Interaction of Platelet Factor Four With Cultured Vascular Endothelial Cells

By Mary Ellen Rybak, Michael A. Gimbrone, Jr, Peter F. Davies, and Robert I. Handin

Platelets secrete a low-molecular-weight protein, platelet factor four (PF-4), which binds to and neutralizes heparin and related sulfated glycosaminoglycans (GAGs). To examine the interactions of PF-4 with the GAGs present on endothelial cell surfaces, we incubated $^{125}$I-PF-4 with cell suspensions derived from confluent monolayers of cultured bovine aortic endothelium. Binding of $^{125}$I-PF-4 was inhibited by a 100-fold excess of nonradioactive PF-4 and varied with duration and temperature of incubation. At 4°C, binding reached equilibrium at 20 minutes with $K_D = 2.87 \mu$mol/L and $B_{max}$ of 63.83 pmol/10^5 cells. Binding capacity was reduced 83.4% by brief incubation of endothelial cells with trypsin and 46.87% by incubation with Flavobacterium heparinase, but was unchanged by chondroitin-ABCase treatment. At 37°C, PF-4 was internalized by confluent monolayer of bovine aortic endothelial cells primarily through low-affinity adsorptive endocytosis. The internalized PF-4 was degraded to amino acids and small peptides with 50% conversion after 18-hour incubation. These studies demonstrate that a secreted platelet protein can bind to and enter endothelial cells. Binding may explain the rapid clearance of released PF-4 from plasma and could have important local effects on endothelial structure and function.

© 1989 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Cell culture. Bovine aortic endothelial cells (BAECs), isolated from the intimal lining of the thoracic aorta of yearling calves, were cultured and characterized by morphologic, immunologic, and biochemical criteria, as previously described.36 For these studies, finite lifespan strains, free of smooth muscle cell contamination, were used in passages 11 through 21. To standardize for potential effects of cell cycle kinetics on expression of surface-associated GAGs,7,8 and endocytic rates,26 density-inhibited endothelial monolayers were studied one day after they reached a stable confluent density.

PF-4 binding assay. Human PF-4 was purified by affinity chromatography on heparin-Sepharose7 and iodinated with a modification of the chloramine-T method7 as previously described. This resulted in incorporation of 30 to 40 mCi $^{125}$I/µg protein. The $^{125}$I-labeled PF-4 retained full antigenicity in our radioimmunoassay8 and bound to heparin with high affinity, and 95% of the radioactivity precipitated in 20% trichloroacetic acid (TCA).

Binding studies were performed on cell suspensions obtained by first washing the monolayer with Hank's balanced salt solution (HBSS) (calcium and magnesium-free; M.A. Bioproducts, Walkersville, MD), then briefly treating the cells with ice-cold 0.2% EDTA in isotonic saline and scraping gently with a rubber policeman. This nonenzymatic treatment resulted in a relatively uniform single-cell suspension. Cells were 86% to 95% viable, as assessed by exclusion of trypan blue. Aliquots of 6 x 10^5 cells were incubated in 400 µL minimal essential media (M.A. Bioproducts) supplemented with 0.1% bovine serum albumin (BSA) and varying concentrations of $^{125}$I-PF-4 at 4°C in plastic tubes (Sarstedt, FRG) in an oscillatory shaker bath. Although PF-4 will specifically bind to endothelial cell monolayers, in previous studies of ligand binding to vascular cells we showed that cell suspensions give more consistent and reproducible results than intact monolayers.27-29 Cell suspensions avoid the potential artifacts associated with binding studies performed with cell monolayers, including trapping of ligand, nonspecific binding to exposed plastic, and nonuniform exposure of the cell surface. The incubation at 4°C was chosen to prolong the viability of cell suspensions and to prevent endocytosis of bound PF-4.

Separation of bound from free ligand was accomplished by layering 25-µL aliquots of the cell suspension with an intervening air space, over 150 µL ice-cold 5% sucrose solution in a 500-µL plastic microfuge tube. After a one-minute centrifugation at 10,000 g in a Beckman microfuge (Beckman Instruments, Menlo Park, CA), the

From the Hemostasis Unit, Hematology Division, Department of Medicine; and Vascular Pathophysiology Laboratory, Department of Pathology, Brigham and Women's Hospital; Harvard Medical School, Boston.

Submitted June 20, 1988; accepted December 19, 1988.

Supported by grants No. HL-17513, HL-22602, HL-24612, training grant HL-07523, and Special Fellowship HL-6037 from the National Institutes of Health, Bethesda, MD.

