Lysophosphatidic Acid Enhances Fibronectin Binding to Adherent Cells

William J. Checovich, Deane F. Mosher

1-Oleoyl lysophosphatidic acid (LPA) enhanced binding of 125I-labeled fibronectin by cultured MG-63 osteosarcoma cells and human fibroblasts in monolayer cultures up to threefold over control levels. For osteosarcoma cells, LPA was minimally active at 0.1 ng/mL (0.2 nmol/L) and reached maximal activity at 10 ng/mL (20 nmol/L). Increased binding was evident within 10 minutes of treatment of cycloheximide-treated cells with LPA and was due to an increase in the number of fibronectin binding sites. LPA also increased the binding of a fragment containing the 70-kDa amino-terminal region of fibronectin that is primarily responsible for the reversible binding of fibronectin to matrix assembly sites on cell surfaces. Removal of LPA resulted in prompt return of fibronectin binding to baseline levels. These results indicate that LPA is an important enhancer of fibronectin-rich matrix deposition by cultured cells, and it may be the active component in serum and lipoprotein fractions that is responsible for enhancing fibronectin deposition. (Arterioscler Thromb. 1993;13:1662-1667.)

KEY WORDS • fibronectin • lysophosphatidic acid

Fibronectin is a dimeric glycoprotein found at high concentrations in plasma and other body fluids.1,2 It also exists in an insoluble form in connective tissues and basement membranes, mediating many important biologic functions including promotion of cellular migration during embryogenesis, wound healing, and tumor metastasis.2-4 Insolubilization of plasma or cellular fibronectin and subsequent deposition into the extracellular matrix are initiated by the binding of soluble fibronectin to cell surfaces in a specific and saturable manner.5,6 Reversibly bound fibronectin is subsequently insolubilized into high-molecular-weight fibronectin multimers.7,9 Serum has long been known to influence elaboration of fibronectin matrix by cultured cells.10-13 We recently identified lipoproteins as potent enhancers of the binding of fibronectin to adherent cells via pathways that do not involve the low-density lipoprotein (LDL) receptor.14 Lipoproteins also increase the deposition of fibronectin as multimers into the extracellular matrix of cells. Our initial study did not ascertain whether the protein component of lipoproteins or the lipid component of lipoproteins or both were the final arbitrators of activity. Recent observations that 1-oleoyl lysophosphatidic acid (LPA), the simplest, naturally occurring phospholipid, accounts for the strong enhancement by serum of actin stress-fiber formation at focal adhesions15 led us to conjecture that LPA is the active component in serum and lipoprotein fractions. We now show that LPA is a potent enhancer of the binding of fibronectin to human osteosarcoma cells and normal fibroblasts and is likely responsible for the enhancement activity attributed to serum and lipoproteins.

Methods

The human osteosarcoma fibroblastic cell lines MG-63, KHOs, and MMNG/HOS were obtained from the American Type Culture Collection, Rockville, Md. Human foreskin fibroblasts (TJ) were a strain derived locally by Dr Lynn Allen-Hoffmann, University of Wisconsin-Madison. All cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, Md) supplemented with 5% or 10% fetal bovine serum (Intergen, Purchase, NY). All cells were seeded at 100 000 cells/2-cm² well in 24-well plates (Costar, Cambridge, Mass) and were used 2 to 3 days after seeding. Phospholipids and phospholipases were obtained from Sigma Chemical Co, St Louis, Mo. Human plasma fibronectin was purified from fibronectin- and fibrinogen-rich by-products of factor VIII production.16 The 70-kDa amino-terminal, gelatin-binding fragment of fibronectin (800 cpm/ng) and the 70-kDa fragment (1160 cpm/ng) were purified by using the chloramine T method as described.17 The purity of labeled proteins was assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by autoradiography. Cell binding studies were done on confluent monolayers as described.14 Unless otherwise indicated, cell layers were washed twice with tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS; Tris HCl, 20 mmol/L, pH 7.4, and NaCl, 150 mmol/L) and were preincubated in the standard medium of Dulbecco's modified Eagle's medium (0.5 mL) containing 0.2% bovine serum albumin (fraction V, Sigma), 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid,
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both. Analysis of fibronectin binding isotherms in fibronectin equilibrium affinity binding constants or MG-63 cells indicated that LPA approximately doubled binding could be due to an increase in the number of fibronectin binding sites or to an alteration of the treatment (data not shown). LPA-enhanced fibronectin binding to human osteosarcoma cell lines MG-63, KHOS, and MMNG/HOS human osteosarcoma cells was determined. Cells were incubated with LPA and 0.3 μg/mL radiolabeled fibronectin for 45 minutes and then washed as described in "Methods." Each point represents the average amount in nanograms of fibronectin specifically bound per milligram of cell protein and the range of duplicate values.

