LPA and PDGF Differentially Regulate Contraction of Collagen Matrices by Human Fibroblasts; the Role of Myosin Light Chain Phosphorylation.

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2000

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General Introduction

Closure of cutaneous wounds involves three fundamentally different processes: epithelization, connective tissue deposition and contraction. In general, epithelization results in resurfacing of the wound; connective tissue deposition serves as the replacement of damaged dermis; and contraction brings the margins of open wounds together (Peacock, 1984; Clark, 1988). Wound contraction is especially important in humans whose skin is firmly attached to underlying tissues. As a consequence of tension the result of contraction can vary from minimal cosmetic scar to major deformation with a loss of joint motion. Therefore, distinction has been made between contraction as a normal process, and contracture as the abnormal result of wound closure (Hunt and Dunphy 1979). Moreover, in some pathologic conditions the general malfunction of wound healing has been found (diabetes, chronic ulcus cruris), while in case of other diseases hyperactive and prolonged repair mechanisms have been reported (keloids, Dupuytren's contacture, lung and liver fibrosis).

Signal transduction pathways which dynamically regulate contraction and wound repair are only beginning to be understood. Therefore, we wanted to learn more about downstream signalling events in order to get closer to the possible therapeutic intervention. We focused our research on the human wound fibroblast, which considered to have a key role in contraction, and in case of numerous connective tissue diseases.

Introduction and Aims

To learn more about the mechanisms of matrix reorganization involved in wound contraction, we have been studying model systems in which fibroblasts contract collagen matrices in vitro. Depending upon whether the matrices are mechanically unloaded or loaded, contraction occurs, respectively, as a consequence of cell migration or smooth muscle-like contraction. We have showed before that in the case of mechanically unloaded (i.e. "floating") collagen matrices, platelet-derived growth factor (PDGF) and

lysophosphatidic acid (LPA) stimulate contraction by different signalling pathways. LPA-dependent contraction was selectively inhibited by pertussis toxin (PTx), whereas PDGF-dependent contraction was selectively inhibited by the protein kinase inhibitor KT5926. KT5926 has been reported to block myosin light chain (MLC) kinase, the enzyme that promotes muscle contraction by phosphorylating MLC. A variety of studies have implicated MLC phosphorylation in the contractile activity of non-muscle cells. One interpretation of our previous observations, therefore, was that PDGF (but not LPA) stimulated contraction by activating MLC kinase leading to MLC phosphorylation. A role for MLC kinase and MLC phosphorylation also was suggested based on the ability of cyclic AMP to inhibit collagen matrix contraction and the assumption that the mechanism of inhibition involved negative regulation of MLC kinase by cyclic AMP-dependent protein kinase. In the present studies, we examined more directly the relationship between MLC phosphorylation and the contraction of floating collagen matrices. Contrary to the above hypothesis, our data suggest that PDGF and LPA stimulation of contraction can be uncoupled from an increase in MLC phosphorylation.

Experimental Procedures

Cell Culture

Fibroblasts from human foreskin specimens (<10th passage) were maintained in Falcon 75-cm2 tissue culture flasks in DMEM supplemented with 10% fetal bovine serum. Fibroblasts were harvested from monolayer culture with 0.25% trypsin and 1 mM EDTA. Trypsin was neutralized with soybean trypsin inhibitor (3.3 mg/ml). For contraction experiments, collagen matrices containing cells were polymerized as described below. For monolayer culture experiments, harvested cells were incubated for 60 min at 37 °C in Falcon T-75 flasks (5 ml, 105 cells/ml) or on 22-mm glass coverslips (0.5 ml, 10^5cells/ml). The culture flasks and coverslips were previously coated for 20 min with 20 μg/ml fibronectin or 50 μg/ml collagen 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). To load fibroblasts in monolayer culture with PTx, the cells were incubated overnight in DMEM and 10% fetal bovine serum containing PTx at the concentrations indicated.

