Fibroblast Quiescence and the Disruption of ERK Signaling in Mechanically Unloaded Collagen Matrices*

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Fibroblasts in mechanically unloaded collagen matrices had low levels of DNA synthesis compared with cells in mechanically loaded matrices. Under the former conditions, the cellular ERK signaling pathway appeared to be disrupted. Also, pharmacologic inhibition of ERK signaling blocked DNA synthesis by fibroblasts in mechanically loaded matrices. These results were consistent with the idea that mechanoregulation of fibroblast DNA synthesis in collagen matrices occurs at the level of the ERK signaling pathway.

During wound repair, fibroblasts go through a sequence of cell proliferation, quiescence, and apoptosis (1–4). To study these processes in vitro, we have been studying growth regulation of fibroblasts cultured in three-dimensional collagen matrices (5). The collagen matrix model provides a unique opportunity to study the transition from cell proliferation to quiescence without pharmacologic intervention or removal of soluble growth factors.

Fibroblasts proliferate in mechanically loaded (“attached”) collagen matrices (6, 7) where the isotropic tension is similar in magnitude to that in skin wounds (8, 9). When, however, mechanically loaded matrices are switched to unloaded conditions (“released”), the cells then become quiescent (7) and apoptotic (10). Also, fibroblasts in collagen matrices prepared in the absence of mechanical load (“floating”) (11) have low levels of DNA synthesis (6, 12) and become apoptotic (13).

The mechanisms accounting for cell quiescence and apoptosis in mechanically unloaded collagen matrices are poorly understood, but cytoskeletal tension has been implicated as an important factor (10, 13). Cytoskeletal tension also has been implicated in anchorage dependence of cell growth in monolayer culture (14, 15), raising the possibility that cessation of DNA synthesis by fibroblasts in mechanically unloaded collagen matrices is analogous to anchorage dependence albeit in a three-dimensional context.

Anchorage dependence of cell proliferation in monolayer culture involves the ERK1 signaling pathway (16, 17), which is required for cell cycle progression (18, 19). It was of interest, therefore, to compare ERK signaling in fibroblasts in mechanically loaded versus unloaded collagen matrices. Our results show that disruption of ERK signaling could explain the onset of quiescence of fibroblasts in mechanically unloaded matrices even though the cells are anchored and well spread.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), restriction enzymes, reverse transcriptase, and deoxynucleotides were purchased from Life Technologies, Inc. MEK inhibitor PD98059 was purchased from Calbiochem (La Jolla, CA). Anti-phospho-MEK1 antibodies were acquired from New England Biolabs (Beverly, MA). Total MEK1 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-active ERK 1/2 was obtained from Promega Corp. (Madison, WI). Total ERK1 antibody was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Nylon (Nytran) membranes for DNA hybridization were purchased from Schleicher & Schuell. Fetal bovine serum (FBS) was purchased from Intergen Co. (Purchase, NY). Rhodamine isothiocyanate-conjugated phalloidin was purchased from Molecular Probes, Inc. (Eugene, OR). Type I collagen (Vitrogen) was purchased from Collagen Corp. (Palo Alto, CA). Guanidinium thiocyanate for Solution D (20) was purchased from Fluka Chemical Co. (Ronkonkoma, NY).

Collagen Matrix Culture—Fibroblasts from human foreskin specimens (<10th passage) were maintained in Falcon 75-cm² tissue culture flasks in DMEM supplemented with 10% FBS. Collagen matrix cultures were prepared using Vitrogen 100 collagen as described previously (21, 22). Neutralized collagen solutions (1.5 mg/ml) contained fibroblasts in DMEM but no serum. The cell/collagen mixture was prewarmed to 37 °C for 3–4 min, after which aliquots (0.2 ml) were polymerized in Corning 24-well culture plates for 60 min at 37 °C in a humidified incubator with 5% CO₂. After polymerization, 1.0 ml of DMEM, 10% FBS and 50 μg/ml ascorbic acid were added to each well. Floating matrices were gently released from the underlying culture dish with a spatula immediately after polymerization. Attached matrices were released at the times indicated in the figure legends.

