PI-3-kinase and MAPK regulate mesangial cell proliferation and migration in response to PDGF

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Ghosh Choudhury, Goutam, C. Karamitsos, James Hernandez, Alessandra Gentilini, John Bardgette, and Hanna E. Abboud. PI-3-kinase and MAPK regulate mesangial cell proliferation and migration in response to PDGF. Am. J. Physiol. 273 (Renal Physiol. 42): F931–F938, 1997.—Proliferation and migration are important biological responses of mesangial cells to injury. Platelet-derived growth factor (PDGF) is a prime candidate to mediate these responses in glomerular disease. PDGF and its receptor (PDGFR) are upregulated in the mesangium during glomerular injury. We have recently shown that PDGF activates phosphatidylinositol 3-kinase (PI-3-kinase) in cultured mesangial cells. The role of this enzyme and other more distal signaling pathways in regulating migration and proliferation of mesangial cells has not yet been addressed. In this study, we used two inhibitors of PI-3-kinase, wortmannin (WMN) and LY-294002, to investigate the role of this enzyme in these processes. Pretreatment of mesangial cells with WMN and LY-294002 dose-dependently inhibited PDGF-induced PI-3-kinase activity assayed in antiphosphotyrosine immunoprecipitates. WMN pretreatment also inhibited the PI-3-kinase activity associated with anti-PDGFRα immunoprecipitates prepared from mesangial cells treated with PDGF. Pretreatment of the cells with different concentrations of WMN resulted in a dose-dependent inhibition of PDGF-induced DNA synthesis. Both WMN and LY-294002 inhibited PDGF-stimulated migration of mesangial cells in a dose-dependent manner. It has recently been shown that PDGF physiologically interacts with Ras protein. Because Ras is an upstream regulator of the kinase cascade leading to the activation of mitogen-activated protein kinase (MAPK), we determined whether activation of PI-3-kinase is necessary for activation of MAPK. Pretreatment of mesangial cells with WMN and LY-294002 significantly inhibited PDGF-induced MAPK activity as measured by immune complex kinase assay of MAPK immunoprecipitates. Furthermore, PD-098059, an inhibitor of MAPK-activating kinase inhibited PDGF-induced MAPK activity and resulted in a significant reduction of mesangial cell migration in response to PDGF. These data indicate that MAPK is a downstream target of PI-3-kinase and that both these enzymes are involved in regulating proliferation and migration of mesangial cells.

Phosphatidylinositol 3-kinase; mesangial cells; migration; mitogenesis; mitogen-activated protein kinase; platelet-derived growth factor

Cell migration and cell proliferation are fundamental responses of mesangial cells to glomerular injury and contribute to hypercellularity observed in a number of glomerular diseases. Mesangial cell migration may also contribute to repopulation of glomerular cells that follows cytolytic lesions as observed in experimental and human forms of glomerulonephritis. Platelet-derived growth factor (PDGF), secreted by glomerular cells as well as activated platelets and macrophages, is the most potent mitogen for mesangial cells in vitro and in vivo (1). PDGF also induces directed migration during inflammatory glomerular disease (3). In immunemediated human glomerulonephritis and in experimental models of glomerular injury, mesangial cell proliferation and migration are accompanied by increased expression of PDGF and its receptor (PDGFR).

Binding of PDGF causes dimerization of its cognate receptor and induces its intrinsic protein tyrosine kinase activity leading to autophosphorylation of the receptor and to the phosphorylation of target substrates on tyrosine residues (2). Tyrosine autophosphorylation of the receptor creates binding sites for a set of proteins characterized by the presence of ~100 amino acid residue sequence motifs known as src homology 2 (SH2) domain. Some of the proteins that associate with PDGFR include phospholipase Cγ1, guanosine triphosphatase activating protein, phosphotyrosine phosphatase (PTP) 1D, and phosphatidylinositol 3-kinase (PI-3-kinase) (2). Tyrosine phosphorylation and association of these enzymes with PDGFR stimulate their enzymatic activity. PI-3-kinase is activated by several growth factors and cytokines, including different tyrosine kinase oncogenes (14). This enzyme is a heterodimer of 110-kDa catalytic and 85-kDa regulatory subunits (2, 6). Activation of this enzyme results in the production of D-3 phosphorylated inositol phosphates, the precise functions of which are not yet clear. Several investigators reported that PI-3-kinase lipid products, the D-3 phosphorylated inositides, are necessary for cell proliferation. Also, activation of this enzyme is necessary for cell migration and PDGFR internalization (13, 16). The 85-kDa regulatory subunit contains two SH2 domains through which it can associate with tyrosine-phosphorylated PDGFR on the plasma membrane, thus stimulating the enzymatic activity of its 110-kDa catalytic subunit.

