Immunohistochemical Localization of Membrane and α-Granule Proteins in Human Megakaryocytes: Application to Plastic-Embedded Bone Marrow Biopsy Specimens

By Jay H. Beckstead, Paula E. Stenberg, Rodger P. McEver, Marc A. Shuman, and Dorothy F. Bainton

Using a new technique for antigen localization, we have demonstrated platelet proteins in megakaryocytes in plastic-embedded biopsy specimens of normal human bone marrow. In a series of 25 specimens, megakaryocytes showed labeling with antibodies to the integral membrane glycoproteins IIa, IIb, and the IIb-IIIa complex; granule membrane protein 140; and five alpha-granule matrix proteins: thrombospondin, factor VIII–related antigen, β-thromboglobulin, platelet factor 4, and fibrinogen. The antibodies to the membrane glycoproteins IIa, IIb, and IIb-IIIa produced diffuse cytoplasmic staining and heavier staining on the plasma membrane, whereas the antibodies to the α-granule matrix proteins produced a distinct granular staining within the cytoplasm. Staining for granule membrane protein 140 was also granular in distribution. Rare mononuclear cells consistent with megakaryocytes were labeled with these markers. Other enzyme histochemical and lectin-binding studies showed that the enzyme α-naphthyl acetate esterase, the lectin Ulex europaeus I, and the periodic-acid Schiff reaction were consistent, but not specific, markers of megakaryocytes. This immunohistochemical technique should facilitate the examination of qualitative and quantitative changes in megakaryocytes in a variety of physiologic and pathologic processes.

MATERIALS AND METHODS

A series of 25 specimens of human bone marrow was obtained after informed consent was given. All were from patients with normal platelet counts who were undergoing evaluation for malignancies or mild anemias and whose biopsy specimens were morphologically normal. The specimens were obtained by Jamshidi needle biopsy of the posterior iliac crest. They were fixed immediately at 4 °C in 1% paraformaldehyde in 0.1 mol/L PO4 buffer, pH 7.4 and were kept in this fixative for one to 12 hours at 4 °C. They were further processed in an Autotechnicon Tissue Processor (Technicon, Tarrytown, NY) in a cold room (4 °C) as follows: (1) fixed for six hours in 4% paraformaldehyde in 0.1 mol/L PO4 buffer; (2) washed in 0.1 mol/L PO4 buffer with 2% sucrose and 50 mmol/L NH4Cl for one hour, repeated twice; (3) washed in 0.1 mol/L PO4 buffer with 50 mmol/L NH4Cl for one hour, repeated once; (4) dehydrated in 50% acetone for one hour, 100% acetone for two hours, 50 parts acetone/50 parts glycol methacrylate monomer for one hour; (5) infiltrated with 100% glycol methacrylate monomer for one hour; (6) changed to 100% glycol methacrylate monomer and infiltrated for approximately four hours. The specimens were then embedded in a mixture composed of glycol methacrylate monomer (20 mL), benzyl peroxide (0.09 g), and polyethylene glycol 400 with N,N-dimethylaniline (0.5 mL) (Polysciences Inc, Warrington, Pa). The embedded tissue was placed under vacuum (15 to 20 mm Hg) at 4 °C and allowed to polymerize overnight. Sections were then cut at 1 to 3 μm with glass knives on a Sorvall JB-4 microtome (Dupont, Wilmington, Del). The sections were transferred via water to cover-slips and air dried at room temperature.

Routine staining procedures and enzyme histochemistry were performed as previously described. These included hematoxylin-eosin stain, Masson’s trichrome, and Azan stains for connective tissue, periodic-acid Schiff reaction for glycogen, and enzyme histochemical and lectin-binding studies. For immunohistochemical staining, the sections were washed in 0.1 mol/L PO4 buffer, pH 7.4 and allowed to polymerize overnight. Sections were then cut at 1 to 3 μm with glass knives on a Sorvall JB-4 microtome (Dupont, Wilmington, Del). The sections were transferred via water to cover-slips and air dried at room temperature.

