Studies of the Release from Human Platelets of the Growth Factor for Cultured Human Arterial Smooth Muscle Cells

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SUMMARY Platelets contain a growth-promoting factor for arterial smooth muscle cells (SMC) that may play a major role in atherogenesis. We have studied some of the effects of the platelet-derived growth factor (PDGF) on human arterial SMC in culture and the release of PDGF from human platelets in relationship to other released substances. Material released from platelets was highly potent in stimulating human SMC to proliferate. A substantial portion of the growth-promoting activity of human serum could be attributed to a factor(s) released from platelets. Similar dose-response patterns to PDGF were observed with human SMC and with mouse 3T3 cells. The time-course of release of PDGF and its concentration dependence on human thrombin were determined in comparison with serotonin, ADP, ATP, an acid hydrolase, platelet factor 4 (PF4), and β-thromboglobulin (βTG). PGDF activity was assayed by stimulation of the incorporation of 3H-thymidine into DNA of 3T3 cells; PF4 and βTG were measured by newly developed radioimmunoassays. PDGF, PF4, and βTG were released from platelets by lower concentrations of thrombin than those required for release of the other components. The results suggest that PDGF, PF4, and βTG are localized in the platelet in granules different from either the dense bodies (that contain serotonin, ADP, ATP) or the acid hydrolase-containing granules, possibly in α-granules. The contents of these PDGF-containing α-granules are actively released during the release reaction and are particularly sensitive to release by low doses of thrombin.

IT IS well established that whole blood serum is essential for the growth of most mammalian cells in culture in vitro. Recent studies have, moreover, demonstrated that a substantial proportion of the growth-promoting activity of serum is derived from blood platelets. Thus, material from platelets has been shown to stimulate the growth of monkey arterial smooth muscle cells (SMC) of monkey and mouse (3T3) fibroblasts, and of human glial cells. Ross and his colleagues have suggested that the growth factor derived from platelets may play a critical role in the pathogenesis of atherosclerosis. Proliferation of intimal SMC in affected arteries appears to be a key element in the development of the early atherosclerotic lesion. Such SMC proliferation is dramatically evident in vivo after endothelial cell injury by mechanical, chemical, or other means (reviewed in Ref. 9). It has been suggested that factors released by platelets are largely responsible for promoting the intimal SMC proliferation associated with the development of experimental or human atherosclerotic disease. Support for this hypothesis was obtained recently from studies in which intimal thickening and arteriosclerotic lesion formation in rabbits were prevented by rendering the animals thrombocytopenic with antiplatelet serum; similar results were obtained in homocysteinemic baboons treated with the antiplatelet drug, dipyridamole.

Limited information is available about the chemical or biological characteristics of the platelet-derived growth factor. The growth factor has been reported to have the properties of a relatively heat-stable, basic protein and to be released from platelets during the release reaction induced by several different agents. We now report the growth-promoting effects of material released from human platelets upon human arterial smooth muscle cells in culture and describe the characteristics of the release of the growth factor from platelets in comparison to other known platelet-released products.

Methods

Human Smooth Muscle Cells

The human arterial smooth muscle cells were grown from explants of segments of media from normal-appearing pieces of lower abdominal aorta obtained within minutes of death from two young male kidney transplant donors. Informed consent for the procedure was obtained in conformity with institutional policy. The method used was similar to that of Ross, as applied by Albers and Bierman for the culture of human arterial SMC. One- to 2-mm explants of the superficial layer of media were prepared as described by Colloff-Schiller et al. Ten to 15 explants were placed into 25-cm² Falcon flasks (Fisher Scientific Co.) containing 1 ml of Dulbecco's modified Eagle's medium (DME), supplemented with penicillin (100 U/ml); streptomycin (100 μg/ml); 1% (wt/vol) non-

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1 This work was supported in part by National Institutes of Health Grants HL 14236, HL 21006, HL 15486, and HL 14595.

