Decreased Level of PDGF-stimulated Receptor Autophosphorylation by Fibroblasts in Mechanically Relaxed Collagen Matrices

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Abstract. The goal of our studies was to characterize the interrelationship between extracellular matrix organization and fibroblast proliferation in response to growth factors. We compared fibroblasts in monolayer culture with cells in contracted collagen matrices that were mechanically stressed or relaxed. In response to platelet-derived growth factor (PDGF), DNA synthesis by fibroblasts in mechanically relaxed collagen matrices was 80-90% lower than in monolayer culture and 50% lower than in mechanically stressed matrices. Fibroblasts in monolayer and contracted collagen matrix cultures contained similar levels of PDGF receptors, but differed in their autophosphorylation response. Cells in mechanically relaxed matrices showed lowest levels of autophosphorylation, 90% less than cells in monolayer culture. Experiments comparing receptor expression and capacity for PDGF-stimulated autophosphorylation showed that cells in mechanically relaxed collagen matrices never developed normal receptor autophosphorylation. Furthermore, when mechanically stressed collagen matrices were switched to mechanically relaxed conditions, capacity for receptor autophosphorylation decreased within 1-2 h and remained low. Based on immunomicroscopic observations and studies on down-regulation of receptors by PDGF binding, it appeared that most PDGF receptors in monolayer or contracted collagen matrix cultures were localized on the cell surface and accessible to PDGF binding. In related studies, we found that EGF receptors of fibroblasts in mechanically relaxed collagen matrices also showed low levels of autophosphorylation in response to EGF treatment. Based on these results, we suggest that mechanical interactions between cells and their surrounding matrix provide regulatory signals that modulate autophosphorylation of growth factor receptors and cell proliferation.

Fibroblasts cultured within or on the surface of a collagen matrix reorganize collagen fibrils and contract the matrix (Bell et al., 1979; Grinnell and Lamke, 1984). After contraction, the cells show a marked decline in DNA synthesis compared to cells in monolayer culture (Sarber et al., 1981; Van Bockxmeer et al., 1984). This decline results in part from a direct effect of collagen on the fibroblasts (Elsdale and Bard, 1972; Schor, 1980; Yoshizato et al., 1985; Nishiyama et al., 1989). In addition, as will be discussed below, decreased proliferation depends on collagen matrix organization.

Collagen matrix organization varies when collagen matrices are anchored or floating during contraction. In the case of anchored matrices, tension exerted by fibroblasts is distributed asymmetrically, and mechanical stress develops during contraction. Collagen fibrils become more closely packed and organized along lines of stress, and fibroblasts spread in a bipolar morphology with prominent actin filament bundles. In the case of floating matrices, tension exerted by fibroblasts is distributed symmetrically and the matrices remain mechanically relaxed during contraction. Collagen fibril density increases without alignment, and fibroblasts spread in a stellate morphology without actin filament bundles (Nakagawa et al., 1989; Mochitate et al., 1991).

Fibroblasts in mechanically stressed collagen matrices continue to proliferate in response to growth factors (Nakagawa et al., 1989). On the other hand, cells in mechanically relaxed matrices become arrested in G0 (Kono et al., 1990) and less responsive to addition of serum or purified growth factors (Fukamizu et al., 1990; Nishiyama et al., 1991). The mechanism underlying decreased responsiveness by fibroblasts in mechanically relaxed collagen matrices to serum stimulation probably involves changes in the PDGF signaling pathway. PDGF is the principle mitogen in serum for mesenchymal cells such as fibroblasts (Ross et al., 1990; Heldin and Westermark, 1990). Binding of PDGF to its receptors causes receptor clustering and activation of receptor tyrosine kinase, which results in autophosphorylation as well as phosphorylation of a group of cytoplasmic signaling proteins (Williams, 1989; Koch et al., 1991). Studies on receptor mutants indicate that receptor tyrosine kinase activity is required for PDGF's mitogenic activity (Mori et al., 1991). Binding of PDGF to PDGF receptors also accelerates receptor internalization and down-regulation (Heldin et al., 1982; Bowen-Pope and Ross, 1982), but this process is separate from mitogenic stimulation (Escobedo et al., 1988; Sorokin et al., 1991).

