Inhibition of Mesangial Cell Proliferation by Platelet Factor 4

Jeffrey L. Barnes, Katherine A. Woodruff, Shirley P. Levine, and Hanna E. Abboud

Platelets contain a large number of secretory products that have a variety of biologic activities. Many of these substances influence cell migration, cell proliferation, and degradation or synthesis of extracellular matrix; all of which are important events in cell remodeling in wound healing and tissue repair (reviewed in Reference 1). The effects of several of these products, including platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF-α) and beta (TGF-β), epidermal growth factor (EGF), and interleukin-1 on cell function are well defined in vitro and have been the subject of intense investigation with regard to their role in the pathogenesis of renal diseases (1–3). On the other hand, the biologic effects of platelet factor 4 (PF4), an abundant alpha-granule secretory protein, on glomerular cells have not been characterized.

PF4 is released from platelets during aggregation as a high molecular tetramer weight complex consisting of 7800-d subunits and a carrier composed of chondroitin-4-sulfate (4,5). PF4 can be easily dissociated from its complex and has a high affinity for glycosaminoglycans with the following order of avidity: heparin > heparan sulfate > dermatan sulfate > chondroitin-6-sulfate > chondroitin-4-sulfate, the binding of which is related to their degree of sulfation (5–7). By virtue of its affinity for glycosaminoglycans, PF4 binds to endothelial cell surfaces presumably through ionic interactions with heparan sulfate (8,9). Similarly, PF4 binds avidly in vitro and in vivo to glomerular structures, including endothelial and epithelial cell surfaces, glomerular basement membrane (GBM), and mesangial matrix (10,11). Because of its high affinity to GBM, PF4 has been implicated to play a role in neutralization of electrostatic charge barrier to circulating macromolecules and influence immune complex deposition in glomerular disease (1,10).

PF4 also localizes to glomerular structures in various models of experimentally induced glomerular disease (11–13) and in clinical forms of glomerulonephritis (14–16). The expression of PF4 is a useful marker of platelet activation and local release of platelet secretory products (1,17). Apart from its potential influence on glomerular permeability, little attention has been directed to PF4 as a mediator of glomerular pathology during the pathogenesis of renal injury. In this study, the effect of PF4 on fetal calf serum (FCS)-, PDGF AB-, and EGF-induced mesangial cell mitogenesis and expression of two genes (PDGF A chain and TGF-β1) involved in the autocrine regulation of mesangial cell growth (18,19) was examined. PDGF AB was used because this cytokine is the major PDGF isoform secreted by platelets and is a potent inducer of mesangial cell proliferation through auto-

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ABSTRACT
Platelet factor 4 (PF4), an abundant platelet secretory product, is a strong candidate for modulating glomerular pathology. Because PF4 might be released from platelets and influence intrinsic cell growth during glomerular injury, the effect of PF4 on fetal calf serum- and platelet-derived growth factor (PDGF)-induced mesangial cell mitogenesis was examined. Mitogenesis was measured as the amount of 3H-thymidine incorporated into acid-precipitable material associated with modulation of the autocrine ways associated with modulation of the autocrine growth factors PDGF and TGF-β1.

Key Words: Mesangial cells, platelet factor 4, proliferation, platelet-derived growth factor

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crine pathways. EGF is also secreted from platelets and is a known mitogen for mesangial cells through autocrine stimulation of PDGF (19).

METHODS

Preparation of PF4

Human PF4 was purified and characterized as previously described (4). In brief, outdated platelet concentrates were washed in citrate saline buffer, then lysed by repeated freeze-thawing. After centrifugation of cell membranes, the PF4-enriched supernatant was passed through a heparin-ε-aminocaproic acid-Sepharose column, and washed extensively with buffer. Purified PF4 was eluted from the column utilizing a gradient of 0.5 to 3.0 M NaCl in 0.005 M sodium barbitral buffer, pH 7.4. Purity and characterization of PF4 were performed utilizing a PF4 activity assay, immunodiffusion and column chromatography, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4) and immunoblotting (20). Purity of PF4 was verified by the formation of a single line of identity in immunodiffusion plates with an antibody to PF4, one band of mobility corresponding to a molecular weight of 11,600 by electrophoresis and Western blotting.

Mesangial Cell Culture

Human mesangial cells were isolated and grown as described previously (18). Cells were determined to be mesangial by positive staining for intermediate filament proteins: desmin, myosin, and vimentin (18). In addition, the cells stained intensely for α-smooth muscle actin, a specific marker for smooth muscle and mesangial cells in culture and activated mesangial cells in vivo (21). The cells were negative for the endothelial cell markers Factor VIII-related antigen and uptake of labeled acetylated low-density lipoproteins (22).

