

Dendritic Fibroblasts in Three-dimensional Collagen Matrices

Frederick Grinnell,* Chin-Han Ho, Elisa Tamariz, David J. Lee,[†] and Gabriella Skuta[‡]

Department of Cell Biology, University of Texas Southwestern Medical School, Dallas, Texas 75390-9039

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Cell motility determines form and function of multicellular organisms. Most studies on fibroblast motility have been carried out using cells on the surfaces of culture dishes. In situ, however, the environment for fibroblasts is the three-dimensional extracellular matrix. In the current research, we studied the morphology and motility of human fibroblasts embedded in floating collagen matrices at a cell density below that required for global matrix remodeling (i.e., contraction). Under these conditions, cells were observed to project and retract a dendritic network of extensions. These extensions contained microtubule cores with actin concentrated at the tips resembling growth cones. Platelet-derived growth factor promoted formation of the network; lysophosphatidic acid stimulated its retraction in a Rho and Rho kinase-dependent manner. The dendritic network also supported metabolic coupling between cells. We suggest that the dendritic network provides a mechanism by which fibroblasts explore and become interconnected to each other in three-dimensional space.

INTRODUCTION

Form and function of multicellular organisms depend on tissue-specific programs of cell motility (Trinkaus, 1984). Motility has been studied extensively using fibroblasts cultured on planar surfaces. Cells migrate over these surfaces using their flattened, ruffling lamellipodia (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Tractional force necessary for migration is exerted at newly formed cell-substratum adhesions (Galbraith and Sheetz, 1997; Oliver *et al.*, 1999; Beningo *et al.*, 2001). Formation and release of these adhesions along with regulation of cell protrusive and contractile activity requires complex molecular interactions between many adhesion, motor, and regulatory molecules (Schoenwaelder and Burridge, 1999; Borisy and Svitkina, 2000; Schwartz and Shattil, 2000; Geiger *et al.*, 2001). Small G proteins are particularly important in the process because of their diverse effects on the actin cytoskeleton (Hall, 1998; Kaibuchi *et al.*, 1999). Activation of Rac (e.g., by platelet-derived growth factor [PDGF]) stimulates cell pro-

trusion, whereas activation of Rho (e.g., by lysophosphatidic acid) inhibits cell protrusion and stimulates cell contraction (Clark *et al.*, 1998; Rottner *et al.*, 1999).

The flattened, lamellar appearance characteristic of fibroblasts on planar surfaces differs markedly from the in situ appearance of mesenchymal cells and connective tissue fibroblasts, which tend to be stellate or dendritic in shape, often with long, slender extensions (Breathnach, 1978; Trinkaus, 1984; Van Exan and Hardy, 1984; Omagari and Ogawa, 1990; Beertsen *et al.*, 2000). In part, the differences in appearance of fibroblasts on planar surfaces compared with tissue may be a reflection of topographic responsiveness (Trinkaus, 1984); cells can detect nanometric substratum surface features (Curtis and Wilkinson, 1999). In addition, however, differences in substratum stiffness likely are important. Cells can modulate the strength of their adhesive interactions (Wang and Ingber, 1994; Choquet *et al.*, 1997), and increased surface stiffness permits increased cell spreading and formation of focal adhesions on flexible, planar surfaces (Pelham and Wang, 1997). Fibroblasts in the adult connective tissue environment rarely, however, are under isometric tension judging from the lack of stress fibers or fibronexus junctions (the in vivo equivalent of focal adhesions) except during fibrotic conditions such as wound repair (Tomasek *et al.*, 2002).

Fibroblasts cultured in collagen matrices develop more in situ like morphology compared with cells on planar surfaces (Elsdale and Bard, 1972). Instead of migration, cell motility in the matrix causes translocation of the flexible collagen

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* Corresponding author. E-mail address: frederick.grinnell@utsouthwestern.edu.

Present addresses: [†] Physicians' Education Resource, 3535 Worth Street, Dallas, TX 75246; [‡] 1750 Kalakaua Avenue 2404, Honolulu, HI 96826.

fibrils of the matrix and global matrix remodeling (contraction), processes important for morphogenesis and wound repair (Bell *et al.*, 1979; Harris *et al.*, 1981; Grinnell, 1994; Tomasek *et al.*, 2002). If matrices are restrained during contraction, then isometric tension develops in the cells (Brown *et al.*, 1998; Tranquillo, 1999; Grinnell, 2000).

Recently, we analyzed formation and maturation of cell-matrix interactions comparing human fibroblasts embedded at low (10^5 /ml) or high density (10^6 /ml) in restrained collagen matrices (Tamariz and Grinnell, 2002). At low cell density, local matrix remodeling occurred as measured by movement of collagen-embedded beads toward the cells but not global matrix remodeling as measured by matrix contraction. High cell density was required for matrix contraction. Initial fibroblast morphology in the matrices appeared to be dendritic and became stellate/bipolar over time. In addition, stress fibers and focal adhesions formed when global matrix remodeling occurred in the presence of growth factors.

In the current research, we studied the morphology and motility of fibroblasts embedded at 10^5 /ml in floating collagen matrices. Under these conditions, cells were observed to project and retract a dendritic network of extensions. These extensions contained microtubule cores with actin concentrated at their tips resembling growth cones. PDGF promoted formation of the network; lysophosphatidic acid stimulated its retraction in a Rho and Rho kinase-dependent manner. The dendritic network also supported metabolic coupling between cells. We suggest that the dendritic network provides a mechanism for fibroblasts to explore and become interconnected to each other in three-dimensional space.

MATERIALS AND METHODS

Cell Culture

Fibroblasts from human foreskin specimens (<10th passage) were maintained in Falcon 75-cm² tissue culture flasks in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Intergen Co., Purchase, NY). Fibroblasts were harvested from monolayer culture with 0.25% trypsin/EDTA (Life Technologies). Trypsin was neutralized with soybean trypsin inhibitor (3.3 mg/ml; Sigma Chemical, St. Louis, MO) or 10% FBS in DMEM. All incubations with cells were carried out at 37°C in a humidified incubator with 5% CO₂.

For experiments with collagen-coated surfaces, harvested cells (2×10^5) were incubated for the times indicated on 22-mm² glass coverslips. The coverslips previously were coated for 20 min with 50 µg/ml collagen (Vitrogen 100; Cohesion Co., Palo Alto, CA) and then rinsed with Dulbecco's phosphate-buffered saline (DPBS; 1 mM CaCl₂, 0.5 mM MgCl₂, 150 mM NaCl, 3 mM KCl, 1 mM KH₂PO₄, 6 mM Na₂HPO₄, pH 7.2). Basal incubation medium was 2 ml of serum-free DMEM containing 5 mg/ml bovine serum albumin (BSA, fatty acid free; Sigma) and growth factors or inhibitors added as indicated. At the end of the incubations, the samples were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS; 150 mM NaCl, 3 mM KCl, 1 mM KH₂PO₄, 6 mM Na₂HPO₄, pH 7.2) for 10 min at 22°C.