DNA Synthesis—Collagen matrices were incubated in 10% FBS DMEM containing 5 μCi/ml [3H]thymidine (specific activity 5–81 Ci/mmol) for 1 h at 37 °C in a humidified incubator with 5% CO₂ and then washed three times for 5 min with PBS (150 mM NaCl, 3 mM KCl, 6 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.2). Subsequently, the cells were harvested from matrices (10), and DNA was precipitated by incubating the cells with 10% trichloroacetic acid in PBS containing 125 μg/ml bovine serum albumin for 20 min at 4 °C. Precipitates were collected on Whatman 2.5-cm glass microfiber filters, washed with 10 and 5% trichloroacetic acid, and transferred to scintillation vials containing 10 ml of Budget Solve. Radioactivity was counted using a Beckman scintillation counter (LS-6000 SC). All experiments were carried out in duplicate, and every experiment was repeated at least two times. Data points and error bars in the figures represent averages and standard deviations, respectively.

Analysis of ERK and MEK—SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (21). Matrices (two matrices/sample) were extracted in 100 μl of extraction buffer (0.2% Nonidet P-40, 150 mM NaCl, 3 mM KCl, 6 mM Na₂HPO₄, 1 mM KH₂PO₄, 0.5 mM MgCl₂, 1 mM CaCl₂, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μM 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), 1 mM CaCl₂, 50 mM NaF, 1 mM Na₂VO₄, and 1 mM Na₂MoO₄, pH 7.4) by homogenization (50 strokes) using a Dounce homogenizer (paddle B; Wheaton Scientific, Millville, NJ). Samples were clarified by centrifuga-
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gation for 5 min at 16,000 × g (Eppendorf microcentrifuge Model 5415 C), and the supernatants were dissolved in 4× reducing sample buffer (250 mM Tris, 4% SDS, 40% glycerol, 20% mercaptoethanol, 0.04% bromphenol blue) and boiled for 5 min. Equal volumes from each sample were subjected to SDS-polyacrylamide gel electrophoresis using 9% acrylamide mini-slab gels. After transfer to polyvinylidene difluoride membranes, samples for ERK analysis were subjected to immunoblotting with anti-active ERK 1/2 (1:10000) and anti-ERK 1 (1:5000). Samples for MEK analysis were subjected to immunoblotting with phospho-MEK (1:1000) and MEK1 (1:500).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Southern Blotting—RNA was isolated as described previously (21). Briefly, collagen matrices (one/sample) were dissolved using Solution D, and total RNA was extracted with phenol/chloroform/isooamyl alcohol and precipitated with isopropanol and 20 µg of glycogen (as carrier) (20).

RNA precipitates were redissolved in 10 µl of autoclaved water. First-strand cDNA synthesis reaction with Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Life Technologies, Inc.) using oligo(dT) (15) primer was carried out according to the manufacturer’s protocol in a final volume of 20 µl. After incubation for 3 h at 37 °C, M-MLV RT enzyme was heat-inactivated by incubation at 70 °C for 10 min.

One µl of each cDNA preparation was used in PCR reactions using c-fos and GAPDH primers (see below). DNA was denatured at 94 °C for 4 min followed by 5 cycles of the following: 30 s denaturation at 94 °C, 30 s annealing at 57 °C, 60 s elongation at 72 °C, and a final elongation phase for 10 min at 72 °C. Reaction mixtures (12 µl) contained 20 mM Tris-HCl (pH 8.4), 0.05 units/µl Taq polymerase, 50 mM KCl, 1.5 mM MgCl2, 200 µM dNTPs, and 1.0 µM of each primer.

