual cell bodies and occasionally between cell processes (Fig. 7B), again with no visible junctional complex present. Cell processes of cultured fibroblasts, when present, were shorter and thicker than those observed in subcutaneous tissue fibroblasts.

To assess the relationship between the confocal localization of anti-connexin 43 and the ultrastructural appearance of membrane contact points, we performed immunoelectron microscopy. Subcutaneous tissue pieces were fixed in a 3% PFA/0.1% glutaraldehyde combination, followed by low temperature dehydration and embedding in the hydrophilic resin Lowicryl K4 M. Antigenic sites for connexin 43 were localized with the protein A-gold technique. With this method, very few intact fibroblast processes were seen and only one membrane contact point was found to be labeled with colloidal gold particles indicative of anti-connexin 43 immunoreactivity. The staining was specifically localized to the point of membrane contact, however, no membrane specialization was observed (not shown). As a positive control, sections from human myocardium processed in an identical manner as the mouse subcutaneous tissue were incubated with the anti-connexin 43 antibody followed by protein A-gold. Very specific gold particle staining was observed along segments of the intercalated disk corresponding to gap junctions (not shown). Taken together, the results from conventional electron microscopy and immunoelectron microscopy suggest that, in subcutaneous tissue fibroblasts, staining for connexin 43 may be localized to membrane contact points displaying no specialized junctional complex.

Discussion

Our findings show that fibroblasts form a cellular network in mouse subcutaneous tissue, and that the cytoplasmic processes of these fibroblasts are extensively interconnected. Furthermore, we found with confocal microscopy that connexin 43 immunoreactivity was present at apparent points of cell-to-cell contact. Electron microscopy revealed that, at contacting points, cell processes were in very close apposition with one another but did not form gap junctions. Our findings in cultured fibroblasts were similar to those in whole tissue. Connexin channels are known to be highly mobile with a short turnover rate (half-life of a few hours; Harris 2001), and connexin immunostaining of cultured cells has proven influenced by plating density and the presence of growth factors such as TGF-β1 (Petridou and Masur 1996; Larson et al. 1997). Our cultured fibroblasts were plated at high density and examined near confluence, which should have maximized connexin expression (Petridou and Masur 1996). Nevertheless, despite extensive positive staining for connexin, no gap junctions were seen by electron microscopy.

One possible explanation for the apparent absence of gap junctions is that gap junctions may have been present before fixation but were damaged by our fixation method. We consider this to be unlikely. Our confocal microscopy results do show that fibroblast processes are sensitive to