Routine staining procedures and enzyme histochemistry were performed as previously described.11-13 These included hematoxylin-eosin stain, Masson’s trichrome, and Azan stains for connective tissue, periodic-acid Schiff reaction for glycogen, and enzyme histochemical and lectin-binding studies.
Control procedures included the substitution of irrelevant antibodies, preimmune serum, or ascites fluid for the primary antibodies and the elimination of primary or secondary reagents. All biopsy specimens were examined using a Zeiss microscope and a 40 × objective. All morphologically identifiable megakaryocytes were counted in each specimen with each antibody, (range, 32 to 174; mean, 87). Morphologically identifiable megakaryocytes were defined as cells with large nuclei (greater than 1.5 times the diameter of a myeloblast) and moderate-to-low nuclear/cytoplasmic (N/C) ratios. Those with low N/C ratios and granular cytoplasm were considered mature megakaryocytes; those with moderate N/C ratios were considered immature megakaryocytes. In addition, rare mononuclear cells the size of myeloblasts or smaller with high N/C ratios were assumed to be megakaryocyte precursors if they showed positive reactions with the antibody reagents. Although these cells are not morphologically identifiable, control preparations showing an absence of staining in myeloid, erythroid, monocytic, or lymphoid precursors make this a reasonable assumption.

Cells for electron microscopy were washed from marrow specimens into 2% paraformaldehyde–0.05% glutaraldehyde in 0.1 mol/L PO buffer, treated with polyclonal antibody to GPIIIa (1:500 dilution), exposed to protein-A gold (5 nm), and then processed for electron microscopy as previously described. Since the reason for some of the technical modifications may not be immediately apparent, the rationale in brief is presented here. The NH4Cl in the buffer washes removes remaining aldehydes in the tissues and thus prevents further fixation; the effect on total antigen survival is modest. Acetone dehydration allows survival of all of the antigens, whereas ethanol dehydration markedly decreases the survival of some antigens, particularly GPIIb and the GPIIIa–IIb complex. Covolumes should dry overnight or longer at room temperature to ensure adhesion through the entire immunohistochemical procedure. We found that trypsin digestion for longer than 15 minutes degraded the morphology and that only a brief trypsin exposure was necessary. Some antigens could be detected without trypsin digestion. We found overnight (4°C) incubation with primary antibody to be the most convenient and reliable procedure; good results were also obtained with a four- to six-hour incubation at room temperature or a two-hour incubation at 37°C.

The large amount of endogenous peroxidase poses a particular problem for bone marrow studies. We approached this problem by combining several techniques. We inhibited with methanol–H2O2 after the biotinylated secondary antibody to prevent interference with antigen–antibody reactions. We selected a lower concentration of these reagents than is generally used because higher concentrations had a tendency to loosen the sections. The addition of sodium azide, which inhibits animal but not plant peroxidases, further improves the inhibition of endogenous peroxidase. The use of this combination usually results in complete inhibition of peroxidase in eosinophils and red cells. Weak persistence of reactivity in these cells is occasionally noted. We have also had some success using phenylhydrazine in a preincubation prior to the primary antibody step. A consistent, apparently nonspecific, reaction was seen in mast cells using all of the primary antibodies. These cells have been shown to bind avidin complexes. There was no other binding suggesting the presence of endogenous biotin or avidin.

RESULTS

The main structural components of human platelets and megakaryocytes have been well defined by ultrastructural studies in recent years. Antibodies to a variety of platelet proteins have been produced in our laboratory and by others. Most of these proteins can be classified by their fine structural localization in platelets into three main groups: (1)
plasma membrane proteins, (2) α-granule matrix proteins, or (3) α-granule membrane proteins. Using antibodies against these antigens and the technique described previously, we were able to precisely localize the proteins within the megakaryocytes of well-preserved intact biopsy specimens of human bone marrow (Figs 1A through 1F). Control preparations showed no evidence of nonspecific staining with the exception of the mast cell labeling previously noted. The results are summarized in Table 1.

Staining With Antibodies and Platelet Plasma Membrane Proteins

GPIIIa. Polyclonal antisera against GPIIIa labeled an average of 99% (range, 97% to 100%) of morphologically identifiable bone marrow megakaryocytes (Figs 1A and 1B). Rare mononuclear cells, presumably megakaryocytic precursors, were also labeled. Staining in megakaryocytes was distributed throughout the cytoplasm but was heaviest on the plasma membrane. This pattern was most readily observed in thin sections (1 μm) at high antibody dilutions and is consistent with localization of the antigen in both the plasma membrane and the demarcation membrane system and/or rough endoplasmic reticulum. In some megakaryocytes, distinct staining around the nucleus suggestive of localization in the perinuclear cisterna could be seen (Fig 1B). The monoclonal antibody (AP-3) showed the same distribution.

The plasma membrane localization of GPIIIa on nonpermeabilized megakaryocytes has been confirmed by electron microscopic examination (Fig 2). Studies on permeabilized cells that will allow the labeling of intracellular structures are in progress.

There was no staining of other hematopoietic cells. The endothelial cells within the marrow showed moderate staining, and osteoclasts showed a striking plasma membrane reaction (Fig 3) with both monoclonal and polyclonal antibodies.