To better understand the interrelationship between extracellular matrix organization and serum stimulation of
fibroblast proliferation, we compared PDGF responsiveness by fibroblasts in monolayer culture with that of cells in contracted collagen matrix cultures that were either mechanically stressed or relaxed. Our results indicate that the decreased ability of PDGF to stimulate DNA synthesis by fibroblasts in contracted collagen matrices may result from decreased levels of PDGF receptor autophosphorylation.

Materials and Methods

Cell Culture

Maintenance of human foreskin fibroblast monolayer cultures and preparation of hydrated collagen matrices from Vitrogen "100" collagen (Celtix Labs, Palo Alto, CA) have been described previously (Nakagawa et al., 1989). Fibroblasts were added to the neutralized collagen solutions (1.5 mg/ml) at a concentration of 5 x 10^5 cells/ml. Aliquots (0.2 ml) of the cell/collagen mixtures were prewarmed to 37°C for 3-4 min and then placed in 24-well culture plates (Costar Corp., Cambridge, MA). Each aliquot occupied an area outlined by a 12-mm-diameter circular score within a well. Polymerization of a collagen matrices required 60 min at 37°C, after which 1.0 ml of DME (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FBS (Intergen Co., Purchase, NY) and 50 µg/ml ascorbic acid were added to each well. The cultures were maintained in a humidified CO2 incubator (5% CO2, 95% air) for 5 days. Each experiment was performed in triplicate, and the results were normalized by cell number.

DNA Synthesis

PDGF isofrom AB (human recombinant) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). DNA synthesis was measured by thymidine incorporation. Cultures were radiolabeled metabolically for 2 h with 5 µCi/ml [3H]thymidine (20 Ci/mole, New England Nuclear, Boston, MA). At the end of the incubations, the cultures were rinsed twice with PBS (GIBCO-BRL) for 10 min at 37°C. Then 0.3 ml of 0.2% NP-40 in DPBS containing proteinase inhibitors (1 µg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride (HCl)) and phosphatase inhibitors (2 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM ammonium molybdate, 0.2 mM microcystin-LR) was added. Samples were homogenized (50 strokes) with a 1 ml Dounce homogenizer (B pestle; Wheaton Scientific, Millville, NJ) at 4°C, and collagen fibrils and nuclei were removed by centrifugation at 11,000 g (Beckman Microfuge) for 5 min.

During the 5-d culture period, cell number increased most in monolayer culture and least in mechanically relaxed collagen matrices. To compare extracts from different cultures, it was necessary to normalize the samples to contain cell equivalents. Preliminary experiments using cells harvested by trypsin and collagenase (see above) showed that levels of lactate dehydrogenase (measured using the Sigma LD Diagnostic kit) were similar per cell regardless whether the cells were cultured in monolayer or collagen matrices. Therefore, aliquots of the cell extracts were analyzed for lactate dehydrogenase activity, and samples to be subjected to SDS-PAGE were adjusted to equivalent lactate dehydrogenase levels.

For SDS-PAGE, samples were mixed with reduced SDS sample buffer (6.25 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% mercaptoethanol, pH 6.8), subjected to electrophoresis in 6% acrylamide mini-gels (Laemmli, 1971), and transferred to PVDF membranes. After blocking the membranes with 76 mM Na phosphate, 68 mM NaCl, 5% casein, 1% polyvinylpyrrolidone 40 (PVP-40), and 10 mM EDTA for 3 h, the membranes were incubated for 2 h with anti-PDGFR β-receptor antibodies (Upstate Biotechnology Inc.) or anti-PDGF β-receptor antibodies (Upstate Biotechnology Inc.) in blocking solution. The blots were washed with 0.03% Tween 20, 76 mM Na phosphate, and 68 mM NaCl following by incubation for 1 h with alkaline phosphatase-conjugated goat anti–mouse or goat anti–rabbit antibodies in blocking solution. Photoactivation was initiated by addition of 50 mM sodium bicarbonate (pH 9.6), 1 mM MgCl2 and 3-(4-methoxyphenyl)2,4-dioxetane-3,2′-tricyclo-[3.3.1.13,7]decane-4-yl-phosphorylphenolphosphate (AMPPD). Light emitted by activated AMPPD was detected with XAR x-ray film (Eastman Kodak, Rochester, NY). PDGF receptor bands were analyzed using a laser scanning densitometer (Ultrascan XL, LKB Instruments Inc., Bromma, Sweden).