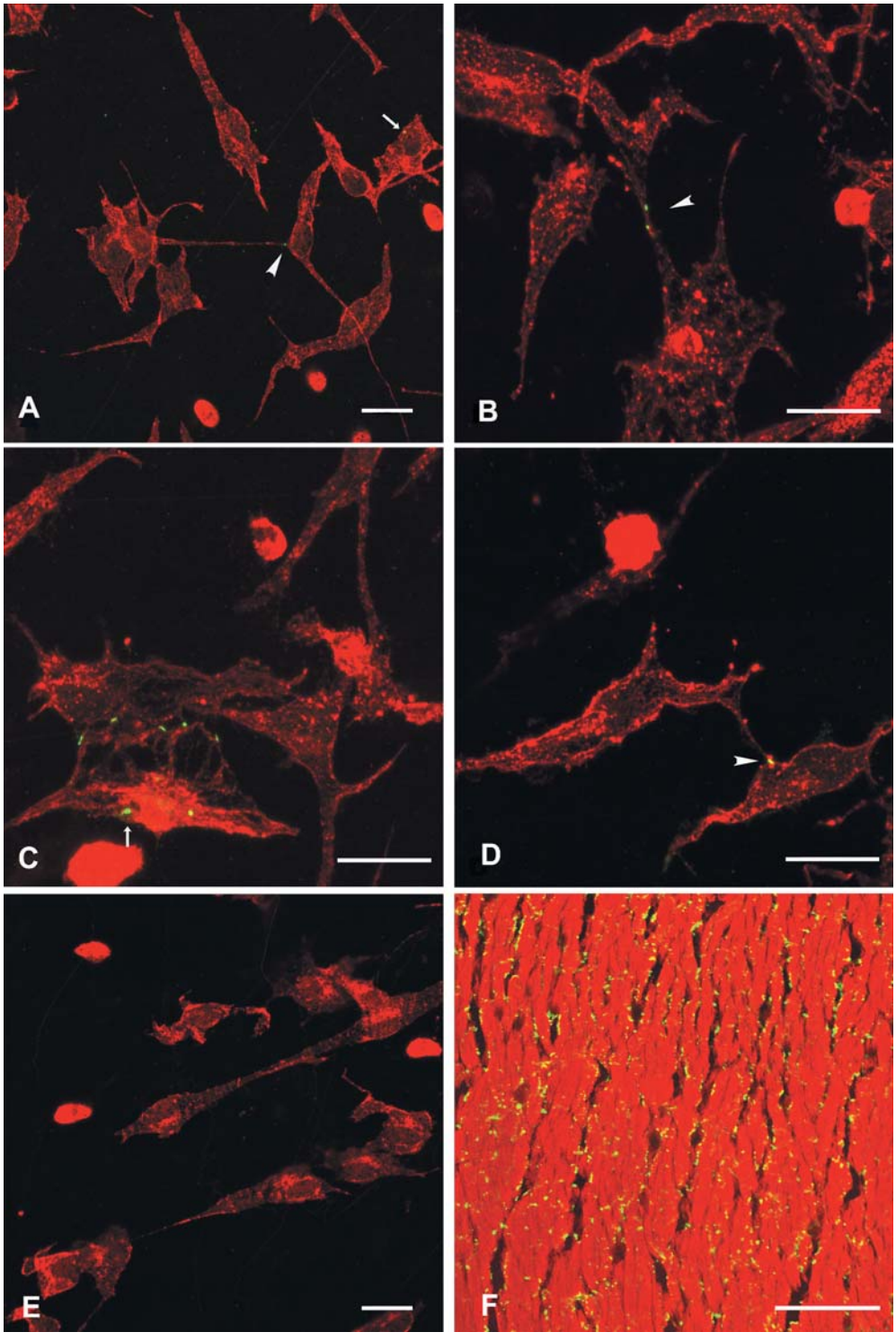

Fig. 4 A–D Mouse subcutaneous connective tissue fibroblasts fixed with 95% ethanol and doubly stained with Texas red-conjugated phalloidin and anti-connexin 43 antibody followed by Alexa 488-conjugated secondary antibody (*green*). Punctate connexin 43 immunoreactivity can be seen at points of cell-to-cell contact (*arrowheads*) and on cell bodies (*arrows*). **E** Negative control omitting anti-connexin antiserum. **F** Positive control demonstrating anti-connexin 43 immunostaining of cardiac muscle intercalated discs using same method as **A–D**. *Scale bars* 40 μm

fixatives, and indeed very few intact processes could be seen in tissue processed for immunoelectron microscopy. With conventional electron microscopy, however, we were able to see abundant cell processes with many points of cell-to-cell contact showing well-preserved membrane structure but no visible membrane specialization. In this regard, it would be of interest to investigate whether gap junctions could be seen using specialized electron microscopic fixation techniques, such as high-pressure physical cryofixation (Studer et al. 2001). Alternatively, it is also possible that gap junctions were not present despite positive connexin staining. Gap junctions often are assumed to exist when connexin immunoreactivity is found at points of cell-to-cell contact (Dolber et al. 1992; McNeilly et al. 1996; Ralphs et al. 1998). It is increasingly recognized, however, that undocked connexin hex-

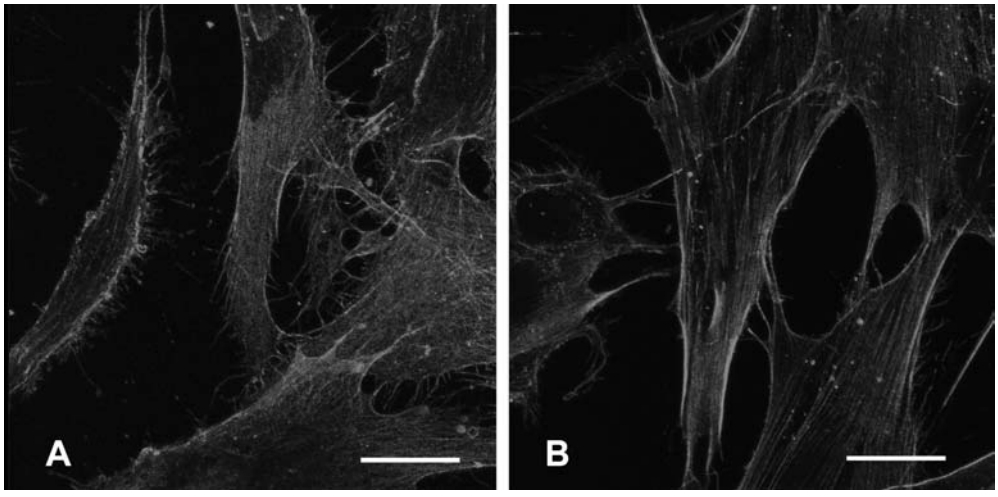

Fig. 5A, B Texas red-conjugated phalloidin-stained human dermal fibroblasts fixed with 95% ethanol (**A**) and 1% PFA (**B**). A greater number of fine fibroblast processes are seen with ethanol than with PFA fixation. Cortical actin and intracellular stress fibers also are seen in finer detail with ethanol than with PFA fixation. *Scale bars* 40 μm

