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 [3H]leucine. Newly synthesized [3H]-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 [3H]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.
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 anesthetized 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 Hank’s balanced salt solution (HBSS; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), containing 50 μM/mL penicillin and 50 μM/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 μM/mL penicillin and 50 μM/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, Oxonard, 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 twice 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 × 107 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, and the bone marrow cell viability was assessed by Trypan blue exclusion.

Marrow suspensions were centrifuged at 140 mm/h. The megakaryocytes from the elutriator chamber were recovered by resuspending the pellet with a 2-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 HBS, 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% CO2 at 37°C for 3 h with 2.5 × 107 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 culture cell 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 the morphological appearance of the cell systems, Trypan blue did not exceed 0.1% of the isolated total cell population. Control cultures contained 0.1% or fewer megakaryocytes.

Morphological Observations of Megakaryocytes:

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Preparation of Purified Rabbit Platelet Factor 4 and Antiserum Against Rabbit Platelet Factor 4:

Rabbit platelet factor 4 was purified by affinity chromatography on columns of direct linked heparin Sepharose (19, 25, 26), as described in detail previously. The protein is homogenously as judged by SDS polyacrylamide electrophoresis and by N-terminal amino acid sequence analysis (27). Anti-rabbit platelet serum 4 was obtained from goats after immunization with purified rabbit platelet factor 4. The antiserum 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 arc observed in immunodiffusion 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).
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 subtracting 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 x 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. 2A). Higher magnifications showed greater detail of these granules (Fig. 2B). 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 antisera 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 [³H]leucine into Platelet Factor 4 Antigen

Platelet factor 4 antigen in disrupted megakaryocytes was precipitated by double antibody immunoprecipitation as de-
were assumed to be nonspecific contaminants, on the basis of

peak of radioactivity (~28-30 min, Fig. 5) were variably found
of this peak has not been definitively established. A very small

megakaryocytes (~14,000 mol wt) was found consistently in gels of
immunoprecipitates from megakaryocyte cultures. The identity

radioactivity into this peak by 77%. Boiled megakaryocytes

and megakaryocyte poor elutriator fractions had <10% of the
radioactive protein peak illustrated in Fig. 4.

Megakaryocytes (6 x 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 x 10^6
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.

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 ^3H 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 antipeptide factor 4 antisera.

A slightly less rapidly migrating protein peak (~46% of ^3H
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.

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 megakary-
cytes synthesize the platelet-specific protein, platelet factor 4.
The purification of a newly synthesized protein which copuri-
fied with homogeneous rabbit platelet factor 4 using heparin
affinity chromatography and which coprecipitated with ho-
mogeneous rabbit platelet factor 4 using specific goat antii-
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 antithromboxane B2 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) [3H]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 thus 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 PSF2 protein, or the Mr ~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 antisem. 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 B2 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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