

# $\alpha$ -Thrombin Induces Release of Platelet-derived Growth Factor-like Molecule(s) by Cultured Human Endothelial Cells

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**Abstract.** Cultured endothelial cells secrete a platelet-derived growth factor-like molecule (PDGFc). We examined the effects of purified human  $\alpha$ -thrombin on the production of PDGFc in cultures of human umbilical vein endothelial cells (HUVE) using a specific radio-receptor assay for PDGF. Addition of physiologically relevant concentrations of  $\alpha$ -thrombin (0.1 to 10 U/ml) induced a time- and dose-dependent increase in the release of PDGFc into the culture medium. Significant stimulation of PDGFc release was observed as early as 1.5 h after addition of  $\alpha$ -thrombin (10 U/ml) with a  $4.9 \pm 1.1$  fold increase at 24 h (mean  $\pm$  SEM of nine experiments,  $P < 0.01$ ).  $\alpha$ -Thrombin treatment of HUVE did not affect cell viability as assessed by trypan blue dye exclusion. The receptor binding of PDGFc secreted by HUVE in response to  $\alpha$ -thrombin was inhibited by monospecific antibody to purified human PDGF indicating that the molecule(s) is closely

related to PDGF.  $\alpha$ -Thrombin inactivated with diisopropylfluorophosphate was without stimulatory effect. Lysis of HUVE by repeated cycles of freeze/thaw released minimal PDGFc ( $<0.3$  ng per  $10^6$  cells) compared to levels of PDGFc released into supernatant medium in response to  $\alpha$ -thrombin ( $>5.0$  ng per  $10^6$  cells after a 24-h incubation with 10 U/ml  $\alpha$ -thrombin). Moreover, incubation of freeze/thaw lysates of HUVE with  $\alpha$ -thrombin failed to release PDGFc. Over a 3-h time course, however,  $\alpha$ -thrombin-induced secretion of PDGFc was not prevented by cycloheximide. We conclude that  $\alpha$ -thrombin induces secretion of PDGFc from HUVE by a nonlytic mechanism requiring the serine esterase activity of the enzyme. Although this effect does not initially require de novo protein synthesis, it does require cell-mediated conversion of PDGFc from an inactive to an active form.

**P**LATELET-DERIVED growth factor (PDGF)<sup>1</sup> is a heat-stable, cationic polypeptide with growth-promoting and chemoattractant activities (28). Cultured endothelial cells constitutively secrete a molecule(s) (PDGFc) which is antigenically related to PDGF and which is able to compete with <sup>125</sup>I-PDGF for binding to the PDGF receptor (8). Although it has been shown that synthesis and secretion of PDGFc is increased in lethally injured endothelial cells (10), factors modulating the release of PDGFc by intact, viable endothelium are unknown.  $\alpha$ -Thrombin is a multifunctional enzyme with diverse effects on endothelial cell function (6, 12–14, 16–19, 21, 24, 25, 33). We show here that at physiologically relevant concentrations purified human  $\alpha$ -thrombin induces a time- and dose-dependent secretion of PDGFc from cultured human umbilical vein endothelial cells (HUVE) by a nonlytic mechanism.

1. *Abbreviations used in this paper:* DIP-thrombin, diisopropylfluorophosphate-treated  $\alpha$ -thrombin; HPDS, human plasma-derived serum; HUVE, human umbilical vein endothelial cells; PDGF, platelet-derived growth factor; PDGFc, PDGF-like molecule(s); RRA, radioreceptor assay.

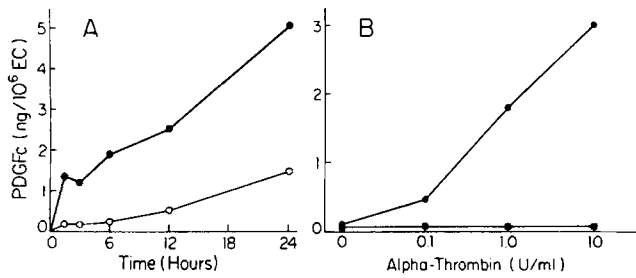
## Materials and Methods

### Cell Culture

HUVE were obtained by collagenase digestions of umbilical cords as previously described (32) and maintained in RPMI-1640 medium (M.A. Bioproducts, Walkersville, MD) containing 20% fetal bovine serum (Hyclone Laboratories, Sterile Systems, Inc., Logan, UT). Passaged cells were supplemented with heparin (90  $\mu$ g/ml) and endothelial cell growth factor (50  $\mu$ g/ml) as described by Thorton et al. (31). Endothelial cell growth factor was prepared from bovine hypothalamus as described by Maciag et al. (22). Human dermal fibroblasts were obtained from explants and subcultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% calf serum (Gibco).