GPIIb. The monoclonal antibody (Tab) that recognizes GPIIb produced strong staining in an average of 97% (range, 89% to 100%) of morphologically recognizable bone marrow megakaryocytes (Fig 1C). Rare mononuclear cells, presumably megakaryocytic precursors, were also stained (inset, Fig 1C). The staining pattern was very similar to that for GPIIIa, ie, present throughout the cytoplasm, prominent on the plasma membrane, and occasionally perinuclear. There was no staining of other hematopoietic or marrow cells, including osteoclasts. Staining with polyclonal antisera against GPIIb showed the same distribution.

GPIIb-IIIa complex. The monoclonal antibody (T-10) that recognizes the GPIIb-IIIa complex produced strong staining in an average of 96% (range, 91% to 100%) of morphologically recognizable bone marrow megakaryocytes (Fig 1D). The localization of staining was similar to that observed with antibodies to GPIIIa and GPIIb. In unstained sections peroxidase was clearly visible along the nuclear membrane in a few megakaryocytes (Fig 1D), indicating that the antigen may be present in the perinuclear cisterna, a compartment of the rough endoplasmic reticulum. As with GPIIIa and GPIIb, diffuse cytoplasmic staining was also present. Rare cells consistent with megakaryocytic precursors were stained, but other hematopoietic cells were negative. There was no staining of other marrow elements. Osteoclasts and endothelial cells did not stain.

Staining With Antibodies to Platelet α-Granule Matrix Proteins

Thrombospondin. Monoclonal antibodies against thrombospondin produced a distinctly granular reaction throughout the cytoplasm of megakaryocytes, consistent with localization of the antigen in the α-granules (Fig 1E). On average, 98% (range, 91% to 100%) of morphologically recognizable megakaryocytes were reactive. Fewer presumed megakaryocyte precursors were labeled with the antibodies against thrombospondin and the other α-granule matrix proteins than with the antibodies against plasma membrane proteins. Rare cells showed perinuclear staining in uncounterstained preparations. No other marrow cells showed reaction product. The absence of staining in macrophages and endothelial cells is of interest since it has been shown that these cells can secrete thrombospondin in culture. However, Jaffe and co-workers, were unable to demonstrate thrombospondin in these cultured cells immunohistochemically.

Factor VIII-related antigen. Both monoclonal and polyclonal antibodies against VIIIIR:Ag produced strong granular staining in an average of 95% (range, 84% to 100%) of morphologically recognizable megakaryocytes. The distribution was identical to that observed with thrombospondin. There was no labeling of other hematopoietic cells, but moderate endothelial cell staining was observed.

β-thromboglobulin. Polyclonal antisera against β-thromboglobulin produced a strong granular reaction similar to that observed with VIIIIR:Ag and thrombospondin in 94% (range, 84% to 100%) of the megakaryocytes. There was some nonspecific background labeling, but other hematopoietic and marrow cells appeared to be negative.

Platelet factor 4. Polyclonal antisera against PF4 produced a granular reaction similar to that observed with the other antibodies to α-granule proteins. An average of 94% (range, 85% to 100%) of the megakaryocytes were positive. There was slight background staining with the antibody and weak staining of endothelial cells and fat cell membranes.

Fibrinogen. Polyclonal antisera against fibrinogen produced a strong granular reaction consistent with α-granule localization. There were somewhat fewer positive cells, 84%, and a wider range (60% to 99%) than with antibodies against the other α-granule proteins. There was strong staining of fibrin strands within tissue and vascular spaces. In addition, many fat cell membranes and endothelial cells appeared to be positive.

Staining With Antibodies to Platelet α-Granule Membrane Protein 140

Both polyclonal and monoclonal (S-12) antibodies developed against the α-granule membrane protein with a molecular weight of 140 kilodaltons produced strong staining of 95% (range, 82% to 99%) of morphologically recognizable marrow megakaryocytes (Fig 1F). The pattern of staining was very similar to that observed with the antibodies against platelet α-granule matrix proteins. Although the granules were generally distributed throughout the cytoplasm, the
MEGAKARYOCYTE ANTIGENS

Table 1. Immunohistochemical Reactions in Megakaryocytes

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Abbreviations: TSP, thrombospondin; Fib, fibrinogen; UEA, Ulex europaeus; ND, not determined.

Results expressed as positive megakaryocytes over total megakaryocytes.

