New Synthesis of a Platelet-specific Protein: Platelet Factor 4 Synthesis in a Megakaryocyte-enriched Rabbit Bone Marrow Culture System

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ABSTRACT The site of synthesis of platelet-specific proteins remains to be established. With the use of short-term megakaryocyte-enriched cultures, direct evidence was obtained to show that megakaryocytes synthesize the platelet-specific protein, platelet factor 4. A megakaryocyte-enriched fraction of rabbit bone marrow for culture was obtained by centrifugal elutriation and cultured with [^{3}H]leucine. Newly synthesized ^{3}H-platelet factor 4 was sought by copurification with added carrier rabbit platelet factor 4, using heparin agarose affinity chromatography and immunoprecipitation with specific goat anti-rabbit platelet factor 4 antisera. SDS PAGE of the washed immunoprecipitates demonstrated a [^{3}H]leucine-containing peak which migrated identically with purified homogeneous rabbit platelet factor 4. A second, slightly larger molecular-weight protein was identified in the gels also, suggesting that rabbit platelet factor 4 may be synthesized as a larger molecular-weight precursor in rabbit megakaryocytes. These results provide direct evidence that the platelet-specific protein, platelet factor 4, is synthesized in rabbit megakaryocytes before it is packaged into α-granules for release in circulating platelets.

Several proteins found in platelet α-granules are unique to platelets and presumably unique to megakaryocytes, the bone marrow precursor cell of platelets. The best known of these proteins are platelet factor 4, β-thromboglobulin, and the platelet-derived growth factor (1–9). During normal blood coagulation, the platelet undergoes the "release reaction", secreting α-granule constituents to the extracellular melieu. While the precise role of the released proteins is unknown, these platelet release proteins have been shown to have the capacity to profoundly influence inflammation, wound healing, DNA synthesis, and cell growth. They may be important also in the development of atherosclerosis in humans (10–17).

Platelet factor 4 and β-thromboglobulin are the most fully characterized of the secreted proteins. The complete amino acid sequence of each protein has been published (18–21). No information is available on the synthesis of these proteins and little is known on how they are packaged into α-granules. Platelets synthesize little if any protein; it is assumed that megakaryocytes synthesize platelet-specific proteins. Alternatively, these proteins may be synthesized elsewhere and actively taken up by platelets for storage and subsequent release. Support for megakaryocytic origin of platelet factor 4 was recently provided by the demonstration in human bone marrow preparations that only megakaryocytes (7) and potential megakaryocyte progenitors (8) showed positive immunofluorescence after incubation with purified fluorescein-labeled rabbit anti-human platelet factor 4; these results do not preclude the uptake of platelet factor 4 by megakaryocytes after its synthesis elsewhere.

The present experiments use short-term rabbit marrow cultures enriched for megakaryocytes by centrifugal elutriation (22, 23) to provide direct evidence that platelet factor 4 is

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synthesized in megakaryocytes.

MATERIALS AND METHODS

Harvesting of Megakaryocytes: Megakaryocytes were harvested from bone marrow from New Zealand white rabbits by methods adapted from those described previously (21, 22). Briefly, 8–10 rabbits were anesthesized with Nembutal (Abbott Laboratories, North Chicago, IL) and were sacrificed by cardiac puncture. The femora, tibiae, and humeri were removed aseptically, cleaned of adherent tissue, and kept in cold Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), containing 50 U/ml penicillin and 50 U/ml streptomycin. After the bones were opened with a rongeur, the marrow was scooped free with a spatula and the bone marrow tissues were immersed in 10 ml of cold phosphate-buffered saline (PBS) (pH 6.7), supplemented with 1.5% bovine serum albumin (BSA), containing 50 U/ml penicillin and 50 U/ml streptomycin, in a 9-cm plastic petri dish (Costar Packaging, Cambridge, MA). The tissues were cut with small scissors into 2-mm pieces and transferred to a 50-ml plastic tube (Falcon Labware, Oxnard, CA). Marrow suspensions were vigorously pipetted 18–20 times with wide-bore pasteur pipettes to break the cell clumps.

