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Cell Biology

Platelets contain a peptide inhibitor of endothelial cell replication and growth

(atherosclerosis/injury/mitogen/growth factor)

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ABSTRACT **Platelets release specific growth factors that stimulate division of aortic endothelial cells. Acidification or heating to 56°C of platelet extracts is required to detect these factors. Nonheated platelet extracts inhibit endothelial growth. To determine if this inhibitory effect is due to a discrete chemical substance, a crude extract obtained by freezing and thawing human platelets was incubated with an endothelial cell mitogen and found to inhibit the endothelial cell incorporation of [^{3}H]thymidine by greater than 95%. The inhibitor was partially purified by ion-exchange and gel-filtration chromatography. The partially purified material inhibited endothelial cell division as well as DNA synthesis. Inhibition caused by the partially purified material was concentration dependent, reversible, and not due to cytotoxicity. Further purification by heparin-Sepharose chromatography resulted in removal of >95% of the contaminating substances without a loss of inhibitory activity. Physical characterization revealed that heparin-Sepharose-purified factor was heat labile, had a molecular size estimated at 35–40 kDa, and was stable between pH 4.5 and 9.0. Treatment with proteolytic enzymes destroyed all biologic activity, suggesting a peptide composition. These data support the conclusion that platelets contain a potent inhibitor of endothelial replication and growth. Since platelet deposition and release occur after endothelial denudation, release of this platelet-derived growth inhibitor may be an important regulator of reendothelialization that occurs after vessel injury.**

Specific growth factors have been purified from platelets that stimulate division of smooth muscle cells (1, 2), endothelial cells (3, 4), and fibroblasts (2, 5). The role of platelet-derived growth factors following endothelial injury *in vivo* has remained undefined, since crude platelet extracts must be subjected to nonphysiologic conditions to purify a given factor and since insufficient quantities of these materials have been available for *in vivo* testing. Our laboratory has reported the presence of an endothelial mitogen in extracts of human platelets (3). During the preparation of these extracts, it was noted that the mitogen could only be detected after the extract was heated to 56°C or acidified to below pH 4.0. Since the unheated platelet extracts actually inhibited endothelial cell replication, we undertook the present studies to determine whether the inhibitory activity was a discrete chemical substance and, if so, to determine some of its physicochemical properties.

MATERIALS AND METHODS

Isolation of Endothelial Cells. Porcine aortic endothelial cells were isolated as described (6). After incubating aortas from young piglets for 30 min at 37°C in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) containing 0.1% type II collagenase (Worthington, lot 42C074; 126 units/mg), detached endothelial cells were cultured in 10-cm dishes (Falcon) in DMEM supplemented with 20% (vol/vol) bovine serum (Colorado Serum, Denver, CO). After allowing sufficient time (6–8 hr) for attachment, this medium was removed and replaced with DMEM supplemented with 10% (vol/vol) citrated human plasma (16 mM sodium citrate/7 mM citric acid, pH 7.4; the American Red Cross) prepared as described (3). The cells were maintained in this medium until confluent growth was reached (usually 7–8 days). The cells were then removed with 0.1% trypsin/0.02% EGTA (GIBCO) and cultured using a 1:3 passage dilution. This procedure was repeated every 7–10 days to avoid long periods of confluent growth. Cells processed in this manner formed a homogeneous monolayer, had no myofilaments by electron microscopy, and stained for factor VIII-related antigen (7). No observable transformation or loss of the endothelial monolayer (8, 9) was noted for up to 6 weeks.

[^{3}H]Thymidine Incorporation into Cultured Endothelial Cells. To quantitate DNA synthesis, porcine endothelial cells were cultured in microtest wells (Falcon) at 5000 cells per well in DMEM containing 10% (vol/vol) citrated human plasma. After 16 hr, the medium was removed and replaced with DMEM/2% (vol/vol) human platelet-poor plasma (PPP) (final volume, 200 μl). The PPP was prepared by a described method (3). After 3 days, the platelet extract or chromatographic fraction to be tested was added directly to duplicate wells with 0.5 μCi of [^{3}H]thymidine (10 Ci/mmol; 1 Ci = 37 GBq) (Schwartz/Mann). To determine the effect of inhibitory material, the cultures were maximally stimulated with either 10% (vol/vol) human serum or 1.0 μg of partially purified platelet-derived endothelial mitogen per ml that had been purified by a published method (3). Test mixtures were incubated for 36 hr in a humidified incubator containing an atmosphere of 5% CO_2/95% air at 37°C. Following termination of the experiment, the wells were washed twice with Ringer's bicarbonate, pH 7.4, at 4°C and precipitated twice (10 min each) with 5% (wt/vol) trichloroacetic acid. Following DNA extraction with two 0.2-ml aliquots of 0.1 M NaOH/1% (wt/vol) $NaDodSO_4$, the radioactivity incorporated into DNA was determined by liquid scintillation counting.

