

## Platelet-Derived Growth Factor Does Not Stimulate Prostacyclin Synthesis by Cultured Endothelial Cells

By Karleen S. Callahan, Anna Schorer, and John M. Harlan

We examined the effect of highly purified platelet-derived growth factor (PDGF) on prostacyclin (PGI<sub>2</sub>) release by cultured human umbilical vein and bovine aortic endothelial cells. PDGF tested at concentrations equal to or exceeding those observed in serum did not increase endothelial cell PGI<sub>2</sub> synthesis as measured by radioimmunoassay of its metabolite, 6-keto-PGF<sub>1α</sub>. In contrast, cells incubated with 20% human whole blood serum (WBS) demonstrated significantly increased PGI<sub>2</sub> production (fivefold stimulation). Addition of anti-PDGF antibody to the 20% WBS did not

attenuate the increased synthesis of PGI<sub>2</sub>. Incubation with 20% plasma-derived serum (PDS) that was deficient in PDGF produced stimulation of PGI<sub>2</sub> release similar to 20% WBS. These results demonstrate that PDGF does not cause increased PGI<sub>2</sub> synthesis in cultured human endothelial cells of human or bovine origin, and further suggest that the stimulation observed with serum is not due to a platelet-release product.

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**T**HE INTERACTION of platelets and endothelium with respect to thromboxane A<sub>2</sub> and prostacyclin (PGI<sub>2</sub>) synthesis by these respective cell types has been extensively investigated. Although platelet-derived endoperoxides may supply substrate for endothelial prostaglandin formation,<sup>1</sup> the effects of platelet-derived proteins on endothelial cell prostanoid synthesis are not well defined.<sup>2-6</sup> Platelet activation by various physiologic stimuli results in the release of platelet  $\alpha$  granule constituents including purified platelet-derived growth factor (PDGF), a potent mitogen which may contribute to wound repair following injury.<sup>7-9</sup> PGI<sub>2</sub>, a major arachidonic acid metabolite produced by endothelium, is a potent vasodilator and inhibitor of platelet aggregation and release.<sup>10</sup> Previous investigations have reported increased prostaglandin synthesis in cultured cell systems including bovine aortic endothelial cells following incubation with PDGF,<sup>11,12</sup> and it was postulated that increased vascular PGI<sub>2</sub> production due to PDGF might act as a negative feedback mechanism for limiting platelet aggregation at sites of vascular injury.<sup>4</sup> The current study was designed to investigate this hypothesis by examining the effects of highly purified human PDGF,<sup>13</sup> as well as specific PDGF antibody, on PGI<sub>2</sub> synthesis in cultured human umbilical vein endothelial cells.

### MATERIALS AND METHODS

**Cell culture.** Human umbilical vein and bovine aortic endothelial cells were isolated by collagenase treatment of vessels as previously described.<sup>14</sup> The human endothelial cells were maintained in 20% fetal calf serum (FCS), and the bovine cells were maintained in 10% FCS (Hyclone Laboratory, Logan, Utah) in either RPMI 1640 (human) or Waymouth's (bovine) medium (Grand Island Biologic Company, Grand Island, NY). Endothelial cells were passaged following trypsinization and plated at confluent density in 16-mm diameter wells (Costar, Cambridge, Mass). The human umbilical vein endothelial cells were used in first passage, and the bovine aortic endothelial cells were used in eighth passage. Bovine aortic smooth muscle cells at fifth passage were provided by Dr Stephen Schwartz, University of Washington, Seattle. Human foreskin fibroblasts were prepared as previously described<sup>15</sup> and were generously provided by Dr Russell Ross, University of Washington, Seattle.

**Radioimmunoassay of endothelial cell 6-keto PGF<sub>1α</sub> release.** Prior to treatment, the monolayers were gently washed twice with 2.0 mL of serumless Neuman-Tytell medium which contains 0.2% lactalbumin (Grand Island). The wash medium was decanted and replaced with 0.5 mL of Neuman-Tytell medium containing

control or test agents for an overnight incubation (24 hours) at 37 °C in 5% CO<sub>2</sub>, 95% air. At the end of 24 hours, an aliquot of cell-free supernatant medium was removed from each well, placed into a polypropylene tube, and immediately frozen in a methanol-dry ice bath for storage at -20 °C until assayed. In selected experiments, the endothelial monolayers were gently washed again, the supernatant medium was decanted, and 0.5 mL of serumless Neuman-Tytell medium with or without purified human  $\alpha$ -thrombin or A23187 was added for an additional 30-minute incubation. At the end of this time, the cell-free supernatant medium was removed and treated as above for later radioimmunoassay. Cell counts were performed on replicate wells by electronic particle counter (Particle Data, Inc, Elmhurst, Ill).