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Received June 20, 1977; accepted for publication September 13, 1977.
essential amino acids; 2 mM L-glutamine (Grand Island Biological Co.); and 20% (vol/vol) normal human serum (freshly prepared). The flasks were incubated undisturbed for 4 days in a humidified Wedco model E2-178, 37°C CO₂ incubator (Wedco, Inc.), after which the medium was changed three times per week. Following 3-4 weeks of growth, the cells (outgrown from the explants) were dissociated for subculturing with 0.05% trypsin-0.02% EDTA (Grand Island Biological Co.). Inocula of 0.75-1.5 x 10⁶ cells were plated into 75-cm² Falcon flasks (Fisher Scientific Co.), using 10 ml of the above-described medium. The cells were allowed to grow to confluency (approximately 1-2 weeks), during which time the human serum concentration was gradually reduced from 20% to 10% (vol/vol). After the second or third passage, a portion of the cells was frozen. The cells used in the experiments were between the third and seventh passage.

The SMC were distinguished from other cell types by their characteristics, as described previously. Compared to fibroblasts, the SMC had a longer lag period before outgrowth from the explant and a slower rate of growth after being established in culture. The pattern of growth was unlike that of fibroblasts in that the SMC tended to pile up into many cell layers in a multicentric proliferative pattern. By electron microscopy, the SMC demonstrated myofilaments and dense bodies, characteristics of SMC in culture.

3T3 Mouse Cells

3T3-Swiss albino (contact-inhibited fibroblasts, no. ATCC CCL 92) mouse cells were purchased from the American Type Culture Collection (ATCC), Rockville, Md. The cells were stored frozen in vials and were grown in stock 75-cm² Falcon flasks according to the instructions provided by the ATCC. The medium was DME supplemented with 10% (vol/vol) fetal calf serum. The medium was changed every other day, and the cells were passed every 3-4 days, with care taken not to allow the cells to reach confluency before passage.

Preparation of Lipoproteins

Human very low-, low-, and high-density lipoproteins (VLDL, LDL, and HDL) and the lipoprotein-deficient (density > 1.21) fraction of plasma or serum, were isolated by sequential ultracentrifugation at densities 1.006, 1.063, and 1.21, according to the method of Havel et al. The lipoprotein and lipoprotein-deficient fractions were dialyzed exhaustively against phosphate-buffered saline (pH 7.4) and then against DME before being added to cells.

Preparation of Supernatant Fluid (PS) from Aggregated Platelets

Platelets from one unit of blood, concentrated into 20-25 ml of plasma, were obtained from the New York Blood Center in fresh 20-unit batches. The platelets were washed free of plasma by centrifugation at 1,200 g for 15 minutes at 22°C, adjusted to a concentration of approximately 10¹⁰ platelets/ml, and aggregated with purified human thrombin, 1 U/ml, (kindly provided by Dr. D. Aronson, Division of Biologics, Bethesda, Md.). After aggregation, the mixture was centrifuged and the supernatant fluid was brought to 56°C for 10 minutes to inactivate complement and defibrinogenate the plasma, as described by Käser-Glanzmann et al. After cooling, the supernatant fluid was centrifuged at 10,000 g for 30 minutes at 4°C to remove any precipitated protein.

Preparation of Platelet-Rich Plasma, and Platelet-Poor Plasma, Heat-Treated (PPPΔ)

Platelet-rich plasma was prepared, as described by Holmsen et al., from venous blood drawn from healthy donors who had not taken aspirin within the preceding 2 weeks. The PPPΔ was prepared from the remainder of the blood (after the removal of the platelet-rich plasma) by two consecutive centrifugations for 30 minutes at 4°C, at 1200 g-max and at 10,000 g-max, to remove any remaining platelets. The platelet-free plasma obtained after the second centrifugation was dialyzed against phosphate-buffered saline, heated at 56°C for 30 minutes (in order to remove the fibrinogen by precipitation and to inactivate complement), cooled, and centrifuged at 10,000 g-max for 30 minutes at 4°C to remove the formed precipitate. The resulting plasma was designated PPPΔ.