Another signal transduction pathway utilized by PDGFR is the Ras-Raf mitogen-activated protein kinase (MAPK or ERK) (17). Activated PDGFR binds the SH2 domain-containing adaptor protein Grb-2, which brings the guanine nucleotide exchange factor, son of sevenless (SOS), to the plasma membrane to replace GDP with GTP in Ras. GTP-bound Ras interacts with Raf serine threonine kinase, localizing it in the plasma membrane to activate its serine threonine kinase. Raf thus initiates the kinase cascade to finally stimulate MAPK, which phosphorylates downstream target proteins including transcription factors (17). Modulation of any component of this kinase cascade including the upstream regulator Ras may have an impact on PDGFR-mediated human glomerulonephritis and in experimental models of glomerular injury, mesangial cell proliferation and migration are accompanied by increased expression of PDGF and its receptor (PDGFR).
stimulated signals and their biological consequences. With the discovery of the drugs wortmannin and LY-294002 as potent inhibitors of PI-3-kinase (28, 29), it is now possible to study the biological role of PI-3-kinase in different cellular responses by directly inhibiting its enzymatic activity after PDGF stimulation of cells. In this study, we demonstrate that wortmannin and LY-294002 dose-dependently inhibit PDGF-induced PI-3-kinase activity in mesangial cells. Inhibition of PI-3-kinase activity leads to inhibition of PDGF-induced chemotaxis and DNA synthesis. In addition, we demonstrate that inhibition of PI-3-kinase blocks activation of MAPK in response to PDGF. These data indicate that PI-3-kinase regulates mesangial cell proliferation and chemotaxis in a MAPK-dependent manner.

MATERIALS AND METHODS

Materials. Tissue culture materials were obtained from Gibco-BRL. Nonidet P-40 (NP-40), phenylmethylsulfonyl fluoride (PMSF), Na2VO3, phosphatidylinositol, and wortmannin were purchased from Sigma. LY-294002 was obtained from Calbiochem. PD-098059 was provided by Parke-Davis Pharmaceutical Research Division. Aprotinin was obtained from Miles Laboratories. Human recombinant PDGF BB was obtained from Amgen. Human PDGF-Rβ monoclonal antibody was obtained from Genzyme. Antiphosphotyroisine and PI-3-kinase antibodies were obtained from Upstate Biotechnology. Protein A-Sepharose CL4B was obtained from Pharmacia. NADP was obtained from Genzyme. Antiphosphotyrosine and PI-3-kinase antibodies were obtained from Amgen. Human PDGFRα was purchased from Pharmacia. LY-294002 was obtained from Sigma. Wt P-32P-ATP was from New England Nuclear. All other reagents were of analytical grade.

Cell culture. Human mesangial cells were propagated in Waymouth’s medium in the presence of 17% fetal calf serum as described (6). Cells were made quiescent by serum starvation in Waymouth’s medium in the presence of 17% fetal calf serum. All other reagents were of analytical grade.

Preparation of membrane and cytosolic fractions. Solubilization buffer (0.5 ml) [20 mM tris(hydroxymethyl)aminomethane (Tris·HCl), pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na2VO3, 1 mM PMSF, and 0.1% aprotinin] is added to the cell monolayer. The cells are collected by centrifugation, and the supernatant is used for PI-3-kinase assay as described (6).

Immunoprecipitation and PI-3-kinase assay. Cells are lysed in RIPA buffer at 4°C for 30 min. The debris is separated by centrifugation at 10,000 g for 30 min and used for the membrane fraction.

Immunoprecipitation and MAPK assay. MAPK assay was performed using a modified method of Kribben et al. (15). Briefly, cleared cell lysate was immunoprecipitated with MAPK-specific antibody, and the immunoprecipitates were resuspended in MAPK assay buffer (10 mM HEPES, pH 7.4, 10 mM MgCl2, 0.5 mM dithiothreitol, and 0.5 mM Na2VO3) in the presence of 0.5 mg/ml myelin basic protein (MBP), 0.5 µM protein kinase A inhibitor, and 25 µM cold ATP plus 1 µC [γ-32P]ATP. The reaction was incubated at 30°C for 30 min followed by a 10-min incubation on ice. The reaction mixture was then separated on 15% SDS polyacrylamide gel. Phosphorylated MBP was visualized by autoradiography.