Mesangial cells were maintained in Waymouth's medium supplemented with 15 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.6 U/mL insulin, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 17% fetal calf serum (FCS). The cells were passed by washing them with Ca²⁺-free Mg²⁺-free Hank’s balanced salt solution (HBBS) followed by incubation with 0.025% trypsin, 0.5 mM EDTA, and resuspension in complete Waymouth’s medium. Cells were used between passages 7 and 11.

Effect of PF4 on ³H-thymidine Incorporation

Mesangial cells were passed into 24-well dishes and rendered quiescent by placing them in serum-free-insulin free (SFIF) medium for 2 days. To examine a direct effect of PF4 on mesangial cell ³H-thymidine incorporation, PF4 (2.5 and 25 µg/mL) was added in to triplicate wells and incubated in a 95% air, 5% CO₂ atmosphere at 37°C. In additional experiments, quiescent cells were stimulated by the addition of FCS (10%), PDGF (10 ng/mL), or EGF (50 ng/mL) in the presence of graded concentrations of PF4 (0 to 25 µg/mL) in triplicate. Controls consisted of wells treated with diluent in place of PF4 to determine basal levels of ³H-thymidine incorporation. Twenty-four hours later, the wells were pulsed with ³H-thymidine (1.0 µCi/mL) for an additional 4 hours. At the end of the pulsing period, the cells were washed twice with 5% trichloroacetic acid to remove unincorporated ³H-thymidine, then solubilized by adding 0.7 mL of 0.25 N NaOH in 0.1% SDS. One half milliliter of this solution was neutralized and counted in scintillation cocktail. To examine the time course when PF4 was effective in inhibiting mesangial cell proliferation, PF4 was added to quiescent cells 24 h before and 1, 4, 8, and 16 hours after the addition of PDGF AB heterodimer. ³H-thymidine incorporation studies were performed as described above.

Measurement of DNA Synthesis by Autoradiography

In a separate study, mesangial cells were plated onto 4-well Lab-Tek chamber slides (Nunc, Inc., Naper, IL) to confluence, made quiescent, then treated with PF4, FCS, or FCS plus PF4. Cells were then pulsed with ³H-thymidine as outlined above. The slides were then processed for autoradiography and microscopic analysis (18). The number of positive cells in each well determined by the presence of silver grains in the adjacent emulsion was counted on the slides and expressed as cell number per cm².

Effect of PF4 on Expression of mRNA Encoding PDGF A Chain and TGF-β₁

To assess if the inhibitory effect of PF4 was mediated through alterations in known autocrine pathways for mesangial cells, expression of mRNA encoding the growth factors PDGF A chain and TGF-β₁ was determined. Mesangial cells were grown to confluence in 100-mm² plastic petri dishes and made quiescent as above. Three sets of triplicate plates were exposed to PDGF AB (10 ng/mL) or PDGF plus PF4 (10 µg/mL) for 3 h. Nonstimulated control cells were also incubated for 3 h with diluted in the absence of PDGF or PF4. Because PF4 induced modest reductions in PDGF A chain and TGF-β₁ mRNA 3 h after addition of PDGF AB (see the Results section), additional studies were performed to examine the time course of PF4 inhibition of PDGF AB-stimulated expression of PDGF A chain and TGF-β₁, mRNA. Mesangial cells were incubated with diluted, PDGF AB, or PDGF AB plus PF4 for 1, 3, 6, and 24 hours. At the end of each time point, total RNA was obtained and Northern analysis performed as outlined above.

Total RNA was isolated from cells in all experiments at the times outlined above according to the method of Chirgwin et al. (23). In brief, the cells from each set of plates were lysed in Tris-HCl buffer containing 0.6 g/mL guanidium thiocyanate and 0.5% sodium N-laurylsarcosinate. The samples were heated at 65°C for 15 min, then centrifuged at 35,000 rpm for 18 h at 21°C through 5.7 M cesium chloride, 0.1 M EDTA pads utilizing a SW 50.1 rotor (Beckman Instruments, Fullerton, CA). The pellet was resuspended in 5 mM sodium citrate, 5 mM EDTA, and 1% SDS, pH 7.5, followed by extraction with chloroform/butanol at a ratio of 4:1 vol/vol. The RNA was precipitated with 100% ethyl alcohol containing 3 M sodium acetate, pH 6.0. The purity and concentration of RNA were determined from the absorbance at 260 and 280 nm wavelengths. This method produced nondegraded preparations of RNA as assessed by electrophoresis in 1% agarose gels and subsequent visualization of 28S and 18S ribosomal bands. Total RNA (15 µg/lane) representing each experiment was fractionated on 1% agarose-formaldehyde gels and transferred to GeneScreen (DuPont, NEN Research Products, Boston, MA). The transferred blots were hybridized with ³²P-UTP-labeled cDNA probes encoding human PDGF A chain (24), TGF-β₁ (25), or the ribosomal protein 36B4 (26) as a control for housekeeping-gene expression.