For experiments with collagen matrices, cells in neutralized solutions of collagen (1.5 mg/ml) were prewarmed to 37°C for 3–4 min, and 0.2-ml aliquots were placed in Corning 24-well culture plates. Cell density was 10^5 /ml (2×10^4 cells/matrix) unless specified differently. Each aliquot occupied an area outlined by a 12-mm-diameter circular score within a well. After 60 min, matrices were gently released from the underlying culture dishes with a spatula and allowed to float in 0.5 ml of basal medium. Growth factors and inhibitors were added at the times indicated. At the end of the

incubations, samples were fixed with 3% paraformaldehyde in PBS for 10 min at 22°C.

Growth Factors, Inhibitors, and Antibodies

PDGF was obtained from Upstate Biotechnology (Lake Placid, NY). Rho kinase inhibitor Y-27632 was a generous gift from Welfide Corporation (Osaka, Japan). Exotransferase C3 was obtained from List Biological Lab. Inc. (Campbell, CA). Lipofectamine Plus reagent was obtained from Invitrogen Life Technologies (Carlsbad, CA). Cytochalasin D, lysophosphatidic acid (LPA), monoclonal anti-β-tubulin, and nocodazole were obtained from Sigma Chemical. Alexa Fluor 488 goat anti-rabbit IgG, calcein AM, DiI, and rhodamine-conjugated phalloidin were obtained from Molecular Probes (Eugene, OR). Fluorescein-conjugated rabbit anti-mouse IgG (H+L) was obtained from Zymed Lab. Inc. (South San Francisco, CA). Rabbit anti-Rho antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-Rac antibodies were from BD Biosciences (Palo Alto, CA).

Fluorescence Microscopy

Cells on coverslips or in collagen matrices were fixed for 10 min at 22°C with 3% paraformaldehyde in PBS, blocked with 2% glycine/1% BSA in DPBS for 30 min, and permeabilized with 0.5% Triton X-100 in DPBS for 20 min. To stain for actin, samples were incubated with rhodamine-conjugated phalloidin (0.8 U/ml) for 30 min at 37°C followed by six washes with DPBS. To stain for tubulin, anti-β-tubulin (1:100 dilution) was added to samples and incubated for 30 min at 37°C before actin staining. After washing in DPBS, slides were mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Observations were made using a Nikon Elipse 400 Fluorescent Microscope, and digital images were collected using a Photometrics SenSys camera and MetaView (Universal Imaging Corporation, West Chester, PA).

Measurement of Small G Protein Activation

GTP-loading of small G proteins was determined as has been described (Ren *et al.*, 1999). Matrices (4/sample; 4×10^5 cells/matrix) were extracted with 300 µl ice-cold $5 \times$ lysis buffer (MLB; Upstate Biotechnology, Lake Placid, NY) containing 10 µg/ml each of leupeptin and aprotinin or with modified RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂) containing 10 µg/ml each leupeptin and aprotinin and 1 mM AEBSF. Lysates were clarified at 16,000 × g (Eppendorf Microfuge; Brinkmann Instruments, Westbury, NY) at 4° for 5 min. Equal volumes of lysates were incubated for 45 min with 10 µg bacterially produced fusion proteins bound to glutathione-agarose beads (GST-TRBD; Rhotekin aa 7–89) for Rho or (GST-PBD; human PAK1 aa 67–150) for Rac. Samples were washed, and pellets were subjected to SDS-PAGE electrophoresis using 12% acrylamide mini-slab gels and transferred to PVDF membranes (Millipore, Bedford, MA). Blots were probed with rabbit anti-Rho antibodies or mouse anti-Rac antibodies followed by HRP-coupled goat anti-rabbit or anti-mouse. SuperSignal Western blotting reagent was obtained from Pierce Chemical Co. (Rockford, IL). The bacterial expression constructs for GST-TRBD and GST-PBD were generous gifts from Dr. Paul Sternweis, UT Southwestern.

Cell Loading with Exotransferase C3

Cells cultured overnight were treated for 30 s with trypsin/EDTA to cause cell rounding. This pretreatment was found to increase efficiency of human fibroblast transfection with lipid carriers containing proteins, plasmids, or oligonucleotides (unpublished data). Rounded cells were incubated with Lipofectamine Plus reagent (see below) containing exotransferase C3 or fluorescent IgG as indicated for 1 h, rinsed with DMEM, and incubated for an additional 30 min with DMEM/10% FBS. Subsequently, cells were harvested with

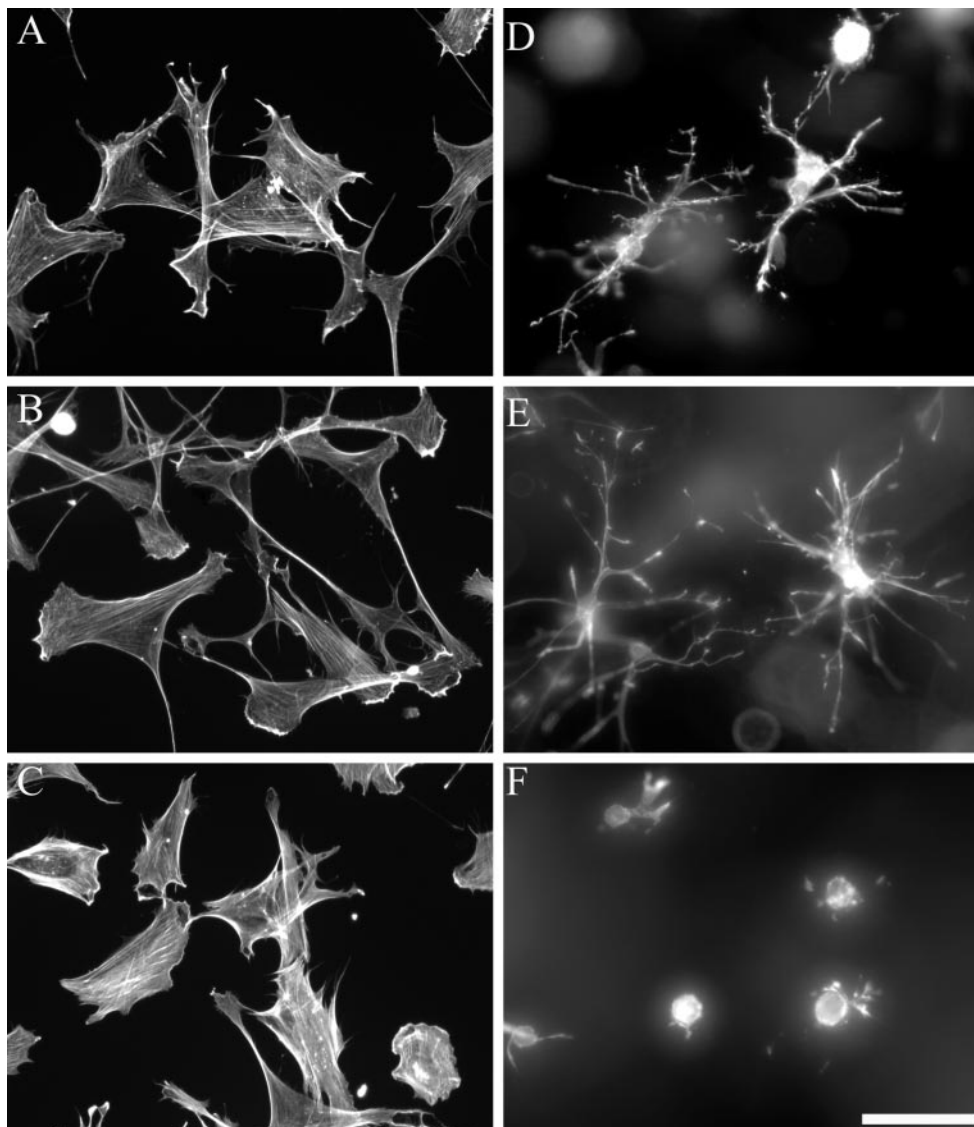


Figure 1. Human fibroblasts project a dendritic network of extensions in collagen matrices but not on collagen-coated coverslips. Fibroblasts were incubated 5 h on collagen-coated surfaces (A–C) or in collagen matrices (D–F). After 1 h, 50 ng/ml PDGF (B and E) or 10 μ M LPA (C and F) was added to the incubations. At the end of the incubations, samples were fixed and stained for actin. Bar, 80 μ m.