Collagen Matrix Contraction

Contraction of floating collagen matrices was carried out as described previously (8). Neutralized solutions of Vitrogen "100" collagen (1.5 mg/ml) were prepared containing fibroblasts (10^6 cells/ml) in DMEM without serum. The cell/collagen mixture was prewarmed to 37 °C for 3-4 min, and 0.2-ml aliquots were placed in Corning 24-well culture plates. Each aliquot occupied an area outlined by an 11-mm diameter circular score within a well. Polymerization of collagen matrices required 60 min at 37 °C. To initiate matrix contraction, matrices were released gently from the underlying culture dish with a spatula into 0.5-1 ml of serum-free DMEM containing 5 mg/ml bovine serum albumin with growth factors and inhibitors added as indicated, after which the matrices were incubated at 37 °C. To determine the extent of floating matrix contraction, 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 20 min at 22 °C. The matrices were washed, placed on a flat surface, and measured with a ruler. For quantitative purposes, contraction data are presented as the change in diameter (starting final) measured in mm. All experiments were carried out in duplicate, and every experiment was repeated two or more times. Data points and error bars in the figures represent averages and standard deviations. Where error bars cannot be seen, the data points overlapped.

Myosin Light Chain Phosphorylation

MLC phosphorylation was determined using the urea/glycerol-PAGE method. Cells in T-75 flasks were treated with ice-cold 10% (w/v) trichloroacetic acid containing 10 mM

dithiothreitol, scraped off the dishes, and homogenized on ice using ~100 strokes with a Dounce homogenizer (Wheaton, tight pestle). After centrifugation at 8,000 rpm (Beckman Microfuge) for 1 min at 4 °C, the pellets were washed three times with diethylether, dissolved in urea-sample buffer (10 mM dithiothreitol, 0.004% bromphenol blue, 8 M urea, 20 mM Tris, and 23 mM glycine, pH 8.6), and resuspended using a water bath sonicator (Branson 2210) for 10 min. Samples were subjected to urea/glycerol-PAGE using 10% gels. Transfer to polyvinylidene difluoride was carried out for 2 h at 50 V. Blots were fixed with 0.4% glutaraldehyde in PBS for 30 min at 22 °C and washed three times in PBS, blocked with 5% milk in TTBS (0.1%Tween 20, 150 mM NaCl, 20 mM Tris, pH 7.5), and then incubated with anti-MLC monoclonal antibody in blocking solution at 4 °C for 12 h. After washing in TTBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG in 3% bovine serum albumin and TTBS at 22 °C for 2 h and then visualized by enhanced chemiluminescence. Quantitative evaluation of the nonphosphorylated, monophosphorylated, and diphosphorylated MLC was performed by densitometric analysis using NIH Image.

Fluorescence Microscopy of the Actin Cytoskeleton

Cells on coverslips were fixed for 20 min at 22 °C with 3% paraformaldehyde in DPBS, blocked with 1% glycine and 1% bovine serum albumin in DPBS for 30 min, and permeabilized with 0.2% Nonidet P-40 in DPBS for 10 min. To stain for actin, samples were incubated with rhodamine-conjugated phalloidin (8 units/ml) for 30 min at 37 °C. After washing in DPBS, slides were mounted with Fluoromount G. Images were observed and captured using a Olympus BH2-RFCA fluorescence microscope equipped with a Hamamatsu C5985-02 cooled CCD camera.

Results

Cyclic AMP Selectively Inhibits PDGF-dependent Matrix Contraction

In a typical floating matrix contraction experiment increasing concentrations of forskolin inhibited PDGF-dependent matrix contraction in a dose-dependent manner. Forskolin also inhibited basal contraction, but had little effect on LPA-induced contraction A similar pattern of inhibition was observed when the cells were treated with dibutyryl cyclic AMP rather than forskolin.