Confluent Microscopy

Cultures were fixed for 10 min at 22°C with 3% paraformaldehyde in DPBS. To block non-specific staining, the samples were treated with 1% BSA (Fraction V; ICN Biochemicals Inc., Irvine, CA) for 30 min. Subsequently, half the cultures were permeabilized with 0.2% NP-40 in DPBS for 10 min. Samples were incubated with anti-PDGF β-receptor antibodies (anti-PDGF β-receptor antibodies in blocking solution. Photoactivation was initiated by addition of 50 mM sodium bicarbonate (pH 9.6), 1 mM MgCl2 and 3-(4-methoxyphenyl)2,4-dioxetane-3,2′-tricyclo-[3.3.1.13,7]decane-4-yl-phosphorylphenolphosphate (AMPPD). Light emitted by activated AMPPD was detected with XAR x-ray film (Eastman Kodak, Rochester, NY). PDGF receptor bands were analyzed using a laser scanning densitometer (Ultrascan XL, LKB Instruments Inc., Bromma, Sweden).

Results

**PDGF-stimulated DNA Synthesis by Fibroblasts in Monolayer and Contracted Collagen Matrix Cultures**

Fig. 1 shows DNA synthesis after PDGF stimulation of fibroblasts in monolayer cultures and collagen matrices (mechanically stressed or relaxed). Cells were cultured in DME/10% FBS for 1 d and then in DME/1% FBS for an additional 3 d. The initial day in 10% serum-containing medium was required to obtain maximal collagen matrix contraction. Mechanically stressed collagen matrices remained attached to underlying culture dishes during contraction. Mechanically relaxed collagen matrices floated in the culture medium during contraction. Culture for 3 d in

1. Abbreviations used in this paper: IGF, insulin-like growth factor; PTPase, protein-tyrosine phosphatase; PVP-40, polyvinylpyrrolidone 40.
Figure 1. PDGF-stimulated DNA synthesis by fibroblasts in monolayer culture and contracted collagen matrices. Fibroblasts were cultured 1 d in DME/10% FBS followed by 3 d in DME/1% FBS in monolayer culture, in collagen matrices that were anchored to culture dishes (mechanically stressed after contraction), or in collagen matrices that were floating in medium (mechanically relaxed after contraction). At the end of the 4-d culture period, PDGF was added to the cultures at the concentrations indicated. DNA synthesis was measured 22 h later by \(^{3}H\)thymidine incorporation.

Regardless of the culture conditions (i.e., monolayer or collagen matrix), DNA synthesis by fibroblasts reached highest levels after 0.5-1 ng/ml PDGF stimulation. The different culture conditions resulted in marked differences, however, in the magnitude of the cellular responses. Fibroblasts in mechanically relaxed collagen matrices showed 80-90% lower levels of PDGF-stimulated DNA synthesis compared to cells in monolayer culture. Fibroblasts in mechanically stressed collagen matrices showed levels of PDGF-stimulated DNA synthesis that were intermediate. Further increases in DNA synthesis were not observed even at PDGF concentrations as high as 100 ng/ml PDGF. In fact, at this level, DNA synthesis was 10-30% lower than with 1 ng/ml PDGF (data not shown).

PDGF Receptor Levels in Fibroblasts in Monolayer and Contracted Collagen Matrix Cultures

Decreased responsiveness to PDGF by fibroblasts in mechanically relaxed collagen matrices might have resulted from a decrease in the number of PDGF receptors. Fig. 2 (see also Figs. 7 and 10) compares PDGF receptor levels in cells harvested from monolayer (M), mechanically stressed (S), and mechanically relaxed (R) matrices after culture for 1 d in DME/10% FBS and 4 d in DME/1% FBS. Cells were extracted by detergent treatment and mechanical disruption, and extracts were centrifuged to remove collagen fibrils and cell nuclei. Under all three culture conditions, the major polypeptide detected by immunoblotting with polyclonal anti-PDGF \(\alpha/\beta\)-receptor antibodies was \(~180\ kD\) (arrow), which corresponded to PDGF \(\beta\) receptors that predominate in fibroblasts (Kazlauskas et al., 1988; Seifert et al., 1989).