trypsin/EDTA and incubated on collagen-coated coverslips or polymerized in collagen matrices as indicated. Lipofectamine plus reagent was used according to the manufacturer's specifications. Precomplexing mixture contained 7.5 μ g of fluorescent IgG (loading marker, FITC-rabbit anti-mouse IgG) and 4 μ g of exotransferase C3 as indicated.

Metabolic Coupling

The dye transfer method to determine metabolic coupling between cells was carried out as has been described (Goldberg *et al.*, 1995). Cultured fibroblasts were rinsed with DPBS and labeled with 10 μ M DiI/5 μ M calcein AM in 1.5 ml of DPBS for 15 min at 37°C. At the end of the incubations, the cells were rinsed with DMEM and then incubated with 10% FBS/DMEM for 60 min at 37°C. Labeled and unlabeled cells were harvested by trypsin/EDTA, mixed together (1:19 ratio), and polymerized in collagen matrices as above.

RESULTS

Human Fibroblasts Project a Dendritic Network of Extensions within Floating Collagen Matrices but not on Collagen-coated Surfaces

Figure 1 shows representative examples of human fibroblasts incubated several hours on collagen-coated coverslips and visualized by phalloidin staining for actin. Cells in basal medium (DMEM containing 5 mg/ml BSA) were well spread with lamellipodia and actin stress fibers (A). PDGF and lysophosphatidic acid (LPA) have been shown to activate the small G proteins Rac and Rho, respectively, and addition of PDGF (50 ng/ml) to cells on coverslips resulted in an increase in actin-containing ruffles along the cell margins as well as greater cell elongation (B), whereas addition of LPA (10 μ M) caused more compact

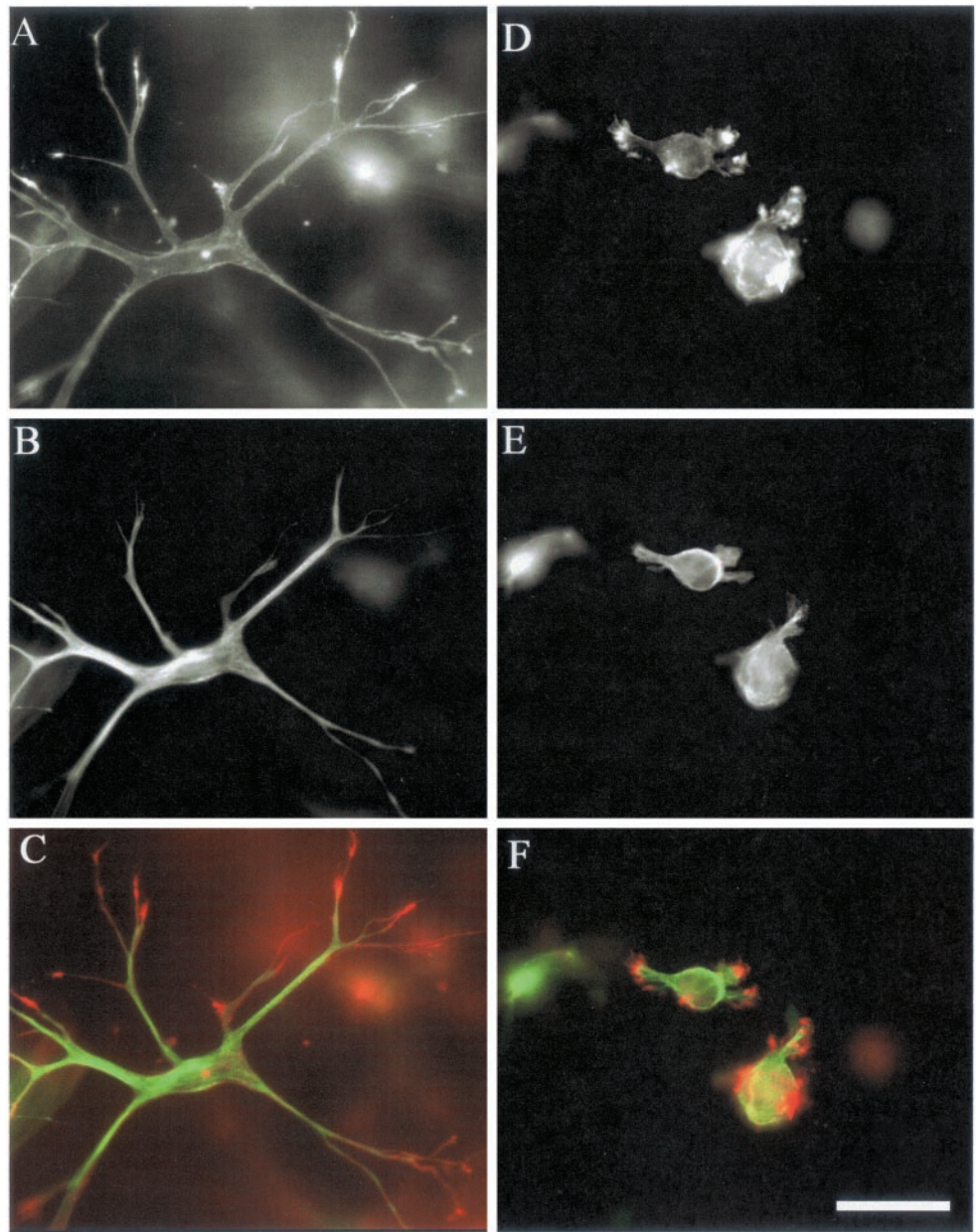


Figure 2. Extensions of the dendritic network contain a core of microtubules with actin concentrated at their tips. Fibroblasts were incubated 4 h in collagen matrices. PDGF at 50 ng/ml (A–C) or 10 μ M LPA (D–F) was added to the incubations after 1 h. At the end of the incubations, samples were fixed and stained for actin and tubulin. (A and D) Actin; (B and E) tubulin; (C and F) overlay. Bar, 40 μ m.

cell morphology and greater density of stress fibers (C; cf. Nobes and Hall, 1995).