Growth Factor Stimulation of Contraction Does Not Correlate with MLC Phosphorylation

Experiments were then carried out to learn if cyclic AMP blocked PDGF-dependent contraction through an effect on MLC phosphorylation. In the absence of growth factors, the basal level of MLC phosphorylation was 10-20%. In response to PDGF stimulation, MLC phosphorylation typically decreased slightly. Even if MLC phosphorylation was measured 5 min after the addition of PDGF, no increase was detected. Therefore, the PDGF signalling pathway in human fibroblasts did not appear to be linked to MLC phosphorylation, which argued against a role for MLC kinase in PDGF-dependent stimulation of collagen matrix contraction. In marked contrast, incubation of cells with LPA resulted in a stimulation of MLC phosphorylation with increased levels of both monophosphorylated and diphosphorylated forms of the molecule. In this case, prior forskolin treatment decreased the stimulatory effect of LPA, even though forskolin had little effect on LPA-stimulated contraction. Therefore, although LPA stimulated MLC phosphorylation, the relationship of this stimulation to collagen matrix contraction was questionable.

Forskolin-induced Arborization of the Actin Cytoskeleton Was Reversed by LPA but Not by PDGF

To correlate the changes in MLC phosphorylation with organization of the cells' actin cytoskeleton, observations also were made on fibroblasts that were fixed and stained with phalloidin. In the absence of added growth factors, fibroblasts were able to attach, spread, and form some stress fibers over 2 h. PDGF increased membrane ruffling. LPA increased actin stress fiber formation. Treatment of the cells with forskolin caused the actin cytoskeleton to develop an arborized appearance. Incubation of forskolin-treated cells with LPA but not PDGF was able to reverse the arborized morphology, although the density of actin stress fibers was less than that observed in cells treated with LPA alone.

Pertussis Toxin Inhibits LPA-dependent Contraction without Blocking LPA-dependent MLC Phosphorylation

The ability of LPA to promote MLC phosphorylation and assembly of stress fibers is consistent with previous studies that demonstrated an LPA-activated, Rho-dependent mechanism of contraction. Rho-dependent contraction has been shown to be PTx-insensitive, however, whereas LPA-dependent contraction of floating collagen matrices was PTx-sensitive. Therefore, experiments were carried out to compare the effects of PTx treatment on matrix contraction and MLC phosphorylation. Prior treatment of fibroblasts with PTx selectively inhibited LPA-dependent contraction but had little effect on PDGF-dependent contraction. On the other hand, unlike forskolin, PTx did not alter the ability of LPA to stimulate MLC phosphorylation. Other experiments demonstrated that PTx treatment did not alter the ability of cells to spread and form stress fibers. These findings suggested that LPA stimulated two sets of receptors, one of which was part of a PTx-sensitive pathway required for matrix contraction, and the other part of a PTx-insensitive pathway leading to MLC phosphorylation and stress fiber formation.

Blocking Phosphatidylinositol 3-Kinase (PI3 Kinase) Selectively Inhibits PDGF-dependent Matrix Contraction

The foregoing results suggested that PDGF and LPA stimulation of contraction could be uncoupled from increased MLC phosphorylation. Consequently, we began to examine the role of other signalling pathways in contraction. We showed before that cell membrane ruffling increased in response to PDGF. Because PDGF-dependent ruffling requires activation of the enzyme PI3-kinase, we tested the possibility that PI3-kinase stimulation might be required for contraction. Wortmannin and LY294002, which blocked PI3-kinase activity, blocked PDGF-dependent collagen matrix contraction. In marked contrast, neither wortmannin nor LY294002 interfered with LPA-dependent collagen matrix contraction. These results suggested a role for PI3-kinase in PDGF- but not LPA-dependent contraction.

Discussion

Our studies were carried out to investigate the relationship between PDGF and LPA-dependent matrix contraction and the ability of these growth factors to stimulate MLC phosphorylation. As discussed in more detail below, increased MLC phosphorylation is neither necessary for PDGF-dependent matrix contraction nor sufficient for LPA-dependent contraction. On the other hand, increased MLC phosphorylation appears to be correlated with formation of stress fibers by cells spreading in monolayer culture. Our findings suggest that the signal transduction pathways required for PDGF- and LPA-dependent matrix contraction involve PI3-kinase and the Gi class of heterotrimeric G proteins, respectively, whereas LPA-dependent stress fiber formation and MLC phosphorylation involve the small G protein Rho. Overall, the results suggest that growth factor-dependent contraction of floating collagen matrices is uncoupled from an increase in MLC phosphorylation. They do not exclude the possibility, however, that MLC phosphorylation plays a permissive role in contraction. A role for MLC