Preparation of Labeled Gel-Filtered Platelets (GFP)

Platelet-rich plasma (12-15 ml) was incubated at 37°C for 15 minutes with 5-hydroxytryptamine-1-C¹⁴, 0.04 μCi/ml (48 mCi/mmol, from Schwarz/Mann). The labeled, platelet-rich plasma was then immediately gel filtered, as described by Lages et al., on a 4-cm diameter column packed with Sepharose 2B (Pharmacia Fine Chemicals) to a bed height of 20 cm, and eluted with Ca²⁺-free Tyrode’s buffer containing 0.1% glucose and 0.2% crystalline human albumin (Sigma Chemical Co.). Approximately 30 ml of GFP were collected, containing 1-3 x 10⁶ platelets/ml.

Aggregation of GFP and Preparation of Samples for Assay

The GFP were aggregated with purified human thrombin (dissolved in phosphate-buffered saline) at the concentrations and for the times described in the figures. In all experiments, buffer blank controls (samples containing only the buffer in which the platelets were suspended, plus the thrombin solvent, phosphate-buffered saline) and freeze-thawed controls (samples of GFP containing the thrombin solvent, frozen and thawed four times) were prepared at the same time. In the time-course experiments, the entire experimental sample of GFP was aggregated as a single unit in a siliconized beaker at 37°C with stirring, and the individual samples were removed at the times indicated. For the dose-response studies, each sample was aggregated separately at 37°C with the indicated dose of human thrombin, using a Payton Dual Channel Aggregation Module with a Riken-Denshi Vertical Two-Channel Recorder. In each study, the samples were removed at the times indicated in the Results section and placed immediately into an ice-water bath. The samples, plus controls, were centrifuged at 10,000 g-max for 10
minutes at 4°C, and the supernatant fluids were decanted and used for the various assays described below.

**Assay for Serotonin Release**

Fifty microliters of each GFP supernatant sample were removed, placed into a scintillation vial containing 10 ml of Insta-Bray (York Town Research) and 0.5 ml of 0.2 N NaOH and assayed for 3H in a Packard model 3003 liquid scintillation spectrometer (Packard Instrument Co.).

**Assays for Cell Growth Stimulation**

Growth stimulation was assayed in SMC and 3T3 cells by both cell counting, with a ZB-1 Coulter Counter, and by the incorporation of thymidine into DNA, by the method described by Gospodarowicz and Rubin, with modifications. 3T3 cells or SMC were dissociated from stock flasks with 1-2 ml of trypsin, and an inoculum of 4 x 10⁴ cells was seeded into plastic dishes (35 mm diameter) (Fisher Scientific Co.) containing 2 μCi of thymidine-methyl-3H (2 Ci/mmol, from New England Nuclear Corp.) and 2 μg of unlabeled thymidine (Sigma Chemical Co.) were added to each dish. Twenty-four hours (3T3) or at 48 and 96 hours (SMC) after the initial medium change, identical changes were made. The cells entered a resting stage at 48 hours after the last medium change; the samples to be tested for growth-promoting activity were added at this time (48 hours). Sixteen hours (3T3) or 32 hours (SMC) after the sample addition, 50 μl of DME containing 2 μCi of thymidine-methyl-3H (2 Ci/mmol, from New England Nuclear Corp.) and 2 μg of unlabeled thymidine (Sigma Chemical Co.) were added to each dish. Four hours (3T3) or 16 hours (SMC) later, the medium was removed from each plate, and the monolayer of cells was washed at ice temperature two times with phosphate-buffered saline, once with 5% trichloroacetic acid for 5 minutes and once with water. The cells were dissolved in 0.5 ml of 0.2 N NaOH at 37°C, after which the dish contents were quantitatively transferred (with a further 0.5 ml wash of 0.2 N NaOH) to scintillation vials containing 12 ml of ScintiVerse (Fisher Scientific Co.) and 100 μl of trichloroacetic acid. The vials were assayed for 3H (representing the incorporation of thymidine into DNA) in a Packard liquid scintillation spectrometer. All samples were assayed for growth factor activity in duplicate or triplicate.

**Assays for Adenine Nucleotides and Acid Hydrolase**

For analysis of ADP, ATP, and β-N-acetylglucosaminidase (β-N) from the GFP supernatant and the appropriate control supernatant fluids, samples were taken and treated as follows: adenine nucleotides: 0.1 ml into 0.1 ml of an ice-cold mixture of ethanol-0.1 M EDTA (pH 7.4), 9:1 (vol/vol); acid hydrolases: 0.8 ml into 0.1 ml 1.8% Triton X-100 in water.