The foregoing results show that our human fibroblast preparations behaved in a typical manner when placed on planar surfaces. Figure 1 also shows the markedly different appearance of human fibroblasts embedded at 10^5 /ml in floating collagen matrices and cultured for a similar time period. During the incubation, no collagen matrix contraction was detected as measured by change in matrix diameter (unpublished data). Under these conditions, fibroblasts projected a dendritic network of extensions (Figure 1D). As observed by time-lapse confocal microscopic observations on green-fluorescent protein (GFP)-expressing cells, these extensions were highly dynamic structures

undergoing projection and withdrawal (Tamariz and Grinnell, 2002).

Addition of PDGF (Figure 1E) to the incubations caused an overall increase in size and branching of the dendritic network compared with BSA (Figure 1D). Table 1 summarizes measurements made on randomly photographed cells. In the presence of PDGF, the average length of the major branches of the network was greater, and the branches showed more complexity. Consequently, when cell outlines were traced and average projected surface areas calculated, fibroblasts in PDGF-containing medium were found to be 50% larger than cells in basal medium. In contrast to PDGF, adding LPA to the incubations caused the network of extensions to retract (Figure 1F).

Extensions of the Fibroblast Dendritic Network Contain a Core of Microtubules with Actin Concentrated at Their Tips and Depend on the Actin Cytoskeleton and Microtubules for Formation and Stability

The above findings indicated that human fibroblasts in floating collagen matrices in basal or PDGF-containing medium developed neuronal-like dendritic morphology distinct from fibroblasts on coverslips and also responded differently to LPA by withdrawal their extensions rather than increasing stress fibers. In neuronal cells, the complex morphology and length of dendrites and axons requires unique mechanisms of stabilization and transport in which microtubules play a key role (Gallo and Letourneau, 1999; Baas and Ahmad, 2001; Scott and Luo, 2001). Consequently, studies were carried out to compare the roles of the actin cytoskeleton and microtubules in the structure and stability of extensions of the fibroblast dendritic network.

Figure 2 shows that cell extensions contained a tubulin core (B) with actin localized cortically and concentrated at the tips (A). Overlaid images demonstrated that actin was localized beyond tubulin resulting in a growth cone-like appearance (C). Figure 2 also shows that the short extensions remaining after LPA stimulation contained tubulin (E), with actin (D) localized in ruffles and at the tips of the extensions as shown by overlaid images (F).

Figure 3A shows that 15 min after preparing collagen matrices containing fibroblasts, cells were still round. By 30 min, cells extensions that appeared to be a mixture of ruffles and filopodia could be observed forming at the cell margins (Figure 3B), and development of the dendritic network was evident by 60 min (Figure 3C). Addition of 10 μM cytochalasin D to disrupt the actin cytoskeleton (Figure 3E) or 5 μM nocodazole to disrupt microtubules (Figure 3F) prevented subsequent development of the dendritic network compared with control cells (Figure 3D). Therefore, both microfilaments and microtubules were required for formation of the extensions.

If the dendritic network of extensions was allowed to elongate for several hours, then addition of cytochalasin D or nocodazole had different consequences for the cells. Disrupting the actin cytoskeleton caused actin to redistribute into clusters (Figure 3G) but the extensions did not retract. These clusters were not a consequence of cell fragmentation. Costaining for actin (Figure 3I) and tubulin (Figure 3J) demonstrated that the clusters were located along intact extensions (overlay, Figure 3K). Disrupting microtubules, on the other hand, caused the dendritic network to retract (Figure 3H). Therefore, intact microtubules were necessary not only for projection of the dendritic network of extensions but also for its continued stability. In other experiments, we found that nocodazole-induced retraction of the network was prevented if the actin cytoskeleton was first disrupted by cytochalasin D (unpublished data).

LPA-stimulated Retraction of the Fibroblast Dendritic Network Depends on Rho and Rho Kinase

Retraction of the fibroblast dendritic network was one of the most striking differences between the response to LPA by fibroblasts in floating collagen matrices compared with cells on planar surfaces. Consequently, it was of interest to learn more about the mechanism involved. Figure 4 shows that

when LPA was added to cells previously incubated 1 h in collagen matrices, no changes in the extensions were observed during the first 5 min (A). Extensions began to shorten, however, by 15 min (B) and were fully retracted after 60 min (C). LPA also was added to fibroblasts that had previously elongated a dendritic network over several hours in medium containing PDGF. Figure 4E compared with 4D shows that retraction of extensions also occurred under these conditions, but small clumps of actin staining—perhaps cell fragments—were left behind. Conversely, addition of PDGF to cells whose network had been retracted in response to stimulation by LPA did not cause reelongation of the extensions over the next 2–3 h (unpublished data). Finally, Figure 4F shows that disrupting the actin cytoskeleton with cytochalasin D blocked LPA-stimulated retraction.

The response of fibroblasts in matrices to LPA resembled retraction of neuronal cell processes, which has been shown to depend on activation of Rho and the Rho effector Rho kinase (Jalink *et al.*, 1994; Hirose *et al.*, 1998; Hall *et al.*, 2001). Consequently, studies were carried out to learn if a similar mechanism was involved. Figure 5, A and B, shows that activated (GTP-loaded) Rho was undetectable in fibroblasts in matrices incubated for 1 h, whereas activated Rac was present. Therefore, the activated Rac/Rho ratio was relatively high. Within 5 min after stimulating cells with LPA (but not BSA or PDGF), Rho activation occurred.

The above findings were consistent with the possibility that Rho activation by LPA stimulation resulted in retraction of the fibroblast dendritic network. To test this possibility further, human fibroblasts were loaded with exotransferase C3 to block Rho function (Narumiya *et al.*, 1988). Cells were loaded with exotransferase C3 using lipofectamine and fluorescent antibody as a loading marker. Before loading, cells were rounded by trypsinization to increase loading efficiency. Figure 6, A, C, E, and G, shows the cellular organization of actin, and Figure 6, B, D, F, and H, shows the loading marker in the same visual fields. The loading process itself had no effect on LPA-stimulated retraction of the fibroblast dendritic network (A and B), but retraction was blocked in cells loaded with exotransferase C3 (C and D) indicating a requirement for Rho. On collagen-coated cov-

Table 1. Projected area of dendritic fibroblasts in collagen matrices and length of major cell branches

Growth factor added	Cell area (μm^2)	Branch length (μm)	Number of branches
BSA	997 \pm 580	36.8 \pm 14.4	9.7 \pm 3.3
PDGF	1628 \pm 536	48.5 \pm 15.0	9.7 \pm 1.6
p value	0.014	0.046	

Fibroblasts were incubated 4 h in collagen matrices in basal medium. PDGF (50 ng/ml) was added after 1 h where indicated. At the end of the incubations, samples were fixed and stained for actin. Ten cells from each preparation were photographed and measured using MetaView (Universal Imaging Corporation). To determine projected cell area, cell outlines were traced. To determine number and length of major branches, the path from the tip of the major branch of each extension to the point at which it left the cell body was marked. Data shown are averages \pm SD. BSA and PDGF values for area and branch length were compared using the Student's *t*-test.