The marrow cells were filtered through a stainless steel sieve (100 mesh) and poured into 50-ml plastic tubes. The bone marrow suspensions were centrifuged at 150 *g* for 5 min at 4°C, and the cells washed two times with PBS supplemented with 1.5% BSA (pH 6.7). The marrow cells were suspended and introduced into the Beckman elutriator (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 1×10^7 cells/ml. The elutriator buffer was PBS supplemented with 1.5% BSA (pH 6.7) and precooled to 4°C. The cell elutriator was washed with 70% ethanol to provide asepsis before starting the separation and maintained at 4°C. Megakaryocytes were harvested with a buffer flow of 30 ml/min and a centrifuge speed of either 2,793 rpm (chamber fractions >10 mm/h) or 1,975 rpm (>20 mm/h). The megakaryocytes from the elutriator chamber were recovered by resuspending the pellet with a 5-ml syringe fitted with an 18-gauge needle. Cells from other fractions of the elutriator run were also collected and used in control cultures. The harvested megakaryocytes were washed two times with calcium- and magnesium-free Hanks' Buffered Salts Solution (HBSS) containing 1.5% BSA (pH 7.2) by centrifugation at 200 *g* for 5 min at 4°C. The total nucleated cell count was electronically determined with a Coulter Counter Model B. Megakaryocytes in cell suspensions were stained with hematoxylin and counted microscopically as described previously (7). $1.2–3.0 \times 10^7$/ml cells were recovered in 4 ml of calcium- and magnesium-free HBSS; megakaryocytes comprised 1–4% of the isolated total cell population. Control cultures contained 0.1% or fewer megakaryocytes. Fig. 1 shows a low power micrograph of the harvested megakaryocytes stained with hematoxylin.

Culture of Megakaryocytes: The isolated megakaryocytes were cultured in HBSS containing 2.3% BSA, pH 7.4, in siliconized glass tubes (19 × 140 mm) covered with caps. Cultured cells were incubated under 5% CO_2 at 37°C for 3 h with $2–5 \times 10^5$ cells in 2.0–2.5 ml of medium. Solutions and culture media were sterilized by passage through 0.22-μm filters (Millipore Corp., Bedford, MA). The cell culture systems were incubated for up to 48 h at 37°C. The morphological appearance of the cell systems was largely unchanged. Trypan blue exclusion was used to assess the bone marrow cell viability. As judged by morphological appearance, up to 50% of the megakaryocytic cells excluded Trypan blue. The percentage of cells excluding Trypan blue did not decrease over 48 h of culture, which supported previous results on liquid cultures of guinea pig megakaryocytes (24). The morphological appearance of the cells remained constant, suggesting that megakaryocytes maintained their viability in culture.

Morphological Observations of Megakaryocytes: Megakaryocytes were washed one time with Tyrode's solution and fixed for 1 h at 4°C with 2.5% glutaraldehyde in Tyrode's solution, postfixed in 1% osmium tetroxide, dehydrated in *continuous* ethanol, and embedded in Epon 812. Sections were prepared and examined with a Philips E.M.201 electron microscope.

Preparation of Purified Rabbit Platelet Factor 4 and Antisera Against Rabbit Platelet Factor 4: Rabbit platelet factor 4 was purified by affinity chromatography on columns of direct linked heparin Sepharose (18, 25, 26), as described in detail previously. The protein is homogeneous as judged by SDS polyacrylamide electrophoresis and by N-terminal amino acid sequence analysis (27). Anti-rabbit platelet factor 4 serum was obtained from goats after immunization with purified rabbit platelet factor 4. The antisera is monospecific for platelet factor 4, on the basis of a single precipitin line of identity with purified rabbit platelet factor 4 and a rabbit platelet lysate in immunodiffusion analysis and on a single precipitin arch observed in immunoelectrophoresis analysis (27).

Iodination of Rabbit Platelet Factor 4: Platelet factor 4 was iodinated by the chloramine T technique (28) as described in detail previously (18).