### Radioreceptor Assay

Levels of PDGFc in endothelial cell-conditioned medium and endothelial cell lysates were measured by radioreceptor assay (RRA) as previously described (4). Briefly, cultures of diploid human fibroblasts were rinsed once with cold PBS, incubated with 1 ml test solution in binding medium for 3 h at 4°C with continuous oscillatory mixing, rinsed once with cold PBS, then incubated with 1 ml binding medium containing 0.5 ng/ml <sup>125</sup>I-PDGF for 1 h at 4°C with continuous oscillatory mixing. The cultures were rinsed three times with cold binding rinse and the cell-bound <sup>125</sup>I-PDGF was solubilized with 1% Triton X-100. Standard curves were obtained using pure



**Figure 1.** Time course and dose response of  $\alpha$ -thrombin-induced release of PDGFc. (A) First passage HUVE were placed in 75-cm<sup>2</sup> flasks in RPMI medium containing 20% fetal bovine serum and grown to confluence. Before the experiment, the cells were washed twice with serum-free RPMI medium. The medium was then replaced with RPMI medium containing 1% HPDS with (●) or without (○) purified human  $\alpha$ -thrombin (10 U/ml). At various intervals, the conditioned medium was decanted for determination of PDGFc concentration by RRA. (B) 25-cm<sup>2</sup> flasks of confluent first passage HUVE were incubated for 24 h at 37°C with 2 ml of RPMI medium supplemented with 1% HPDS or the same medium containing varying concentrations of  $\alpha$ -thrombin. The conditioned medium was then decanted and assayed for PDGFc by RRA in the presence (■) or absence (●) of 100  $\mu$ g/ml of monospecific goat anti-human PDGF IgG. Values represent individual flasks.

PDGF standards (0.025–0.8 ng/ml) and 20 ng/ml pure PDGF to determine non-specific binding (10–20% of total binding). In each case, three or more different dilutions of the conditioned media were used to determine the level of the PDGF-like growth factor present in the conditioned media. Values obtained from dilutions falling within the reliable range of the standard curve were averaged to give the reported value.

### Reagents

Highly purified human PDGF and <sup>125</sup>I-labeled PDGF were prepared as previously described (3, 4, 26). The IgG fraction of monospecific goat antiserum to human PDGF was purified by sodium sulphate precipitation and chromatography by DEAE-Sephacel and is specific for PDGF (5, 30).

Purified human  $\alpha$ -thrombin, and diisopropylfluorophosphate-treated  $\alpha$ -thrombin (DIP-thrombin) were generously provided by Dr. John Fenton II, Albany, NY (9). The specific activity of  $\alpha$ -thrombin was 3,425 NIH U/mg. The specific activity of the DIP-thrombin was 0.379 NIH U/mg.

Pooled human plasma-derived serum (HPDS) was prepared from whole blood obtained by venipuncture of healthy normal donors. The whole blood was drawn into cold 3.8% sodium citrate (1:10) and cell-free plasma obtained by centrifugation of whole blood at 3,100 g at 4°C for 20 min. Plasma-derived serum was prepared from cell-free plasma by recalcification with 1 M CaCl<sub>2</sub> (1:50), followed by incubation at 37°C for 2 h, and centrifugation at 25,000 g at 4°C for 20 min. The HPDS was decanted, and then filtered (0.22  $\mu$ m), aliquoted, and stored at –80°C until use. The concentration of PDGF in HPDS was <0.001 ng/ml as assessed by RRA.

Cycloheximide, heparin, and aprotinin were obtained from Sigma Chemical Co., St. Louis, MO.