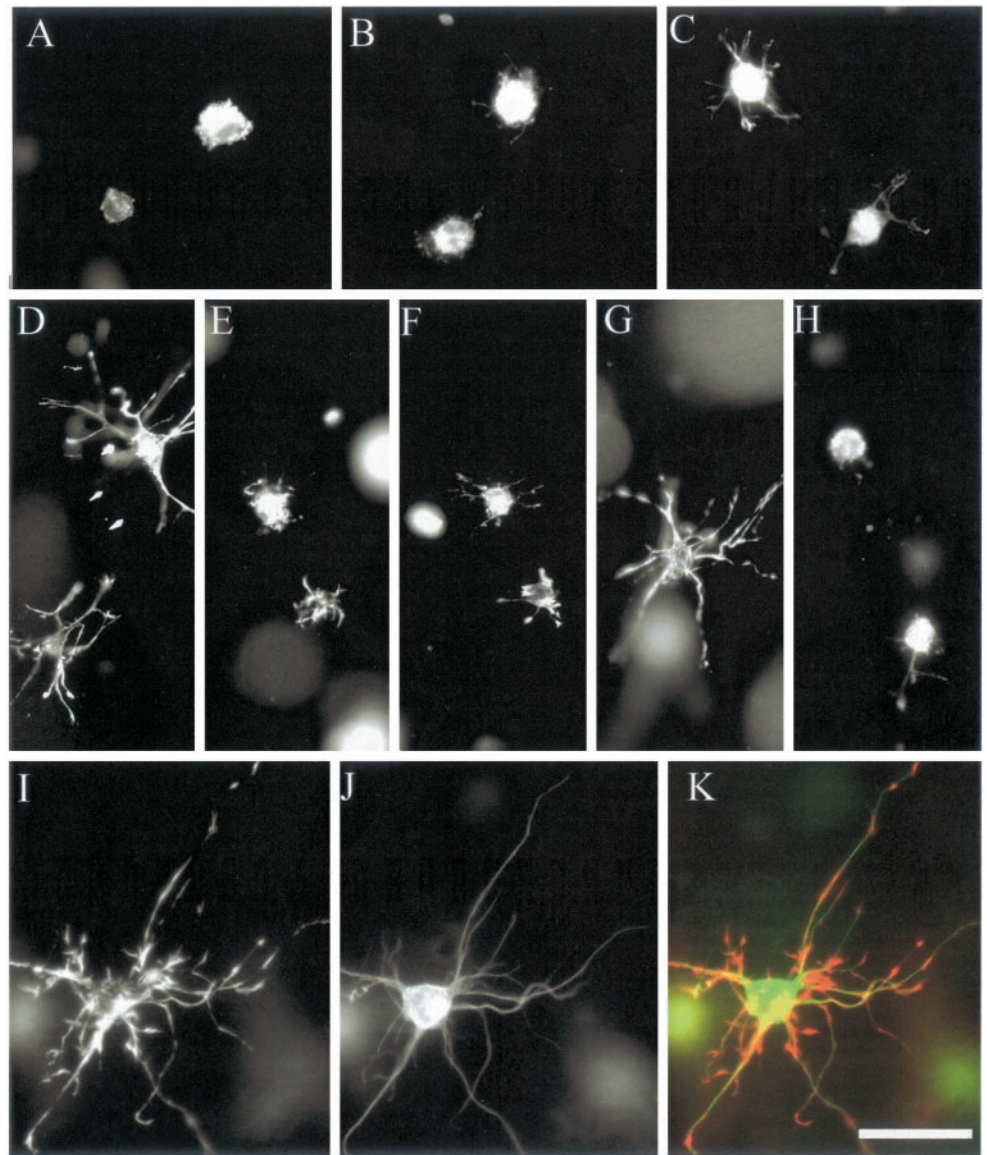


Figure 3. The dendritic network depends on actin and tubulin for formation and motility. Fibroblasts were incubated in collagen matrices for 15 min (A), 30 min (B), or 60 min (C). In some samples (D–K), 50 ng/ml PDGF was added to the incubations after 60 min, which were continued for an additional 4 h. Cytochalasin D (10 μ M) was added after 15 min (E) or 4 h (G and I–K). Nocodazole (5 μ M) was added after 15 min (F) or 4 h (H). At the end of the 5-h incubations, samples were fixed and stained for actin (A–H) or actin and tubulin (I, actin; J, tubulin; K, overlay). Bar, 80 μ m for A–H and 40 μ m for I–K.

erslips, the loading process also had no effect on the ability of cells to attach and spread (E and F), but exotransferase C3-loaded cells plated on coverslips in LPA-containing medium lacked stress fibers and developed distorted cell extensions (G and H).

In other experiments, we found that the fibroblast dendritic network in PDGF-containing medium was not disrupted in control (Figures 7, A and B) or exotransferase C3-loaded cells (Figure 7, C and D). On the other hand, cells loaded with exotransferase C3 and plated on coverslips in PDGF-containing medium lacked stress fibers and developed distorted cell extensions (Figures 7, E and F). The appearance of distorted extensions in C3-loaded cells on planar surfaces but not in matrices (see also Figure 6) indicated that disrupting Rho signaling had different consequences for the cells under these two sets of conditions.

To learn if Rho kinase also was required for LPA-stimulated retraction of the fibroblast dendritic network, experiments were carried out with the Rho kinase inhibitor Y27632 (Narumiya *et al.*, 2000). In unstimulated fibroblasts, the appearance of cell extensions was similar with (Figure 8B) or without (Figure 8A) the inhibitor. Addition of LPA stimulated retraction of extensions (Figure 8C), which was inhibited by the Rho kinase inhibitor (Figure 8D). In contrast, retraction of extensions simulated by nocodazole (Figure 8E) was only partially prevented by blocking Rho kinase (Figure 8F).

The Fibroblast Dendritic Network Can Metabolically Couple Fibroblasts

The foregoing studies demonstrated that fibroblasts embedded at low density in floating collagen matrices projected

and retracted a dendritic network of extensions. Mesenchymal cells in developing connective tissue, fibroblasts in dermis, and myofibroblasts in wound tissue all become metabolically coupled (Gabbiani *et al.*, 1978; Salomon *et al.*, 1988; Warner, 1999). Because fibroblasts within collagen matrices develop gap junctions (Bellows *et al.*, 1982; Ehrlich *et al.*, 2000), it was of interest to learn if the dendritic network of extensions was able to support metabolic coupling between cells. To accomplish this, donor cells preloaded with calcein and DiI were mixed with unlabeled recipient cells. Calcein is able to pass through gap junctions; DiI is not (Goldberg *et al.*, 1995). Coupling was allowed to proceed in basal medium for 1 h after which PDGF or LPA was added for an additional hour. Figure 9, A and B, shows that DiI/calcein donor fibroblasts transferred calcein through dendritic extensions to recipient cells in basal medium. A close association between interconnected cells also was maintained in the presence of PDGF (Figure 9, C and D) or LPA (Figure 9, E and F).

DISCUSSION

Most studies on fibroblast motility have used conditions in which cells develop isometric tension as indicated by formation of cellular stress fibers and focal adhesions. On planar surfaces, fibroblasts develop a flattened, lamellar appearance (Trinkaus, 1984). In collagen matrices, cells under isometric tension become bipolar or stellate (Brown *et al.*, 1998; Tranquillo, 1999; Grinnell, 2000; Tamariz and Grinnell, 2002). Except during fibrotic conditions such as wound repair, however, fibroblasts in the adult connective tissue environment lack stress fibers and fibronexus junctions, the focal adhesion equivalent (Tomasek *et al.*, 2002).