Purification of Platelet-Derived Growth Inhibitor. Six outdated 10-unit platelet packs (American Red Cross) were combined, washed thoroughly with 0.15 M NaCl/0.01 M Na_2HPO_4, pH 7.4, and centrifuged at 5000 × g for 15 min to remove the residual plasma. The platelet pellet was resuspended in 2 vol of 0.01 M Na_2HPO_4/0.9 M NaCl, pH 7.4, and freeze/thawed three times. After centrifuging the mixture at 20,000 × g for 30 min, the supernatant was delipidated by filtration through Whatman no. 1 paper. The filtrate was dialyzed against 4 liters of 0.01 M Na_2HPO_4, pH 7.4, for 24

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Abbreviations: PDECM, platelet-derived endothelial cell mitogen; PDGI, platelet-derived growth inhibitor; PPP, platelet poor plasma; PDGF, platelet-derived growth factor.

hr with 2 changes then stirred with 1.0 g of DEAE-Sephadex beads (Sigma) at 4°C for 24 hr. A column (5 × 10 cm) was poured and washed with two column volumes of 0.01 M $(NH_4)_2CO_3$, pH 7.4. The column was eluted with 0.01 M $(NH_4)_2CO_3$/1.0 M NaCl, pH 7.4, and the fractions were assayed for inhibitory activity in the presence of partially purified platelet-derived endothelial cell mitogen (PDECM) and 2% (vol/vol) human PPP (3). Biologically active platelet-derived growth inhibitor (PDGI)-containing material obtained from the two most active fractions was dialyzed using Spectropor 3 membranes (Spectrum Medical Industries, Los Angeles, molecular weight cutoff 3500) for 18 hr against 0.01 M $(NH_4)_2CO_3$, pH 7.4, and concentrated 5-fold by lyophilization. The concentrated material (1.2 ml) was applied to a Sephadex G-150 (Sigma) column (160 × 1.2 cm) that had been equilibrated with 0.01 M $(NH_4)_2CO_3$, pH 7.4, and eluted with the same buffer at a flow rate of 14 ml/hr. The column had been calibrated using bovine serum albumin, cytochrome c, and ovalbumin (Sigma). Fractions (5 ml) were collected, and 10 μl of each fraction was assayed for its capacity to inhibit [^{3}H]thymidine incorporation.

The PDGI was also further purified by heparin-Sepharose chromatography. Protein (3.5 mg) was dialyzed against 0.1 M $(NH_4)_2CO_3$, pH 7.4, for 18 hr at 4°C then applied to a 10 × 0.3 cm column containing heparin-Sepharose that was a gift from M. Griffith. The column was washed with two column volumes of the equilibration buffer and eluted using a step gradient from 0.1 to 1.5 M NaCl. The 2.5-ml fractions were tested directly for PDGI bioactivity.

Other Mitogens and Cell Types. Platelet-derived growth factor (PDGF) was prepared as described (10). BALB/c 3T3 cells (clone A31) were a gift from W. J. Pledger. Smooth muscle cells were isolated from porcine aortas (11). DNA synthesis in each of these cell types was quantitated by plating the cells at a density of 5000 cells per well in 96-well microtest plates (Falcon) in either Eagle's minimal essential medium (MEM) plus 10% (vol/vol) bovine serum (fibroblasts) or DMEM plus 10% (vol/vol) fetal bovine serum (smooth muscle cells). After 5 days of incubation the medium was removed and replaced with fresh medium containing 2% (vol/vol) PPP, 0.5 μCi [^{3}H]thymidine and test reagents. After a 36-hr (fibroblasts) or a 30-hr (smooth muscle cells) incubation, [^{3}H]thymidine incorporation was determined.