To test the activity of phospholipase A2, 20 ng LPA or 1,2-dioleoyl phosphatidic acid (PA) was treated with 0.14 U enzyme and 2.5 mmol/L CaCl2 for 60 minutes at 37°C in TBS; this mixture was then added directly to cycloheximide-treated MG-63 cells for 45 minutes at a final concentration of 40 ng/mL. For phospholipase B, LPA and PA were treated identically except that 0.12 U enzyme was used. Nonspecific binding in the presence of 500 μg/mL unlabeled fibronectin was also determined and subtracted from the total bound values. Nonspecific fibronectin binding did not vary between treatments. Calcium chloride was added to a final concentration of 2.5 mmol/L in all enzyme treatments.

**Results**

LPA enhanced the binding of fibronectin to human MG-63 osteosarcoma cells in a dose-dependent manner (Fig 1). The average percent increase in binding over several experiments was 220±20% (mean±SD; n=5). In MG-63 cells, LPA was minimally active at 0.1 ng/mL (0.2 nmol/L) and began to reach maximal activity at 10 ng/mL (20 nmol/L). Normal human foreskin fibroblasts were also sensitive to LPA, but the percent increase was more variable, from 50% to 300% (Fig 1). In normal fibroblasts, LPA was maximally active at more than 100 ng/mL (200 nmol/L). Virally or chemically transformed human osteosarcoma cell lines KHOS and MMNG/HOS, which bind very little fibronectin, were not sensitive to LPA (Fig 1). Binding of fibronectin to KHOS and MMNG/HOS cells was also insensitive to LDL treatment (data not shown). LPA-enhanced fibronectin binding could be due to an increase in the number of fibronectin binding sites or to an alteration of the fibronectin equilibrium affinity binding constants or both. Analysis of fibronectin binding isotherms in MG-63 cells indicated that LPA approximately doubled the number of fibronectin binding sites without altering the affinity constants (Fig 2). The results were similar for normal fibroblasts (data not shown). Enhancement of the binding of fibronectin to MG-63 cells was observed within 10 minutes after the addition of LPA to MG-63 cells (Fig 3) or normal fibroblasts (data not shown). MG-63 cells preincubated with LPA and then washed before the addition of 125I-labeled fibronectin

**Fig 1.** Line graph showing enhancement of the binding of 125I-labeled fibronectin to osteosarcoma cells and normal fibroblasts by increasing concentrations of 1-oleoyl lysophosphatidic acid (LPA). The ability of LPA to enhance the binding of fibronectin to normal fibroblasts and MG-63, KHOS, and MMNG/HOS human osteosarcoma cells was determined. Cells were incubated with LPA and 0.3 μg/mL radiolabeled fibronectin for 45 minutes and then washed as described in "Methods." Each point represents the average amount in nanograms of fibronectin specifically bound per milligram of total cell protein and the range of duplicate values.

**Fig 2.** Graph showing isotherm of 125I-labeled fibronectin to MG-63 osteosarcoma cells in the presence (●) or absence (○) of 20 ng/mL 1-oleoyl lysophosphatidic acid (LPA). The total fibronectin binding curves were deconvoluted into their specific and nonspecific component curves as described in "Methods." The hyperbolic isotherms represent the curves of best nonlinear least-squares fit, whereas the straight lines represent calculated nonspecific binding (in nanograms fibronectin bound per microgram free fibronectin) in the presence (—) or absence (——) of LPA.