In the current studies, we examined the features of human fibroblasts embedded at 10^5 cells/ml in floating collagen matrices. Under these conditions, neither stress fibers nor focal adhesions formed, and no matrix contraction occurred. We observed a new type of "normal" (i.e., uninduced by pharmacologic or genetic intervention) fibroblast morphology. Cells projected and retracted a dendritic network of extensions and developed the appearance of neuronal cells. PDGF stimulated formation of the network; LPA induced its retraction.

The unusual appearance of fibroblast in floating collagen matrices could be attributed to the balance between the small G proteins Rac and Rho. Activation of Rac stimulates cell protrusion, whereas activation of Rho inhibits cell protrusion and stimulates cell contraction (Clark *et al.*, 1998; Hall, 1998; Rottner *et al.*, 1999). After 1 h, by which time the dendritic network was evident, the ratio of activated Rac/Rho was relatively high. Within 5 min after stimulating cells with LPA (but not BSA or PDGF), Rho activation occurred; and extensions were retracted. Although LPA was able to stimulate retraction of the fibroblast dendritic network that had formed in the presence of PDGF, PDGF did not (within a few hours) stimulate reelongation of the network after it had retracted in the presence of LPA. Therefore, for fibroblasts in floating collagen matrices the Rho signal was dominant.

Differences in cell adhesion and matrix stiffness may play a critical role to determine the cellular response to LPA. On planar surfaces, contractile force stimulated by LPA results in an increase in stress fibers and focal adhesions (Nobes and

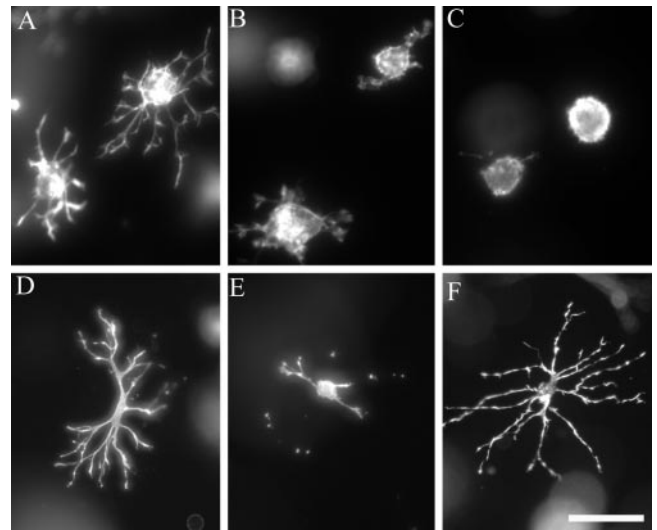


Figure 4. LPA stimulates retraction of the fibroblast dendritic network. Fibroblasts were incubated in collagen matrices for 60 min at which time LPA ($10 \mu\text{M}$) was added and the incubations were continued for an additional 5 min (A), 15 min (B), or 60 min (C). In some samples (D–F), fibroblasts were incubated in collagen matrices for 60 min at which time PDGF (50 ng/ml) was added. The latter samples were incubated for an additional 4 h, at which time LPA ($10 \mu\text{M}$) was added for 60 min (E and F). In F, cytochalasin D ($10 \mu\text{M}$) was added 15 min before LPA. At the end of the incubations, samples were fixed and stained for actin. Bar, $80 \mu\text{m}$ for A–C and $40 \mu\text{m}$ for D–F.

Hall, 1995; Burrige and Chrzanowska-Wodnicka, 1996). Similarly, fibroblasts in restrained collagen matrices form focal adhesions and stress fibers in response to LPA under conditions in which global remodeling of restrained matrices occurs (Tamariz and Grinnell, 2002). Formation of focal adhesions and stress fibers implies that the substratum is stiff enough to allow isometric tension to develop in the cells. Collagen matrices containing 10^5 /ml fibroblasts that are floating (this study) or restrained (Tamariz and Grinnell, 2002) do not undergo global matrix reorganization (i.e., contraction). Consequently, the low matrix stiffness and/or distance between collagen fibrils may result in formation of cell adhesions that are more easily reversed or broken. As a result, stimulation of cellular contractile force by LPA results in retraction of the fibroblast dendritic network rather than development of isometric tension. Retraction was Rho and Rho kinase-dependent, similar to LPA-stimulated retraction of neurites (Jalink *et al.*, 1994; Hirose *et al.*, 1998; Hall *et al.*, 2001).

Differences in the cell adhesion might also explain the different cellular responses to inhibition of Rho function by exotransferase C3. For fibroblasts in matrices, blocking Rho prevented LPA from causing retraction of the fibroblast dendritic network, and the extensions that formed appeared undistorted compared with control cells. For fibroblasts on coverslips in the presence of LPA or PDGF, on the other hand, blocking Rho caused cells to form extensions that were collapsed and distorted. Small G protein activation and its consequences for cell behavior have been shown to be regulated not only by growth factors but also by cell adhe-

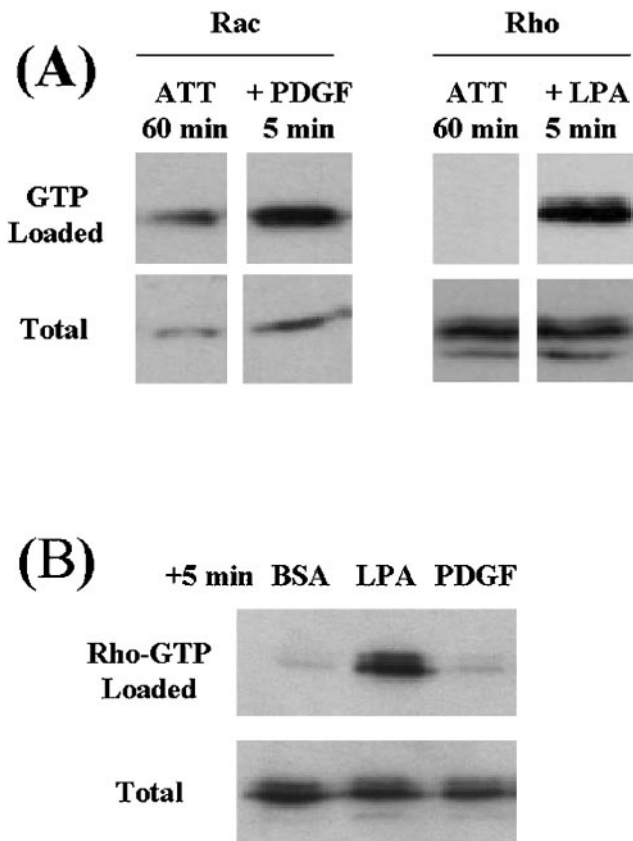


Figure 5. Rho activation occurs in fibroblasts in collagen matrices after LPA stimulation. Fibroblasts were incubated in collagen matrices for 60 min (ATT) followed by 5 min in basal medium (DMEM/5% BSA) (BSA) containing LPA (10 μ M) or PDGF (50 ng/ml) as indicated. Samples for each lane represented four matrices containing 4×10^5 cells each. At the end of the incubations, samples were lysed, and aliquots were used to determine GTP-loaded and total levels of Rac and Rho as shown.

sion (Hall, 1998; Price *et al.*, 1998; Kaibuchi *et al.*, 1999; Ren *et al.*, 1999; Arthur *et al.*, 2000; del Pozo *et al.*, 2000; Cox *et al.*, 2001). Our findings suggest that Rho signals generated by fibroblast adhesion on planar surfaces impact cells differently from signals generated by fibroblast adhesion in the three-dimensional collagen matrices.