Measurement of unextracted, supernatant medium from the endothelial cell cultures for PGI<sub>2</sub> production was determined by radioimmunoassay (RIA) of the stable PGI<sub>2</sub> metabolite using [<sup>3</sup>H]6-keto-PGF<sub>1α</sub> (Amersham, Arlington, Ill), authentic 6-keto-PGF<sub>1α</sub> standard kindly provided by Douglas McCarter and Dr John Pike, The Upjohn Company, Kalamazoo, Mich, and antiserum generously supplied by Dr William B. Campbell, University of Texas Health Science Center at Dallas. This anti-6-keto-PGF<sub>1α</sub> antiserum cross-reacted <0.9% with PGE<sub>1</sub>, PGE<sub>2</sub>, PGF<sub>1α</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, PGA<sub>2</sub>, and TxB<sub>2</sub>, 3.5% with 6, 15-diketo PGF<sub>1α</sub>, and 70% with 6-keto-PGE<sub>1</sub>. The level of sensitivity was 10 pg/300  $\mu$ L, and assays were run in duplicate and at more than one dilution when necessary. The proteins in the Neuman-Tytell medium did not interfere in the RIA and recovery of exogenous 6-keto-PGF<sub>1α</sub> added to Neuman-Tytell medium was 106.7%  $\pm$  5.8%.

**Preparation of whole blood serum (WBS) and plasma-derived serum (PDS).** Human WBS was prepared following venipuncture from a single normal donor by allowing the blood to sit at room temperature until clotted (~two hours). The blood was then centrifuged at 2000 g for 20 minutes, and the cell-free serum was collected and passed through a 0.2  $\mu$ m filter (Millipore, Bedford, Mass) in order to obtain a sterile preparation. The WBS was stored at 4 °C until used. PDS was prepared by collecting normal human blood from several donors into precooled syringes containing 3.5% citrate at a final dilution of 1:10 (vol/vol). The sample was immediately

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*From the Departments of Pathology and Medicine (Hematology), University of Washington, Seattle.*

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*Address reprint requests to Karleen S. Callahan, PhD, Division of Hematology, Harborview Hall ZA-34, 325 Ninth Ave, Seattle, WA 98104.*

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centrifuged at 2500 g for 20 minutes at 4 °C. The cell-free and platelet-free plasma was recalcified, dialyzed against phosphate-buffered saline (PBS) to remove citrate, centrifuged to remove the clot, and stored at 4 °C.

**Radioreceptor assay of PDGF.** Levels of PDGF in WBS and PDS were determined by radioreceptor assay using human foreskin fibroblasts and <sup>125</sup>I-labeled PDGF as described by Bowen-Pope and Ross.<sup>15</sup> In brief, confluent fibroblast monolayers were put on ice and rinsed with cold PBS containing 0.1% bovine serum albumin (BSA) and 0.5 mmol/L of CaCl<sub>2</sub>, pH 7.4. This binding rinse was aspirated from the cells and replaced with 1.0 mL per well of test solution for a three-hour incubation at 4 °C with constant shaking. Next, the supernatant was removed and replaced with 1.0 mL per well of 25 mmol/L of HEPES buffer, pH 7.4, containing 0.25% BSA and 0.5 ng <sup>125</sup>I-PDGF. The fibroblasts were incubated at 4 °C with shaking for one hour, the supernatant was aspirated, and the cells were washed twice with the binding rinse to remove unincorporated <sup>125</sup>I-PDGF. Finally, the cells were solubilized with 0.1% Triton X-100 (New England Nuclear, Boston) in water containing 0.1% BSA and were collected for gamma counting. This radioreceptor assay detects levels of >0.1 ng PDGF/1.0 mL.