Most of the receptors were solubilized during the extraction procedure since the pellets contained little antibody-reactive material (data not shown). Polypeptides \(~160-170\ kD\) occurred variably from experiment to experiment, and these probably corresponded to PDGF \(\alpha\) receptors and/or PDGF \(\beta\) receptor precursors. In general, no significant differences in receptor levels were observed regardless of cell culture conditions.

PDGF-stimulated Receptor Autophosphorylation by Fibroblasts in Monolayer and Contracted Collagen Matrix Cultures

Since fibroblasts in collagen matrix cultures accumulated similar levels of PDGF receptors, decreased PDGF-dependent mitogenic responsiveness by fibroblasts in mechanically relaxed matrices could not be explained in terms of receptor numbers. Next, we studied the ability of PDGF to stimulate receptor autophosphorylation. Fig. 3 A shows dose–response studies for PDGF stimulation of tyrosine kinase activity detected by immunoblotting with anti-phosphotyrosine antibodies, and quantification of the 180-kD PDGF receptor band by scanning densitometry can be found in Fig. 3 B. In the absence of PDGF stimulation, little staining by the antibodies was observed. After PDGF stimulation, cells in mechanically relaxed matrices showed the lowest levels of autophosphorylation. For instance, at 10 ng/ml PDGF, there was a 90% lower level of autophosphorylation by fibroblasts in mechanically relaxed collagen matrices compared to monolayer cells. Even at 100 ng/ml PDGF, autophosphorylation by fibroblasts in mechanically relaxed collagen matrices was markedly suppressed.

In the experiment described in Fig. 3, fibroblasts were stimulated with PDGF for 10 min at 37°C. It was possible that the different levels of autophosphorylation occurred because different times were required for maximal stimulation. This was not the case, however, as shown in Fig. 4. After the addition of 50 ng/ml PDGF, peak phosphorylation occurred by 10 min (Fig. 4 A), and analysis by scanning densitometry (Figure 4B) showed that the pattern of activation and subse-
Figure 3. Dose response of PDGF-stimulated receptor autophosphorylation by fibroblasts in monolayer and contracted collagen matrix cultures. Fibroblasts were cultured 1 d in DME/10% FBS followed by 4 d in DME/1% FBS in monolayer culture (M), in collagen matrices that were anchored to culture dishes (mechanically stressed after contraction) (S), or in collagen matrices that were floating in medium (mechanically relaxed after contraction) (R). At the end of the 5-d culture period, cultures were stimulated with PDGF at the concentrations indicated for 10 min at 37°C, and cells extracts were prepared by homogenization in DPBS containing 0.2% NP-40 and proteinase and phosphatase inhibitors. Samples were subjected to SDS-PAGE, and phosphorytrosine containing polypeptides were detected by immunoblotting with anti-phosphotyrosine antibodies. Equivalent cell numbers were loaded onto each lane. (A) Immunoblotting; arrow, 180-kD PDGF receptors. (B) Results in A analyzed by scanning densitometry.

Consequent decline in receptor phosphorylation levels was similar for monolayer and collagen matrix cultures.

Changes in PDGF Receptor Autophosphorylation as a Function of Receptor Synthesis and Collagen Matrix Organization

The foregoing results indicated that the PDGF receptors of fibroblasts in collagen matrices—especially mechanically relaxed matrices—had low autophosphorylation levels in response to PDGF compared to cells in monolayer culture. Since activation of PDGF tyrosine receptor kinase activity has been shown to be required for PDGF mitogenic stimulation (Mori et al., 1991), the decreased ability of fibroblasts in collagen matrices to respond to PDGF by receptor autophosphorylation may explain decreased PDGF stimulation of DNA synthesis by these cells.