Determination of Growth Velocity. To determine the rate of growth, the porcine endothelial cells were plated at 6–7000 cells per well in 24-multiwell plates (Falcon) in DMEM supplemented with 10% (vol/vol) human citrated plasma (2). The medium was changed after allowing 14 hr for attachment and replaced with DMEM supplemented with 3% (vol/vol) human PPP. Various test reagents were added to triplicate wells, and the incubation was continued for 72 hr. At that time cell number was determined by detaching the cells with 0.1% trypsin/0.02% EDTA and counting them in a particle data counter (Coulter model ZBI). The remaining wells received fresh medium with test reagents and were incubated for an additional 72 hr before the procedure was repeated.

Assessment of Physical Properties of the PDGI. To determine temperature stability partially purified (DEAE-Sephadex) inhibitor was heated to 56°C for 1 or 2 hr or 100°C for 10 min. To determine if the inhibitor was susceptible to proteolysis, an aliquot was incubated with a protease preparation attached to CM-Sephadex beads (Sigma) at 37°C for various times (0.5, 1, 2, and 4 hr). At those times the preparation was centrifuged 3000 × g for 2 min, and the supernatant was removed and tested for inhibitory activity. Control tubes that did not contain the inhibitor and inhibitor controls that did not contain protease-CM-Sephadex were included for each time point. To exclude the possibility that the active inhibitory material was a type of heparin, material that had been purified by heparin-Sepharose chromatography was incubated with *Flavobacterium* heparinase (10 units/ml, Sigma) for 1 hr at 37°C and then added directly to test cultures to determine if the inhibitory activity had been destroyed.

To determine if the inhibitory activity was due to a proteolytic enzyme, crude platelet freeze/thaw supernatant and DEAE-Sephadex- and heparin-Sepharose-purified inhibitor were tested for proteolytic activity using Bio-Rad protease-substrate gel tablets. The test samples were added at concentrations that were 10- to 100-fold greater than that required to achieve maximal inhibition in the bioassay and incubated at 22°C for 24 hr. The results were compared to trypsin standards.

RESULTS

Biological Activity of the PDGI. Crude freeze/thaw extracts contained material that when incubated with quiescent endothelial monolayers in the presence of 2% (vol/vol) PPP and a highly purified preparation of the PDECM inhibited [^{3}H]thymidine incorporation (3). Partially purified (through the DEAE step) PDGI retained the inhibitory activity. Increasing concentrations of the DEAE-step material induced an increased degree of inhibition that was maximal at 8 μg/ml (Fig. 1). In an additional experiment a concentration of 8 μg of partially purified inhibitor per ml reduced the effect of 10% (vol/vol) human serum on [^{3}H]thymidine incorporation into endothelial cultures by 85% (data not shown). To exclude the possibility that this inhibition was simply due to cytotoxicity, a lower concentration of the inhibitor (2 μg/ml) was incubated with various concentrations of PDECM. The inhibitory effect could be partially overcome by adding sufficient mitogen (Fig. 2, *Left*) so that PDECM at 1.0 μg/ml could stimulate the rate of DNA synthesis to 76% of the maximal rate of DNA synthesis. To determine if transient exposure to the inhibitor resulted in a sustained inhibition, endothelial cultures were exposed to the inhibitor transiently for various periods of time from 2 to 31 hr (Fig. 2, *Right*). Following inhibitor removal, the cultures were exposed to PDECM plus 2% (vol/vol) PPP, and the incubation was continued for 36 hr. Transient exposure for intervals of 2–4