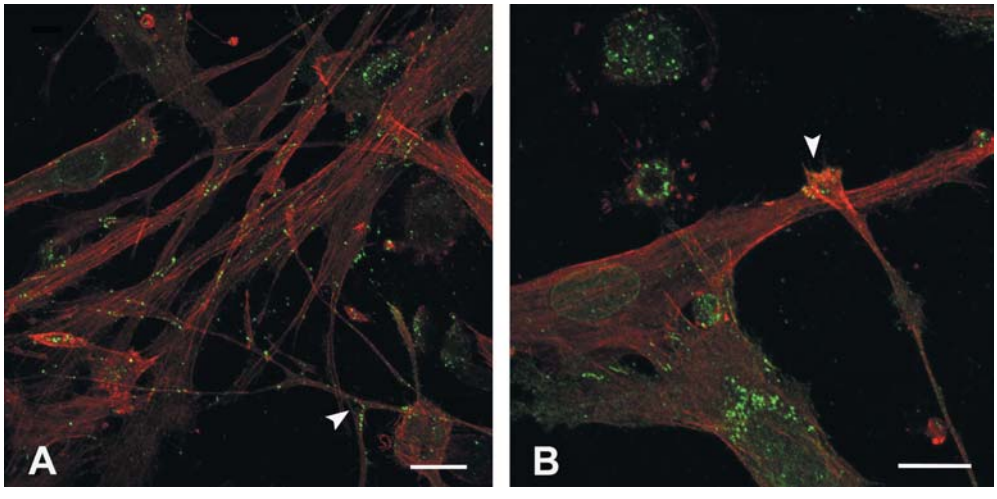

Fig. 6A, B Cultured human dermal fibroblast stained with Texas red-conjugated phalloidin (*red*) and anti-connexin 43 antibody followed by Alexa 488-conjugated secondary antibody (*green*). Punctate connexin 43 immunoreactivity is particularly abundant at points of cell-to-cell contact (*arrowheads*), although cytoplasmic staining is also apparent. *Scale bars* 40 μm

amers (hemichannels) are often present along the cell membrane outside of gap junctions (Li et al. 1996; Lal and Lin 2001) and that these hemichannels can have important paracrine functions via extracellular release of ATP, NAD, and glutamate (Ebihara 2003; Ye et al. 2003). In astrocytes, connexin 43 expression has been associated with cell-to-cell propagation of mechanically induced calcium waves and spontaneous calcium oscillations (Charles et al. 1992). These calcium oscillations were associated with extracellular release of ATP through connexin hemichannels (Stout et al. 2002), suggesting that connexins may participate in cell-to-cell signaling even in the absence of gap junctions. Our data, therefore, raise the interesting possibility that connections between connective tissue fibroblasts may involve connexins, but not gap junctions, and that fibroblasts may communicate via some form of intercellular signaling.

Another intriguing possibility is that contacting fibroblast processes could be precursors of tunneling nanotubes (TNTs). TNTs were recently described in cultured pheochromocytoma cells as 50- to 200-nm-diameter actin-containing tubular structures connecting adjacent cells and allowing the passage of vesicles and organelles from one cell to another (Rustom et al. 2004). Although we did not observe TNT-like structures in our EM samples, the mean±SD width of the processes measured on electron micrographs was 113±62 nm (range 31–498 nm, based on 1,054 measurements of 33 processes in 10 cells), which is similar to that reported by Rustom for TNTs.