In the experiments described above, fibroblasts were incubated in collagen matrices for 5 d and then receptor autophosphorylation was determined. Additional studies were carried out to compare the timing of PDGF receptor expression with the capacity for PDGF-stimulated autophosphorylation of the receptors. Fig. 5 shows that after the first day of culture, during which cells were in medium containing 10% FBS, only a low level of residual receptors were observed. Subsequently, after cells were switched to medium containing 1% FBS, PDGF receptor levels increased reaching a maximum at day 3–4 in all the cultures.

Fig. 6 shows that receptor phosphorylation occurred for fibroblasts in monolayer cultures and in mechanically stressed matrices. In mechanically relaxed matrices, however, even newly appearing PDGF receptors lacked the capacity to be
autophosphorylated in response to PDGF. This result suggested that receptor autophosphorylation did not occur initially and then disappear after culture. Rather, loss of autophosphorylation appeared to be a function of mechanical forces on the cells. To test this possibility more directly, collagen matrices were cultured 5 d allowing stress to develop and then switched to floating conditions allowing stress-relaxation. After 1 h (data not shown) or 2 h (Fig. 7A), there was a marked decrease in PDGF-stimulated receptor autophosphorylation with no change in PDGF receptor levels (Fig. 7B). Similar results were observed after release for 2 d. A decrease in PDGF-stimulated receptor autophosphorylation could be detected as early as 5 min after release but the result was variable compared to release for 1-2 h. These findings show that a change in the capacity for receptor autophosphorylation occurred when the cells were switched from mechanically stressed to mechanically relaxed conditions, i.e., what has been previously called “stress-relaxation” (Mochitate et al., 1991).

**Localization of PDGF Receptors at the Cell Surface**

Although changes in total receptor number could not account for decreased receptor autophosphorylation by fibroblasts in mechanically relaxed matrices, there could have been differences in PDGF receptor accessibility. For instance, fibroblasts in mechanically relaxed matrices may have internalized their PDGF receptors thereby making the receptor unaccessible to PDGF stimulation. Ideally, ^4^C-binding studies with radiolabeled PDGF would have determined the number of accessible receptors. For fibroblasts in mechanically stressed or relaxed collagen matrices, however, saturable binding of PDGF did not occur even at PDGF concentrations as high as 500 ng/ml. Moreover, nonsaturable binding of PDGF to contracted collagen matrices occurred after the preparations were extracted with NP-40-containing buffer. On the other hand, parallel studies with cells in monolayer culture showed typical binding kinetics with half-maximal PDGF binding ~14 ng/ml and binding was lost after NP-40 treatment (data not shown).

Since direct PDGF binding studies were uninformative for the reasons described above, anti-PDGF receptor antibodies were used as an alternative approach to determining accessibility of PDGF receptors on fibroblasts in collagen matrices. Fig. 8 shows the distribution of PDGF receptors in fibroblasts in mechanically stressed or relaxed matrices observed by immunostaining and confocal microscopy. Non-permeabilized cell preparations (Fig. 8, A and C) were used to ob-
serve receptors on the cell surface, whereas permeabilized cell preparations (Fig. 8, B and D) were used to detect both cell surface and intracellular receptors. Control incubations using non-immune IgG in place of anti-receptor antibodies showed little background staining (data not shown). In general, most receptors appeared to be present on the cell surfaces since the staining pattern was similar with (Fig. 8, B and D) or without (Fig. 8, A and C) permeabilization. Moreover, the distribution of PDGF receptors on fibroblasts in mechanically relaxed matrices (Fig. 8, C and D) was indistinguishable from that observed on cells in mechanically stressed matrices (Fig. 8, A and B). Therefore, the cell surface distribution of PDGF receptors did not appear to change after stress--relaxation.

As an additional control, experiments were carried out to determine if antibodies bound to PDGF receptors on fibroblasts in collagen matrices could be internalized. After an initial 20-min incubation with anti-receptor antibody, the preparations were washed and incubated an additional 80 min at 37°C. Subsequently, the cells were fixed and stained with secondary antibodies. In this case, non-permeabilized cell preparations (Fig. 9, A and C) were used to localize anti-receptor antibodies remaining on the cell surface, whereas permeabilized cells (Fig. 9, B and D) were used to identify cell surface and internalized anti-receptor antibodies. There was decreased staining of anti-receptor antibodies localized on the cell surfaces (Fig. 9, A and C), compared to anti-receptor antibodies observed in permeabilized cells (Fig. 9, B and D). Therefore, the anti-receptor antibodies originally bound at the cell surface appeared to have been internalized by fibroblasts in mechanically stressed or relaxed matrices. Similar results were found with fibroblasts in monolayer culture (data not shown).