ATP and ADP were analyzed by the firefly-luciferase method of Holmsen et al., using an Aminco microphotometer (American Instruments Co.), as described by Weiss et al.

β-N was determined by the liberation of p-nitrophenol from the appropriate substrates, as described by Holmsen et al. The results were expressed as units of acid hydrolase activity, where 1 U is defined as the hydrolysis of 1 μmol of p-nitrophenol per minute from the appropriate substrate.

**Results**

**Response of Human Arterial Smooth Muscle Cells to Various Serum and Platelet-Poor Plasma Fractions**

Initial studies were carried out with human arterial SMC to investigate the growth-promoting activity of various human serum components. Some of the results obtained are shown in Figure 1. SMC growth and proliferation were maximal when the cells were grown in the presence of 10% whole human serum. Most (approximately two-thirds) of the growth-promoting activity of whole serum was found to be present in the lipoprotein-deficient (density > 1.21) fraction of the serum. In contrast to serum, 10% heat-defibrinogenated platelet-poor plasma (PPPΔ) showed only slight growth-promoting activity, and the lipoprotein-deficient fraction of the PPPΔ appeared able to maintain the cells in a resting stage but unable to stimulate new cell growth.

Lipoproteins alone were not able to maintain the cells in a resting stage, and the cell count decreased when the SMC were incubated with LDL (see Fig. 1), VLDL, or HDL (data not shown) prepared from either whole human serum or PPPΔ. When lipoproteins were added to the lipoprotein-deficient fraction prepared from PPPΔ, some improvement in cell growth was apparent (data not shown in Figure 1), especially with addition of VLDL and LDL. However, the improvement was not large, and the addition of lipoproteins could not compensate for the loss in growth-promoting activity seen in the PPPΔ, compared to whole serum.

**Response of Human Arterial Smooth Muscle Cells and Mouse 3T3 Fibroblasts to Platelet Supernatant (PS) Material**

Since serum contained growth-promoting activity not present in platelet-poor plasma (Fig. 1), experiments were carried out to investigate directly whether this activity resided in material released from human platelets during their aggregation. The studies employed PS preparations, comprising substances released from washed platelets...
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Figure 1 The effect of whole human serum (WHS), platelet-poor plasma heat-treated (PPPA), and lipoprotein-deficient human plasma (density $>1.21$) fraction of WHS and of PPPA, the LDL fraction from WHS, and incubation medium alone (0% serum) on the proliferation of human arterial smooth muscle cells. All of the fractions used in this study were prepared from a sample of blood from a normal donor. One-half the blood was clotted in glass centrifuge tubes and the serum separated (WHSS). The remaining blood was used for the preparation of PPPA (see Methods). Portions of the WHS and PPPA were separated into various lipoprotein and lipoprotein-deficient fractions by ultracentrifugation. The various fractions were further prepared for addition to the cells, as described under Methods for the preparation of PPPA, except that the lipoprotein fractions were not heat inactivated. The cells were plated at $3 \times 10^4$ cells/35-mm diameter dish in 2 ml of standard growth medium containing 1% WHS, and incubation medium alone (0% serum) on the proliferation of human arterial smooth muscle cells. All of the fractions used in this study were prepared from a sample of blood from a normal donor. One-half the blood was clotted in glass centrifuge tubes and the serum separated (WHSS). The remaining blood was used for the preparation of PPPA (see Methods). Portions of the WHS and PPPA were separated into various lipoprotein and lipoprotein-deficient fractions by ultracentrifugation. The various fractions were further prepared for addition to the cells, as described under Methods for the preparation of PPPA, except that the lipoprotein fractions were not heat inactivated. The cells were plated at $3 \times 10^4$ cells/35-mm diameter dish in 2 ml of standard growth medium containing 1% WHS, with medium changes every 48 hours throughout the experiment. On day 7, the medium was changed to the appropriate test medium, with the PPPA and lipoprotein-deficient (density $>1.21$) samples adjusted to the same protein content as that of the 10% WHS, and the LDL concentration equal to that found for LDL in the 10% WHS. Cell counts were made on duplicate plates on the days indicated. Duplicate samples agreed closely with each other, and the data points shown represent the mean values for each duplicate pair.