Structurally, fibroblast extensions that made up the dendritic network contained microtubule cores with actin concentrated at their tips resembling growth cones. Disrupting the actin cytoskeleton prevented formation of the dendritic network, consistent with a role for actin polymerization in projection of cell extensions (Borisov and Svitkina, 2000; Polard *et al.*, 2000). If the network already was formed, however, then disrupting the actin cytoskeleton did not cause withdrawal of extensions but did cause actin clustering. Disrupting the actin cytoskeleton of bipolar chick embryo fibroblasts in collagen matrices (Tomasek and Hay, 1984) or neurites on planar surfaces was shown to have a similar effect on actin organization (Joshi *et al.*, 1985). The appearance of these actin clusters suggests that the extensions are

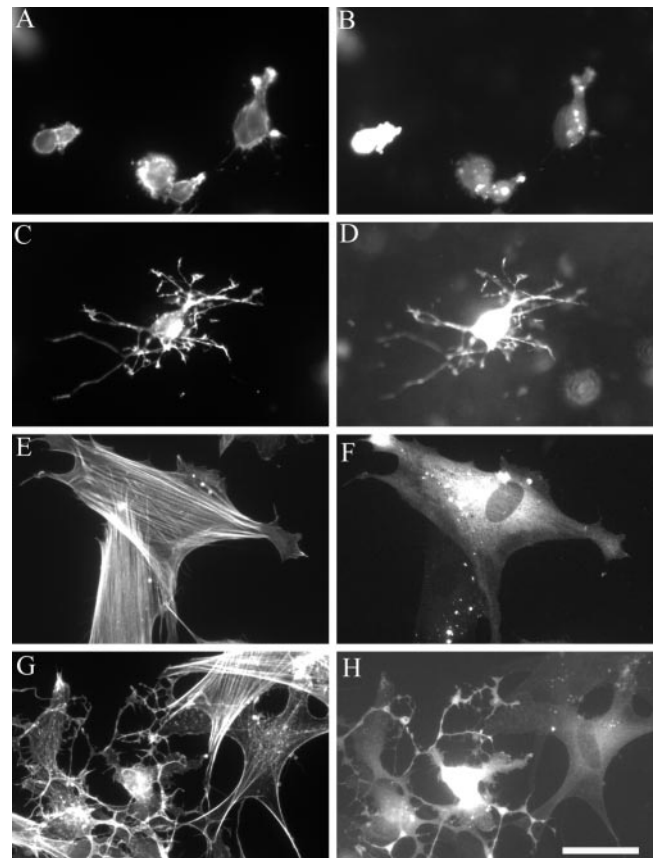


Figure 6. In LPA stimulated cells, exotransferase C3 inhibits retraction of the fibroblast dendritic network but causes distortion cell extensions on coverslips. Fibroblasts loaded with FITC-rabbit IgG (A, B, E, and F) or with FITC-rabbit IgG and exotransferase C3 (C, D, G, and H) were incubated on collagen-coated coverslips (E-H) or in collagen matrices (A-D) for 4 h. LPA (10 μ M) was added after 1 h. At the end of the incubations, samples were fixed and stained for actin (A, C, E, and G) and with FITC-conjugated goat anti-rabbit IgG to intensify the IgG signal (B, D, F, and H). Bar, 80 μ m.

subject to some kind of as yet unexplained topographic organization. Although the length of the extensions was unchanged after cytochalasin treatment, the dendritic network was inhibited from retracting in response to LPA or nocodazole treatments.

Microtubules were found to play a key role in the fibroblast dendritic network. Previously, microtubules have been implicated in maintenance of fibroblast polarity, and their disruption leads to abnormal formation and protrusion of cell surface extensions (Bershadsky *et al.*, 1991; Omelchenko *et al.*, 2002). Also, disrupting microtubules of bipolar chick embryo fibroblasts in collagen matrices was reported to induce abnormal pseudopodia (Tomasek and Hay, 1984). Loss of fibroblast polarity caused by disrupting microtubules typically results in an increase in cellular isometric tension and actin stress fibers (Danowski, 1989; Kolodney and Elson, 1995). For fibroblasts in floating collagen matrices, however, rather than formation of stress fibers, the

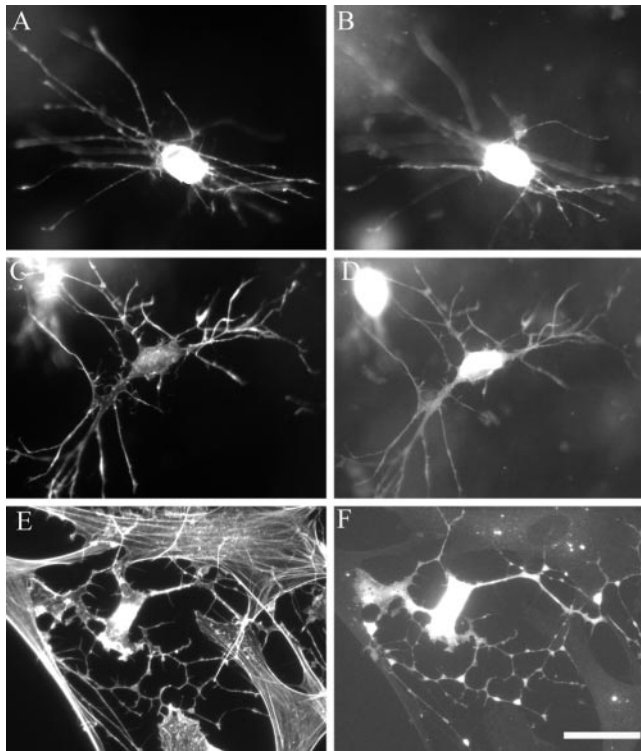


Figure 7. In PDGF stimulated cells, exotransferase C3 does not inhibit the fibroblast dendritic network but causes distortion of fibroblast extensions on coverslips. Fibroblasts loaded with FITC-rabbit IgG (A and B) or with FITC-rabbit IgG and exotransferase C3 (C–F) were incubated on collagen-coated coverslips (E and F) or in collagen matrices (A–D) for 4 h. PDGF (50 ng/ml) was added after 1 h. At the end of the incubations, samples were fixed and stained for actin (A, C, and E) and with FITC-conjugated goat anti-rabbit IgG to intensify the IgG signal (B, D, and F). Bar, 80 μm .

consequence of disrupting microtubules was complete loss of the dendritic network.

The increase in cell contractility caused by disrupting microtubules has been attributed to activation of Rho and Rho kinase (Bershadsky *et al.*, 1996; Liu *et al.*, 1998). Our evidence, indicates that retraction of dendritic processes caused by disrupting microtubules does not require Rho kinase, a finding consistent with the recent report that loss of cell polarity and activation of Rho-dependent contractility are independent consequences of disrupting microtubules (Omelchenko *et al.*, 2002). The role of microtubules in stability of the fibroblast dendritic network requires further study and might depend on structural support as implied by the tensegrity hypothesis (Ingber, 1997), a transport mechanism such as dynein-driven net forward movement of cytoskeletal elements (Nabi, 1999; Baas and Ahmad, 2001) or a regulatory mechanism such as stimulation of Rac activation by microtubule assembly (Waterman-Storer *et al.*, 1999).