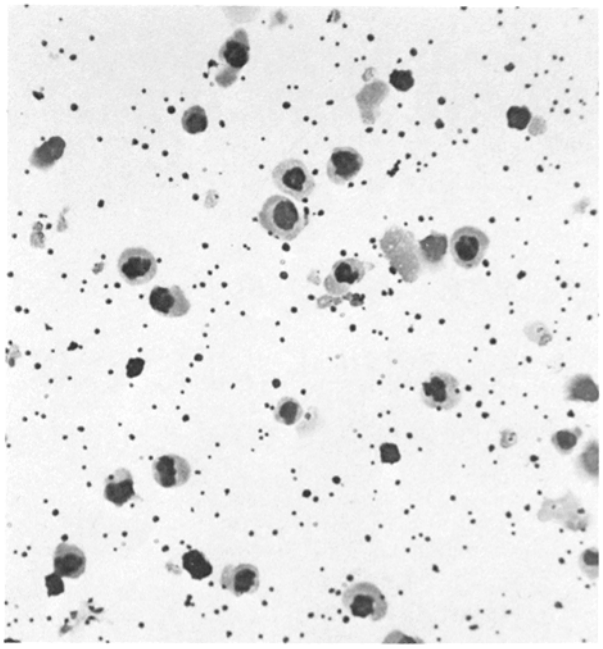

FIGURE 1 Photomicrograph taken from megakaryocyte-enriched cultures and stained with hematoxylin. × 50.

Immunofluorescence Studies: Smears prepared from megakaryocyte-enriched suspensions were dried and fixed in neutralized formalin in PBS (pH 7.2) for 30 min. Fluorescent staining of smears with monospecific goat anti-rabbit platelet factor 4 was performed by the method of Ortega and Mellors (29), using an indirect technique as described in detail previously (6). Preparations of nonimmune serum and specific antiplatelet factor 4 antisera adsorbed with purified rabbit platelet factor 4 were used to establish the specificity of the immunofluorescence observed.

Synthesis of Platelet Factor 4 in the Culture of Megakaryocytes: The cultured megakaryocytes were incubated at 37°C with HBSS containing 2.3% BSA, pH 7.4, and 5 μCi/ml or 10 μCi/ml [^{3}H]leucine (New England Nuclear, Boston, MA). After an appropriate labeling period, the reaction was stopped by addition of one-tenth volume of 10 mM leucine in HBSS and by cooling to 0°C. The radiolabeled megakaryocytes were washed three times by centrifuging at 200 *g* for 5 min at 4°C with cold PBS containing 1.5% BSA (pH 6.7). The megakaryocytes were resuspended in 0.15 M NaCl, 10 mM phosphate (pH 7.5), containing 0.5% Triton X-100 and 100 U/ml Trasylol (FBA Pharmaceuticals, New York) and disrupted for 10 s with sonication. The final volume of labeled megakaryocyte-enriched cell suspensions was adjusted at $2–6 \times 10^6$ cells/ml. After 15-min extraction at 0°C, the extract was centrifuged at 10,000 *g* for 30 min at 4°C, and the small pellet of Triton X-100-insoluble material was discarded.

Purification of Newly Synthesized Heparin Neutralizing Protein with Heparin Sepharose Column: The Triton X-100 treated soluble supernatant was diluted 1:10 with 0.15 M NaCl, 10 mM phosphate (pH 7.4). The diluted cell extract was applied to a 1 × 5 cm column containing 1.5 ml of heparin-Sepharose equilibrated with 0.15 M NaCl, 10 mM Tris, HCl pH 8.6. The column was washed with 200 ml of 0.5 M NaCl, 10 mM Tris HCl (pH 8.7), and eluted in a linear gradient between 10 ml of 0.5 M NaCl, 10 mM Tris HCl (pH 8.7), and 10 ml of 3 M NaCl, 10 mM Tris HCl (pH 8.7). The eluates were collected in 1-ml aliquots in plastic tubes containing 2 μg/ml heparin. Conductivity was measured with a conductivity meter. The radioactivity in the eluates was counted with Dimilume-30 (Packard Instrument Co., Inc. Downers Grove, IL) in a Searle Mark III liquid scintillation spectrometer (Amersham Corp., Arlington Heights, IL).

