Constitutive Expression of Platelet Glycoproteins by the Human Leukemia Cell Line K562

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The human leukemia cell line K562 was derived from a patient with chronic granulocytic leukemia. This cell line has subsequently been shown to possess phenotypic markers typical of erythroid and myeloid cells. Using a rabbit antisem directed against purified platelet glycoproteins (PGPs), we have obtained evidence for the constitutive expression of PGP's on the surface of K562 cells. PGP's expressed have been tentatively identified as IIa and IIIb based on their apparent migration in a 7% sodium dodecyl-sulfate polyacrylamide gel. K562 may become an important tool for the study of early events involved in megakaryocytic differentiation.

**a-PGP Source**

Preparation and specificity determination of a-PGP have been previously reported. Briefly, New Zealand white rabbits were immunized with purified human PGP prepared by Marchesi and Chasis and kindly supplied to us by J. A. Chasis. One milligram of material was mixed in Freund's complete adjuvant and injected subcutaneously, followed by intramuscular injection of 1 mg of material 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. Specificity was proven by loss of ability to stain bone marrow megakaryocytes after immunoblotting of a-PGP with purified PGP bound to cyanogen-bromide-activated sepharose 4B beads and by SDS-PAGE analysis of immunoprecipitated authentic PGP with a-PGP.

**Immunofluorescent Analysis of K562 and HL60 Cell Surfaces**

A quantity of 10⁶ K562 or HL60 cells, grown in RPMI 1640 medium with 10% FCS, were obtained 4 days after initial seeding at 2 x 10⁶ cells/ml. Cells were washed 3 times in phosphate-buffered saline (PBS) and then incubated in rabbit antiplatelet glycoprotein antiserum (a-PGP) or normal rabbit serum (NRS) (Accurate Chemical and Scientific Corp., Westbury, N.Y.) at a final concentration of 1/20 for 20 min at room temperature (RT). Cells were washed 3 additional times in PBS, incubated in fluorescein-conjugated goat anti-rabbit antiserum (Meloy Laboratories, Inc., Diagnostic Division, Springfield, Va.) at RT for 20 min at a final concentration of 1/20, washed 3 times in PBS, and then analyzed in a FACScan IV cell sorter (Becton-Dickinson, Mountain View, Calif.). Prior to each sorting run, the FACScan IV sorter was calibrated and laser drift controlled for by first running standard fluorescent beads in the machine. Approximately 20,000 cells were accumulated for every analysis. The area under each histogram curve is therefore equal, allowing for their direct comparison.

**Cell Line**

K562 cells from two sources were utilized. These two subcultures have been described in detail previously.4 “Clone R” was obtained as a generous gift from Dr. Timothy Rutherford and J. B. Clegg; these cells have also been described previously.5 “Clone 6" was the kind gift of Dr. C. G. Gahmberg and provided to us by Dr. H. Furthmayr. These cells have been previously described as well.1,2 Results obtained from both subcultures of K562 were indistinguishable with respect to PGP expression and yielded results essentially identical to those described herein. The cells were maintained in RPMI 1640 medium supplemented with 10% newborn calf serum and maintained at 37°C in a humidified atmosphere containing 5% CO₂.
experiments were repeated 5 times with essentially identical results.

Tritium Labeling of K562 Surface Glycoproteins

A quantity of 10^6 K562 cells were grown and harvested as described above. Surface glycoproteins were tritiated with sodium borohydride (NaB\(_{3}\)H\(_4\)) (New England Nuclear, Boston, Mass., 347 mCi/mmol) as described by Gahmberg et al. Briefly, the cells were washed 3 times in PBS and then incubated in 10 ml of 1 mM sodium m-periodate in PBS on ice for 5 min. The reaction was terminated by the addition of 2 ml of 0.1 M glycerol. Cells were washed again 3 times in PBS, resuspended in 5 ml of PBS to which was added 5 mCi of NaB\(_{3}\)H\(_4\). After 30 min at room temperature, the cells were washed extensively in PBS until radioactivity in the wash solution returned to baseline. The cell membranes were then solubilized in 1 ml of 1% Triton X-100, 1% ETOH, 2 mM phenylmethylsulfonyl fluoride, and 0.02% Na azide in PBS (hereafter called buffer A) for 0.5 hr on ice and then spun for 10 min at 4000 rpm. The clear supernatants were stored at −80°C until needed.