During the thrombin-induced platelet release reaction, the protein concentration of the PS preparations averaged approximately 1 mg/10$^8$ platelets. Before this work was developed, experiments were conducted to ensure that the purified human thrombin used did not itself provide significant growth stimulation in the system under study, since Chen and Buchanan have reported the stimulation of chick embryo fibroblasts by thrombin. In two experiments with 3T3 cells, purified human thrombin, tested at concentrations varying from 0.01 to 24 U/ml of incubation medium, gave very little stimulation (less than 20% of that seen with 10% fetal calf serum) of the incorporation of $^3$H-thymidine into DNA.

Figure 2 shows the effect of increasing concentrations of PS material on the stimulation of the incorporation of $^3$H-thymidine into DNA of SMC. A dose-related increase in stimulation was observed, particularly over the range of 0.5-5 $\mu$g of PS protein/ml. At a level of 5 $\mu$g/ml, the stimulation of human SMC by PS was 67% as great as that seen with 10% whole human serum (which gave maximal stimulation).

Similar data on the mitogenic effects of PS on 3T3 mouse cells are also shown in Figure 2. The proliferation of 3T3 cells was effectively stimulated by human PS, with a dose-response pattern similar to that observed with the human SMC. Both 3T3 cells and SMC showed the sharpest increase in stimulation between the concentration range of from 1 to 5 $\mu$g of PS protein/ml of medium, when tested over a concentration (dose) range of from 0 to 50 $\mu$g of PS protein/ml.

Since both SMC and 3T3 cells responded similarly to the growth-promoting factor(s) released from human platelets, further studies on the characteristics of the release of the growth factor from platelets were carried out using 3T3 cells for the assay of the growth factor activity of various samples. This provided a more rapid and simpler standard assay than that which could be carried out with SMC.

Time Course of the Release of Platelet-Derived Growth Factor (PDGF) from Human Platelets

Four pilot experiments were carried out to explore the rate of release of PDGF in comparison with that of serotonin. These experiments used platelets washed either by serial centrifugation or by gel filtration on Sepharose 2B and aggregated with different doses of purified human thrombin. In addition, an experiment was conducted to measure the rate of release of PDGF and platelet-bound $^3$H-serotonin from platelet-rich plasma on addition of 4 $\mu$g of collagen/ml. In each of these initial studies, the release of PDGF and of serotonin followed a generally similar pattern. Thrombin-induced release of PDGF and...
serotonin (from washed platelets) was rapid, mainly occurring during the first 30 seconds. Collagen-induced release (platelet-rich plasma) of both components was slower and occurred mainly between 1 and 3 minutes.

More extensive experiments were then carried out to compare the time course of the release of PDGF with that of a number of other platelet-released products, namely ADP, ATP, PF4, βTG, and β-N. Three experiments were conducted. The results of one experiment are shown in Figure 3, with similar results being obtained in the other two experiments. PDGF, PF4, βTG, serotonin, and ADP all showed a rapid pattern of release during thrombin-induced aggregation. ATP (data not shown) showed the same pattern of release as did ADP and serotonin, while β-N (data not shown) showed a much slower rate of release, as previously described by Holmsen et al. 30, 33

Dose-Response Studies of Thrombin-Induced Release of Platelet-Derived Growth Factor

Detailed studies were carried out on the effect of thrombin concentration on the release of PDGF from human platelets in comparison with a number of other released platelet components. We performed two detailed dose-response experiments using GFP and concentrations of thrombin ranging from 0.01 to 0.5 U/ml. We also determined the total amount of each component present in the platelets (using platelets that were frozen and thawed four times) and the maximal amount releasable by thrombin (5 U/ml for 5 minutes). The amount of each component released at each concentration of thrombin studied was then expressed in two ways, both as the percent of the total amount released by repeated freeze-thawing and as the percent of the total amount released after 5 minutes by thrombin, 5 U/ml. The results of the two detailed experiments were similar, with the major findings (see below) being confirmed by the results of four additional, more limited experiments.