Hybridization conditions and autoradiography were performed as previously described (18). Each blot was reused by
stripping each probe by boiling followed by rehybridization with a different probe. Expression of mRNA in each lane was analyzed by measurement of optical density of the appropriate bands in the developed autoradiographs using image analysis software (Image-Pro, Media Cybernetics, Silver Spring, MD). All data was normalized to measurements obtained from bands hybridized with the housekeeping gene 36B4. Differences in expression of mRNA after incubation with PDGF A or PDGF A plus PF4 were calculated by measuring the optical density of each band relative to diluent controls. The nonstimulated control samples showed nearly identical basal expression of PDGF A and TGF-β mRNA, thus, their intensity measurements were averaged for subsequent calculation of relative optical densities.

**Effect of PF4 on PDGF Binding to Mesangial Cells**

Mesangial cells were plated in 24-well dishes until confluence. Cells were then deprived of serum for 48 h to maximally express PDGF receptors. The cells were incubated for 2 h on ice in binding buffer containing a 1:1 mixture of Dulbecco’s modified Eagle medium and Ham’s F-12 medium (DVF-12), 25 mM HEPES, 0.2% BSA, pH 7.4. PF4 was then added in graded concentrations from 1 to 10 μg/mL to triplicate wells, immediately followed by the addition of 5 ng/mL of 125I-PDGF BB (40 μCi/μg; NEN, DuPont) to each well and incubated for an additional 3 h on ice. Nonspecific binding was determined by preincubation of cells with unlabeled PDGF, 2 h before addition of 125I-PDGF. Another set of wells was treated with diluent without PF4 followed by 125I-PDGF BB to determine maximum binding of the growth factor. All dishes were incubated for 3 h with constant agitation. The wells were washed twice with PBS containing 1 mM calcium chloride and 0.2% BSA then solubilized with 1% Triton X-100. Radioactivity was counted using a gamma counter and specific binding was calculated by subtracting nonspecific binding from total CPM bound per well.

**RESULTS**

**Effect of PF4 on Mesangial Cell DNA Synthesis**

PF4 reduced basal DNA synthesis in quiescent mesangial cells as indicated by reductions in 3H-thymidine incorporation after incubation with 2.5 and 25 μg/mL of PF4 compared with control levels (388 ± 31 and 187 ± 24 versus 580 ± 94 CPM, respectively). PF4 had a dose-dependent inhibitory effect on FCS (Figure 1), PDGF AB (Figure 2), and EGF (Figure 3)-induced mesangial cell DNA synthesis. The maximum inhibitory effect of PF4 on 3H-thymidine incorporation was 67%, 93%, and 40% for FCS, PDGF and EGF, respectively. The concentration of PF4 that caused 50% inhibition of FCS- and PDGF-induced 3H-thymidine incorporation was 8 μg/mL and 5 μg/mL, respectively. PF4 was effective in inhibiting mesangial cell DNA synthesis when added 24 h before and at 1, 4, and 8 h; but not 16 h after addition of FCS (Figure 4), indicating that the effect of PF4 is not related to very early events in cell-cycle regulation.

The above results were verified by analysis of 3H-thymidine incorporation into nuclear DNA by autoradiography. FCS-induced a 6.6-fold increase in the number of positive cells from basal counts of 35 ± 5.0 (SE) cells/cm² to 236 ± 23.7 cells/cm² (P < 0.01). PF4 inhibited FCS-induced 3H-thymidine incorporation, showing 76.4 ± 19.1 positive cells/cm² or a 2.1-fold increase over basal levels, significantly less that ob-
Figure 3. Inhibition of EGF-induced mesangial cell proliferation by PF4. 3H-thymidine incorporation is expressed as mean counts/minute (cpm) ± SE. * values are significantly different from EGF alone (P < 0.05).

Figure 4. Effect of PF4 on mesangial 3H-thymidine incorporation when added 24 h before and 0, 1, 4, 8, and 16 h after addition of 10% fetal calf serum. Data are expressed as mean counts/minutes (cpm) ± SE. * values are significantly different from serum alone (P < 0.05).