pH 7.4, and 10 μg/mL cycloheximide (Sigma). The cycloheximide was used routinely to avoid the possibly confounding effect of differences in synthesis of endogenous fibronectin and other proteins. After 2 hours, the cells were washed once with TBS before the binding mixtures were added. The mixtures contained radiolabeled fibronectin (0.3 μg/mL, or approximately 150 000 cpm/well) and unlabeled phospholipids at various concentrations in 0.5 mL of the same standard medium. After 45 minutes (except in the case of time course experiments) at 37°C, the cell layers were washed three times with cold TBS and solubilized with 0.2N NaOH, and the amount of total cell and matrix-associated radioactivity was measured. Nonspecific binding in the presence of 500 μg/mL unlabeled fibronectin (approximately 10% to 30% of total binding for intact fibronectin and the 70-kDa fragment) was subtracted from total binding to calculate specific binding. This amount of unlabeled ligand is 25 times greater than the fibronectin equilibrium dissociation constant (Kd), and it inhibits 96% of the specific binding of the labeled ligand. Fibronectin dose-response data were analyzed separately by using the weighted nonlinear least-squares program LIGAND, as modified by G.A. McPherson (Elsevier-BIOSOFT, Cambridge, UK). This analysis allowed for the deconvolution of the total binding curve to its specific and nonspecific components. Data are represented as nanograms bound per milligram of cell protein as determined by the method of Lowry et al.18

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Fig 3. Line graph showing the time course of fibronectin binding to MG-63 osteosarcoma cells in the presence or absence of 1-oleoyl lysophosphatidic acid (LPA). Cells received 0.3 μg/mL radiolabeled fibronectin in the presence or absence of 22.5 ng/mL LPA, and cell-associated 125I-labeled fibronectin was measured at the indicated times. Incubation conditions are described in "Methods." Each point represents the average amount in nanograms of fibronectin specifically bound per milligram of total cell protein and the range of duplicate values.

did not exhibit enhanced fibronectin binding (Fig 4). All of these studies were done in the presence of cycloheximide. The results suggest that LPA exerts a rapid effect on the regulation of fibronectin binding sites that is independent of protein synthesis.

The enhancement effect was specific for LPA, in accordance with the phospholipid specificity observed by others.\(^\text{19,20}\) The serine, ethanolamine, choline, and inositol derivatives of LPA were not active (Fig 5). PA was active at approximately 2000 ng/mL (4000 nmol/L), possibly due to contamination with LPA, as discussed by Jalink et al.\(^\text{20}\) To confirm LPA as the active agent, LPA and PA were treated with phospholipases B and A\(_2\) (Table). Phospholipase B hydrolyzes both ester bonds that link the fatty acids to the glycerol backbone of phospholipids and lysophospholipids. Phospholipase A\(_2\) hydrolyzes only the ester bond at the C2 position in phospholipids, converting them to lysophospholipids. Treatment of LPA or PA with phospholipase B caused loss of activity (Table). In contrast, phospholipase A\(_2\) did not alter the activity of LPA, which already lacks an acyl group at the C2 position, but it did increase the activity of PA, presumably by virtue of its conversion to LPA.

Fig 4. Bar graph showing effect of incubation order on the binding of 1-oleoyl lysophosphatidic acid (LPA)-enhanced fibronectin to MG-63 osteosarcoma cells. Cells were preincubated for 2 hours, washed quickly three times with tris(hydroxymethyl)aminomethane-buffered saline over the course of 5 minutes, and then incubated for 1 hour with radiolabeled fibronectin, 0.3 μg/mL. LPA concentration during the preincubation or incubation period was 18 ng/mL. Each value is the average amount bound and SD (n=3).

Binding of soluble fibronectin to cell surfaces is mediated principally by the interaction of the 70-kDa amino-terminal region of fibronectin with a hypothetical

Fibronectin Binding Induction by Phospholipids After Treatment With Phospholipases