Formation of a metabolically coupled, fibroblast dendritic network in collagen matrices is consistent with the in situ appearance of mesenchymal cells and skin fibro-

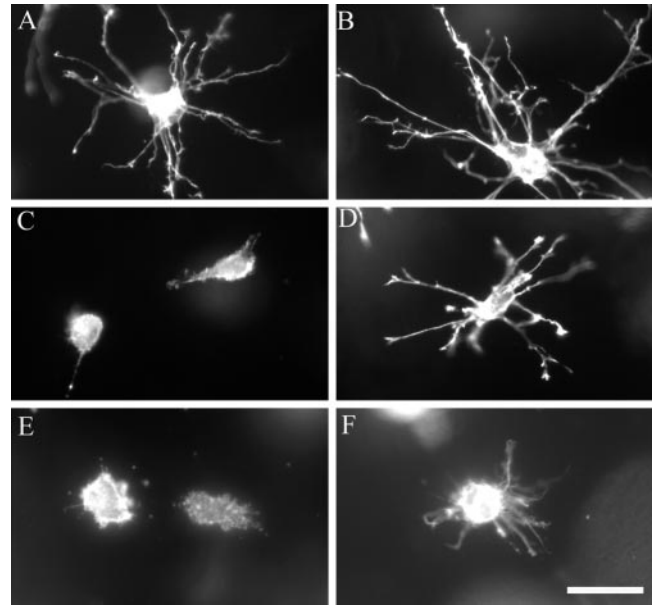


Figure 8. Rho kinase inhibitor blocks LPA-stimulated retraction of the fibroblast dendritic network. Fibroblasts were incubated 4 h in collagen matrices. LPA (10 μM) or nocodazole (5 μM) was added to the incubations after 3 h. In some samples (B, D, and F), Rho kinase inhibitor Y-27632 (10 μM) was added for 15 min before adding LPA or nocodazole. At the end of the incubations, samples were fixed and stained for actin. Bar, 40 μm .

blasts (Breathnach, 1978; Trinkaus, 1984; Van Exan and Hardy, 1984; Omagari and Ogawa, 1990), which also are interconnected by gap junctions (Salomon *et al.*, 1988; Warner, 1999). The fibroblast dendritic network has not been appreciated from earlier studies of cells in floating or restrained collagen matrices (Brown *et al.*, 1998; Tranquillo, 1999; Grinnell, 2000; Tomasek *et al.*, 2002), which were concerned primarily with the matrix contraction (i.e., global matrix remodeling).

Given their supportive function, connective tissues need to be highly dynamic structures, mechanically and biosynthetically responsive to their surroundings. Formation of a dendritic network of extensions provides fibroblasts with a mechanism to explore and become interconnected to each other in three-dimensional space, potentially playing a role in the homeostatic responsiveness of soft connective tissues similar to the mechanosensory function of the dendritic network of extensions utilized by osteocytes in bone (Burger and Klein-Nulend, 1999; Noble and Reeve, 2000).

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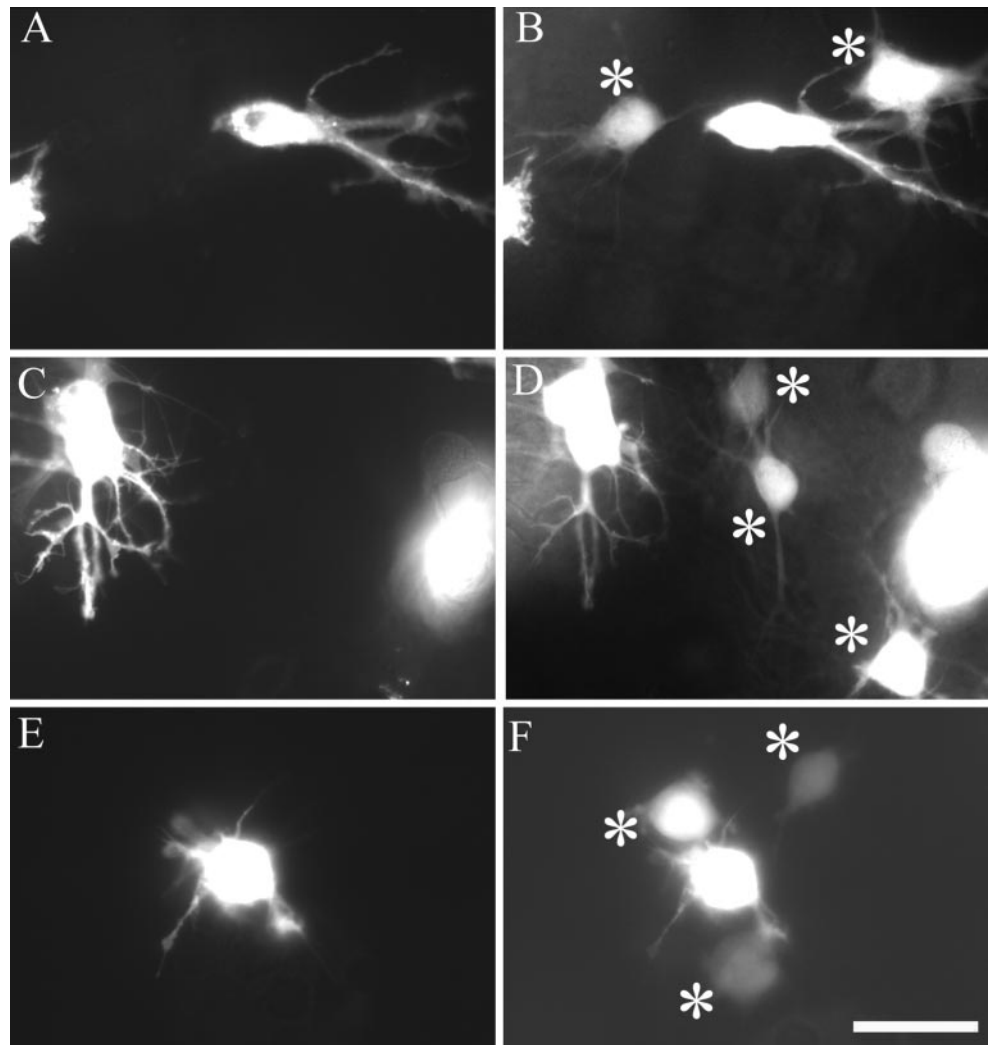


Figure 9. Metabolic coupling of cells through the fibroblast dendritic network. Donor fibroblasts (labeled with DiI and Calcein AM) and unlabeled cells were incubated 2 h in collagen matrices. After 1 h, 50 ng/ml PDGF (C and D) or 10 μ M LPA (E and F) was added. At the end of the incubations, samples were visualized without fixation for DiI (donor cells; A, C, and E) or Calcein AM (donor and recipient cells; B, D, and F). Asterisks indicate recipient cells. Bar, 80 μ m.

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