Immunoprecipitation of Newly Synthesized Protein with Anti-Rabbit Platelet Factor 4: A double antibody immunoprecipitation procedure was used with Triton X-100-treated supernatant or eluates from the heparin Sepharose column. Immunoprecipitations were done immediately after preparing the samples. Heparin column eluates were mixed with 0.02 ml of antiserum (or normal goat serum), 50 μl of 0.15 M NaCl, 50 mM phosphate (pH 7.5), containing 2.5% Triton X-100 and 2.5% deoxycholate, 100 μl of 150 μl of 0.25 M NaCl, 50 mM phosphate (pH 7.5), containing 5 mg/ml BSA and 2 μg/ml heparin, in a volume of 250 μl. The reaction mixtures were incubated for 24 h at 4°C in Eppendorf microtest tubes previously soaked in 1% BSA. 8 μl of rabbit

anti-goat γ-globulin (Cappel Laboratories Inc., Cochranville, PA) was then added to reaction mixtures and the reaction mixture was incubated for 20 h at 4°C.

Immunoprecipitates were recovered by centrifugation at 8,000 *g* for 5 min in a Beckman microfuge (Beckman Instruments, Inc.) and were washed three times with ice-cold 0.15 M NaCl, 0.5% Triton X-100, 10 mM phosphate (pH 7.4), and once with 0.15 M NaCl, 10 mM phosphate (pH 7.4). The washed precipitates were dissolved with 0.2 ml of NCS (New England Nuclear, Boston, MA), and the radioactivity was counted with neutralizer (Research Products International Corp., Mt. Prospect, IL) in a liquid scintillation spectrometer. A correction for nonspecific precipitation was made by substracting the radioactivity in precipitates derived from the normal serum control. The radioactivity in nonspecific precipitates did not exceed 10% of the total precipitate radioactivity in any analysis.

Analysis of Labeled Immunoprecipitates on SDS Gel: The washed immunoprecipitate was subjected to electrophoresis in a 10% SDS polyacrylamide gel according to the modification of Weber and Osborn (30). The washed pellet was resuspended in 20 μl of 0.15 M NaCl, 10 mM phosphate and dissolved in 20 μl of 20 mM Tris, 2 mM EDTA, 2% SDS, 15% sucrose, 80 μg/ml Pyramin and 80 mM dithiothreitol. It was then boiled for 3 min and applied to the tops of gels (0.5 × 7 cm). The gels contained 10% acrylamide and were equilibrated with a gel buffer composed of 1% SDS and 100 mM Tris, pH 7.4. Protein bands were stained with Coomassie Blue, and 2-mm slices of gels were cut for analysis. Slices were transferred to scintillation vials and solubilized as described by Ames (31). Radioactivity of the sliced gels were measured in a liquid scintillation spectrometer.

RESULTS

Morphological Observations on Harvested Megakaryocytes

Preliminary experiments were done to seek α-granule-like particles in cultured megakaryocytes. Electron micrographs show that the megakaryocytes harvested after 48 h of culture had abundant cytoplasmic granules (Fig. 2*A*). Higher magnifications showed greater detail of these granules (Fig. 2*B*). The granules observed appear in electron micrographs to be identical to granules found in platelets; isolation and biochemical characterization of the megakaryocytic granules are required to definitively establish the precise relationship of megakaryocyte and platelet granules.

Immunofluorescence Studies

Experiments were done to detect platelet factor 4 antigen in the megakaryocyte preparations. When the megakaryocyte-enriched suspensions and cultured megakaryocytes were incubated with goat anti-rabbit platelet factor 4 antiserum and fluorescein-conjugated rabbit anti-goat γ-globulin, prominent immunofluorescent staining was observed in all megakaryocytes, similar to that reported previously using identical staining techniques on human bone marrow smears (7). No megakaryocytes were stained when normal goat serum was substituted for goat anti-platelet factor 4 serum. The positive staining of megakaryocytes was sharply reduced by prior adsorption of goat anti-platelet factor 4 serum with purified platelet factor 4. The platelet factor 4 antigen in megakaryocytes appears roughly equal in fluorescent staining to that in platelets.