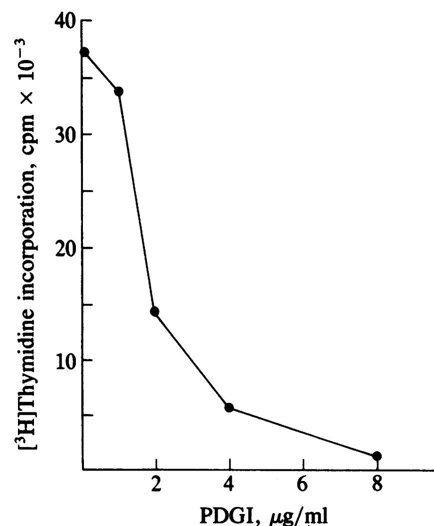

FIG. 1. Inhibition of [^{3}H]thymidine incorporation by a platelet-derived factor. Various concentrations of partially purified PDGI were added to quiescent endothelial monolayers growing in 1.0 μg of PDECM per ml, 0.2 ml of DMEM, 2% (vol/vol) PPP, and 0.5 μCi [^{3}H]thymidine. Following a 36-hr incubation, the cells were precipitated twice with 5% (wt/vol) trichloroacetic acid, and the DNA was extracted with 0.1 NaOH/1% $NaDodSO_4$. The [^{3}H]thymidine that was incorporated into DNA was determined by liquid scintillation counting.

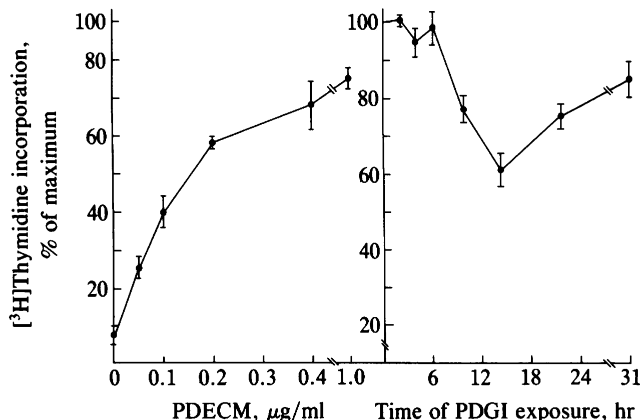

FIG. 2. Reversibility of the inhibitory effect of PDGI. Various concentrations of PDECM were added to quiescent cells cultured in PDGI (1.5 μg/ml), 0.2 ml of DMEM, 2% (vol/vol) PPP, and 0.5 μCi [^{3}H]thymidine (*Left*). After a 36-hr incubation [^{3}H]thymidine incorporation into DNA was determined as for Fig. 1. Control cultures received the concentration of PDECM listed plus 3% (vol/vol) PPP, and the results are expressed as the percentage of maximum [^{3}H]thymidine incorporation at each concentration. In a separate experiment (*Right*), quiescent endothelial monolayers were transiently exposed to PDGI (4 μg/ml) in 0.2 ml of DMEM. At the times listed, the medium was aspirated, the cultures were washed twice with phosphate-buffered saline, and 0.2 ml of DMEM containing PDECM (0.4 μg/ml) and 3% (vol/vol) PPP, and 0.5 μCi [^{3}H]thymidine was added. Following an additional 36-hr incubation [^{3}H]thymidine incorporation was determined. The results are presented as the mean ± 1 SD of the percent of maximal [^{3}H]thymidine incorporation that could be stimulated in control cultures receiving serum-free DMEM for each of the times listed.

hr had no effect. An interval of 14 hr resulted in maximal inhibition (39%), and exposure for longer intervals allowed some restoration of the rate of DNA synthesis toward control levels (e.g., 14% inhibition at 31 hr). This restoration appeared to be due to degradation of PDGI. When medium containing inhibitor that had been incubated with cells for 31 hr was added to fresh endothelial cultures, its capacity to inhibit [^{3}H]thymidine incorporation was reduced by 55% (data not shown).

To determine if the inhibitor could influence mitogenesis, endothelial cells were cultured at a density of 6000 cells per well in DMEM containing 10% (vol/vol) human citrated plasma. Following a 14-hr incubation to allow for attachment, the medium was aspirated and replaced with fresh DMEM containing either PPP alone or PPP in combination with PDECM with or without PDGI. Cell number doubled every 58 hr in the PDECM-exposed wells, whereas those exposed to inhibitor showed only a minimal increase (Fig. 3). In a separate experiment, an identical concentration of the inhibitor that had been heated to 56°C for 2 hr was shown to have no effect.

To obtain PDGI of higher purity, DEAE-Sephadex-purified inhibitor was further purified using heparin-Sepharose chromatography. The biologic activity was eluted with 1 M NaCl, and several contaminant peaks were removed (Fig. 4). To determine the effect of the inhibitor on other cell types, cultures of porcine aortic smooth muscle cells or BALB/c 3T3 fibroblasts were exposed to either PDGF plus PPP or human serum plus various concentrations of inhibitor. Crude preparations of PDGI (DEAE-Sephadex purified) reduced [^{3}H]thymidine incorporation that had been stimulated by either human serum or PDGF in each of these cell types. Following heparin-Sepharose chromatography, however, the effect of the inhibitor was specific for endothelial cells and did not inhibit replication in smooth muscle or BALB/c 3T3 cells.