Analysis of Tritiated K562 Membrane Supernatants by Lectin-Affinity Column Chromatography, Immunoprecipitation, and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The supernatants obtained after the 3H-labeling and solubilization procedures were applied to an affinity column containing wheat germ lectin (from Triticum vulgaris) attached to sepharose 6MB (Sigma Chemical Co., St. Louis, Mo.; cat. NO. L6257, 5 mg lectin/ml of gel). The column (1 ml bed volume) was washed with buffer A until the column effluent radioactivity returned to baseline. Bound material was then eluted with 2 ml of 0.1 M N-acetylglucosamine (Sigma Chemical) in buffer A. Seven-hundred-fifty microliters of this material was preabsorbed with 15 μg of mouse IgG (Sigma Chemical) and 15 μg of rabbit anti-mouse globulin (Sigma Chemical) for 10 min, after which 50 μl of a 1:1 slurry of staph-A protein fixed to sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, N.J.) was added for 30 min. The reaction mixture was agitated every 5–10 min and the beads subsequently removed by centrifugation. In this manner, material nonspecifically adherent to staph-A beads was eliminated. NRS or a-PGP was then added to the supernatant to a final concentration of 1/10. This material was incubated for 2 hr at RT and then overnight with 150 μl of a 1:1 slurry of staph-A beads in buffer A at 4°C with constant gentle shaking, followed by 3 washes in 1 ml of buffer A containing 1 mg/ml egg ovalbumin. Immunoprecipitated material was removed from the beads by boiling for 5 min in 50 μl of a solubilizing buffer containing 6% SDS, 0.125 M Tris, and 0.004 M EDTA, pH 6.9, reduced with 2% (v/v) mercaptoethanol and applied to a 7% resolving polyacrylamide gel with a 5% stacking gel. High molecular weight marker standards (Bio-Rad Laboratories, Richmond, Calif.) were run simultaneously in the gel to serve as reference markers and as a control for shrinking and drying artifact. Two lambda of the marker standard in 50 μl of solubilizing buffer was boiled for 5 min with 1% (v/v) mercaptoethanol and then applied to the gel. Markers consisted of 5 μg of myosin (mol wt 200,000), β-galactosidase (mol wt 116,250), phosphorylase-B (mol wt 92,500), bovine serum albumin (mol wt 66,200), and ovalbumin (mol wt 45,000).

RESULTS

Histograms generated by the FACS IV cell sorter of the fluorescein-labeled K562 and HL60 cells are shown in Fig. 1. Panel A shows the superimposed histograms of the K562 cells reacted with a-PGP, the curve labeled A, and with NRS, the curve labeled B. It is readily apparent that minimal cell fluorescence is obtained when the cells are reacted with NRS, while a large number of cells are intensely labeled after incubation with a-PGP. In fact, if one excludes from PGP positively those a-PGP-labeled K562 cells that fluoresce with the same intensity as 99% of NRS-labeled cells, then approximately 35% of a-PGP-labeled cells subjected to sorter analysis may be said to be brightly fluorescent and therefore express PGPs on their surface. Staining the cells with PI, a dye that fluoresces red when excited by light of the appropriate wavelength, allowed for the easy detection of nonviable cells as these cells cannot exclude PI. Once identified, dead cells could then be eliminated from the actual sorter analysis. Comparison of histograms generated with or
without PI yielded essentially identical results, making it highly unlikely that nonspecific adherence of antibody to dead cells in any way influenced our results. It is also unlikely that our results were influenced by cell aggregation or solely by cell size, as is shown in Fig. 2. It is apparent from the light scatter plots that there is no bias in cell size distribution between the NRS (panel A) or a-PGP (panel B) treated groups. If the intensity of fluorescence was strictly a function of cell size, there should be no difference in fluorescence intensity between the two groups, but in fact, many of the a-PGP-reactive cells fluoresce at a higher intensity. In addition, in the a-PGP-treated group, it can be seen that the brightly fluorescent cells are not restricted to the higher light scatter ranges. In fact, these bright cells can be seen throughout the light scatter distribution.

The specificity of this reaction is demonstrated in panel B of Fig. 1. This panel is a composite of the histograms obtained when K562, represented by curve A, and HL60, represented by curve B, were treated with a-PGP and analyzed under identical conditions. It may be seen here that HL60 fluoresces weakly when reacted with a-PGP and in fact generates a curve that is almost identical to that seen when K562 is treated with NRS.