Data analysis. Significance of the data was determined by unpaired Student’s t-test.

RESULTS

Inhibition of PI-3-kinase by wortmannin in mesangial cells. PDGF stimulates PI-3-kinase activity in
antiphosphotyrosine-associated protein fraction from mesangial cells (6). We tested the effect of wortmannin on PDGF-induced PI-3-kinase activity in these cells. Cleared cell lysate from PDGF-stimulated mesangial cells pretreated with different concentration of wortmannin was immunoprecipitated with antiphosphotyrosine monoclonal antibody. The washed immunobeads were assayed for PI-3-kinase activity using phosphatidylinositol as substrate in the presence of [γ-32P]ATP. As shown in Fig. 1, PDGF-induced PI-3-kinase activity that associates with antiphosphotyrosine immunoprecipitates was inhibited by wortmannin in a dose-dependent manner. At 100 nM wortmannin, >90% of the activity was inhibited.

PI-3-kinase activation requires translocation of this enzyme to the plasma membrane and its association with tyrosine-phosphorylated proteins that include tyrosine-phosphorylated growth factor receptors. We have recently demonstrated that, in mesangial cells, PDGF stimulates association of PI-3-kinase with PDGFR, confirming translocation of this enzyme to the plasma membrane in response to PDGF (6). To confirm direct translocation of PI-3-kinase, we isolated membrane and cytoplasmic fraction from mesangial cells treated with PDGF. Both these fractions were immunoprecipitated with antiphosphotyrosine antibody, and the immunoprecipitates were used in PI-3-kinase assay. The data show that PDGF-stimulated PI-3-kinase activity is associated with the membrane fraction (Fig. 2). To confirm direct translocation of PI-3-kinase, we isolated membrane and cytoplasmic fraction from mesangial cells treated with PDGF. Both these fractions were immunoprecipitated with antiphosphotyrosine antibody, and the immunoprecipitates were used in PI-3-kinase assay. The data show that PDGF-stimulated PI-3-kinase activity is associated with the membrane fraction (Fig. 2). Next we tested the effect of wortmannin on PDGF-associated PI-3-kinase activity, which is also a measure of membrane-associated PI-3-kinase activity. Lysates from PDGF-treated mesangial cells preincubated with wortmannin were immunoprecipitated with PDGFR monoclonal antibody. The immunoprecipitates were assayed for PI-3-kinase activity. The data show that wortmannin inhibits the PDGFR-associated PI-3-kinase activity (Fig. 3).

Role of PI-3-kinase in PDGF-induced DNA synthesis in mesangial cells. We and others have previously shown that PDGF is a potent mitogen for mesangial cells in culture (1, 6). However, the requirement of PI-3-kinase in PDGF-mediated DNA synthesis in mesangial cells has not yet been investigated. To explore the potential involvement of PI-3-kinase in PDGF mitogenic signaling in mesangial cells, we measured PDGF-induced DNA synthesis in the presence of the PI-3-kinase inhibitor wortmannin. The data in Fig. 4 show that wortmannin inhibits PDGF-induced DNA synthesis in a dose-dependent manner. These data...
indicate that inhibition of PI-3-kinase completely blocks mesangial cell DNA synthesis in response to PDGF.

Effect of PI-3-kinase inhibition on PDGF-mediated mesangial cell migration. Mesangial cell migration is an important biological response during glomerular injury. We tested the involvement of PI-3-kinase in PDGF-induced mesangial cell migration. Quiescent cells were treated with different concentrations of wortmannin and subsequently used in chemotaxis assay in the presence of PDGF. The results show that wortmannin inhibits PDGF-induced mesangial cell migration in a concentration-dependent manner similar to that observed for PI-3-kinase inhibition and DNA synthesis inhibition (Fig. 5). These data suggest that mesangial cell migration involves PI-3-kinase activation.

Although wortmannin has been extensively used as an inhibitor of PI-3-kinase, other enzymes are also inhibited by this fungal metabolite in different cell types. For example, wortmannin inhibits bombesin-induced phospholipase A₂ in Swiss 3T3 cells and anti-CD3-stimulated phospholipase D in J urkat T cells (4, 18). This drug also inhibits myosin light chain kinase and the biological effect mediated by this kinase (20). To address the involvement of PI-3-kinase in mesangial cells, we used the chromone derivative, LY-294002, which is known to block the activity of this enzyme in different cells (28). Lysates from PDGF-treated mesangial cells preincubated with LY-294002 were immunoprecipitated with antiphosphotyrosine antibody followed by measurement of PI-3-kinase activity in these immunoprecipitates. The data show that 50 µM and 100 µM of LY-294002 significantly inhibit PDGF-stimulated PI-3-kinase activity (Fig. 6).