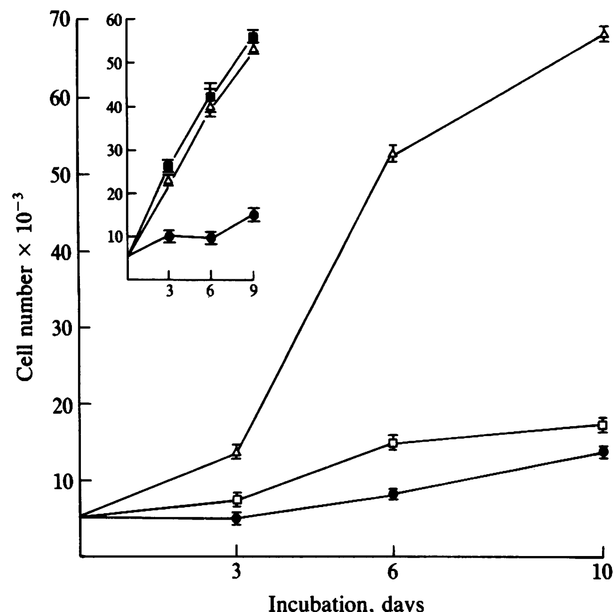

FIG. 3. Inhibition of endothelial cell growth by PDGI. Porcine endothelial cells were plated at a density of 6000 cells per well in DMEM containing 10% (vol/vol) citrated human plasma. After allowing 14 hr for attachment, the medium was removed, and fresh DMEM containing PDECM (0.5 μg/ml) plus 3% (vol/vol) PPP (△); PDECM (0.5 μg/ml), PDGI (2.0 μg/ml) plus 3% (vol/vol) PPP (□); or 3% (vol/vol) PPP alone (●) was added in a final volume of 0.75 ml. After a 72-hr incubation, the cell number was determined in triplicate cultures. The remaining cultures received fresh medium, and after 3 days the cell number was again determined in triplicate cultures. In a separate experiment (*Insert*), the endothelial cultures were plated in an identical manner but received PDECM (0.5 μg/ml) plus 3% (vol/vol) PPP (△); PDECM (0.5 μg/ml), 3% (vol/vol) PPP, and PDGI (■) that had been heat inactivated (56°C for 2 hr); or 3% (vol/vol) PPP alone (●). Cell number was determined as described above.

Physical Properties of PDGI. Material that had been purified through the heparin-Sepharose step was subjected to several physicochemical treatments to determine its properties (Table 1). The material was heat labile, and its inhibitory activity was destroyed by boiling for 10 min or heating to 56°C for 2 hr. Incubation for 4 hr in 0.01 M Na_2HPO_4, pH 7.4, at 37°C, however, resulted in no loss of activity. It appeared to have a net negative charge, since it adhered to DEAE- but not CM-Sephadex. Acidification and alkalinization of the mate-

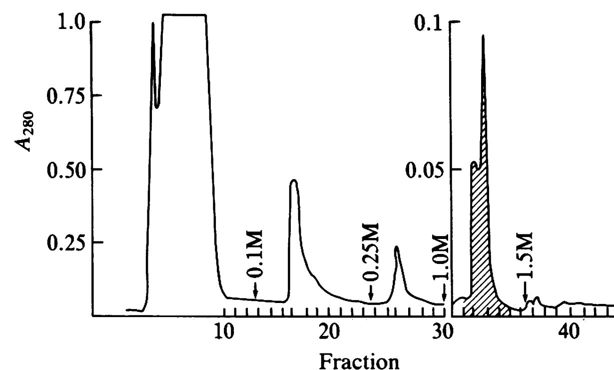

FIG. 4. Heparin-Sepharose chromatography of the inhibitor. Protein (2.5 mg) that had been retained on the DEAE-Sephadex was dialyzed against 0.01 M $(NH_4)_2CO_3$ (pH 7.4) then applied to a 10 × 0.3 cm heparin-Sepharose column. The column was washed with two column volumes of the loading buffer and eluted with a step gradient from 0.1 to 1.5 M NaCl. The inhibitory activity of each fraction was determined as in Fig. 5. The hatched peaks denote the fractions containing biologically active PDGI.