Figure 5. Northern analysis of PDGF A chain mRNA expression 3 h after addition of PDGF AB or PDGF AB plus PF4. Basal levels represent untreated control cells (receiving diluent alone).
Despite the lack of change in TGF-β1 mRNA expression after PDGF A chain in our original 3-h study, a transient enhancement TGF-β1 mRNA expression was observed 3 h after addition of PDGF AB in the time-course study. Elevations in TGF-β1 mRNA above control samples were not observed at the 0.5, 6, and 24-h time points after addition of PDGF AB; thus, the apparent discrepancy between the two experiments could be explained by biological variation, if RNA was obtained before or after peak expression of PDGF-induced TGF-β1 mRNA as observed in Figure 7. PF4 inhibited the PDGF-induced expression of TGF-β1 mRNA (Figure 7, Table 1) at 3 h and reduced expression below baseline levels at 6 h after addition of conditions, contrary to expected results if the inhibitory effect of PF4 on cell proliferation is modulated by a negative autocrine loop involving enhanced expression of TGF-β.

**Effect of PF4 on PDGF Receptor Binding**

Because inhibition of expression PDGF mRNA and 3H-thymidine incorporation could be modulated by PF4 through an interference between the growth factor and its surface receptor, the effect of PF4 on

![Figure 6. Northern analysis of TGF-β1 mRNA expression 3 h after addition of PDGF AB or PDGF AB plus PF4. Basal levels are untreated control cells incubated with diluent alone.](image)

**Figure 6.** Northern analysis of TGF-β1 mRNA expression 3 h after addition of PDGF AB or PDGF AB plus PF4. Basal levels are untreated control cells incubated with diluent alone.

**Table 1. Effect of PF4 on PDGF AB-Induced mesangial cell expression of PDGF A chain and TGF-β1 mRNA**

<table>
<thead>
<tr>
<th>Factor/Time Point</th>
<th>PDGF AB</th>
<th>PDGF AB + PF4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 h</td>
<td>0.8b</td>
<td>1.2</td>
</tr>
<tr>
<td>3.0 h</td>
<td>7.5</td>
<td>3.6</td>
</tr>
<tr>
<td>6.0 h</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>24 h</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>TGF-β1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 h</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>3.0 h</td>
<td>2.3</td>
<td>1.2</td>
</tr>
<tr>
<td>6.0 h</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>24 h</td>
<td>0.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a PF4, platelet factor 4; PDGF, platelet-derived growth factor; TGF, transforming growth factor.
b Values represent optical densities of transcripts (PDGF A chain or TGF-β1) in autoradiographs shown in Figure 7, in response to PDGF AB or PDGF AB + PF4 relative to the averaged intensity of untreated controls (see the Methods section). All data were normalized to the housekeeping-gene expression of 36B4.

**125I-PDGF binding to mesangial cells was examined.**

**125I-PDGF, when added alone to mesangial cells, resulted in specific binding of 20,508 ± 683 cpm of the label (Figure 8) (after subtraction of nonspecific background). Addition of PF4 from 1 to 10 μg/mL concentrations that significantly inhibited DNA synthesis, did not have an effect on 125I-PDGF BB binding to mesangial cells (Figure 8).**

![Figure 7. Northern analysis of mRNA encoding PDGF A chain, TGF-β1, and ribosomal protein 36B4 over a time course from 0.5 to 24 h after addition of PDGF AB or PDGF AB plus PF4. Lanes 1, 6, and 9 represent diluent control cells; Lanes 2, 4, 7, and 10 are from PDGF AB-treated cells; and Lanes 3, 5, 8, and 11 are from PDGF AB plus PF4-treated cells. A lane for the 3-h control sample is missing because of loss and insufficient recovery of RNA. Expression of PDGF A chain and TGF-β1 is maximum at 3 h after addition of PDGF AB. PF4 inhibits peak expression of mRNA encoding both cytokines by approximately half (see Table 1).](image)
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regarded as a stimulator of mesangial cell proliferation, whereas TGF-β1 is inhibitory (19,36,37). PDGF is generally regarded as a stimulator of mesangial cell proliferation, whereas TGF-β1 is inhibitory (19,36,37). The inhibition of PDGF-induced elevations in PDGF A

