Expression of the Human Monocyte Membrane Antigen gp55 by Murine Fibroblasts After DNA-Mediated Gene Transfer

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Human DNA sequences that contain the gene encoding gp55, a cell surface glycoprotein expressed exclusively on mature human monocytes and monocyctic leukemia cells, were isolated in a mouse genetic background. DNA from mature human monocytes was cotransfected with DNA from a molecularly cloned feline sarcoma virus containing the v-fms oncogene into NIH-3T3 cells. Transformed mouse fibroblasts that expressed gp55, based on their reactivity with the MY4, B44.1, or LeuM3 monoclonal antibodies, were selected by fluorescence-activated cell sorting. Regardless of which antibody was used for selection, equivalent binding of all three antibodies was observed for positive transformants. Secondary and tertiary mouse cell transformants were obtained after additional rounds of transfection and cell sorting with the use of DNA from primary and then secondary transformants. Southern blot analysis of the cellular DNA from two independently derived tertiary subclones revealed a limited complement of human sequences, thus indicating that the gene encoding gp55 is included in fewer than 50 kilobases of human DNA. Independently derived tertiary subclones displayed concordant patterns of reactivity with 13 monocyte-specific monoclonal antibodies, thus indicating that each recognized an epitope on the product (gp55) of a single human gene. The 55-kilodalton cell surface polypeptide was specifically immunoprecipitated with a representative monoclonal antibody, 26/11, from lysates of enzymatically radiiodinated peripheral blood monocytes and tertiary transformants. We conclude that gp55 is highly immunogenic and that a large number of independently derived monoclonal antibodies specific for human monocytes react with epitopes on this one molecule.

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MANY MONOCLONAL ANTIBODIES recognize epitopes on the surface of mature cells of the monocyte-macrophage lineage and therefore have proved valuable for studying normal mononuclear phagocytes and their malignant counterparts. With few exceptions, the functions of cell surface antigens recognized by these reagents are unknown, but it is reasonable to speculate that such molecules participate in key cellular activities mediated by monocytes such as antigen processing or cell-mediated cytotoxicity.

One of the monocyte-specific monoclonal antibodies, MY4, precipitates a 55-kilodalton (kd) cell surface glycoprotein (gp55) that is present on monocytes, macrophages, and cells from patients with acute monocytic or myelomonocytic leukemia. At least two other monoclonal antibodies including Mo23 and AML-2-23 display similar patterns of reactivity and immunoprecipitate proteins with similar apparent molecular weights. None of these antibodies reacts with lymphocytes, lymphoid leukemia cells, platelets, or erythrocytes. The antigen recognized by AML-2-23 was shown to be a glycoprotein secreted by cultured human monocytes and by HL-60 cells induced to differentiate along the monocyte lineage, thus suggesting that it may represent an as-yet-uncharacterized monokine.

We recently used DNA-mediated gene transfer and cell-sorting techniques to isolate and molecularly clone the gene encoding a myeloid cell surface glycoprotein, gp150. Mouse NIH-3T3 cells were cotransfected with high-molecular-weight human cellular DNA and DNA from cloned feline sarcoma virus containing the v-fms oncogene. The v-fms-transformed cells that expressed gp150 were selected by fluorescence-activated cell sorting of transformed cell populations stained with a monoclonal antibody specific for the antigen. Antigen-expressing primary transformants isolated in this way typically contained approximately 1,500 kilobases (kb) of human DNA. Successive rounds of cotransfection and cell sorting permitted the isolation of transformants that contained limited human DNA sequences, including those that encoded gp150.

Capitalizing on these methods, we have now isolated transformed mouse cell populations that express gp55 and display concordant reactivities with each of 13 distinct monoclonal antibodies. Independently derived tertiary subclones shown to express gp55 contained a limited subset of human DNA sequences, which indicated that the epitopes recognized by our panel of antibodies were restricted to a product of a single human gene.