### Results

Cultured HUVE constitutively released PDGFc into supernatant medium during a 24-h incubation (Fig. 1 A). Addition of  $\alpha$ -thrombin at 10 U/ml produced a marked increase in PDGFc in conditioned medium (Fig. 1 A). A significant effect of  $\alpha$ -thrombin on PDGFc release was observed as early as 1.5–3 h (Fig. 1 A). In nine separate experiments, the mean fold increase ( $\pm$  SEM) in PDGFc release after a 24-h incubation with  $\alpha$ -thrombin (10 U/ml) was  $4.9 \pm 1.1$  ( $P < 0.01$ ). ( $\alpha$ -Thrombin-induced release of PDGFc was observed in passaged as well as primary HUVE (see Table V).

Addition of  $\alpha$ -thrombin alone to the binding medium did not affect the RRA for PDGF (data not shown). Furthermore, addition of  $\alpha$ -thrombin to supernatant medium after it was decanted from the endothelial cell monolayer did not produce an increase in the amount of PDGFc detected. Conditioned medium (3-h incubation) from untreated endothelial cells contained 0.24 ng of PDGFc per 10<sup>6</sup> cells and 0.30 ng per 10<sup>6</sup> cells after subsequent incubation of the decanted medium with  $\alpha$ -thrombin (10 U/ml at 37°C for 3 h), whereas in the same experiment the conditioned medium from  $\alpha$ -thrombin-treated endothelial cells (10 U/ml for 3 h) contained 1.41 ng of PDGFc per 10<sup>6</sup> cells.

At concentrations of from 0.1 to 10 U/ml,  $\alpha$ -thrombin-induced release of PDGFc from HUVE was dose dependent (Fig. 1 B). At these concentrations,  $\alpha$ -thrombin-treated cells remained viable throughout the incubation with >95% trypan blue dye exclusion in both control and  $\alpha$ -thrombin-treated cells. The PDGF-binding competitor detected in medium from HUVE treated with increasing concentrations of  $\alpha$ -thrombin was antigenically related to human PDGF in that it was neutralized by antibodies raised against purified human PDGF (Fig. 1 B). The anti-human PDGF IgG does not neutralize PDGF in fetal bovine serum (Table I).

Thrombin inactivated at the serine esterase site with diisopropylfluorophosphate (DIP) did not induce an increase in PDGFc release even at concentrations as high as 5,000 nM. A 50-fold excess of DIP-thrombin did not prevent  $\alpha$ -thrombin-induced release of PDGFc (Table II).

In a 3-h incubation, the presence of cycloheximide (10  $\mu$ g/ml) did not prevent  $\alpha$ -thrombin-induced release of PDGFc (Table III), despite the fact that protein synthesis was

**Table I. Species Specificity of Antibody Neutralization of PDGF**

	No antibody	Anti-human PDGF IgG	Non-immune IgG
	ng of PDGF	ng of PDGF	ng of PDGF
Conditioned medium from $\alpha$ -thrombin-stimulated HUVE	1.40	0.11	1.47
0.20 ng/ml purified human PDGF	$0.22 \pm 0.02$	<0.02	$0.21 \pm 0.03$
20% fetal bovine serum	$0.15 \pm 0.02$	$0.14 \pm 0.04$	$0.13 \pm 0.03$
60% fetal bovine serum	$0.29 \pm 0.11$	$0.28 \pm 0.07$	$0.30 \pm 0.04$

Samples containing conditioned medium from  $\alpha$ -thrombin-stimulated HUVE, 0.20 ng/ml purified human PDGF, or 20% and 60% fetal bovine serum were preincubated for 1 h at 4°C with or without 256  $\mu$ g/ml anti-PDGF IgG or non-immune IgG. PDGFc activity was then determined by radioreceptor assay. Results are the means  $\pm$  SEM of three separate experiments.