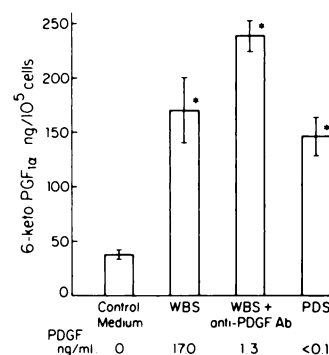
**Reagents.** Highly-purified PDGF<sup>13</sup> and goat anti-human PDGF antibody were generously supplied by Dr Elaine Raines and Dr Russell Ross, University of Washington, Seattle. The PDGF preparation was a 500,000-fold purification from serum and consists of four components with mol wts of 27,000 to 31,000 in the nonreduced state using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). PDGF was stored in 10 mmol/L of acetic acid containing 0.25% BSA, which was diluted a 100-fold or more in Neuman-Tytell media for experiments. A comparable volume of this acetic acid solution was added to control cultures. The IgG fraction of goat anti-human PDGF antiserum was prepared by 18% Na<sub>2</sub>SO<sub>4</sub> precipitation and diethylaminoethanol (DEAE)-sephacel chromatography. The anti-PDGF IgG (at a concentration of 20 μg/mL) completely inhibits 1 ng/mL purified PDGF or an equivalent amount of PDGF in WBS as determined by mitogenic activity or ability to compete with <sup>125</sup>I-PDGF in the radioreceptor assay (E. R., personal communication). Purified human α-thrombin (12,000 U/mg protein) was generously provided by Dr John Fenton II, (Buffalo). A23187 was obtained from Sigma Chemical Company.

**Statistical analysis.** The results were evaluated by analysis of variance with the Neuman-Keul's multiple comparisons test.

## RESULTS

Figure 1 shows the effects of WBS and PDS on endothelial PGI<sub>2</sub> synthesis as measured by RIA of 6-keto-PGF<sub>1α</sub>. Incubation of the human endothelial cell monolayers with 20% human WBS resulted in a significant increase in 6-keto-PGF<sub>1α</sub> release when compared with incubation in serumless medium alone. Comparable stimulation was observed with WBS prepared from a pool of donors. Addition of the anti-PDGF IgG to 20% WBS did not attenuate the stimulatory effect. Anti-PDGF IgG alone did not increase 6-keto-PGF<sub>1α</sub> synthesis over that observed with medium alone (data not shown). Addition of 20% PDS, however, caused a significant increase over medium alone (Fig 1). There was no further stimulation over that observed with PDS alone when PDGF was added to 20% PDS (data not shown). The concentration of PDGF in WBS was 17.0 ng/mL, 1.3 ng/mL in WBS containing anti-PDGF IgG, and <0.1 ng/mL in PDS.

In contrast to the results obtained with WBS or PDS, a 24-hour incubation of human endothelial cells with purified

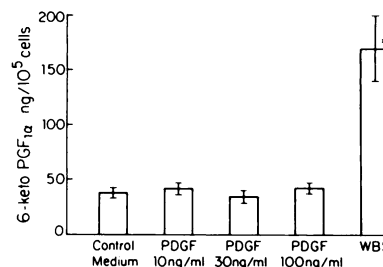


**Fig 1. Effect of whole blood serum (WBS) and plasma-derived serum (PDS) on 6-keto-PGF<sub>1α</sub> release.** Human umbilical vein endothelial cell monolayers were incubated with medium alone (n = 16), medium containing 20% WBS (n = 12), 20% WBS with anti-platelet-derived growth factor (PDGF) antibody (Ab) (n = 8), or 20% PDS (n = 8). Aliquots of supernatant medium were removed after 24-hour incubation for determination of 6-keto-PGF<sub>1α</sub> levels by RIA. Values represent means ± 1 SE from quadruplicate wells in two to four individual experiments (\* P < .01). The levels of PDGF in the serum preparations were determined by radioreceptor assay.

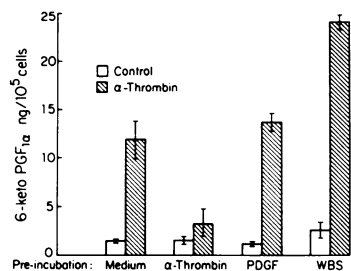
PDGF (10 to 100 ng/mL) did not result in stimulation of 6-keto-PGF<sub>1α</sub> production (Fig 2). The reported values of PDGF in human whole blood serum are 17.5 ng/mL by radioreceptor assay<sup>16</sup> or 50.0 ng/mL by radioimmunoassay.<sup>17</sup>