After PCR, 7.5 µl was removed from each sample, and 1.5 µl of 6× loading buffer II (23) was added and the mixture loaded onto 2% agarose gels containing 1× Tris-acetate-EDTA (23). Samples were subject to electrophoresis for 30 min at 100 V. The gel was then soaked in 0.5 M NaOH, 1.5 M NaCl for 45 min to denature the DNA and then pH-neutralized in 20× saline/sodium phosphate/EDTA. After transfer, DNA was cross-linked with a Stratalinker UV light source (Stratagene) at a setting of 1,200 µJ of radiation/cm2. c-fos and GAPDH PCR products were then detected under aqueous conditions at 65 °C according to standard protocols (23). DNA probes for hybridization were generated with a Roche Molecular Biochemicals random-primed labeling kit. Gel-purified PCR products using c-fos and GAPDH primers served as templates. c-fos primers were synthesized by the University of Texas southwestern Molecular Biology Core Facility as follows: 5′-atgatgttctcgggcctcaacgcagcag-3′ and 5′-cttcgagatcaggttcgacttgc-3′. GAPDH primers were purchased from CLONTECH.

Actin Distribution—Collagen matrices were fixed for 30 min at 22 °C with 3% paraformaldehyde in DPBS, washed 2 × 10 min with DPBS (1 mM CaCl2, 0.5 mM MgCl2, 150 mM NaCl, 3 mM KCl, 6 mM Na2HPO4, 1 mM KH2PO4, pH 7.2), blocked for 30 min with DPBS containing 2% bovine serum albumin and 1% glycine, and then permeabilized for 10 min with DPBS containing 0.5% Triton X-100. Subsequently, the samples were incubated with rhodamine isothiocyanate-conjugated phallolidin (8 units/ml) for 30 min at room temperature followed by six washes with DPBS. After mounting the samples on glass slides with Fluoromount G, observations and photographs were made under a Bio-Rad MRC 1024 Laser Sharp Confoilc microscope.

RESULTS

Differences in DNA Synthesis by Fibroblasts in Attached, Floating, and Released Collagen Matrices—Fig. 1A shows that in mechanically loaded (i.e. attached) collagen matrix cultures, the rate of total DNA synthesis increased over 72 h. When matrices that had been attached for 48 h were released for 4 h, DNA synthesis in the cultures was unchanged, as shown in Fig. 1B (A48,R4). By 10 h, however, DNA synthesis was released for 48 h and released for 24 h and then released for 24 h. Rates of DNA synthesis were measured at the indicated times.

Fig. 1. DNA synthesis by fibroblasts in attached and released collagen matrices. A, fibroblasts in attached (A) matrices were cultured for 24, 48 or 72 h in DMEM, 10% FBS. B, fibroblasts in attached matrices were cultured in DMEM, 10% FBS for 48 h and then released (R) for either 4 or 10 h. C, fibroblasts were cultured for 48 h in attached and released for 24 h and released for 24 h. Rates of DNA synthesis were measured at the indicated times.

Inhibition of the ERK Signaling Pathway in Collagen Matrices Switched from Mechanically Loaded to Unloaded Conditions—Cessation of DNA synthesis after loss of cell anchorage in monolayer culture has been suggested to result, at least in part, from the inability of cells to phosphorylate ERK in response to growth factor stimulation (24–26). Consequently, studies were carried out to examine whether fibroblasts in released collagen matrices were altered in the ERK signaling pathway similar to fibroblasts lacking suitable anchorage.