DISCUSSION

These studies indicate that PF4 is a negative regulator of mesangial cell mitogenesis on the basis of the observation that PF4 reduced basal ³H-thymidine incorporation and inhibited FCS, PDGF, and EGF-induced mesangial cell proliferation. Such an inhibitory effect of PF4 on cell proliferation has been shown for megakaryocytes (27,28), endothelial cells (29–31) fibroblasts (32), tumorigenesis (29,33,34), and angiogenesis (29,30). Maione et al. (30) reported that the inhibition of tumorigenesis and angiogenesis is probably related to a direct effect of PF4 on endothelial cell proliferation, as indicated by an inhibition of growth factor-induced endothelial cell proliferation, but not for fibroblasts, keratinocytes, and several murine tumor cell lines (30). The authors suggested a specificity of the inhibition toward cell types closely associated with capillaries and suggested a possible physiological role for PF4 in the regulation of vessel development. Mesangial cells are pericytes associated with glomerular capillaries and are analogous to vascular smooth muscle cells (35). This study with mesangial cells is the first report that PF4 is inhibitory to vascular cells other than endothelial cells and is consistent with the above hypothesis.

The cellular mechanisms by which PF4 inhibits cell proliferation are not known. Our experiments indicate that PF4 interferes with two well-defined autocrine growth regulatory pathways for mesangial cells involving PDGF and TGF-β1 (19,36,37). PDGF is generally regarded as a stimulator of mesangial cell proliferation, whereas TGF-β1 is inhibitory (19,36,37). The inhibition of PDGF-induced elevations in PDGF A

Recent studies demonstrate that PF4 can inhibit endothelial cell migration and proliferation by blocking growth factor-receptor interactions as for FGF and TGF-β1, and their receptors (38,39). Our studies specifically rule out this mechanism since PF4 did not alter PDGF binding to mesangial cell receptors, yet inhibited PDGF-induced mesangial cell DNA synthesis. Time-course studies in which PF4 was added to FCS-stimulated mesangial cells indicated that PF4 was effective when added up to 8 h after the stimulus, suggesting that inhibition of DNA synthesis might be the result of mechanisms distal to early signal-transduction pathways. Early-transduction pathways usually occur from minutes to 1 to 2 h after addition of stimulus in vitro, as has been shown for induction of phospholipid hydrolysis, phosphorylation pathways, and oncogene expression (40,41). This is also supported by our observation that PF4 inhibited DNA synthesis induced by several mitogens that may not share early signal-transduction mechanisms. Studies to identify specific regulatory pathways in which PF4 might be modulating cell proliferation are currently underway in our laboratory.

Platelets and their secretory products have been implicated as mediators of glomerular disease in experimental and clinical settings (1). PDGF and TGF-β1 have been implicated as major mediators of proliferative glomerular disease and glomerulosclerosis (2,3). Mechanisms whereby platelet secretory products could mediate alterations in glomerular disease include alterations in glomerular permeability, leading to enhanced immune complex deposition (1,10) glomerular cellularity, and matrix metabolism. The mechanisms by which platelet secretory products mediate glomerular cellularity may involve modulation of glomerular extracellular matrix metabolism, migration, proliferation, and redistribution of resident glomerular cells (1). Similar processes have been postulated in cell modeling during embryogenesis, intimal hyperplasia in atherogenesis, and during formation of granulation tissue in wound healing (42,43). A precise role for PF4 in glomerular disease has not been defined; however, several studies suggest a potential role for PF4 as a mediator of glomerular injury by several of the mechanisms listed above. For example, PF4 binds avidly to glomerular structures, presumably to hepatan sulfate and other anionic moieties in GBM and glomerular cell surfaces, and has been implicated as a mediator of enhanced glomerular permeability (1,10). Such nonspecific "targeting" of PF4 to glomerular structures could also be operational during glomeru-
lary disease and is supported by the observations of glomerular localization of PF4 in clinical (14-16) and experimental (13) settings of glomerulonephritis in vitro. PF4 has been observed in glomerular structures in proliferative forms of glomerular disease and may have a role in regulation of glomerular cellularity (12,14). PF4 has chemotactic properties and is known to enhance monocyte, neutrophil, eosinophil, and fibroblast migration in vitro (44-47). Similarly, inflammatory cell migration has been induced at PF4 injection sites in vivo (48) and therefore could influence inflammatory cell infiltration in glomerular disease as suggested by Mezzano et al. (16) in post-streptococcal glomerulonephritis. PF4 may have negative regulatory functions for intrinsic glomerular cells by inhibition of glomerulonephritis. PF4 may have a role in regulation of glomerular cellularity and is known that inflammatory cell infiltration in glomerular disease as is supported by National Institutes of Health Grants DK38758 and DK43986 from the National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases) and the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

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