Incorporation of [^{3}H]leucine into Platelet Factor 4 Antigen

Platelet factor 4 antigen in disrupted megakaryocytes was precipitated by double antibody immunoprecipitation as de-

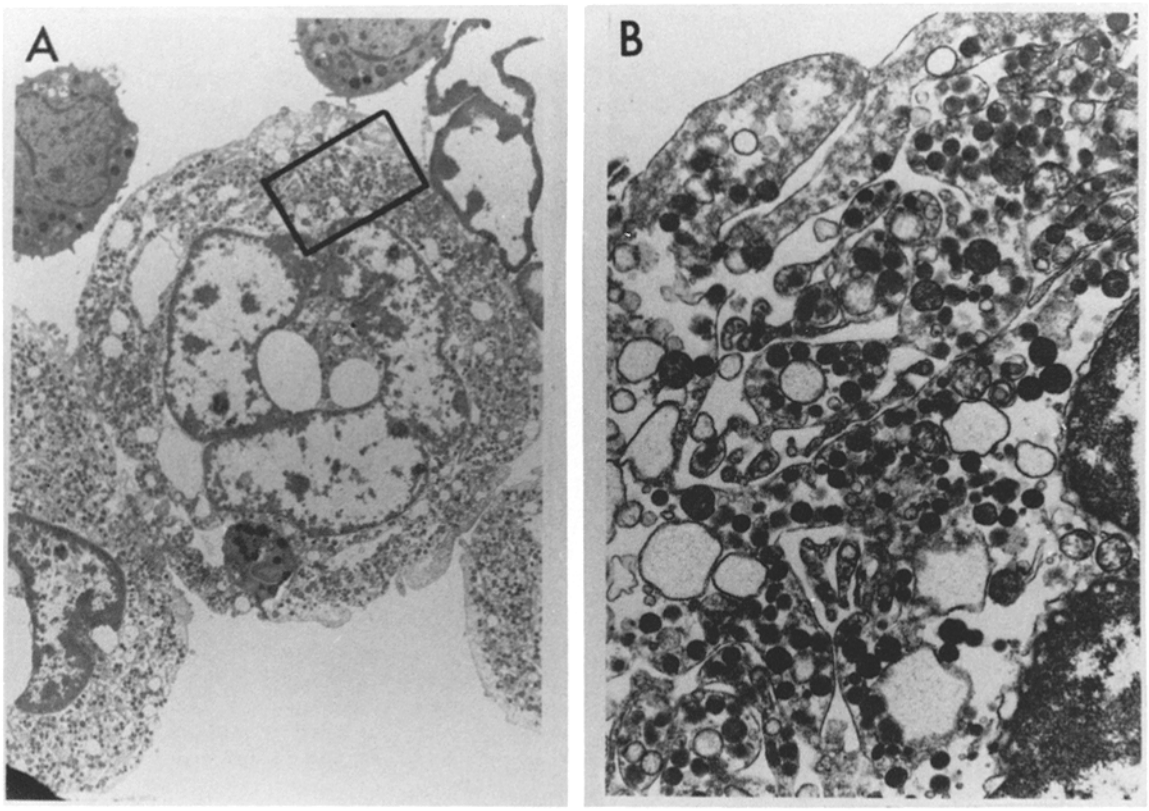

FIGURE 2 An electron micrograph of megakaryocytes from a preparation of megakaryocyte-enriched cultures. The figure on the left (*A*) demonstrates an intact megakaryocyte (× 5,000) and on the right (*B*) is a picture at higher power (× 50,000) of the section outlined in the intact megakaryocyte. The darkly stained granules (*A*) are identical in appearance to platelet α-granules, the subcellular locus of PF4 in intact platelets.

scribed in Materials and Methods. Incorporation of [^{3}H]leucine into platelet factor 4 antigen varied linearly with time (Fig. 3). A correction for nonspecific precipitation was made by subtracting the radioactivity incorporated after precipitation with normal goat serum. This correction was never >10% of the total radioactivity precipitated.

Elution Pattern of Newly Synthesized Product on a Heparin Affinity Column

Megakaryocytes (6×10^5/ml) were cultured as noted above with [^{3}H]leucine (10 μCi/ml) for 2 h. The megakaryocytes were then washed three times with cold PBS containing 1.5% BSA at 4°C, and disrupted by sonication. This extract (6×10^5 megakaryocytes/ml, 2 ml) was applied to 1.5-ml heparin affinity column as described in Materials and Methods. A linear salt gradient was used to elute labeled protein. Fig. 4 shows a single peak of radioactivity which appeared at a molarity of 1.0–1.5 M NaCl. Recovery of [^{3}H]leucine in this fraction was 0.6%. Cycloheximide (100 μg/ml) inhibited incorporation of radioactivity into this peak by 77%. Boiled megakaryocytes and megakaryocyte poor elutriator fractions had <10% of the radioactive protein peak illustrated in Fig. 4.