phosphorylation in collagen matrix contraction was suggested previously based on inhibition of contraction by cyclic AMP. The current studies confirm that cyclic AMP can regulate contraction, at least contraction dependent on PDGF, but the mechanism of regulation does not appear to require MLC phosphorylation, because an increase in MLC phosphorylation did not occur when the cells were treated with PDGF. In fact, PDGF treatment caused a slight decrease in MLC phosphorylation, at least transiently. It should be noted that fibroblasts in collagen matrices can be stimulated to increase their level of MLC phosphorylation when they exert increased isometric force, and under these conditions the cells form prominent stress fibers. In marked contrast, fibroblasts in floating collagen matrices remain mechanically unloaded throughout contraction, unable to form stress fibers or fibronexus junctions. Cell membrane ruffling stimulated by PDGF may play an important role in the migratory activity required for floating matrix contraction. PDGF-induced membrane ruffling depends on PI3-kinase, and blocking PI3-kinase also prevented matrix contraction. At high concentrations of PDGF, the inhibition by PI3-kinase inhibitors appeared to be overcome, at least partially. Interestingly, smooth muscle contraction that is independent of MLC phosphorylation has been reported to involve the protein kinase PAK, and PAK has been implicated in cell membrane ruffling as well. Unlike PDGF, LPA stimulated both matrix contraction and MLC phosphorylation. The evidence suggests that different signalling pathways were involved, however. LPA activates receptors linked to PTx-sensitive (Gi) and -insensitive (Gq and G12/13) heterotrimeric G proteins. Matrix contraction appeared to require the former; MLC phosphorylation the latter. LPA-dependent, PTx-insensitive, MLC phosphorylation and stress fiber formation have been shown to depend on activation of the small G protein Rho by G13-coupled LPA receptors. In agreement with these previous findings, we observed a correlation between MLC phosphorylation and stress fiber formation; that is, LPA stimulated both MLC phosphorylation and stress fiber formation. Moreover, both the basal level of MLC phosphorylation and stress fiber formation were decreased when cells were treated with forskolin, presumably a result of

negative regulation of Rho. As in the case of smooth muscle, where agonist-stimulated MLC phosphorylation is transient although contractile force is sustained, LPA stimulation of MLC phosphorylation in fibroblasts appeared to decline by 4 h after stimulation. In marked contrast to the above pathway, little is known about the LPA-dependent, PTx-sensitive mechanism of floating matrix contraction other than the presumed involvement of the Gi class of heterotrimeric G proteins. Whatever the precise mechanism, the pathway also is likely to be important in other aspects of fibroblast migration and chemotaxis. This pathway clearly differs from routine cell spreading and stress fiber formation, however, because fibroblasts were able to spread and form stress fibers on protein-coated surfaces under conditions that matrix contraction did not occur (e.g. in the absence of added growth factors or with the combination of LPA and PTx). Cyclic AMP inhibition of PDGF but not LPA-dependent matrix contraction provided further evidence that these growth factors regulate collagen matrix contraction by different signalling mechanisms. The site at which cyclic AMP regulates PDGF-dependent contraction is likely downstream of PI3-kinase. We cannot exclude, however, the possibility that cyclic AMP regulation of PDGF-dependent contraction also occurs at the level of Rho because PDGF-dependent contraction was inhibited by C3 exotransferase. In summary, our studies suggest that extracellular matrix reorganization leading to contraction of floating collagen matrices occurs by a cell migratory mechanism that can be uncoupled from MLC phosphorylation. Although much is known about the regulatory mechanisms that control cell migration, most of our understanding comes from monolayer experiments in which there is a competition between adhesion and migration. In mechanically unloaded collagen matrices, where stress fibers and fibronexus junction cannot form, far different constraints on cell migration likely apply.

Publications

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