Table 1. Physicochemical properties of PDGI

Factor	Maximum [^{3}H]thymidine incorporation, %
Control*	100
PDGI (4.0 μg/ml) + PDECM (0.5 μg/ml)†	9
Heated PDGI (56°C, 1 hr)	54
Heated PDGI (56°C, 2 hr)	93
Heated PDGI (37°C, 2 hr)	71
Heated PDGI (100°C, 10 min)	110
Exposure to pH 4.5	90
Exposure to pH 9.0	99
Exposure to protease (2 hr, 37°C)	96
Exposure to protease (2 hr, 24°C)	29
Exposure to protease + 0.02% EGTA (2 hr, 37°C)	13
Exposure to *Flavobacterium* heparinase (10 units/ml, 1 hr, 37°C)	16

*All preparations were tested in the presence of control medium containing 2% (vol/vol) PPP and PDECM (0.5 μg/ml).
†All PDGI preparations tested had been purified by DEAE-Sephadex and heparin-Sepharose chromatography.

rial showed that it was stable over a sharp range between pH 4.5 and 9.0. It appeared to be a peptide, since there was a progressive loss of inhibitory activity during a 2-hr incubation at 37°C with protease attached to CM-cellulose. Control incubations of the inhibitor with this protease preparation at 23°C or at 37°C in the presence of 0.02% EGTA resulted in only minimal losses of inhibitory activity. Following exposure to *Flavobacterium* heparinase for 1 hr at 37°C, there was no loss of inhibitory activity.

To exclude the possibility that the effect of the inhibitor was due to proteolysis of growth factors, crude platelet freeze/thaw lysate and DEAE-Sephadex- and heparin-Sepharose-purified material were analyzed for proteolytic activity. Although the crude lysate contained proteolytic activity equivalent to 2.5 μg of trypsin per ml, DEAE-Sephadex-purified material had no detectable proteolytic activity using a concentration that was 10-fold greater than that required for maximal inhibition in the endothelial bioassay test system, and heparin-Sepharose-purified material had no activity using a 100-fold excess.

To obtain a preliminary estimate of the molecular size of the inhibitor, DEAE-purified PDGI was chromatographed on Sephadex G-150 in 0.01 M $(NH_4)_2CO_3$, pH 7.4. The bioactivity migrated with a distribution coefficient (Kd) of approximately 0.55 and had an estimated molecular weight of 37,000 (Fig. 5). Material of this purity retained full biologic activity if stored at −70°C; however, if stored at −20°C, a progressive loss of material occurred over a 3-month period.

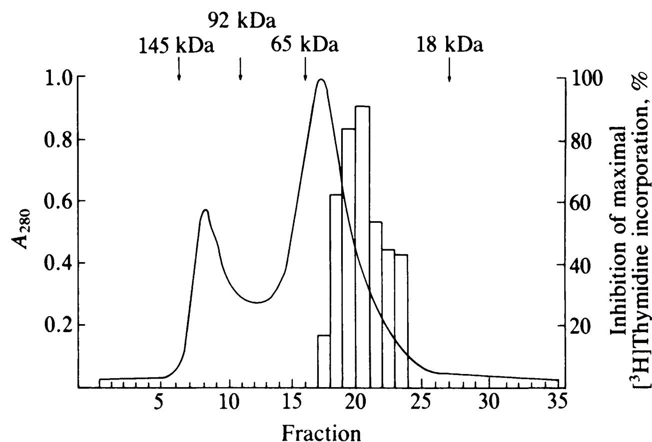

FIG. 5. Gel-filtration chromatography of PDGI. Material (6.0 ml) that had been eluted from the DEAE-Sephadex column was concentrated to 1.6 ml and applied to a 1.6 × 95 cm Sephadex G-150 column that had been equilibrated in 0.01 M $(NH_4)_2CO_3$, pH 7.4. The column was eluted at a flow rate of 14 ml/hr, and the 2.0-ml fractions were lyophilized to dryness and reconstituted in 1.0 ml of DMEM. The activity of each fraction was determined by adding 10 μl directly to quiescent endothelial cultures containing 2% (vol/vol) PPP, and 0.2 ml of DMEM and measuring [^{3}H]thymidine incorporation after a 36-hr incubation as described in Fig. 1. The results (open bars) are expressed as the percent inhibition of maximal [^{3}H]thymidine incorporation that was present in control culture receiving PDECM at 0.5 μg/ml plus 2% (vol/vol) PPP.