To test whether this chromone derivative inhibits PDGF-induced chemotaxis, mesangial cells were incubated with LY-294002 and then used in chemotaxis assay in the presence of PDGF. The results show that 50 µM and 100 µM LY-294002 significantly inhibit PDGF-induced chemotaxis of mesangial cells (Fig. 7).

PI-3-kinase regulates PDGF-induced MAPK activity in mesangial cells. It is well established that PDGF activates Ras, which induces translocation of Raf kinase to the plasma membrane to bind physically with Ras protein (9, 17). This translocation of Raf increases its intrinsic kinase activity to initiate the kinase cascade to finally stimulate MAPK. Although PI-3-kinase can physically associate with Ras protein, the upstream regulator of MAPK (24), it is not clear whether
PI-3-kinase can regulate MAPK activation. To address this issue, we measured kinase activity in MAPK immunoprecipitates of PDGF-stimulated mesangial cells pretreated with wortmannin and LY-294002. As shown in Fig. 8, both these compounds significantly inhibited PDGF-induced MAPK activity in mesangial cells. These data indicate that in mesangial cells, PI-3-kinase activity stimulated by PDGF regulates MAPK activity.

MAPK regulates PDGF-induced mesangial cell chemotaxis. To study the role of MAPK in PDGF-induced mesangial cell chemotaxis, we used the MEK inhibitor PD-098059. Mesangial cells were preincubated with this compound followed by treatment with PDGF. The cell lysates were immunoprecipitated with MAPK antibody and used in an in vitro immunocomplex kinase assay to determine MAPK activity. As shown in Fig. 9, the MEK inhibitor abolished PDGF-stimulated MAPK activity. Next, we treated mesangial cells with PD-098059, and the cells were used in chemotaxis assay in response to PDGF. The data show that inhibition of MAPK activity significantly inhibits PDGF-induced chemotaxis of mesangial cells (Fig. 10). Note that, despite complete inhibition of MAPK activity by PD-098059 (Fig. 9), PDGF-stimulated chemotaxis was inhibited by only 41%.

**DISCUSSION**

Studies in human and experimental animals suggest that PDGF plays a key role in proliferative and inflammatory glomerular disease (1, 2). PDGF stimulates pleiotropic effects in cells of mesenchymal origin including glomerular mesangial cells. Addition of PDGF to cultured mesangial cells stimulates early signal transduction pathways leading to DNA synthesis and PDGF A- and B-chain gene induction (1). We have recently shown that PDGF activates PI-3-kinase as one of the early signaling pathways (6). Activation of PI-3-kinase...
PI-3-KINASE IN MESANGIAL CELL PROLIFERATION AND MIGRATION

Mesangial cell migration has been implicated in the pathology of different glomerular diseases (1, 2). Cytokines and growth factors are the principal mediators of mesangial cell migration during inflammation. PI-3-kinase has recently been implicated in regulated on activation normal T-expressed and presumably secreted (RANTES)-mediated lymphocyte migration (26). Also hepatocyte growth factor (HGF)-mediated mitogenesis, measured by chemotaxis, of renal inner medullary collecting duct (IMCD) cells was inhibited by wortmannin suggesting the involvement of PI-3-kinase in this process (5). However, unlike the effect of wortmannin on PDGF-mediated mitogenesis in mesangial cells, in IMCD cells, HGF-stimulated mitogenesis was inhibited to a lesser extent by wortmannin (5). These data indicate that PI-3-kinase regulates growth factor-induced mitogenesis in a cell type-specific manner. By mutagenesis studies of PDGFR, the role of PI-3-kinase in PDGF-mediated cell migration is controversial (16, 30). Using wortmannin to inhibit PI-3-kinase enzymatic activity, we now show complete inhibition of mesangial cell migration (Fig. 5). Because wortmannin inhibits other enzymes such as phospholipase A2, phospholipase D, and myosin light chain kinase (4, 18, 20), we confirmed the role of PI-3-kinase in PDGF-stimulated mesangial cell migration using another PI-3-kinase inhibitor LY-294002. This chromogen inhibited PDGF-induced PI-3-kinase activity assayed in the antiphosphotyrosine immunoprecipitates (Fig. 6). The same concentration of LY-294002 also significantly inhibited PDGF-induced mesangial cell chemotaxis (Fig. 7). These data taken together with the results obtained with wortmannin indicate that activation of PI-3-kinase in PDGF-stimulated mesangial cells is an essential enzymatic pathway that mediates cell migration. Of interest is the recent observation that PI-3-kinase can physically bind to Rac1, which is a member of Rho family of small GTP binding proteins (25). It has been shown that these proteins play important role in cytoskeletal organization during formation of focal adhesion and cell migration (10, 22, 23).