Address reprint requests to Mary Ellen Rybak, MD, University of Massachusetts Medical Center, 53 Lake Ave, N, Worcester, MA 01605.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc. 0006-4971/89/7306-0010$3.00/0
supernatant was aspirated and the cell pellet in the end of the
microfuge tube was amputated and counted in a γ-8000 Spectrometer
(Beckman Instruments). Nonspecific binding was defined as the
cell-bound radioactivity not displaced by a 100-fold excess of nonradioactive ligand. Binding data were analyzed by Marquardt-Levenberg algorithm for nonlinear parametric estimation.29

Enzyme treatment. The effect of enzymatic modification of the
cell surface was assessed by preincubation of the cell monolayer for
four hours with either 50 μg/mL TPKC-treated trypsin (284 U/mg;
Worthington Biochemicals, Freehold, NJ) or 100 μg/mL protease-
free. F heparinase prepared by the method of Linker and Horink30
or 2 U/mL chondroitin-ABCase (Seikagaku Kogyo, Tokyo). The
heparinase was provided by Dr Robert D. Rosenberg (Dana-Farber
Cancer Institute, Boston). After enzymatic treatment, the cells were
removed from the culture dish as described above. Cell viability
remained at >80% by trypan blue exclusion.

Quantitative endocytosis. Confluent endothelial cell mono-
layers in 35-mm dishes were incubated with 125I-PF-4 at 37°C for
varying times. After incubation, the confluent monolayers were
washed eight times with 2 mL HBSS containing 0.2% serum
albumin and 30 mg/mL heparin sulfate (Sigma Chemicals, St
Louis) and then treated with 500 μg/mL trypsin (GIBCO Laborato-
ries, Grand Island, NY). The detached cells were centrifuged (200 g
for seven minutes at 4°C), the cell pellet was solubilized in 1 mL
0.1% sodium dodecyl sulfate (SDS) and counted in a Gamma 8000
spectrometer. For studies of fluid endocytosis, 10 μCi/mL 3C-
sucrose (New England Nuclear, Boston) was added to the culture
media. In preliminary experiments, PF-4 did not interact with
sucrose and did not affect the basal rate of pinocytosis.

To determine the % of intracellular PF-4, monolayers were
incubated with 125I-PF-4 solution (2.5 μmol/L, 10 μCi/mL), then
washed with HBSS containing serum albumin and heparin as
described above. The washed monolayers were then incubated with
PF-4-free culture media at 4°C for up to 72 hours. Triplicate dishes
of cells were removed at various times. Cells were suspended, washed
and centrifuged at 200 g for seven minutes at 4°C. The cell pellet was
counted for residual 125I-PF-4 radioactivity.

The medium was assayed for degradation products of PF-4 by
measurement of TCA-soluble–labeled material by adapting the
procedure of Bierman et al.31 To 200 μL medium, 0.25 mL 50% TCA
was added. The tube was placed on melting ice for five minutes and
then centrifuged at 10,000 for five minutes at 4°C. The TCA-soluble
material was then analyzed for water-soluble (presumably small
peptides and amino acids) and chloroform-soluble (molecular io-
dine) radioactivity. To each milliliter of TCA supernatant, 0.01 mL
40% KI and 0.05 mL of 30% hydrogen peroxide were added. After
five minutes, the mixture was extracted with 3 vol chloroform, and
the water phase was removed. Additional sequential extractions
revealed no further evidence of any chloroform-soluble radioactivity
(molecular iodine) in the aqueous phase. As a control, identical
medium containing 125I-PF-4 was incubated with cells at 4°C.

The influence of dextran sulfate, an inhibitor of phagolysosome
fusion,32 on the fate of intracellular PF-4 was assessed by incubating
cells with 50 μg/mL dextran sulfate and serum-free tissue culture
medium supplemented with 1% albumin for 15 hours at 37°C before
the cells were exposed to PF-4 as described above.