The above results showed by immunostaining procedures that PDGF receptors on fibroblasts in mechanically stressed or relaxed collagen matrices were accessible on the cell surface. Since down-regulation of PDGF receptors requires PDGF binding to the receptor but occurs independently of autophosphorylation (Escobedo et al., 1988; Sorkin et al., 1991), it was possible to compare receptor down-regulation of fibroblasts in mechanically stressed and relaxed collagen matrices as another indicator of cell surface accessibility of PDGF receptors. To make this comparison, fibroblasts in monolayer or collagen matrix cultures were treated 24 h with 100 ng/ml PDGF in the presence of 10 µg/ml cycloheximide to prevent new receptor synthesis. Subsequently, PDGF receptors were analyzed by immunoblotting. Fig. 10 shows that under all culture conditions, PDGF receptors (arrow) were markedly reduced in cells that were treated with PDGF. Therefore, most PDGF receptors of fibroblasts in mechanically relaxed collagen matrices were available for and responsive to PDGF binding.

**EGF-stimulated Receptor Autophosphorylation by Fibroblasts in Monolayer and Contracted Collagen Matrix Cultures**

Finally, to determine if the change in PDGF receptor auto-
phosphorylation was unique to PDGF receptors or if other growth factor receptors were altered as well, we analyzed EGF-stimulated autophosphorylation by fibroblasts in mechanically relaxed collagen matrix cultures. Like PDGF, EGF binding to its receptor activates the receptor's kinase activity (Ullrich and Schlessinger, 1990; Carpenter and Cohen, 1990). Fig. 11 shows autophosphorylation of 170-kD EGF receptors (arrow) detected by anti-phosphotyrosine antibodies after stimulation of cells with 0–20 ng/ml EGF for 10 min at 37°C. Fibroblasts in mechanically relaxed collagen matrices showed little autophosphorylation compared to fibroblasts in monolayer culture or mechanically stressed collagen matrices. In other experiments, we found that, as was observed for PDGF receptors, the change in capacity for EGF stimulated autophosphorylation could be detected within 1 h after stress-relaxation (data not shown). Therefore, autophosphorylation of a second growth factor receptor appeared to be unresponsive to growth factor-stimulation when the cells were switched from mechanically stressed to mechanically relaxed collagen matrices.

Discussion

The goal of our studies was to understand the interrelationship between extracellular matrix organization and fibroblast proliferation in response to growth factors. Culturing fibroblasts in mechanically relaxed (i.e., floating) collagen matrices induces a quiescent cell state (Sarber et al., 1981; Van Bockxmeer et al., 1984; Kono et al., 1990), whereas fibroblasts in mechanically stressed collagen matrices proliferate in response to serum factors (Nakagawa et al., 1989; Mochitate et al., 1991).

Since PDGF is the principle mitogen in serum for mesenchymal cells such as fibroblasts (Ross et al., 1990; Heldin and Westermark, 1990), we compared PDGF-stimulated mitogenic response by fibroblasts in monolayer culture with that of cells in contracted collagen matrices that were mechanically stressed or relaxed. PDGF-stimulated DNA synthesis by fibroblasts in mechanically relaxed collagen ma-
ternatively, PDGF may have been sequestered in the collagen min for cells in collagen matrices and monolayer culture. Al-
cneically relaxed collagen matrices could have resulted
ture, and 50% lower than that of cells in mechanically
trices was 80-90% lower than that of cells in monolayer cul-
ty of these cells to respond to PDGF stimulation.