To further exclude the possibility that nonspecific cross-reactivity between our rabbit antiserum and K562 cell surfaces accounted for the cell sorter positivity, further evidence of PGP expression by K562 was obtained by other immunochemical methods. K562 surface glycoproteins were specifically labeled with tritiated sodium borohydride, solubilized in buffer containing 1% Triton X-100, and then concentrated on a wheat germ lectin column. Material eluted from this column was immunoprecipitated with a-PGP and then analyzed by SDS-PAGE according to the method of Laemmli. An autoradiograph of this gel is shown in Fig. 3. SDS-polyacrylamide gel autoradiograph of lectin affinity column concentrated radiolabeled K562 surface glycoproteins immunoprecipitated with a-PGP antibody. Lane 1 demonstrates the autoradiograph obtained after 14-days exposure of a 50-μl aliquot of the NaB3H4-labeled surface glycoproteins eluted from the wheat germ lectin column. Lane 2 demonstrates that no immunoprecipitable material was recovered from the NRS-reacted material. Lane 3 reveals two heavy weight bands precipitated with a-PGP corresponding to molecular weights of ~157,000 and 117,000, respectively. According to previous reports, these bands correspond to the reduced forms of PGP Ila and Ill. When reacted with purified authentic PGPs, the anti-PGP antiserum yields three bands of apparent molecular weights 148,000, 125,000, and 95,000 (cf., ref. 9, Fig. 1).
Fig. 3. Lane 1 is the autoradiograph of the entire material eluted from the lectin column. In lane 2 we demonstrate that no immunoreactive bands are formed when the lectin eluate is reacted with NRS. Examination of lane 3 clearly shows two heavy weight immunoprecipitate bands, mol wt ~157,000 and 117,000, which were formed when the lectin eluate was reacted with a-PGP. Identification of these bands is based on their apparent weight (compared to known standards run simultaneously on the gel) and those previously reported for these proteins. These bands correspond in weight to those reported for the reduced forms of PGP IIa and III. A smaller unidentified band of mol wt ~60,000, labeled X, is seen as well.

DISCUSSION

Our work strongly suggests that PGPs are constitutively expressed by K562 cells. This finding is seemingly contradictory to an earlier report from our laboratory that suggested that PGPs were not expressed by K562. However, for these earlier FACS studies we employed a-PGP at a dilution of 1/200, which was chosen because at this dilution our antibody had previously been shown to label normal human megakaryocytes. Reasoning that PGPs might be expressed in low density, or be otherwise poorly accessible on the cell membrane, we increased our working concentration of antibody at least tenfold and obtained the results detailed in Fig. 1 and the confirmatory data shown in Fig. 2.

We are aware that a previous attempt from another laboratory to demonstrate PGP on K562 by immunochimical methods was also unsuccessful. However, these studies used monoclonal antibody at a dilution of 1/500, which, as we have noted above, may have been too dilute for detection. It is also possible that attempts to identify PGP on K562 with monoclonal antibodies may result in failure because of their highly restricted antigenic recognition. PGPs expressed on this neoplastic cell line may have altered patterns of glycosylation that render them unrecognizable to monoclonal but not polyclonal antibody. For example, Fukuda has demonstrated that K562 cells express the fetal type (i) antigen on distinctly different glycoproteins from those of erythrocytes. In addition, the PGP II–III complex may be assembled differently on the membrane, which would also make it unrecognizable to a monoclonal antibody raised against the normal complex. We are reluctant, however, to identify the immunoprecipitable PGP bands shown in Fig. 2 as specific analogues of PGP components expressed on mature platelets. Further studies will be required to characterize the structures of normal PGPs in order to determine whether the PGP-like elements we have demonstrated on K562 are either normal or aberrantly constructed.

The observation that K562 expresses PGP on its surface is of potential importance for at least two reasons. First, K562 should become a valuable tool for the study of the cellular and molecular events involved in early megakaryocyte differentiation. A compound that increases the percentage of K562-expressing platelet specific peroxidase has already been described and would be a logical substance with which to pursue such studies. Second, isolation of PGP mRNA from K562 would allow for the cell-free synthesis of these important glycoproteins and thereby make them available in sufficient quantity for study of their role in thrombosis, atherosclerosis, and tumor cell metastasis. Such work has been extremely difficult to carry out because of the marked difficulty encountered in isolating large numbers of intact human megakaryocytes from bone marrow.

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