The release of lactate dehydrogenase (LDH EC 1.1.1.27) from
cells was used as an indicator of cytotoxicity throughout the experi-
ments. LDH was determined by the Worthington assay (Worthing-
ton Biochemicals) with a skeletal muscle LDH (type II; Sigma) as a
standard. No cytotoxicity due to PF-4 was detected. In all experi-
ments, aliquots of each SDS cell lysate were removed for determina-
tion of cellular protein by the procedure of Lowry et al.33

RESULTS

PF-4 binding to endothelial cells. PF-4 bound toendo-
thelial cells in a time-dependent, saturable, reversible manner.
As shown in Fig 1, when endothelial cells were incubated
with 0.3 μmol/L 125I-PF-4 at 4°C, specific binding increased
over time, with 50% of total binding achieved at three
minutes and equilibrium achieved at 20 minutes. After
apparent equilibrium was reached, the addition of 300

![Fig 1](image1.png)

**Fig 1.** Time course of total and nonspecific binding of 125I-PF-4
to endothelial cells at 4°C. Endothelial cells (2 x 10^4) were
incubated with 0.3 μmol/L 125I-PF-4 (O—O) or with 300 μmol/L
nonradiolabeled PF-4 plus 0.3 μmol/L 125I-PF-4 (O—O) with
constant agitation for up to 40 minutes. Aliquots were removed
at indicated times. Arrow: Nonradiolabeled PF-4 (300 μmol/L) was
added to the incubation mixture.

![Fig 2](image2.png)

**Fig 2.** Dissociation of 125I-PF-4 from the cell surface induced
by heparin. Endothelial cell suspensions were equilibrated with
125I-PF-4, washed, and resuspended in the original volume of HBSS.
Then either 10 μmol/L heparin (O—O), 1 μmol/L heparin (O—O),
or an equivalent volume of HBSS (O—O) was added, and cell-
associated radioactivity was determined at successive times.
μmol/L PF-4, a 1,000-fold molar excess, rapidly reversed this binding. Nonspecific binding did not increase during the 20-minute incubation and represented 10% ± 3% (mean ± SEM) of the total binding at equilibrium. Addition of heparin to cells previously equilibrated with $^{125}$I-PF-4 also rapidly reduced binding, with complete reversal after five-minute incubation (Fig 2). The rate of dissociation of PF-4 from cells in the presence of heparin was dependent on heparin concentration.

Specific PF-4 binding to endothelial cells with increasing concentrations of PF-4 is shown in Fig 3. At saturation, 50 pmol PF-4/10^6 cells was bound. The binding isotherm in Fig 3A shows that half the binding sites were occupied after incubation with 2 μmol/L PF-4. Binding data were analyzed with both two-site and single-site assumption by the Marquardt-Levenberg algorithm for nonlinear parametric estimation. This analysis demonstrated that these data best satisfy a single-site model with $K_d = 2.87$ μmol/L and $B_{\text{max}} = 63.83$ pmol/10^6 cells (Fig 3B).

Treatments of the cells with trypsin reduced the number of PF-4 binding sites by 83.4% with minimal change in $K_d$. Single-site analysis of posttrypsin binding yields a $K_d$ of 0.17 μmol/L and $B_{\text{max}} = 10.6$ pmol. Treatment with heparinase resulted in a 46.6% reduction in binding sites and no significant change in $K_d$. Single-site analysis after heparinase indicates a $K_d$ of 1.58 μmol/L and $B_{\text{max}} = 34.1$ pmol.

Treatment of cells with chondroitin-ABCAsew caused no change in the number or affinity of cell-surface PF-4 binding sites. The post-ABCAsew binding data were virtually identical to that for untreated cells.

Internalization. To examine the internalization of surface-bound PF-4 by endothelial cells, intact confluent monolayers were incubated at 37°C. Under these conditions, there was a time-dependent increase in cell-associated $^{125}$I-PF-4, which could not be removed by incubation with heparin or HBSS or by treatment with trypsin. This trypsin- and heparin-resistant increase in cell-associated radioactivity did not occur if monolayers were incubated with the same concentration of PF-4 at 4°C, suggesting that PF-4 was internalized by the cells at 37°C.

The amount of fluid pinocytosed by the cell monolayer was determined by incubation with $^{14}$C-sucrose and compared with the uptake of $^{125}$I-PF-4. If most of the PF-4 was taken up in the bulk fluid, the rate of fluid pinocytosis as measured by the two tracers should be similar. As shown in Table 1, this was clearly not the case, indicating that PF-4 was internalized by an adsorptive endocytic mechanism. The microliters of fluid internalized as measured by $^{125}$I-PF-4 increased over the concentration range of 0.05 to 2.5 μmol/L PF-4 while the uptake of $^{14}$C-sucrose remained constant. Since we know from other experiments that PF-4 did not stimulate bulk fluid uptake, the data demonstrate that $^{125}$I-PF-4 became concentrated in each endocytic vesicle by absorption to the invaginating plasma membrane. At 2.5 μmol/L PF-4, ~220 pmol PF-4 are bound per 10^6 cells at equilibrium. Correcting for specific activity, ~2 x 10^6 pmol PF-4/10^6 cells was internalized after 36-hour incubation, with ~80% entering by adsorptive endocytosis.