**Table II.  $\alpha$ -Thrombin But Not DIP-Thrombin Induces Release of PDGFc**

Addition to HUVE monolayer	PDGFc (ng per 10 <sup>6</sup> cells)
Medium alone	1.02
	0.63
$\alpha$ -Thrombin	9.60
	10.40
DIP-thrombin	1.87
	1.10
$\alpha$ -Thrombin + DIP-thrombin	8.30
	9.69

25-cm<sup>2</sup> flasks of confluent first passage HUVE were incubated for 24 h in RPMI medium containing 1% HPDS or the same medium containing  $\alpha$ -thrombin (100 nM), DIP-thrombin (5,000 nM), or both  $\alpha$ -thrombin and DIP-thrombin. The concentration of PDGFc in the conditioned medium was determined by RRA. Values represent individual flasks.

inhibited by >95% as assessed by trichloroacetic acid-precipitable <sup>3</sup>H-amino acid incorporation (data not shown). The role of protein synthesis in  $\alpha$ -thrombin-induced release of PDGFc during incubations longer than 3 h could not be assessed due to the fact that concentrations of cycloheximide sufficient to reduce protein synthesis by greater than 75% (>1  $\mu$ g/ml) were toxic to HUVE after a 4–6 h incubation.

Endothelial cell lysis by repeated cycles of freezing and thawing and sonication in the presence of protease inhibitor released minimal PDGFc (consistently <0.3 ng per 10<sup>6</sup> cells). During the 3-h period in which  $\alpha$ -thrombin-stimulated release of PDGFc was independent of de novo protein synthesis,  $\alpha$ -thrombin-treated cells released considerably more PDGFc activity than was detectable in lysates of untreated cells (Table IV). Similar results were observed during 24-h incubations; levels of PDGFc in conditioned medium of  $\alpha$ -thrombin-treated cells were consistently 5- to 10-fold greater than levels of PDGFc in lysates of untreated cells.

$\alpha$ -Thrombin-induced secretion of PDGFc by intact cells was not accompanied by a significant decrease in this small intracellular pool as assessed by measuring the PDGFc content in lysates of control and thrombin-stimulated HUVE. In three separate experiments, total intracellular PDGFc in lysates of  $\alpha$ -thrombin-treated cells (10 U/ml for 24 h) was 140, 200, and 230% of untreated cells.

Addition of  $\alpha$ -thrombin to HUVE previously disrupted by

**Table III. Cycloheximide Does Not Prevent  $\alpha$ -Thrombin-induced Release of PDGFc**

Addition to HUVE Monolayer	PDGFc (ng per 10 <sup>6</sup> cells)
Medium alone	0.16
	0.16
$\alpha$ -Thrombin	2.16
	2.40
Cycloheximide	0.14
	0.25
$\alpha$ -Thrombin and cycloheximide	2.76
	3.62

75-cm<sup>2</sup> flasks of confluent first passage HUVE were incubated for 3 h at 37°C with RPMI medium supplemented with 1% HPDS or the same medium containing  $\alpha$ -thrombin (10 U/ml), cycloheximide (10  $\mu$ g/ml), or  $\alpha$ -thrombin (10 U/ml) and cycloheximide (10  $\mu$ g/ml). The conditioned medium was then decanted and assayed for PDGFc by RRA. Values represent individual flasks.

**Table IV. Effect of  $\alpha$ -Thrombin on Intracellular vs. Released PDGFc**

Addition to HUVE monolayer	Supernatant medium	Cell lysate
	PDGFc (ng per 10 <sup>6</sup> cells)	
Medium alone	0.38	0.30
	0.32	0.05
$\alpha$ -Thrombin	0.85	0.06
	1.90	0.06

75-cm<sup>2</sup> flasks of confluent first passage HUVE were incubated for 3 h at 37°C with 5 ml of RPMI medium supplemented with 1% HPDS or the same medium containing  $\alpha$ -thrombin (10 U/ml). The conditioned medium was then decanted and assayed for released PDGFc by RRA. After incubation, the cell layer of each flask was washed twice with cold 0.02% EDTA in isotonic saline (Irvine Scientific, Santa Ana, CA). The cell layer was then harvested by incubation at 4°C with 0.05% trypsin and 0.02% EDTA in Hanks' balanced salt solution (Gibco) and the cell pellet resuspended in 1.0 ml of cold RPMI medium containing 1% HPDS and aprotinin (10 mg/ml). An aliquot was removed for determination of cell number by electronic particle counter. The cells were then disrupted by three cycles of rapid freezing and thawing, followed by sonication. The cell lysate was then centrifuged and the supernatant decanted for determination of intracellular PDGFc by RRA. Values represent individual flasks.

repeated cycles of freezing and thawing did not produce a significant increase in PDGFc in supernatant medium, whereas in the same experiment incubation of intact HUVE with  $\alpha$ -thrombin produced a threefold increase in PDGFc in the supernatant medium (Table V). The recovery of purified PDGF added to the cell lysates was >85%.