It has recently been shown that the SH3 domain of Grb2 can bind the proline-rich region of the 85-kDa subunit of PI-3-kinase, thus bringing this enzyme in the vicinity of Ras (31). In another study, it has been reported that PI-3-kinase binds Ras protein directly suggesting that this lipid kinase can modulate Ras function (24). These observations provide two independent mechanisms for PI-3-kinase translocation to the plasma membrane away from its binding to the activated PDGFR, which is also a means of translocation to the plasma membrane. It is known that this translocation is required for PI-3-kinase activity. We have also shown that PDGF-stimulated PI-3-kinase activity resides in the membrane fraction of mesangial cells (Fig. 2). In the PDGF signaling pathway, Ras is the upstream regulator of the kinase cascade that ultimately stimulates MAPK (17). In the present study, we have shown that inhibition of PI-3-kinase activity by wortmannin reduced MAPK activity (Fig. 8). Another PI-3-kinase inhibitor, LY-294002, also significantly inhibited PDGF-induced MAPK activity in mesangial cells. Neither wortmannin nor LY-294002 inhibits the enzymatic activity of MAPK in an in vitro MAPK assay (data not shown).

Fig. 10. Effect of MEK inhibitor on PDGF-induced chemotaxis of mesangial cells. Quiescent mesangial cells were pretreated with 20 µM PD-098059 for 45 min. Cells were then tested for directed migration in response to 10 ng/ml of PDGF as described in Fig. 5. *P < 0.05 vs. untreated cells. +P < 0.05 vs. PDGF alone.
to inhibit signals transduced simultaneously or in a kinase-targeting mechanism may provide a convenient mechanism to modulate PDGF directly by injection of antibodies attenuates the biological activity. Alternatively, the D-3 phosphorylated products produced by activated PI-3-kinase may directly or indirectly modulate MAPK activity. These observations of regulatory role of PI-3-kinase in activation of MAPK in mesangial cell migration indicate that MAPK is also involved in this PDGF-induced biological response. Our data showing that indirect inhibition of MAPK activity by PD-098059 (Fig. 9), a potent inhibitor of the MAPK-activating kinase MEK, is associated with significant inhibition of PDGF-induced mesangial cell chemotaxis provide the first evidence that MAPK modulates PDGF-mediated mesangial cell migration (Fig. 10). However, it is important to emphasize that, although treatment of mesangial cells with the MEK inhibitor PD-098059 caused complete inhibition of MAPK activity in these cells, PDGF-induced chemotaxis was inhibited by only 41%. These data suggest that additional signaling pathway(s) are involved in PDGF-induced chemotaxis in mesangial cells.

Mention of mesangial cells in the glomerulus contributes to structural remodeling in proliferative glomerulonephritis. PI-3-kinase is a central downstream signaling enzyme for many growth factor and cytokine receptors including PDGF. Neutralization of PDGF or PDGF directly by injection of antibodies attenuates the pathological lesion during the course of anti Thy-1- induced glomerulonephritis (11, 12). However, during glomerular injury, several inflammatory cytokines and growth factors besides PDGF may be expressed. PI-3-kinase targeting may provide a convenient mechanism to inhibit signals transduced simultaneously or in an overlapping fashion by several inhibitory cytokines.

We thank Sergio Garcia for help with the cell culture. This study was supported in part by the Dept. of Veterans Affairs Medical Research Service and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-50190 (to G. Ghosh Choudhury). H. E. Abboud is supported by a Dept. of Veterans Affairs Medical Research Service grant and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-43988 and DK-33665. Address for reprint requests: G. Ghosh Choudhury, Div. of Nephrology, Dept. of Medicine, Univ. of Texas Health Science Center, San Antonio, TX 78284-7882.

Received 13 November 1996; accepted in final form 29 July 1997.

REFERENCES