DISCUSSION

The results of this study provide evidence that an inhibitor of porcine endothelial cell DNA synthesis and growth is present in human platelets. This material can be detected in platelet supernatants after the freeze/thaw treatment indicating that degranulation results in its release. The factor is heat labile, anionic, and appears to be a peptide that is stable over a sharp pH range from pH 4.5 to 9.0. Following further purification, the factor has been shown to have a molecular weight of ≈37,000 and to be stable for 2 hr at 37°C. The addition of various concentrations of the partially purified material results in a concentration-dependent inhibition of porcine endothelial cell replication. In summary, it appears that the freeze/thaw procedure results in the release of a peptide that inhibits endothelial cell replication.

The inhibitor not only lowers the basal rate of [^{3}H]thymidine incorporation in nonstimulated cultures but also blocks the stimulatory effects of human serum and PDECM. This inhibition is reversible, however, and can be partially overcome either by adding higher concentrations of PDECM or by adding the inhibitor transiently and then removing it from the incubation medium.

The inhibitor causes significant inhibition of porcine endothelial cell division as well as [^{3}H]thymidine incorporation. This finding excludes the possibility that the inhibitor is merely acting by altering the specific activity or the distribution of intracellular [^{3}H]thymidine pools (12, 13).

The inhibitory effect of the PDGI that was purified through the heparin-Sepharose step was limited to endothelium, since it did not inhibit DNA synthesis in smooth muscle cells or BALB/c 3T3 fibroblasts. These results suggest that the platelets contain more than one inhibitory factor and that the peptide we have characterized in this report is specific for endothelium.

Other inhibitors of endothelial growth have been reported. Two of these have been detected in crude extracts from tissues characterized by their relative avascularity—i.e., cartilage (14) and vitreous (15). The chemical composition of the cartilage inhibitor was not reported; however, the bioactive vitreous material was destroyed by proteolytic enzymes. Two other laboratories have reported that inhibitors are released by confluent endothelial cell cultures (16, 17). One of these substances is endothelial cell-derived heparin (17). Our findings indicate that the bioactivity of the partially purified inhibitor is not destroyed by incubation with heparinase and, therefore, it is not a heparin type of substance.

Earlier studies have reported that crude platelet extracts contain material that can inhibit endothelial cell replication (3, 18, 19). Although further chemical characterization of these factors was not reported, these reports provide further evidence to support the existence of platelet-derived inhibitors of endothelial growth. B-transforming growth factor has been shown to inhibit DNA synthesis in ARK 2-B and BSC-1 cells (20). Since B-transforming growth factor is a peptide contained in platelets, it is possible the inhibitor described in

these studies is the same substance. However, B-transforming growth factor is acid and heat stable and has a molecular weight of ≈22,000, indicating that the two peptides have very distinct chemical properties (20). In summary, there appear to be several classes of substances that inhibit endothelial growth. Definition of the exact number of compounds that fit in each of these categories will require further purification and structural characterization of each chemically distinct substance.

The role of specific molecular substances derived from platelets in controlling the rate of reendothelialization following injury is unknown. These findings suggest a mechanism that could regulate endothelial growth following injury. After platelet adherence, three classes of substances would be released into the microenvironment: PDECM, PDGI, and proteolytic enzymes. Since the inhibitor can directly block the effects of the mitogen, it is possible that it functions locally to regulate the expression of its effects. The susceptibility of the inhibitor to proteolysis suggests that proteolytic enzymes in the microenvironment could have a direct effect on its available concentration and, thereby, indirectly modulate growth at discrete sites. This conclusion is strengthened by data from other laboratories that have shown that proteases present in the microenvironment after wounding, such as thrombin, are mitogenic for cultured endothelial cells (21, 22). Substantiation of this hypothesis will require purification of each factor to homogeneity and detailed analysis of the effects of proteolytic enzymes that are present in the injured vessel wall microenvironment.

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