A unique aspect of "loose" connective tissue is its ubiquitous presence throughout the body. Subcutaneous tissue forms a body-wide "sheet" of loose connective tissue. This connective tissue sheet is itself continuous with loose connective tissue planes separating muscle, and with interstitial connective tissues surrounding all blood vessels, nerves, and organs. The significance of finding a connective tissue fibroblast network in subcutaneous tissue, therefore, is that such a cellular network indeed may permeate the entire body. Known physiological systems that transmit information signals throughout the body include the nervous, immune, and endocrine systems. The idea that connective tissue may form an additional distinct body-wide signaling system is suggested by the ancient practice of acupuncture. Acupuncture is based on the traditional theory that acupuncture "meridians" form a network throughout the body

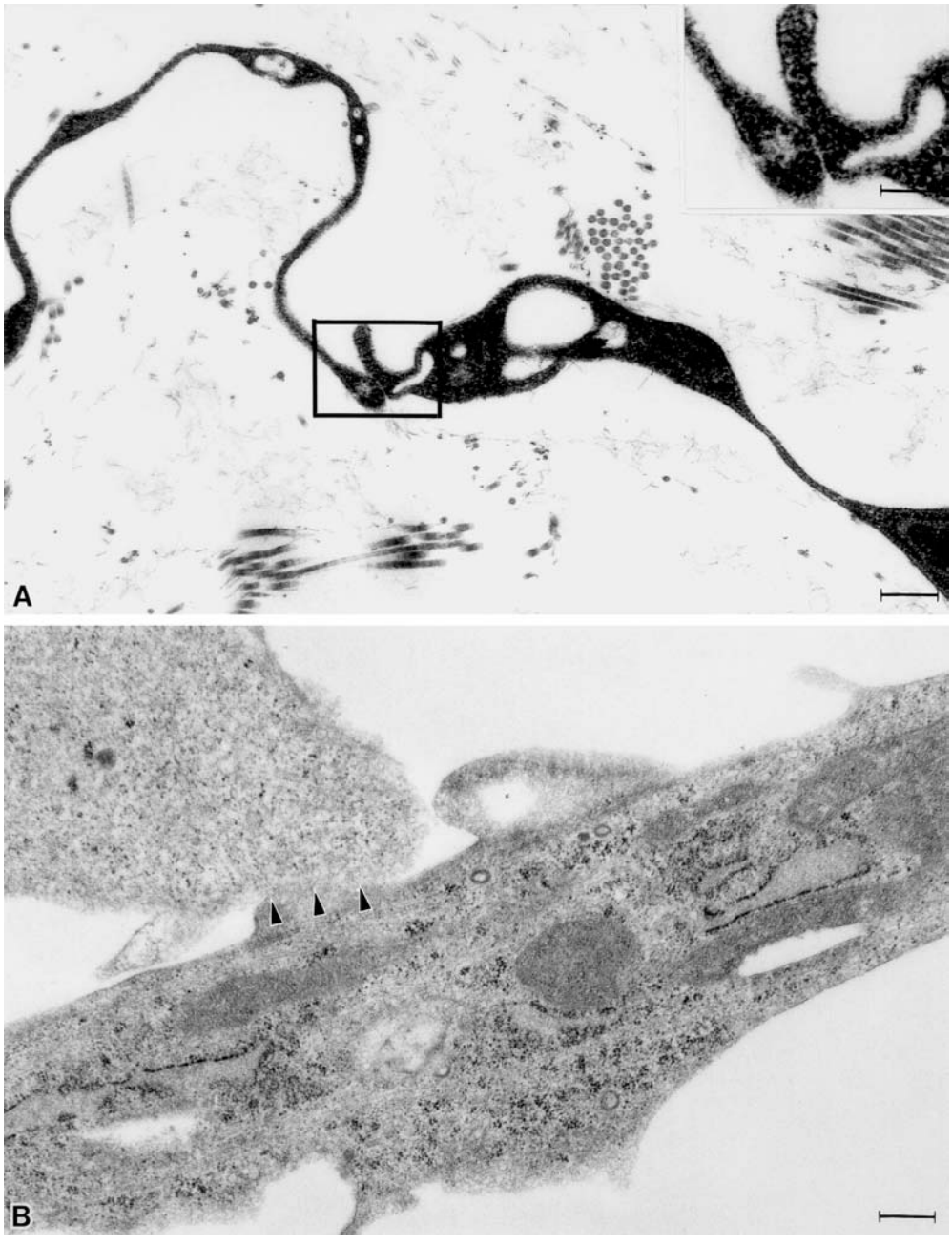

Fig. 7A, B Electron microscopic visualization of mouse subcutaneous connective tissue (**A**) and human dermal fibroblasts (**B**). In subcutaneous connective tissue, long, thin cellular processes emanating primarily from the ends of fibroblasts traverse the extracellular matrix and contact another cell or process (**A**). At higher magnification (*inset* in **A**) no specialized junctional contact is observed where one process abuts another. In cultured dermal fibroblasts (**B**), long cellular processes are not present. Points of contact are observed mostly between cell bodies and occasionally between relatively shorter and thicker processes. *Scale bars* 0.34 μm in **A**; 0.16 μm in *inset* ; 0.4 μm in **B**

through which flows a form of movement, communication, or energy exchange termed "meridian qi" (Cheng 1987; Kaptchuk 2000). Recent evidence suggests that the acupuncture meridian system corresponds to connective tissue planes (Langevin and Yandow 2002), and that acupuncture needle stimulation sends a mechanical signal through this tissue network (Langevin et al. 2001a, b, 2002). Connective tissue consists both of a delicate web with fine branches penetrating all tissues, and major "trunks" forming connective tissue planes and linking all parts of the body with each other. This description fits remarkably well with the definition of acupuncture meridians.