The low level of responsiveness to PDGF by fibroblasts in
reduced collagen matrices could have resulted from
duced PDGF diffusion, but this seemed unlikely since PDGF-stimulated autophosphorylation peaked at 10
in cells in collagen matrices and monolayer culture. Alter-
atively, PDGF may have been sequestered in the collagen matrix. Components of the extracellular matrix have been shown to bind growth factors and regulate their activity (Gospodarowicz and Cheng, 1976; Vlodavsky et al., 1987; Ruoslahti and Yamaguchi, 1991), and PDGF has been re-
ported to interact with heparin (Fager et al., 1992) and the
glycoprotein SPARC (Raines et al., 1992). If a portion of the
PDGF were sequestered in the matrix, then we would have
expected differences in the concentrations required for op-
timal stimulation and saturation of the cellular response to
PDGF. Since fibroblast DNA synthesis reached maximal
levels after stimulation of cells with 0.5-1 ng/ml PDGF stim-
ulation, and further increases in DNA synthesis were not ob-
erved even at PDGF concentrations as high as 100 ng/ml
PDGF, it seemed unlikely that PDGF was sequestered.
Therefore, we suspected that fibroblasts in collagen ma-
tices, especially mechanically relaxed matrices, were de-
cient in some aspect of their PDGF signaling pathway.

We found marked differences between fibroblasts in mono-
layer and contracted collagen matrix cultures in the ability of the cells' PDGF receptors to become autophosphorylated in response to PDGF. Cells in mechanically relaxed matrices showed the poorest autophosphorylation response, 90% lower than cells in monolayer culture. Even at 100 ng/ml PDGF, the extent of autophosphorylation was 60% lower for cells in mechanically relaxed matrices compared to cells in monolayer culture. When fibroblasts under mechanical stress were switched to mechanically relaxed conditions, the cells lost the capacity to become phosphorylated within 1-2 h. Loss of autophosphorylation could not be attributed to PDGF receptor numbers since immunoblotting studies showed no significant differences in PDGF receptor levels regardless of culture conditions, and no differences in receptor accessibility on the cell surface were observed by immu-
nostaining studies and down-regulation studies.

The various assays for PDGF-stimulated autophosphory-
lation and mitogenesis require different time periods and
different concentrations of PDGF, which makes direct com-
parisons impossible. Nevertheless, given the correlation be-
tween decreased autophosphorylation and mitogenesis in
mechanically relaxed collagen matrices and previous studies showing that PDGF stimulated mitogenesis required PDGF receptor autophosphorylation (Williams, 1989; Mori et al., 1991; Koch et al., 1991), it seems likely that in mechanically
relaxed collagen matrices fibroblasts become quiescent at least in part because their PDGF receptors no long can be-
come autophosphorylated and initiate subsequent down-
stream signaling events.

The cause of decreased autophosphorylation of PDGF
receptors in mechanically relaxed collagen matrices is un-
known. Since EGF receptors showed a similar response to
PDGF receptors, it seems likely that some sort of generic
effect is involved. One possibility is activation or de-repres-
sion of a protein-tyrosine phosphatase (PTPase). PTPases
have been implicated in controlling protein phosphotyrosine
levels (Hunter, 1989; Fischer et al., 1991), and a membrane
tyrosine phosphatase has been implicated in growth arrest
caused by cell contact (Pallen and Tong, 1991). Typically,
growth factor–stimulated receptor autophosphorylation ac-
tivates receptor kinase function leading to downstream
events. Therefore, PTPases could play a role in growth regu-
lation by dephosphorylating growth factor receptors thereby
lowering their kinase activity (Shenolikar and Nairn, 1991).
Recently, this hypothesis has been tested directly by trans-
fected a mouse mammary tumor cell line with transmem-
brane PTPase CD45 (Mooney, et al., 1992). In response to
PDGF or insulin-like growth factor (IGF-I), transfected cells
showed markedly reduced levels of receptor autophosphory-
lalion and the mitogenic response of the cells was decreased
as well.

Regardless whether increased PTPases or some other
mechanism turns out to account for decreased growth factor
receptor autophosphorylation by fibroblasts in collagen ma-
trices, the effect appeared to be closely linked to mechanical
stress on the cells. When mechanically stressed collagen ma-
trices were switched to mechanically relaxed conditions, ca-
cacity for receptor autophosphorylation decreased within
1-2 h and remained low. One possibility described below is
that changes in integrin receptor occupancy accompanying
stress–relaxation provide the initial regulatory signals that
lead to subsequent alteration in the capacity for growth fac-
tor receptor autophosphorylation.