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Bound Fibronectin, ng/mg</th>
<th>Control, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no addition)</td>
<td>41.8±2.1</td>
<td>100</td>
</tr>
<tr>
<td>LPA, 40 ng/mL</td>
<td>87.6±4.9</td>
<td>210</td>
</tr>
<tr>
<td>LPA+phospholipase A(_2)</td>
<td>93.3±5.0</td>
<td>223</td>
</tr>
<tr>
<td>LPA+phospholipase B</td>
<td>47.2±2.0</td>
<td>113</td>
</tr>
<tr>
<td>PA, 40 ng/mL</td>
<td>52.0±2.9</td>
<td>124</td>
</tr>
<tr>
<td>PA+phospholipase A(_2)</td>
<td>87.8±1.7</td>
<td>210</td>
</tr>
<tr>
<td>PA+phospholipase B</td>
<td>40.8±2.1</td>
<td>98</td>
</tr>
</tbody>
</table>

LPA indicates 1-oleoyl lysophosphatidic acid, and PA, 1,2-dioleoyl phosphatidic acid. Values are mean±SD. LPA and PA were incubated alone, with phospholipase A\(_2\), or with phospholipase B as described in "Methods" and were then added to MG-63 cells for 45 minutes with radiolabeled fibronectin. Values indicate the average amount bound and SD (n=3).
matrix assembly receptor on substrate-attached cells.\textsuperscript{5,17,21} We previously showed that LDL specifically increased the binding of the 70-kDa fibronectin fragment to MG-63 cells.\textsuperscript{14} To determine whether LPA affected the same region of fibronectin, we assayed the binding of the \textsuperscript{125}I-labeled 70-kDa fragment in the presence and absence of LPA. LPA enhanced the binding of the \textsuperscript{125}I-labeled 70-kDa fragment to MG-63 cells approximately threefold (Fig 6), which is comparable to the enhancement of whole fibronectin binding. The dose response, however, was somewhat different, with minimal activity at 5 ng/mL (10 nmol/L) and maximal activity at 50 ng/mL (100 nmol/L).

**Discussion**

We have shown that LPA enhances the binding of fibronectin to MG-63 osteosarcoma cells and normal fibroblasts in a rapid and specific manner. The activities we attribute to LPA are identical to those that we recently ascribed to lipoproteins.\textsuperscript{14} The similarities in response suggest that LPA carried by lipoprotein particles is responsible for lipoprotein-dependent enhancement of the binding of fibronectin to adherent cells. Whereas we speculated in our earlier report that lipoproteins enhance the binding of fibronectin by virtue of a direct interaction between the lipoproteins and fibronectin, the current studies indicated that LPA, and therefore lipoproteins, are instead potent modulators of fibronectin binding sites.

Cellular expression of both the \(\alpha_5\beta_1\) integrin that recognizes the Arg-Gly-Asp (RGD)–containing cell-adhesion region of fibronectin\textsuperscript{22} and binding molecules that recognize the 70-kDa amino-terminal non-RGD-containing region of fibronectin are important for the binding and assembly of fibronectin into the extracellular matrix.\textsuperscript{23} The 70-kDa amino-terminal fragment can bind to fibroblast monolayers and competes for the binding of soluble full-length fibronectin.\textsuperscript{17,24} In addition, the 70-kDa fragment and a monoclonal antibody to this fragment are able to block the matrix assembly of fibronectin.\textsuperscript{25} Similarly, both a 105-kDa RGD-containing cell-binding fragment of fibronectin and a monoclonal antibody against the fibronectin cell attachment site block matrix assembly of endogenously synthesized fibronectin.\textsuperscript{25} Rat monoclonal antibodies to \(\alpha\) and especially to \(\beta_1\) block fibronectin matrix formation\textsuperscript{26} and the binding of exogenous fibronectin or the 70-kDa fragment to confluent cell monolayers.\textsuperscript{23}

Fibronectin matrix assembly is tightly regulated. One of the earliest reports of regulation was that serum enhances fibronectin binding twofold to fibroblastic monolayer cultures.\textsuperscript{11} This correlates with the finding that transformed hamster Nil HSV cells grown in 5% serum exhibit increased fibronectin deposition compared with cells grown in 0.3% serum, which do not.\textsuperscript{12,13} Cholera toxin, epinephrine, isoproterenol, and forskolin, all stimulators of adenylate cyclase, lead to a rapid decrease in the number of fibronectin matrix assembly sites and an accompanying decrease in fibronectin deposition into extracellular matrix.\textsuperscript{27} Transforming growth factor–\(\beta\), on the other hand, increases cell surface binding and assembly of exogenous plasma fibronectin by fibroblasts.\textsuperscript{28} Our results suggest that LPA is a potent and important regulator of fibronectin binding.