SDS Gel Electrophoretic Profile of an Immunoprecipitate of Newly Synthesized Antigen by Cultured Megakaryocyte

Platelet factor 4 containing fractions from heparin agarose chromatography were immunoprecipitated as noted above. [^{3}H]leucine recovered in the washed immunoprecipitate of material recovered from the heparin agarose column averaged ~10–15%. The radioactive immunoprecipitable platelet factor 4 antigen (120-min incubation) was dissolved in buffer (see Materials and Methods) and boiled for 3 min at 100°C and analyzed by SDS polyacrylamide gels as described also in Materials and Methods. This analysis consistently showed two peaks of radioactivity (Fig. 5). The most rapidly migrating peak (~44% of ^{3}H applied, ~10,000 mol wt) co-migrated precisely with purified rabbit platelet factor 4 shown in the gel before slices were prepared for measurement of [^{3}H]labeled protein (Fig. 5). This material co-eluted with carrier platelet factor 4 from heparin agarose and coprecipitated with carrier platelet factor 4 with specific antiplatelet factor 4 antisera.

A slightly less rapidly migrating protein peak (~46% of ^{3}H applied, ~14,000 mol wt) was found consistently in gels of immunoprecipitates from megakaryocyte cultures. The identity of this peak has not been definitively established. A very small peak of radioactivity (~28–30 min, Fig. 5) were variably found in different preparations. These minor peaks (~10% of total) were assumed to be nonspecific contaminants, on the basis of the small amounts of each found and/or the failure of these small radioactive peaks to be found in repeat experiments.

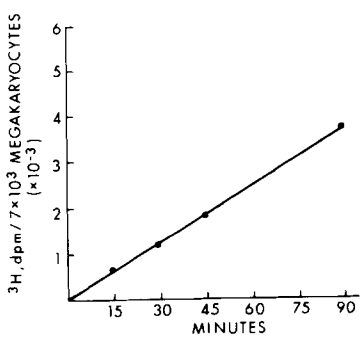

FIGURE 3 Demonstration of the incorporation of [^{3}H]leucine into platelet factor 4 antigen. Linear incorporation is found for 90 min and, in other experiments, for up to 120 min.

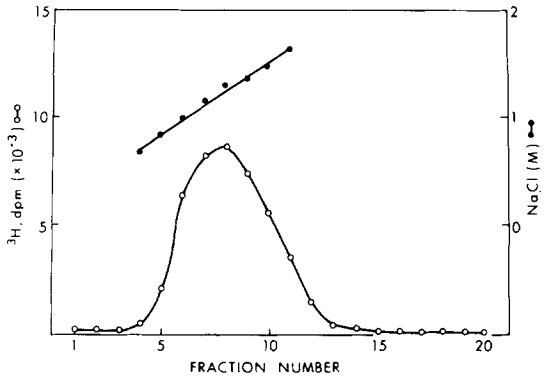

FIGURE 4 The elution pattern of newly synthesized protein from the megakaryocyte-enriched culture system from a heparin-agarose column. The column was washed extensively with 500 mM NaCl and eluted with a linear 0.5–1.5 M NaCl gradient. A large peak of radiolabeled material elutes in the range of 1.3 M NaCl and co-elutes with carrier rabbit platelet factor 4. The elution pattern of newly synthesized protein was the same as that of the ^{125}I-labeled purified rabbit platelet factor 4.

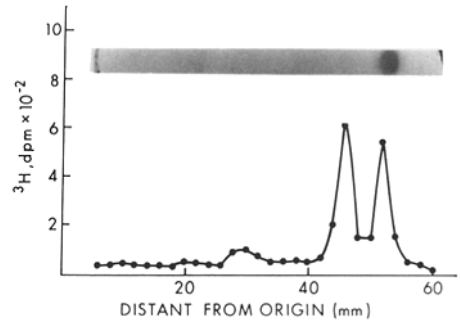

FIGURE 5 SDS gel electrophoresis of the material eluting from the heparin agarose column which has been subsequently precipitated by a double antibody technique, using goat anti-rabbit platelet factor 4 and rabbit anti-goat IgG. DPM in slices of the gel is compared with a photograph of the gel containing carrier purified rabbit platelet factor 4 stained with Coomassie Blue before slicing of the gel for measurement of ^{3}H-labeled protein. A peak of radioactivity is found in the SDS gel which co-migrates with purified platelet factor 4. The migration of marker proteins is shown for reference. Bovine serum albumin (68,000), carbonic anhydrase (30,000), lysozyme (14,300), and PF4.