Figure 4 shows the results of one of the detailed experiments, with the data for each component expressed as the percent of the total amount released from repeatedly freeze-thawed platelets. The thrombin concentration-dependence of the release of PDGF, PF4, and βTG was found to be different from that of the release of the other components measured. Thus, the major release of PDGF, PF4, and βTG occurred between 0.02 and 0.05 U/ml of thrombin/ml of GFP, with 70-80% of each of these three components being released by a concentration of 0.025 U/ml. In contrast, the major portions of the known dense granule components (serotonin, ADP, and ATP) were released at thrombin concentrations between 0.02 and 0.05 U/ml, with only 15-20% of these components being released at 0.025 U/ml. The dose-response curve of acid hydrolase (β-N) release was different from that of any of the other components, and demonstrated both a higher thrombin concentration necessary to achieve equivalent release and less complete (30%) release with the highest concentration (0.1 U/ml) shown. These latter findings are similar to those described previously. 30, 33

The difference between the thrombin concentration-dependence of the release of PDGF, PF4, and βTG and that of the other released components was also evident when the data were expressed as the percent of the maximal amount of each component releasable by thrombin. Table 1 presents the percent of maximal release obtained with 0.025 U of thrombin/ml in each of the two detailed experiments, with the data expressed in each of the two ways described. It is evident that, when either method was used to express the data, the three compo-
served stimulation of SMC growth by lipoproteins, particularly LDL, under appropriate conditions, in addition to the stimulation provided by platelet factors. Taken together, these and other reports suggest that lipoproteins may play an important role in stimulating arterial SMC proliferation, although differences in the systems employed and the results found make it difficult to be more certain about this role at the present time.

Various other substances in serum may also be involved in stimulating the proliferation of cultured SMC. Insulin was observed recently to stimulate the proliferation of monkey arterial SMC. In addition, it has been reported that diabetic rabbit serum, but not normal rabbit serum, contains a factor other than insulin or lipoproteins that stimulates growth of rabbit arterial SMC.

The contribution of platelet-released substances to the growth-promoting activity of whole serum can be estimated from the dose-response curves of growth stimulation observed on adding increasing amounts of serum to cells (curves determined during the course of this work but not reported here in detail), compared to those observed for PS (Fig. 2). From these curves, assuming that the platelet count in the blood from which the serum was derived was 300,000/mm³, and having found that 10¹⁰ platelets yield approximately 1 mg of PS protein, we estimate that approximately two-thirds of the growth-promoting activity of normal serum can be attributed to a factor(s) released from platelets.

Material released from human platelets is highly potent in stimulating human SMC to proliferate, as shown by the experiments summarized in Figure 2. A dose-response pattern similar to that observed with the human SMC was also found for mouse 3T3 cells. Only limited information is available, however, about the range of types of cells that may respond with growth stimulation to platelet-released material. Growth stimulation by platelet factors has been observed with monkey SMC, mouse (3T3) fibroblasts, and with human glial cells. Studies with other cell types would be of considerable interest.

A possible similarity between the growth-promoting activity of platelets and of fibroblast growth factor (isolated from bovine pituitary or brain) has been suggested by Gospodarowicz et al. Several other growth factors from serum have been under study by many investigators, including somatomedins, nonsuppressible insulin-like activity, serum factors SI and S2, and multiplication stimulating activity (MSA) (as discussed broadly in Ref. 39).
The possible relationships between these and other growth factors and the growth factor derived from platelets warrant further investigation. Rutherford and Ross refer to the growth-promoting activity of platelets simply as the platelet factor(s), PF, whereas Busch et al. refer to it as platelet MSA. In view of the evidence (Refs. 8, 9; the present work; and L. Witte, unpublished observations) suggesting that there is a definite substance released from platelets that is responsible for most of the growth-promoting activity seen with cultured cells, we propose the term "platelet-derived growth factor (PDGF)" for this material.