To assess the fate of the internalized PF-4, cells were incubated with $^{125}$I-PF-4, washed extensively, and incubated in PF-4-free media. A progressive decrease occurred in cell-associated $^{125}$I to ~14% of the original content (Fig 4), with a t½ of ~18 hours. The culture medium was analyzed

Table 1. Bulk Fluid-Phase Endocytosis by Confluent Monolayers of Bovine Aortic Endothelial Cells as Measured by Internalization of $^{125}$I-PF-4 and $^{14}$C-Sucrose: Demonstration of Absorptive Endocytosis of PF-4

<table>
<thead>
<tr>
<th>Concentration of PF-4 (μmol/L)</th>
<th>Microliters of Fluid by $^{125}$I-PF-4/mg Cell Protein/h</th>
<th>Microliters of Fluid by $^{14}$C-Sucrose/mg Cell Protein/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.75 ± 0.3</td>
<td>0.82 ± 0.4</td>
</tr>
<tr>
<td>0.25</td>
<td>2.0 ± 0.6</td>
<td>0.79 ± 0.3</td>
</tr>
<tr>
<td>2.5</td>
<td>5.2 ± 0.8</td>
<td>0.80 ± 0.5</td>
</tr>
</tbody>
</table>

Cells were incubated with $^{125}$I-PF-4 and $^{14}$C-sucrose as described in the Materials and Methods section. Data are mean ± SEM for four separate experiments with triplicate cultures used for each PF-4 concentration.
PF-4, ENDOTHELIAL CELLS

Fig 4. Cell-associated 125I cpm after exposure to 125I-PF-4. Cells were incubated with 125I-PF-4 (curve A) or preincubated with 50 μg/mL dextran sulfate for 15 hours before identical exposure to 125I-PF-4 (curve B) and then washed extensively with HBSS and heparin to remove surface-bound 125I-PF-4. Cell-associated radioactivity over time was then recovered at successive times.

for radiolabeled material which was precipitable with 20% TCA (intact protein and larger polypeptide fragments) and for TCA-soluble radiolabeled material which partitioned in the aqueous phase after several chloroform extractions (PF-4-derived amino acids and small peptides). There was a progressive increase in TCA-soluble 125I in the medium, equivalent to the loss of radioactivity from the cells. Less than 10% of the radioactivity in the media was precipitated by TCA. These data suggest that after internalization by the cell most of the PF-4 is degraded by lysosomal enzymes. That pretreatment of cells with dextran sulfate, an inhibitor of phagosome-lysosome fusion, prevented the loss of cell-associated radioactivity (Fig 4) and inhibited excretion of 125I-labeled polypeptides and amino acids further supports this hypothesis.

DISCUSSION

We analyzed the interaction between cultured vascular endothelial cells and purified PF-4. Cultured cells bound up to 50 pmol PF-4/10^5 cells at saturating concentrations of PF-4. Thus, because the vascular intima is a broad area in vivo, the surface could potentially bind large quantities of PF-4. Endothelial cell binding may result from electrostatic interactions between lysine groups on the carboxy-terminus of the PF-4 molecule and sulfated GAGs on the cell surface, analogous to the previously described interaction between PF-4 and heparin.1-10

Binding of PF-4 to endothelial cells is of significantly lower affinity (kd = 2.87 × 10^{-6} mol/L) than the binding of PF-4 to heparin in solution (kd = 2 × 10^{-8} mol/L).7 This may be due to steric considerations at the cell surface or electrostatic environment fully sulfated heparin-like mole-
cules on the cell. Endothelial cell binding of PF-4 is, however, dependent on the presence of heparinlike sulfated GAGs since binding is reduced by treatment with heparinase but not by treatment with enzymes which selectively cleave chondroitin 4 or 6 sulfate and dermatan sulfate. Lack of complete inhibition of PF-4 binding by enzyme treatment may be explained by incomplete removal of heparin molecules from the inferior surface of the monolayer before cell suspension binding studies. Although the data best describe a single class of binding sites, the statistically less likely possibility of two binding sites cannot be entirely excluded. The binding capacity of bovine aortic endothelial cells exceeds the number of active heparin sequences per endothelial cell according to the studies of Marcum et al.17,18 Thus, sequences without anticoagulant activity must bind PF-4.