At least two types of integrin-mediated interactions have
been implicated in fibroblast interactions with collagen ma-
trices. On one hand, cell to collagen binding required for

Figure 11. Dose response of EGF-stimulated receptor autophos-
phorylation by fibroblasts in monolayer and contracted collagen
matrix cultures. Fibroblasts were cultured 1 day in DMEM/10%
FBS followed by 4 days in DME/1% FBS in monolayer culture (M),
in collagen matrices that were anchored to culture dishes (mechani-
cally stressed after contraction) (S), or in collagen matrices that
were floating in medium (mechanically relaxed after contraction)
(R). At the end of the 5-d culture period, cultures were stimulated
with EGF at the concentrations indicated for 10 min at 37°C, and
cells extracts were prepared by homogenization in DPBS contain-
ing 0.2% NP-40 and protease and phosphatase inhibitors. Sam-
plewere subjected to SDS-PAGE, and phosphorytrosine containing
polypeptides were detected by immunoblotting with anti-phospho-
tyrosine antibodies. Equivalent cell numbers were loaded onto each
lane. Arrow, 170-kD EGF receptors.
contraction has been shown to depend on α2β1 integrins (Schiro et al., 1991; Klein et al., 1991). In addition, however, fibroblasts in contracted collagen matrices develop a cell surface fibronectin matrix and fibronexus junctions (Mochitate et al., 1991; Tomasek et al., 1992), which probably involve α5β1 integrins. Allowing mechanically stressed matrices to relax results in disappearance of the junctions along with loss of cell surface fibronectin (Mochitate et al., 1991; Tomasek et al., 1992). Therefore, when fibroblasts in contracted collagen matrices are switched from mechanically stressed to mechanically relaxed conditions, their α5β1 integrin receptors may become disengaged. Since adhesion of integrin receptors can trigger a tyrosine kinase cascade (Guan et al., 1991; Kornberg et al., 1992), differences in integrin receptor engagement by cells within mechanically stressed or relaxed collagen matrices have the potential to provide a mechanically sensitive regulatory mechanism.

The importance of mechanical force for cell function pertains to a wide range of biological organisms including bacteria and plants. Recent interest in this subject is indicated by reviews on “tensegrity” (Ingber and Folkman, 1989), “mechanogenetic” (Erdos et al., 1991), and “mechanogenic” (Vandenburgh, 1992) models of cell growth regulation. Even the well-known dependence of cell growth on cell shape (Folkman and Moscona, 1978) may turn out to be an effect of cell tension (Curtis and Seeher, 1978; O’Neill et al., 1990). Our results suggest a novel mechanism by which cell proliferation can be regulated by reciprocal mechanical forces exerted between the cells and their surrounding matrix. As described below, such a mechanism may play an important role in the regulation of fibroplasia during wound repair.

In the skin, dermal fibroblasts are quiescent, biosynthetically active cells. Within days after cutaneous wounding, fibroblasts migrate towards the fibronectin-fibrin wound interface where granulation (i.e., wound) tissue forms. The activated fibroblasts proliferate and synthesize a new collagen-containing matrix (Peacock, 1984; Grinnell, 1984; Clark, 1985). In addition, fibroblasts in granulation tissue exert tension on the extracellular matrix, develop stress, and contract the matrix thereby bringing the wound margins moving close together (Gabbiani et al., 1972; Singer et al., 1984; Eddy et al., 1988; Welch et al., 1990). Once the wound defect is replaced, the expanded fibroblast population stops dividing and regresses and extracellular matrix remodeling begins.

Activation of fibroblasts during the early stages of repair is believed to depend on locally released growth factors such as PDGF (Ross et al., 1986). Little is known, however, about fibroblast growth at the end of wound healing. Our studies suggest that once new matrix replaces the wound and the tissue becomes mechanically relaxed, fibroblasts would no longer be able to respond PDGF and perhaps other growth factors as well. Consequently, cessation of cell proliferation would occur even in the continued presence of potentially activating growth factors. Consistent with this interpretation, wounds that heal under tension show increased fibroplasia (Bunting and Eades, 1926; Burgess et al., 1990).

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