## Discussion

DiCorleto and Bowen-Pope (8) reported that ~30% of the mitogenic activity previously detected in endothelial cell-conditioned medium (11) represented a platelet-derived growth factor-like molecule(s) (PDGFc) that was antigenically related to purified human PDGF and that competed with labeled purified human PDGF for specific binding to the PDGF receptor. Cultured human umbilical endothelial cells have also been shown to express mRNA encoding the  $\beta$ -chain of PDGF (2, 7). The factors modulating production of PDGFc by cultured endothelial cells have not been well defined. Fox and DiCorleto showed that the synthesis and

**Table V.  $\alpha$ -Thrombin-induced Release of PDGFc Requires Intact HUVE**

	PDGFc (ng total)
A. Addition to HUVE monolayer	
Medium alone	4.1
	4.7
$\alpha$ -Thrombin	12.1
	13.7
B. Addition to HUVE lysate	
Medium alone	0.55
$\alpha$ -Thrombin	0.75

Second passage HUVE were plated in 150-cm<sup>2</sup> dishes and grown to confluence. Immediately before the experiment, the dishes were washed once with RPMI medium. In A, cells were incubated for 24 h in 10 ml of RPMI medium containing 1% HPDS with or without  $\alpha$ -thrombin (10 U/ml). In B, cells were first harvested from the dishes by scraping with a rubber policeman and then resuspended in 10 ml of RPMI medium containing 1% HPDS. The resuspended cells were lysed by three cycles of rapid freezing and thawing. The freeze-thaw lysate was then incubated for 24 h with or without  $\alpha$ -thrombin (10 U/ml). Conditioned medium from A and B were assayed for PDGF by RRA. Values represent individual dishes.

secretion of PDGFc increased when cultured bovine aortic endothelial cells were lethally injured by phorbol esters or endotoxin (10). Using DNA hybridization, Barrett et al. (2) observed a large increase in *c-sis* transcript in proliferating, cultured endothelial cells compared to quiescent, confluent endothelium in vivo (2). More recently, Jaye et al. (15) demonstrated that *c-sis* transcript in cultured HUVE decreased when the monolayers organized into three-dimensional tubular structures under conditions that limited proliferation and increased when they subsequently converted from the organized structure to a proliferative monolayer (15). These observations suggest that the *c-sis* gene may be activated in endothelial cells as a result of increased cell proliferation in vitro.

$\alpha$ -Thrombin is a multifunctional enzyme generated at sites of vascular injury. It enhances synthesis and secretion of a number of endothelial cell products in vitro including prostacyclin (33), adenine nucleotides (24), von Willebrand factor (18, 19), plasminogen activator (17) and plasminogen activator-inhibitor (14), platelet-activating factor (6, 25), fibronectin (12), and thromboplastin (13). In view of these diverse effects of  $\alpha$ -thrombin on endothelial cell function in vitro, we hypothesized that  $\alpha$ -thrombin may be an important endogenous physiologic mediator of PDGFc release from endothelial cells.

Our studies show that addition of  $\alpha$ -thrombin to cultured HUVE results in a marked increase in PDGFc in the medium. A significant increase in PDGFc in the conditioned medium occurs as early as 1.5 h after the addition of  $\alpha$ -thrombin. At physiologically relevant concentrations of  $\alpha$ -thrombin (0.1 to 10 U/ml) (29),  $\alpha$ -thrombin-induced release of PDGFc is dose dependent. In contrast to phorbol esters and endotoxins (10), the release of PDGFc by  $\alpha$ -thrombin-treated HUVE is not accompanied by a decrease in endothelial cell viability. The binding of molecules from  $\alpha$ -thrombin-stimulated conditioned medium to the PDGF receptor is inhibited by antibody to human PDGF indicating that PDGFc detected in the conditioned medium is antigenically similar to human PDGF. Since the anti-human PDGF antibody does not neutralize PDGF in the fetal bovine serum in which the HUVE are cultured (Table I), PDGFc released by  $\alpha$ -thrombin must be derived from the HUVE. The fact that  $\alpha$ -thrombin also releases PDGFc from passaged HUVE that have undergone many population doublings after initial isolation strongly suggests that PDGFc released by  $\alpha$ -thrombin is synthesized by HUVE rather than simply taken up and stored by HUVE in vivo and later released in culture.