We also examined the effects of platelet products on subsequent thrombin-induced PGI<sub>2</sub> synthesis as shown in Fig 3. The cells were incubated for 24 hours with various treatments, washed once, and incubated with fresh medium with or without α-thrombin for 30 minutes. α-Thrombin induced a marked increase in 6-keto-PGF<sub>1α</sub> synthesis in cells preincubated in medium alone and in cells preincubated with either PDGF or WBS. In contrast, monolayers previously incubated with α-thrombin were refractory to subsequent stimulation by α-thrombin, as reported by others.<sup>18</sup>

Because the previous report of PDGF-stimulated endothelial cell PGI<sub>2</sub> release used bovine aortic endothelial cells,<sup>4</sup> we



**Fig 2. Effect of platelet-derived growth factor (PDGF) on 6-keto-PGF<sub>1α</sub> release.** Human umbilical vein endothelial cell monolayers were incubated with medium alone (n = 16), medium containing PDGF 10 ng/mL (n = 16), PDGF 30 ng/mL (n = 8), PDGF 100 ng/mL (n = 4), or medium containing 20% WBS (n = 8). Aliquots of supernatant medium were removed after 24-hour incubation for determination of 6-keto-PGF<sub>1α</sub> levels by RIA. Values represent means ± 1 SE from quadruplicate wells in one to four experiments (\* P < .01).



**Fig 3. Effect of platelet-derived proteins on thrombin-stimulated 6-keto-PGF<sub>1α</sub> release.** Human umbilical vein endothelial monolayers were incubated for 24 hours with medium alone, medium containing 8 U/mL α-thrombin, 10 ng/mL PDGF, or 20% WBS. The monolayers were then washed and incubated with medium alone or medium containing α-thrombin (8 U/mL). Aliquots of supernatant medium were removed after 30-minute incubation for determination of 6-keto-PGF<sub>1α</sub> levels by radioimmunoassay. Values represent means ± 1 SE from quadruplicate wells in two experiments.

repeated similar experiments using our PDGF preparation and bovine aortic endothelial cells (Table 1). Although PGI<sub>2</sub> synthesis in these multiple passages of bovine endothelial cells was considerably less than in the human endothelial cells, addition of PDGF (10 to 100 ng/mL) did not increase 6-keto-PGF<sub>1α</sub> release when compared with serumless medium alone. These same bovine cells were able to respond to subsequent stimulation using calcium ionophore A23187 or 20% WBS (data not shown).

Finally, we examined the effect of PDGF on PGI<sub>2</sub> synthesis in another vessel wall cell type. In contrast to human or bovine endothelial cells, bovine aortic smooth muscle cells demonstrated increased 6-keto-PGF<sub>1α</sub> synthesis following incubation with PDGF (Table 1). PDGF caused a greater than tenfold increase in 6-keto-PGF<sub>1α</sub> release in smooth muscle cells. In addition, 6-keto-PGF<sub>1α</sub> synthesis was significantly increased in smooth muscle cells exposed to 20% WBS when compared with those incubated in 20% PDS. Although

**Table 1. Effect of PDGF on Bovine Vessel Wall Cell PGI<sub>2</sub> Release**

		6-keto-PGF <sub>1α</sub> ng/10 <sup>5</sup> Cells*
Addition to bovine aortic endothelial cells		
Control	(3)	1.34 ± 0.22
PDGF		
10 ng/mL	(3)	0.80 ± 0.06
50 ng/mL	(3)	0.96 ± 0.06
100 ng/mL	(3)	0.99 ± 0.13
Addition to bovine aortic smooth muscle cells		
Control	(4)	0.32 ± 0.04
PDGF		
10 ng/mL	(4)	4.02 ± 0.14*
20% WBS	(4)	18.84 ± 0.68*
20% PDS	(4)	5.14 ± 0.50*
20% WBS + anti-PDGF Ab	(4)	14.24 ± 0.88*

PDGF, platelet-derived growth factor; WBS, whole blood serum, PDS, plasma-derived serum.

6-Keto-PGF<sub>1α</sub> levels in supernatant medium were determined by RIA after a 24-hour incubation. Values represent means ± 1 SE of (n) replicate wells.

\*P < .01.

addition of anti-PDGF IgG to WBS resulted in decreased PGI<sub>2</sub> synthesis as compared with WBS alone, the amount released was still significantly higher than with medium alone (Table 1).