DISCUSSION

While it is generally assumed that platelet-specific proteins are synthesized in megakaryocytes for packaging and subsequent release in circulating platelets, the direct demonstration of the synthesis of platelet-specific proteins has not been reported. These studies were designed to determine whether megakaryocytes synthesize the platelet-specific protein, platelet factor 4. The purification of a newly synthesized protein which copurified with homogeneous rabbit platelet factor 4 using heparin affinity chromatography and which coprecipitated with homogeneous rabbit platelet factor 4 using specific goat anti-rabbit platelet factor 4 provides strong direct evidence that megakaryocytes have synthesized platelet factor 4 and that megakaryocytes are the site of synthesis of the platelet-specific

proteins. Non–megakaryocyte-enriched rabbit bone marrow cultures did not synthesize this protein, providing additional evidence that megakaryocytes alone synthesize platelet factor 4. This view is strengthened by our previous finding that fluorescein-labeled specific antiplatelet factor 4 antisera reacted with megakaryocytes but not with other cells in rabbit bone marrow (7).

Criteria used to establish that the newly synthesized, purified protein is platelet factor 4 are: the protein co-elutes from heparin agarose at the same ionic strength as purified platelet factor 4; the protein is precipitated by a specific goat anti-rabbit platelet factor 4 antisera; and the newly synthesized protein migrates in polyacrylamide gels identically with rabbit platelet factor 4 purified to apparent homogeneity. Cycloheximide, an inhibitor of protein synthesis, blocks the synthesis of this platelet factor 4-like protein when added to the megakaryocyte-enriched culture systems.

A slower migrating (SDS gel electrophoresis) [^{3}H]leucine-labeled protein copurifies with platelet factor 4. This protein binds to heparin agarose, is eluted from heparin agarose with carrier platelet factor 4, and is precipitated together with platelet factor 4 by specific anti-rabbit platelet factor 4 antisera, and seems likely to be the precursor of platelet factor 4 as synthesized in the megakaryocyte. Such a precursor would be important to identify and would provide important information on the mechanism of synthesis of platelet α-granule proteins. We have not established that the larger-molecular-weight peak is the precursor of the smaller-molecular-weight peak. Data from preliminary pulse-chase experiments over an extended incubation period did not support a precursor/product relationship between these two peaks of radioactivity. It is possible that the antiserum to rabbit platelet factor 4 reacts with a second, newly synthesized protein in cell extracts, such as the PSP_2 protein, or the M_r ~12,000 heparin-binding protein purified from rabbit platelets by Muggli et al. (32). Further studies are required to elucidate the identity of this higher-molecular-weight protein precipitated by our anti-PF4 antiserum. Testing of the anti-rabbit platelet factor 4 antisera by immunodiffusion did not demonstrate cross-reacting protein species.

As a result of the abnormally low frequency of megakaryocytes in marrow (<0.1% of nucleated cells), biochemical studies of megakaryocytes have been possible only following isolation and purification on the basis of cell size (22, 23, 33–36), density (37), or both (1, 9, 38–41). Megakaryocytes isolated by these procedures have been shown to contain factor VIII antigen (1), fibrinogen (39), 5-hydroxytryptamine (40), and a platelet-derived growth factor-like material (9, 39). Cultures of megakaryocytes have been established (24, 42) and used to demonstrate the synthesis of thromboxane B_2 from rat marrow (22) and actin (43), and Factor VIII (1) antigen from guinea pig marrow. Platelets and megakaryocytes also share microtubules, microfilaments, and a system of invaginated membranes (44–48), compatible also with the precursor cell, the megakaryocyte, synthesizing organelles found in the product cell, the platelet. The results presented here provide direct evidence that the platelet-specific α-granule protein, platelet factor 4, is synthesized in megakaryocytes for ultimate packaging and subsequent release by platelets.

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