Immunofluorescent Identification of Human Megakaryocyte Colonies Using an Antiplatelet Glycoprotein Antiserum

By Eric M. Mazur, Ronald Hoffman, Joel Chasis, Sally Marchesi, and Edward Bruno

The development of a satisfactory in vitro assay system for human megakaryocyte colony forming progenitor cells has been delayed by the lack of a suitable marker for cells of the human megakaryocyte lineage. For this purpose we raised an antiserum directed against a purified human platelet glycoprotein preparation. In conjunction with indirect immunofluorescent staining of human bone marrow, this antiserum labeled only platelets, megakaryocytes, and an infrequent population of small mononuclear cells. These small mononuclear cells, not otherwise identifiable as members of the megakaryocyte series, constituted 22.9% of the total fluorescein positive nucleated bone marrow cells. This antiserum was also used to label colonies cultured from human peripheral blood mononuclear cells using a modified plasma clot technique. A mean of 13 fluorescein-labeled colonies were cloned per 10^6 mononuclear cells cultured. Granulocyte-macrophage and erythroid burst colonies did not label using this method. No augmentation of colony numbers was found with varying concentrations of erythropoietin, human embryonic kidney cell conditioned media (a source of thrombopoietin), or media conditioned by a human T lymphoblast cell line (a source of both colony stimulating and burst promoting activities). Immunofluorescent labeling for platelet glycoproteins is a convenient phenotypic marker for cells of the human megakaryocyte lineage useful in the study of in vitro human megakaryocytogenesis.

MATERIALS AND METHODS

Bone Marrow Smears

Human bone marrow aspirations obtained in the course of routine clinical evaluations were collected in EDTA and smeared directly onto glass slides. The normal character of the samples was established by evaluation of concomitant Wright-Giemsa stained smears. Specimens for use in immunofluorescent staining were fixed in acetone:methanol (9:1) for 20 min, washed with distilled water, air dried, and stored at -20°C.

Plasma Clot Megakaryocyte Cultures

Peripheral blood mononuclear cells from five normal volunteers were obtained by Ficoll-Paque (specific gravity = 1.077 g/cc, Pharmacia Fine Chemicals, Piscataway, N.J.) density centrifugation. Peripheral blood was diluted 1:1 with alpha medium minus nucleosides (Gibco Laboratories, Grand Island, N.Y.) containing preservative-free sodium heparin at 20 U per ml and layered over an equal volume of Ficoll-Paque. Centrifugation was performed at 500 g for 25 min at 4°C in a Beckman Model J-6B Centrifuge. The interface mononuclear cell layer was removed and washed with alpha medium minus nucleosides containing 2% fetal calf serum. 10^6 mononuclear cells/ml were cultured in 1-ml volumes in 35 mm petri dishes. The plasma clot technique of McLedd et al. was modified by the substitution of heat inactivated human AB serum for fetal calf serum and alpha medium without ribosides and deoxyribosides (Gibco Laboratories, Grand Island, N.Y.) for both NCTC-109 and Eagle's

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Supported in part by USPHS Grants CA22697-03 and HL07262-02 from the National Institutes of Health.
Presented in part at the Symposium on Megakaryocytes In Vitro, May 1, 1980, Atlanta, Georgia and at the AACP/ACSI/APCR National Meetings, May 12, 1980, Washington, D.C.
Submitted June 16, 1980; accepted September 23, 1980.
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minimal essential medium (MEM) with Hanks’ balanced salt solution. A single batch of AB serum was used for all cultures. In the final 1-cc aliquot of each culture were the supplements MEM nonessential amino acids—0.02 mmole/ml, l-glutamine—0.4 mmole/ml, and sodium pyruvate—0.2 mmole/ml. Culture additives consisted of human urinary erythropoietin (EPO, Pool ARG-332-TaSL 46.0 U per mg protein), human embryonic kidney cell conditioned medium13 (HEKM, donated by Dr. T.P. McDonald), and a human T-lymphoblast cell line conditioned medium4 (TC-CM, donated by Dr. David Golde). The cell line, Mo, was established from the spleen cells of a patient with a T-cell phenotype hairy-cell leukemia. This medium has been shown to augment erythroid burst colonies in methylcellulose cultures two- to three-fold14 and to have significant colony stimulating activity.15 Culture dishes were incubated for 12 days at 37°C in a 100% humidified atmosphere of 5% CO2 in air. Harvesting was performed by fixation in situ with methanol:acetone (1:3) for 20 min, washing with 0.01 M phosphate buffered saline (PBS) pH 7.2, distilled water, and then air drying. Plasma clots were stored frozen at −80°C until immunofluorescent staining was performed.