Other investigators have speculated about the possible normal and abnormal physiological roles played by platelet-derived growth factor. It has been suggested that PDGF may play a role in maintaining normal vascular integrity and in vascular and extravascular repair processes. Pathologically, PDGF may play a critical role in the pathogenesis of atherosclerosis, by promoting the intimal smooth muscle cell proliferation that appears to be a key element in the development of the early atherosclerotic lesion,

Human blood platelets are known to contain two morphologically distinct types of granules, the dense bodies and the α-granules. The dense bodies contain serotonin, ADP, ATP, and calcium. Earlier studies identified the α-granules as the storage site for lysosomal enzymes (acid hydrolases and cathepsins). More recent evidence suggests that the platelet lysosomal enzymes are stored in vesicular structures distinct from α-granules. During the platelet release reaction, granule-bound substances are extruded from the cell, while the contents of membranes, cytoplasm, and mitochondria are retained. Different mechanisms may be involved in the release of substances from different types of granules. For example, the dense granule-bound substances can be released rapidly by weak release inducers, such as ADP and epinephrine, whereas acid hydrolases are released more slowly and only by "strong" release inducers, such as collagen and thrombin.

There is no evidence available from cell fractionation studies about the localization of PDGF or βTG within the platelet. There is, however, indirect evidence from studies of the release of these materials that they are contained in granules different from those containing acid hydrolases. Thus, Busch et al. found that the growth-promoting activity for glial cells in culture was released by ADP and epinephrine, and Kaplan et al. observed that βTG was released by ADP; acid hydrolases, in contrast, are not released by ADP or epinephrine. Most of the studies of the subcellular localization of PF4 have been conducted with assays based on its heparin-neutralizing activity. Initially, heparin-neutralizing activity was thought to be localized in the dense bodies, since it could be released by ADP and epinephrine, and its release was inhibited by aspirin. However, studies of patients with storage pool deficiency have shown normal amounts of heparin-neutralizing activity in the platelets of most of these patients, and cell fractionation studies have localized heparin-neutralizing activity to the α-granule fractions and not the dense bodies. With specific assays for PF4, there is indirect evidence that PF4 is in different granules from acid hydrolases, since PF4 is released by ADP. Cell fractionation studies have not been performed, using specific assays for PF4.

The results presented here demonstrate that PDGF is a product of the platelet release reaction and provide detailed information about the characteristics of PDGF release as induced by varying concentrations of thrombin. It was found that the dose-response pattern of release of PDGF was similar to that of both PF4 and βTG and different from that of ATP, ADP, and serotonin, or of the acid hydrolase β-N. Thus, in 3 minutes, the major release of PDGF, PF4, and βTG occurred at thrombin concentrations of 0.015-0.025 U/ml, whereas the dense body substances were mainly released during the same interval at thrombin concentrations of 0.02-0.05 U/ml. These findings suggest that PDGF, PF4, and βTG are localized in granules other than the dense bodies or the acid hydrolase-containing granules, possibly in α-granules.

Recent studies in patients with storage pool disease support this latter possibility. The platelets of patients with this disorder have diminished amounts of ATP, ADP, serotonin, and calcium and show decreased numbers of dense bodies. In the majority of patients, the number of α-granules and the content of PF4, βTG, and PDGF has been found to be normal. One unique patient, however, showed a decreased content of all three substances and a strikingly diminished number of α-granules. Four platelet lysosomal enzymes were previously shown to be normal. Thus, present evidence suggests that ATP, ADP, serotonin, and calcium are stored in dense granules, while PDGF, PF4, and βTG are stored in α-granules. The storage site of lysosomal enzymes is still uncertain. As reported here, the contents of the α-granules (PDGF, PF4, βTG) are particularly sensitive to release by low doses of thrombin. At present, it is not known whether these various α-granule substances are all present in a single granule or in α-granules of varying composition. However, the parallel release of PDGF, PF4, and βTG in vitro suggests that these substances might be released in parallel in vivo, and, thus, an assay of PF4 and βTG in patient samples might reflect release of PDGF as well. Further studies, including those using subcellular fractionation techniques, in both normal and abnormal platelets will be required to define in more detail the properties of this population of granules.

Acknowledgments

We are grateful to B. Adams, H. Brand, M. Drillings, and G. Lessnik for expert technical assistance.

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Studies of the release from human platelets of the growth factor for cultured human arterial smooth muscle cells.
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doi: 10.1161/01.RES.42.3.402

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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