Our study confirms and extends a previous study of Busch et al.32 in which binding of PF-4 (complexed with heparin and chondroitin-4-sulfate) to human endothelial cell monolayers was measured. They observed saturable, specific binding of the radio-iodinated ligand complex at room temperature and reduced binding capacity by pretreatment with platelet heparitinase.34 Direct quantitative comparison of the binding data between these studies is difficult because of differences in experimental design. These differences include cell type: human umbilical vein cells vs bovine aortic endothelial cells, monolayers v cell suspensions, and PF-4 complexed to carrier v PF-4 alone. The results of these two studies are qualitatively consistent despite these methodologic differences.

Although binding of PF-4 to the endothelial cell surface is of relatively low affinity, this association nonetheless serves to facilitate internalization of the macromolecule by adsorptive endocytosis. As expected, internalization of PF-4 by adsorptive endocytosis greatly exceeded that which occurred by fluid-phase pinocytosis. By this process, endothelium presumably can remove PF-4 rapidly and efficiently from its surface. We demonstrated that the primary fate of endocy-
tosed PF-4 in endothelium is degradation to TCA-soluble amino acids in the lysosomal system of the cell, although a small fraction (~10%) appears to avoid degradation and is released into the culture medium through unknown pathways. Aulinikas et al.31 described a similar “retroendocytosis” pathway for low-density lipoproteins in cultured vascular smooth muscle cells, and Connolly et al.32 presented evidence for a “short-circuit” pathway of endocytosis–exocy-
tosis of glycosides that avoids degradation in hepatocyte lysosomes. Dextran sulfate, an inhibitor of endosome-lysosome fusion, blocked the degradation of PF-4 but did not redirect the intact molecule out of the cell (ie, an exocytic pathway for PF-4 was not activated as an alternative to lysosomal degradation in dextran sulfate-treated cells). Instead, PF-4 remained associated with the cell, a situation similar to that described for internalized horseradish peroxi-
dase in the presence of dextran sulfate.37

Although the functional consequences of the interaction of PF-4 with the endothelial cell surface are not yet clear, several different effects might be hypothesized. First, PF-4 binding could reduce the net negative charge of surface-associated GAGs nonspecifically and alter the reactivity of the endothelial cell with circulating blood cells and blood.
proteins. This effect would be analogous to the observed inhibitory action of PF-4 in binding of low-density lipoproteins to fibroblasts. A second type of PF-4 effect might result from specificity of binding to heparin-like domains on the endothelial cell surface. Heparin-like activity on vascular endothelial cells has been described in several studies. Busch et al described antithrombin cofactor activity on cultured mouse capillary endothelium. Marcum et al demonstrated heparin-like molecules on the surface of vascular endothelium in a rat hindlimb perfusion model and cultured vascular endothelial cells. They showed that microvascular endothelial cells synthesize cell-surface-associated heparin-like molecules attached to hydrophobic proteins which contain elements of the tetrasaccharide region important in binding of heparin to antithrombin. These heparin-like species accelerate thrombin-antithrombin complex formation and exhibit anticoagulant activity virtually indistinguishable from that of the commercial mucopolysaccharide. As suggested by Busch et al, binding of the PF-4, released at a site of vascular injury to endothelial cell surfaces could block the anticoagulant activity of surface-associated heparin activity and thus promote local thrombus formation. Third, ultrastructural studies by Simionescu et al demonstrated that various sulfated GAGs, including heparin sulfate, exist in microdomains on the endothelial cell surface. Selective interaction of a ligand, like PF-4, with these heparin-like domains, could exert a localized effect on endothelial membrane structure. Clearly, further studies will be needed to define the functional significance of PF-4/endothelial interactions for vascular pathology.

ACKNOWLEDGMENT

We acknowledge the excellent technical assistance of Shiela Cruise and Ethel Shefton in cell culture.

REFERENCES


From www.bloodjournal.org by guest on November 15, 2017. For personal use only.
41. Marcum JA, Rosenberg RD: Heparin-like molecules with anticoagulant activity are synthesized by cultured endothelial cells. Biochem Biophys Res Commun 126:365, 1985
Interaction of platelet factor four with cultured vascular endothelial cells

ME Rybak, MA Jr Gimbrone, PF Davies and RI Handin