Anti-PGP Antiserum Preparation

Purified human PGP was prepared by lithium diiodosalicylate-phenol extraction of pooled human platelet concentrates as described by Marchesi and Chasis.16 New Zealand white rabbits were immunized by subcutaneous injections of 1 mg of PGP in Freund’s complete adjuvant initially and intramuscular injections (1 mg of PGP in Freund’s incomplete adjuvant) at 2 and 4 wk. Serum was harvested at 6 wk by cardiac puncture and stored in aliquots at −80°C.

Immunoprecipitation Assay

The lithium diiodosalicylate extracted platelet glycoproteins were iodinated by the Bolton-Hunter method17 with the following modifications: 1 mg of protein solubilized in 1-ml borate buffered saline, pH 8.6, was incubated with 0.5 μCi 125I Bolton-Hunter reagent (New England Nuclear, Boston, Mass.) for 2 hr at 4°C. 200 μl of 50 mM Tris 0.1 M glycine was added and incubated for 30 min at 4°C. The unreacted 125I was then separated by gel filtration on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) and dialysis in 0.01 M sodium phosphate buffer, pH 7.5, containing 0.02% sodium azide and 30 μM phenylmethylsulfonylfluoride. A specific activity of 3 × 106 cpm/μg was obtained.

For the immunoprecipitation assay, 10 μl of radiolabeled lithium diiodosalicylate extracted glycoproteins was added to 10 μl rabbit immune or nonimmune serum and 190 μl PBS, pH 7.5, and incubated for 24 hr at 4°C. Then 40 μl of sheep anti-rabbit IgG antiserum was added and the mixture incubated for 24 hr at 4°C. Precipitates were collected at 45,000 g for 10 min. Both precipitates and supernatants were counted in a Beckman gamma counter.

The precipitates were resolubilized in 3% sodium dodecyl sulfate (SDS), 2 M urea, 1 mM EDTA, 3% beta-mercaptoethanol, and by heating at 100°C for 3 min. The samples were then electrophoresed on 5.6% acrylamide slab gels in a modified system of Fairbanks et al.18 with the gels containing 1% SDS, 0.5 M urea, and the buffer containing 1% SDS, 40 mM Tris, 2 mM EDTA, and 20 mM sodium acetate, pH 7.4. The gels were dried and autoradiographed on Kodak X-OMAT R film.

Immunoadsorption

Lithium diiodosalicylate extracted platelet glycoproteins were bound to cyanogen bromide activated sepharose 4B beads as previously described.19 The antiserum was run into a PGP-sepharose column and incubated for 2.5 hr at 4°C. The fraction which then passed directly through the column using a PBS pH 7.5 eluent was re-concentrated to its original protein concentration and tested for megakaryocyte immunofluorescent labeling activity.

Immunofluorescent Staining and Scoring

Whole rabbit anti-PGP antiserum was diluted in PBS (1/200) and layered over the specimens to be studied. Human bone marrow smears and plasma clots were incubated with the antiserum for 30 and 60 min, respectively, at 37°C in 5% CO2 100% humidified air. After washing three times with PBS, the specimens were re-incubated with fluorescein conjugated goat anti-rabbit IgG (Meloy, Springfield, Va.) diluted in PBS, final concentration 0.36 mg protein/ml, for an equivalent period of time. After washing again with PBS, the specimens were counterstained with 0.125% Evan’s blue. Immunofluorescent staining was performed.

**Fig. 1.** SDS-acrylamide gel electrophoreses. (A) Periodic acid-Schiff stain of the purified platelet glycoprotein immunogen. (B) Autoradiograph of the solubilized immunoprecipitate derived from the reaction of immune rabbit serum with radiolabeled platelet glycoprotein.
RESULTS

Antibody Activity and Specificity

With the immunoprecipitation assay it was found that immune rabbit serum precipitated 80% of the radiolabeled lithium diiodosalicylate extracted platelet glycoproteins while nonimmune rabbit serum precipitated only 15% of the radioactivity. Autoradiographs of the slab gels electrophoresed with the solubilized immunoprecipitates demonstrated bands corresponding to platelet glycoproteins of estimated molecular weights of 145,000, 125,000 and 95,000 (Fig. 1), demonstrating activity of the antiserum for the original immunogen.