LPA is a critical intermediate in de novo lipid biosynthesis,\textsuperscript{29} and it is produced rapidly on cell\textsuperscript{30,31} and platelets\textsuperscript{32,33} activation. Recently, Eichholtz and colleagues\textsuperscript{34} have shown that LPA is released from activated platelets and that platelets are the primary source of serum LPA. Extracellular LPA has many biologic actions, including \(Ca^{2+}\) mobilization\textsuperscript{20,35}; inhibition of adenylate cyclase in a G protein–dependent manner; stimulation of phospholipase C, leading to phosphoinositide hydrolysis, and activation of protein kinase C;\textsuperscript{32} induction of smooth muscle cell contraction;\textsuperscript{20,35} and stimulation of focal adhesion and stress-fiber formation.\textsuperscript{35} Recently, van der Bend and colleagues\textsuperscript{37} identified a putative LPA membrane receptor by covalent cross-linking. They speculate that the receptor is a G protein–coupled receptor, consistent with the role that LPA plays in phosphoinositide hydrolysis and adenylate cyclase inhibition. The idea of a receptor is consistent with the unfavorable transbilayer LPA “flip-flop” required if extracellular LPA were to bind an inner leaflet or cytosolic target site, the failure of microinjected LPA to mimic the action of extracellular LPA,\textsuperscript{38} and the failure of intracellularly produced LPA to mobilize \(Ca^{2+}\).\textsuperscript{39}

LPA enhances the binding of fibrinogen to isolated platelet glycoprotein IIb-IIIa.\textsuperscript{39} The doses of LPA required, however, are orders of magnitude greater than those required to stimulate actin stress-fiber formation\textsuperscript{45} or to enhance binding of fibronectin. The inhibition of adenylate cyclase by LPA is in accord with the action of agents that increase cAMP levels and reduce the number of fibronectin matrix assembly sites.\textsuperscript{27} Recent findings from our laboratory suggest that stimulators of protein kinase C increase the binding and deposition of fibronectin (C.E. Somers, PhD, and D.F. Mosher, MD; in press; J Biol Chem, November 1993), consistent with LPA stimulation of phospholipase C and phosphoinositide hydrolysis. We speculate, therefore, that the stim-
These authors report that LPA induces shape changes and the subsequent activation of protein kinase C and another report, this group found that LPA regulated the p60<sup>(src)</sup> in neuronal cells and that these changes are accompanied by activation of p21<sup>(ras)</sup> by way of a new pathway involving a heterotrimeric G protein of the G<sub>11</sub> subfamily. Whether any of these signaling pathways is responsible for the enhancement of fibronectin matrix deposition remains to be determined.

Ridley and Hall<sup>11</sup> suggest that LPA causes increased GTP-rho concentrations in Swiss 3T3 cells and is responsible for enhanced formation of actin stress fibers at sites of integrin clustering in the plasma membrane. Other determinants of focal adhesion and stress-fiber formation include protein kinase C<sub>2</sub>,<sup>22</sup> tyrosine kinases including focal adhesion–associated kinase,<sup>43–45</sup> kinase substrates such as paxillin,<sup>9</sup> and integrins.<sup>2</sup> Integrin-mediated adhesive contacts of motile fibroblasts on a substrate demonstrate limited fluctuation in size, density, and shape.<sup>46</sup> Our studies were done on confluent cultures in which the binding sites were on the dorsal (upper and lateral) surfaces of cells (References 5, 17, and 47). Checovich, PhD, D.F. Mosher, MD, and D.M.P. Peters, PhD (unpublished data, April 1993). The rapidity with which assembly sites are upregulated and downregulated on the addition or withdrawal of LPA suggests that the same “machinery” responsible for focal adhesions is used in a much more dynamic fashion to control the display of matrix assembly sites. It is intriguing to speculate that LPA is a primary signal for a complex series of downstream events that control this machinery. If so, the pathways by which extracellular LPA levels are systemically and locally regulated are probably tightly controlled.

**Acknowledgments**

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