Fig. 2 shows that cells cultured in attached collagen matrices for 52 h had low levels of active ERK. The addition of serum to these cells for 15 or 40 min resulted in an acute ERK phosphorylation response detected by immunoblotting with antibodies specific for the phosphorylated form of ERK. If, however, attached matrices were released 4 h before serum was added, then the ability of serum to stimulate ERK phosphorylation was reduced. Therefore, after mechanical unloading, the sig-
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Inhibition of the ERK Signaling Pathway in Floating Collagen Matrices—In the preceding section, ERK phosphorylation by serum and phorbol ester was examined in cells before and after mechanical unloading (i.e. attached and released matrices). In related experiments, we studied stimulation of ERK phosphorylation in fibroblasts in floating collagen matrices, which also have low levels of DNA synthesis (Fig. 1C). Fig. 6 shows that phorbol treatment of cells cultured for 24 h in attached collagen matrices resulted in acute ERK phosphorylation (see also Fig. 2). If, however, the cells had been cultured overnight in floating collagen matrices, then little ERK phosphorylation occurred in response to phorbol ester. Therefore, the ERK signaling pathway appeared to be disrupted in fibroblasts in floating collagen matrices as well as in matrices that were attached and released.

Inhibition of DNA Synthesis by Disruption of the ERK Signaling Pathway in Fibroblasts in Mechanically Loaded Collagen Matrices—The preceding experiments suggested that disruption of ERK signaling occurred in mechanically unloaded collagen matrices, conditions under which cessation of fibroblast DNA synthesis was observed. To learn whether inhibition of ERK signaling was sufficient to block DNA synthesis by
fibroblasts in collagen matrices, additional experiments were carried out using the ERK signaling pathway inhibitor, PD98059 (35). Fig. 7 shows that the addition of PD98059 for 1 h resulted in marked inhibition of DNA synthesis, measured in 48- and 72-h attached collagen matrix cultures. The effect of the inhibitor was completely reversible after a 24-h washout (W24, Fig. 7), however. It could be concluded, therefore, that blocking the ERK signaling pathway in these cells was sufficient to block DNA synthesis.

Differences in Cell Spreading and Actin Cytoskeletal Organization of Fibroblasts in Attached, Floating, and Released Collagen Matrices—Most studies on the anchorage dependence of fibroblasts in monolayer culture have utilized poorly adhesive substrata or suspension cultures, resulting in poor cell adhesion and spreading, from which comes the original idea of anchorage dependence (36–38). By contrast, fibroblasts in collagen matrices are surrounded by the substrata. Consequently, it was of interest to compare cell shape and actin cytoskeletal organization of fibroblasts under proliferative and quiescent conditions. Fig. 8a shows by confocal microscopy that fibroblasts cultured for 48 h in attached collagen matrices were elongated and under a mechanical load as indicated by the prominent stress fibers. Cells that were attached for 24 h and then released for an additional 24 h (Fig. 8b) lost their stress fibers but remained spread in an elongated shape. Fibroblasts cultured for 48 h in floating matrices (Fig. 8c) also lacked actin stress fibers but had stellate morphology with round cell bodies and long pseudopodia. These results show that fibroblasts in collagen matrices are attached and well spread under all conditions. There was no correlation between DNA synthesis and cell spreading per se, but only cells under a mechanical load had actin stress fibers.

DISCUSSION

The collagen matrix model provides a unique opportunity to study the transition from cell proliferation to quiescence without pharmacologic intervention or removal of soluble growth factors. In the current studies, we analyzed ERK signaling after switching fibroblasts from mechanically loaded to unloaded conditions. Based on the decrease in acute ERK phosphorylation in response to serum or phorbol ester and on the inability of phorbol ester to acutely stimulate c-fos transcription, it could be concluded that the ERK signaling pathway was disrupted in fibroblasts after mechanical unloading.

In collagen matrices switched from mechanically loaded to unloaded conditions, ERK signaling was disrupted after 4 h. DNA synthesis was reduced more than 50% after 10 h and 90% after 24 h. ERK signaling also was disrupted in fibroblasts in floating collagen matrices where only low levels of DNA synthesis occurred. Moreover, inhibition of the ERK signaling pathway by the inhibitor PD98059 reversibly inhibited DNA synthesis in fibroblasts in mechanically loaded collagen matrices (see also Ref. 39). Taken together, these studies are consistent with the idea that disruption of the ERK signaling pathway.