When normal bone marrow smears were examined...
using indirect immunofluorescent labeling, only platelets, megakaryocytes (Figs. 2 and 3), and an infrequent population of lymphoid-like cells fluoresced intensely. Red cells, granulocytes, monocytes, and lymphocytes fluoresced little or none. Blast cells obtained from patients with several different lymphoid and myeloid leukemias as well as cells derived from the human leukemic blast cell lines HL60 and K562 demonstrated no fluorescent labeling (Table 1). HL60 is a promyelocytic leukemia cell line while the K562 cell line has recently been shown to possess a number of erythroid phenotypic markers. Activity of the anti-PGP antiserum using indirect immunofluorescent staining could be demonstrated at dilutions to 1/1280. Pre-adsorption of the antiserum through the PGP-sepharose affinity column removed all platelet and megakaryocyte labeling activity. Indirect immunofluorescent staining using a control pre-immune rabbit serum also failed to label any hematopoietic bone marrow elements.
Table 1. Intensity of Fluorescent Labeling by Cell Type with Antiplatelet Glycoprotein Antiserum

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Platelets</td>
<td>4+</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>4+</td>
</tr>
<tr>
<td>Red cells</td>
<td>0</td>
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<tr>
<td>Lymphocytes</td>
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<td>Neutrophils</td>
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<tr>
<td>Eosinophils</td>
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</tr>
<tr>
<td>Monocytes</td>
<td>0</td>
</tr>
<tr>
<td>Leukemic blast cells</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td></td>
</tr>
<tr>
<td>CGL (blast crisis)</td>
<td>0-trace</td>
</tr>
<tr>
<td>CMML</td>
<td></td>
</tr>
<tr>
<td>T-cell ALL</td>
<td>0-trace</td>
</tr>
<tr>
<td>ALL (Ph' + )</td>
<td></td>
</tr>
<tr>
<td>Leukemic cell lines</td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td>0</td>
</tr>
<tr>
<td>HL60</td>
<td>0</td>
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</table>

Bone Marrow Small Platelet Glycoprotein-Bearing Cells

On all normal bone marrow smears, small nucleated cells, approximately 7–14 μm in diameter, demonstrated intense fluorescence when labeled with anti-PGP antiserum (Fig. 4). Fluorescent staining of these cells was generally homogeneous, often obscuring the nucleus. These small cells were most commonly located in or near the bone marrow stromal particles, areas also demonstrating the highest density of large megakaryocytes (Fig. 5). When expressed as the percentage of total fluorescein positive nucleated cells, these small cells, not otherwise identifiable as megakaryocytes, comprised 22.9% ± 2.0% (mean ± SEM) of the total. Since morphologically recognizable megakaryocytes constitute about 0.04% of nucleated bone marrow cells in normal marrow, the overall frequency of these cells can be estimated as approximately 0.01%.

Clonogenic in Vitro Megakaryocyte Cultures

Intensely fluorescent PGP-bearing cellular colonies were cultured from the peripheral blood mononuclear cells of five normal volunteers (Figs. 6 and 7). Colonies universally were dispersed and small in number with less than 20 cells per colony. They were comprised of large and small fluorescein labeled cells, both in combination and alone. Fluorescein positive colonies were generally not mixed with other unlabeled cell clusters such as granulocytes, normoblasts, and macrophages. However, occasional mixed megakaryocytic, granulocyte-macrophage colonies were seen. No mixed megakaryocytic-erythroid colonies were detected in erythropoietin containing cultures.

When fluorescein-labeled colonies were mapped...
Fig. 5. Fluorescein-antibody labeled megakaryocyte and a small platelet glycoprotein bearing cell in human bone marrow. (x380)

Fig. 6. Twenty-cell megakaryocyte colony in plasma clot culture labeled with anti-PGP antiserum. Note the intense fluorescence of the colony and only faint labeling of the background cells. (x80)
Fig. 7. Serial immunofluorescent (A) and Giemsa staining (B and C) of a single megakaryocyte colony in plasma clot culture. The outlined area in B is further enlarged in C revealing the megakaryocytic morphology of some of the fluorescein-positive component cells. (A and B ×100), (C ×380)
and subsequently Giemsa-stained, they were found to consist both of large cells with multilobulated nuclei that resembled megakaryocytes and smaller cells resembling mature lymphocytes (Fig. 7). Pure colonies of both types of cells were seen.