$\alpha$ -Thrombin inactivated at the serine esterase site with DIP does not induce an increase in PDGFc release by HUVE (Table II). In all studies in which it has been examined (13, 16-19, 21, 25), the serine esterase activity of  $\alpha$ -thrombin is required for  $\alpha$ -thrombin-mediated effects on endothelial cell function. Although apparently inactive biologically, DIP-thrombin binds to a high-affinity binding site on HUVE with the same affinity as  $\alpha$ -thrombin (1). Moreover, excess DIP-thrombin inhibits the binding of  $\alpha$ -thrombin to the high-affinity binding sites (1). Nevertheless, a 50-fold excess of DIP-thrombin does not prevent  $\alpha$ -thrombin induced release of PDGFc (Table II).  $\alpha$ -Thrombin-mediated release of prostacyclin (20), von Willebrand factor (18, 19), and plasminogen activator (17) are also unaffected by concentrations of DIP-thrombin that inhibit  $\alpha$ -thrombin binding to the high-

affinity binding sites. It thus appears that several important biologic effects of  $\alpha$ -thrombin on HUVE do not involve the high affinity, active site-independent binding sites. It is not known whether  $\alpha$ -thrombin stimulates these responses by binding to an active site-dependent receptor that is distinct from the receptor that recognizes both  $\alpha$ - and DIP-thrombin or by proteolytically cleaving a plasma membrane substrate (20).

Although the serine esterase activity of  $\alpha$ -thrombin is required for  $\alpha$ -thrombin to induce release of PDGFc, our results suggest that  $\alpha$ -thrombin does not simply act as a protease to convert an inactive secreted precursor into active PDGFc or to liberate membrane-bound PDGFc. Addition of  $\alpha$ -thrombin to conditioned medium from untreated HUVE does not release PDGFc indicating that  $\alpha$ -thrombin does not act proteolytically on secreted precursor. Robbins et al. (27) reported that *v-sis*/PDGF-2 gene product in Simian sarcoma virus-transformed cells localizes to intracellular and plasma membranes. In our studies, prior disruption of HUVE by repeated cycles of freezing and thawing prevents subsequent  $\alpha$ -thrombin-mediated release of PDGFc (Table V). This apparent requirement for an intact, viable cell argues against  $\alpha$ -thrombin proteolytically releasing PDGFc from cell membranes. If this were the case, addition of  $\alpha$ -thrombin to the freeze-thaw lysate containing membrane fragments would also be expected to release PDGFc.

$\alpha$ -Thrombin-induced release of PDGFc is not dependent on de novo protein synthesis (at least during the first three hours) (Table III). Release of von Willebrand factor (18, 19) and fibronectin (12) by  $\alpha$ -thrombin-stimulated HUVE is also protein synthesis independent. For von Willebrand factor it has been proposed that there is a pre-formed storage pool that is mobilized in response to  $\alpha$ -thrombin (18, 19). Our studies, however, indicate that HUVE do not contain significant stores of pre-formed, active PDGFc (Table IV). What then is the source of  $\alpha$ -thrombin-released PDGFc, if it is not present in pre-formed stores, and if it is not newly synthesized? We postulate that HUVE contain stores of molecules that are not recognized by the PDGF receptor. In response to  $\alpha$ -thrombin, these molecules are converted to PDGFc and then released. Whether PDGFc that is released early after  $\alpha$ -thrombin stimulation is derived from an intracellular precursor (23) or from PDGFc complexed to an intracellular binding protein (30) remains to be determined.

In view of the potent mitogenic and chemoattractant activities of PDGF, we speculate that PDGFc released by endothelial cells in response to  $\alpha$ -thrombin generated at sites of vascular injury may play a role in inflammation, wound healing, and atherogenesis. In these situations, endothelial-derived PDGFc could serve as a longer term supplement to growth factors released by platelets that adhere and degranulate during the initial (transient) phase of vascular trauma.

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