Fig. 7. DNA synthesis in fibroblasts cultured in attached collagen matrices treated with PD98059. Fibroblasts in attached (A) matrices were cultured for 48 or 72 h in DMEM, 10% FBS. Cells were then incubated for either 1 or 24 h with 100 μM PD98059 as indicated or an equivalent volume of Me2SO vehicle, after which the DNA synthesis rates were measured by [3H]thymidine incorporation. In samples with 1-h PD98059 (PD) treatment, the drug was added at the same time as the [3H]thymidine. In samples where PD98059 was washed out (W), collagen matrices were washed three times for 5 min with DMEM and incubated for 24 h in DMEM, 10% FBS before the addition of [3H]thymidine.

Fig. 8. Cell shape and actin cytoskeletal organization in fibroblasts cultured in attached, released, and floating collagen matrices. Fibroblasts were cultured for 48 h in attached collagen matrices (a), 28 h in attached matrices followed by 24 h in released matrices (b), or 48 h in floating matrices (c). At the end of the incubation times, the cells were fixed and stained with rhodamine isothiocyanate-phalloidin to show the distribution of actin. Bar = 30 μm.
pathway contributes to the low levels of DNA synthesis by fibroblasts in mechanically unloaded matrices.

The mechanism responsible for loss of ERK signaling in mechanically unloaded matrices remains to be determined but probably occurs upstream of the ERK activator MEK because MEK responded to mechanical unloading similarly to ERK. Receptor tyrosine kinases could be involved because platelet-derived growth factor receptors have been reported activated by increased mechanical stress (40) but desensitized after mechanical unloading (27, 28). In the current experiments, however, the ERK signaling pathway in mechanically unloaded fibroblasts did not function normally even when tested with phorbol ester, which stimulates the signaling pathway downstream of cell surface receptors (29, 30, 41). Therefore, in addition to receptor activity, loss of signaling likely depends on downstream factors. Physical organization of the signaling pathway itself also could be important. Scaffolding proteins are required for proper functioning of mitogen-activated protein kinase signaling modules (42). In endothelial cells, caveolae have been reported to play a role in organizing the signaling molecules leading to ERK activation, and mechanoregulated signaling can be blocked by disrupting caveolae (43). Finally, mechanostimulation has been shown to cause Shc association with integrins, which is followed by recruitment of the Grb2-Sos complex leading to ERK signaling (44). Therefore, mechanical unloading could alter the association of Shc or other adapter proteins with the cell surface matrix receptors.

Whatever the precise mechanism accounting for the loss of ERK signaling, it is interesting that this loss and concomitant cell quiescence in mechanically unloaded collagen matrices resembles the regulation of cells in monolayer cultures by anchorage dependence. Although it has been possible through the use of micropatterned substrata to make a distinction between the effects of cell tension versus shape on cell proliferation (45), such studies cannot replicate the situation of cells engaged in three-dimensional adhesive interactions over their entire surfaces such as occurs in tissue. Use of a three-dimensional environment is similar to that reported for cells in monolayer culture (14–17).

Finally, the current studies offer a novel mechanism to account for cell growth regulation at the end of wound repair. Before wounding, tissue fibroblasts are stellate, quiescent, and sessile. After wounding, a highly cellular wound tissue develops through the migration and proliferation of fibroblastic cells from the wound margins (1, 2, 4). These cells differentiate into myofibroblasts that are under a mechanical load (isometric tension) as shown by their elongated shape, prominent stress fibers, and fibronexus junctions (3, 47). After the wound defect has been replaced through a combination of cellular contractile activity and extracellular matrix biosynthesis, the tissue returns to a mechanically unloaded state, and myofibroblast regression occurs through apoptosis (3). Based on our experiments with fibroblasts in collagen matrices, we speculate that quiescence at the end of wound repair depends on mechanically regulated disruption of the ERK signaling pathway, which leads to cessation of DNA synthesis even in the continued presence of soluble growth factors in the wound environment.

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