In the absence of any specific megakaryocyte growth promoting factor, $13 \pm 6$ (mean $\pm$ SEM) megakaryocyte colonies were cloned per $10^6$ mononuclear cells cultured.

Erythropoietin in concentrations ranging from 0.5 to 4 U/ml did not enhance colony formation (Fig. 8). Neither did human embryonic kidney cell conditioned medium, a source of thrombopoietic stimulating factor, in concentrations of 75 to 600 pg of protein per ml (Fig. 9). Suppression of colony formation was observed at the higher concentrations of both substances.

The addition of media conditioned by a human T-lymphoblast cell line (final concentration 10%), a known source of burst promoting and colony stimulating activities, also failed to enhance colony formation, both alone and with the concomitant addition of erythropoietin (Fig. 10). Thus, no substance tested significantly increased megakaryocyte colony formation above that present in the unstimulated cultures.

**DISCUSSION**

In this report, we demonstrate that platelet glycoproteins are useful phenotypic markers for cells of megakaryocyte lineage, results that are similar to those previously reported by Rabellino et al. With a rabbit antiserum to human PGP, human bone marrow megakaryocytes and platelets labeled intensely using indirect immunofluorescent staining. No significant labeling of other identifiable human hematopoietic cells or in vitro human cell lines was seen. The activity and specificity of this antiserum for platelet glycoproteins was further demonstrated by immunoprecipitation and affinity chromatography.

Using anti-PGP immunochemical labeling of intact human bone marrow, we have also defined a population of small mononuclear cells not otherwise recognizable as megakaryocytes. These cells are approximately $1/4$ as frequent as the morphologically identifiable megakaryocytes. Their fluorescein labeling is intense and homogeneous, similar to the fluorescence of the rodent small acetylcholinesterase positive cells simultaneously labeled with antiplatelet antiserum. We suspect that these small human PGP-bearing cells are indeed the human counterparts of the rodent small acetylcholinesterase positive cells and are progeny of the CFU-M that mature into single megakaryocytes.

Indirect immunofluorescent labeling of PGP-bearing cells is also useful in identifying CFU-M derived colonies in a modified plasma clot culture system of human peripheral blood mononuclear cells. Such labeling permits easy quantitation of these human megakaryocyte colonies and circumvents the uncer-
tainties of morphological enumeration. In addition, this method has the capacity to identify colonies comprised exclusively of small platelet glycoprotein-bearing cells that would not otherwise be included in such a quantitation.

The growth of megakaryocyte colonies in the absence of a specific megakaryocyte growth promoting factor that is described here has been reported previously. The factors triggering CFU-M proliferation and differentiation in these systems are unknown. The failure of erythropoietin to enhance CFU-M derived colony growth in our system contrasts with the work of Vainchenker et al. in man and that of McLeod et al. in the mouse, but is in agreement with the murine studies of Erslv et al. In vivo observations suggest that erythropoietin is unlikely to have an important physiologic regulatory role in early CFU-M proliferation and differentiation. Clinical states associated with high endogenous erythropoietin levels such as hypoxia are not generally accompanied by sustained thrombocytosis or megakaryocytic hyperplasia.

We found no augmentation of CFU-M derived colony formation with human embryonic kidney cell conditioned medium. HEKM, a source of thrombopoietic stimulating factor, has been previously shown to increase 75SeM platelet incorporation in thrombocytotic animals. Effects previously demonstrated in clonogenic assay systems have been conflicting. In animal studies, HEKM has been reported both to promote CFU-M derived colony growth as a primary stimulant and to potentiate such colony formation only in the presence of another primary growth promoting substance. In the latter study, HEKM alone did not support colony growth, results substantially in agreement with our own.

The lack of colony stimulation using TC-CM, a source of both burst promoting and colony stimulating activities, suggests that these substances are also not active at the level of the CFU-M. Further, it supports the contention that immunofluorescent PGP labeling is indeed limited exclusively to megakaryocytic colonies. In our experiments, the numbers of erythroid bursts and granulocyte-macrophage colonies were significantly augmented in the presence of TC-CM and erythropoietin without concomitant enhancement of labeled megakaryocyte colonies.

Thus, no substance tested significantly increased CFU-M derived colony formation above baseline. Further studies will be needed to define those factors important in human megakaryocytopoiesis. Immunofluorescent labeling of membrane PGP should provide a new tool for such studies.

ACKNOWLEDGMENT

We would like to thank Drs. T.P. McDonald and D. Golde for supplying the conditioned media utilized in this investigation.

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