## DISCUSSION

Previous investigations have demonstrated that PGI<sub>2</sub> production by endothelial cells is stimulated by unidentified factor(s) present in normal plasma and serum.<sup>19-23</sup> Coughlin et al,<sup>4</sup> using cultured bovine aortic endothelial cells, demonstrated that serum prepared from platelet-rich plasma produced a greater increase in PGI<sub>2</sub> release than did serum prepared from platelet-poor plasma. The serum factor described in that report was similar to PDGF in being heat-stable and nondialyzable; they also found that addition of partially purified PDGF resulted in significant enhancement of PGI<sub>2</sub> synthesis within 24 hours. These investigators suggested that PDGF or other products released by activated platelets might provide feedback inhibition of further platelet aggregation by stimulating endothelial PGI<sub>2</sub> production.<sup>5</sup>

We did not observe an increase in PGI<sub>2</sub> production when cultured human umbilical vein or bovine aortic endothelial cells were incubated with purified PDGF at concentrations equal to or exceeding normal serum levels (Fig 2 and Table 1). Although incubation with WBS significantly increased PGI<sub>2</sub> synthesis, PDS that lacked PDGF produced a similar increase. Furthermore, addition of specific anti-PDGF IgG to the WBS did not attenuate the enhanced PGI<sub>2</sub> generation (Fig 1). These observations demonstrate that PDGF is not the stimulating factor in serum responsible for enhanced PGI<sub>2</sub> synthesis by cultured endothelial cells.

We also found that prior incubation of the endothelial cells with either PDGF or WBS did not attenuate subsequent stimulation of PGI<sub>2</sub> induced by α-thrombin (Fig 3). These observations suggest that platelet-derived products do not down regulate endothelial PGI<sub>2</sub> synthesis as has been reported for β-thromboglobulin.<sup>2</sup> PGI<sub>2</sub> synthesis by bovine aortic smooth muscle cells was stimulated by purified PDGF, and 20% WBS resulted in greater production of PGI<sub>2</sub> than did 20% PDS. In addition, PGI<sub>2</sub> production was reduced by the addition of anti-PDGF IgG to WBS. Thus, as previously reported by Coughlin et al,<sup>5</sup> smooth muscle cell PGI<sub>2</sub> synthesis is sensitive to platelet-derived products, including PDGF. In all of these studies, it should be noted that WBS and PDS differ in more than platelet release products. Products of leukocytes activated during clotting ex vivo could contribute to the PGI<sub>2</sub> stimulatory activity of WBS.

Although our results on endothelial cell response to PDGF differ from those of Coughlin et al,<sup>4</sup> they are similar to those of Poggi and co-workers.<sup>6</sup> These latter investigators examined cultured bovine aortic endothelial cells and reported that partially purified PDGF had an insignificant effect on PGI<sub>2</sub> synthesis at 24 hours (140.7% of control). Both of these earlier investigations<sup>4,6</sup> utilized partially purified PDGF prepared according to the method of Antoniadis et al,<sup>24</sup> whereas the present study used more highly purified PDGF prepared according to the method of Raines and Ross.<sup>12</sup> Consequently, discrepancies among the studies may be due in part to differences in the PDGF preparations and the possibility of a

contaminating serum factor in the earlier studies. Our preparation of PDGF was active as a mitogenic factor in fibroblasts and did stimulate PGI<sub>2</sub> production by cultured smooth muscle cells.

The fact that cultured human umbilical vein and bovine aortic endothelium lack PDGF receptors<sup>25,26</sup> would argue against PDGF-mediated effects on PGI<sub>2</sub> release from these cells although it is possible that PDGF could act by receptor-independent mechanisms. For example, although only native PDGF binds to its receptor and stimulates mitogenesis,<sup>27</sup> both native and reduced PDGF stimulate leukocyte chemotaxis.<sup>28</sup> This observation suggests that receptor binding may not be essential for all cell responses to PDGF.

In conclusion, purified PDGF did not stimulate PGI<sub>2</sub>

production in cultured endothelial cells of either human or bovine origin, and an anti-PDGF antiserum did not inhibit PGI<sub>2</sub> generation induced by WBS. Plasma-derived serum with an undetectable level of PDGF also caused significant enhancement of endothelial PGI<sub>2</sub> synthesis, providing further evidence that PDGF is not responsible for the stimulatory effect of human serum on endothelial cell PGI<sub>2</sub> synthesis.

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