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Fig 1. Biopsy specimens of normal human bone marrow embedded in plastic and tested immunohistochemically for a variety of platelet antigens. The brown peroxidase reaction is present at sites of antigen localization. Scattered eosinophils show partial persistence of endogenous peroxidase. The cells were counterstained with hematoxylin except in Fig 1D. Sections A through D show the distribution of the plasma membrane with the surrounding cells other hematopoietic cells were negative. Some marrow endothelial cells were reactive.

Other Markers

The enzyme histochemical and lectin reactions were consistent, but not specific, markers in human megakaryocytes.
Fig 2. Transmission electron micrograph of part of the cytoplasm of a human megakaryocyte illustrating the presence of GPIIbα (arrows) on the plasma membrane as revealed by an immunogold probe. The plasma membrane is quite irregular. Note that the immunogold label does not penetrate into the demarcation membrane system despite the fact that this membrane system has been shown to be open to the extracellular space when using small tracers such as tannic acid. This may be due to the larger size of the primary antibody or the secondary immunogold probe. Abbreviations: α, α-granules; m, mitochondrion; er, endoplasmic reticulum; dms, demarcation membrane system; gl, glycogen. Original magnification ×31,000.

The lectin Ulex europaeus produced strong, diffuse cytoplasmic labeling in most of the megakaryocytes, but also stained the membranes of early myeloid cells and later erythroid precursors and red cells. All endothelial cells showed strong staining. Most megakaryocytes were positive for PAS, α-naphthyl acetate esterase, and acid phosphatase. The PAS reaction was abolished by digestase; the acid phosphatase reaction was completely inhibited by tartaric acid; and the α-naphthyl acetate esterase reaction was partially inhibited by sodium fluoride. The enzyme α-naphthyl butyrate esterase was present but weak. (This observation differs from our previous observation and is a result of a change in the fixation procedure.) It was also partially inhibited by sodium fluoride. No megakaryocytes were reactive for alkaline phos-
MEGAKARYOCYTE ANTIGENS

In the present study we have shown that a variety of antibody probes can be used to identify normal megakaryocytes in intact biopsy specimens. With the exception of fibrinogen, more than 90% of mature megakaryocytes were labeled by antibodies directed against platelet α-granule constituents or platelet membrane glycoproteins. Similar results have been obtained with antibodies against fibrinogen, VIIIIR:Ag, myosin, and membrane GPIb and GPIIIa using immunofluorescence techniques.36 One should note that some morphologically normal megakaryocytes in our specimens failed to label. Rabellino et al37 observed a similar lack of labeling in some megakaryocytes. This finding suggests several possibilities, including variability in the production of platelet proteins by normal megakaryocytes, loss of these substances by degranulation, and technical variability. These possibilities should be considered when one is examining megakaryocytes in pathologic states.

Rare small mononuclear cells, probably megakaryocyte precursors, were also labeled with many of the antibody probes. Using immunofluorescence techniques and antibodies against VIIIIR:Ag, PF4, and membrane GPIb, GPIIb, and GPIIIa, Rabellino et al3 also detected label in a small number of mononuclear cells, which they regarded as early megakaryocytes. Using immunofluorescence and immunogold procedures, Breton-Gorius and associates have detected certain glycoproteins on the plasma membranes of immature megakaryocytes in both normal and abnormal human bone marrow specimens41,42 and tissue culture cells.38 Detection of such early marrow megakaryocytes in intact biopsy specimens should prove extremely useful in the diagnosis of pathologic conditions involving the megakaryocyte cell lineage.

An additional aspect of our technique that should be emphasized is the resolution allowed by the thinness of the plastic sections. For example, the granular appearance of antigen for GMP140 and the α-granule matrix proteins is consistent with their fine structural localization in α-granules.16,36 In addition, the perinuclear localization of certain antigens is consistent with the synthesis of these proteins in the rough endoplasmic reticulum (RER). These results suggest that the technique may be valuable as a preliminary step for organelle localization of antigens in studies where sampling by electron microscopy is difficult.

We did not find GPIIb-IIIa, GPIIb, or GPIIIa in monocytes or macrophages in bone marrow. This is in agreement with the findings of Clementson et al.43 The identification of GPIIIa in human osteoclasts was an unexpected finding, as was the identification of GMP140 in some endothelial cells. The finding of GPIIIa in endothelial cells confirms the observation of Thiagarajan et al.44 Neither osteoclasts nor endothelial cells showed evidence of staining for the GPIIb-IIIa complex. The presence of these proteins in endothelial cells suggests that they play a more complex role than had been previously suspected. The finding of the GPIIIa in osteoclasts is a particularly interesting observation that needs further study.

The data presented here demonstrate that mature and immature megakaryocytes can be reliably characterized in intact bone marrow biopsy specimens by use of markers for a
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