Traditionally, fibroblasts have been thought to play a mostly supportive role in connective tissue, including the synthesis of extracellular matrix constituents. In the presence of tissue injury, however, myofibroblasts have long been known to play a mechanically active role exerting tractional force within tissue and promoting wound closing (Gabbiani et al. 1978). Recent evidence also suggests that fibroblasts respond to mechanical forces even in the absence of a wound. Fibroblasts grown in tissue culture have been shown to react within minutes to a variety of mechanical stimuli (stretch, pressure, traction, shear forces) with cellular responses ranging from changes in intracellular calcium and ATP release, to signaling pathway activation, actin polymerization, and

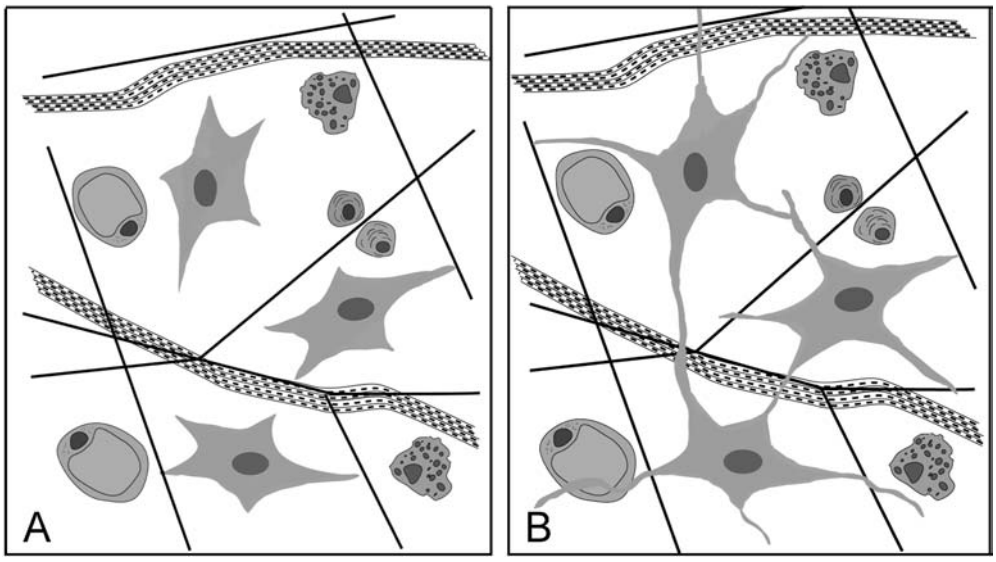

Fig. 8 A Customary model representing fibroblasts as discrete cells embedded in extracellular matrix. **B** Proposed modification of this model representing fibroblasts as part of a cellular network within connective tissue

gene expression (Banes et al. 1995; Stoltz et al. 2000). Altered gene expression and modification of extracellular matrix composition have been demonstrated in response to mechanical signals (Gutierrez and Perr 1999; Skutek et al. 2001). Indeed, change in extracellular matrix composition is itself recognized as an important mode of communication between the many cell types intimately associated with connective tissue (Swartz et al. 2001). We recently have shown that the biomechanical properties of loose connective tissue are closer to that of cells than to that of high load bearing connective tissues such as tendons and ligaments (Iatridis et al. 2003). This whole tissue mechanical behavior supports the notion that fibroblasts may not be as "stress-shielded" from mechanical forces in loose compared with load-bearing connective tissues (Brown et al. 1998). Thus externally applied mechanical forces in loose connective tissue are likely to have substantial effects on fibroblast mechanical signal transduction processes. The description of a cellular network within connective tissue therefore opens up the possibility that fibroblasts may participate in a body-wide signaling system responding to mechanical forces and influencing other physiological systems.

In summary, our results indicate that the traditional view of fibroblasts as discrete cells should be modified to reflect the concept of fibroblasts forming a complex cellular network (Fig. 8). The global nature of this network is unique to fibroblasts and suggests that these cells may have important and so far unsuspected integrative functions at the level of the whole body.

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