MATERIALS AND METHODS

Isolation of peripheral blood leukocyte fractions enriched for monocytes, lymphocytes, and granulocytes. Heparinized peripheral blood was obtained by venipuncture from normal adult volunteers with their informed consent. Mononuclear cell fractions were selected after centrifugation in Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) as described by others. To separate adherent from nonadherent cells, the mononuclear fraction was washed once in Hanks' balanced salt solution (HBSS), resuspended in RPMI 1640 supplemented with 5% fetal calf serum (FCS), and incubated at 37 °C for two hours in plastic tissue culture flasks. Adherent cells were removed from the flask by scraping after incubation for five minutes at 37 °C in 3 mL of 10 μg/mL buffered...
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Tryptsin (GIBCO, Grand Island, NY). Nonadherent cell fractions typically contained >90% lymphocytes, with the remainder consisting of monocytes as determined by microscopic examination of cytopsin slide preparations. Adherent cell fractions included 50% to 90% monocytes and contaminating lymphocytes.

Erythrocytes and granulocytes in the Ficoll-Hypaque pellet were resuspended in an equal volume of 0.4% Dextran T-500 (Sigma Chemical Co, St Louis) in HBSS and allowed to sediment for one hour on ice at unit gravity. The granulocyte-rich supernatant was withdrawn and washed twice in hypotonic lysis buffer consisting of 10 mmol/L KHCO₃ (pH 7.4), 155 mmol/L NH₄Cl, and 0.1 mmol/L EDTA to lyse residual erythrocytes. The remaining cells, typically >90% granulocytes as determined by morphological analysis of cytopsin preparations, were resuspended in RPMI 1640 medium with 5% FCS.

Transfection of high-molecular weight DNA. DNA from the cloned McDonough strain of feline sarcoma virus containing the v-fms gene was mixed with 5 µg of sheared cellular DNA (ten passages through a 25-gauge needle) and cotransfected into NIH-3T3 cells after precipitation with calcium phosphate as previously described. Foci of transformed cells in multiple flasks were counted after ten days, allowed to overlap the contact-inhibited untransformed NIH-3T3 cells, removed with trypsin, and pooled into groups for fluorescence-activated cell sorting. Control plates of NIH-3T3 cells were transfected with NIH-3T3 DNA alone and with donor DNA alone.

Monoclonal antibodies. A panel of monoclonal antibodies (Table 1) was used to detect epitopes on the surface of human monocytes. The monoclonal antibodies MY4, B44.1, and LeuM3 were used to stain transformed recipient NIH-3T3 cells by fluorescence-activated cell sorting. Transformed cell populations selected with one of the antibodies were tested for reactivity with the entire panel. Antibodies Mo1, Mo2, MY4, MY7, and MY9 were purchased from Coulter Immunology (Hialeah, FL). Antibodies LeuM3 and LeuM2 were purchased from Becton Dickinson Immunocytochemistry Systems (Mountain View, CA). Antibodies 61D3 and 63D3 were purchased from Bethesda Research Laboratories (Gaithersburg, MD). All other antibodies were gifts from the investigators who derived them. In addition, the monoclonal antibodies W6/32 (Accurate Chemical and Scientific Corp, Westbury, NY) and I2 (Coulter Immunology), specific for human HLA class I and class II molecules, respectively, were included in the test panel. Mouse myeloma protein UPC10 (IgG2a; Litton Bionetics, Kensington, MD) was used as a control for nonspecific binding.

Flow cytometry. Cell cultures were washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) and treated with 3 mL of 10 µg/mL buffers Trypsin (GIBCO) for five minutes at 37°C to release the adherent cells. The cells were collected, centrifuged, and resuspended in staining medium (Dulbecco’s modified Eagle’s medium with 5% FCS, 6 mmol/L L-glutamine, 10 mmol/L HEPES, and 2 mmol/L sodium azide). Aliquots of 50 µL containing 1 x 10⁶ cells were washed once in the same medium and incubated for 30 minutes on ice with 50 µL of medium that contained a titered excess of monoclonal antibody. After two washes, the cells were incubated for 30 minutes on ice in 50 µL of medium containing a titered excess of affinity-purified, fluorescein-conjugated goat antibody to mouse immunoglobulins G and M (Tago Diagnostics, Burlingame, CA). The cells were then washed twice and resuspended at 2 x 10⁶ cells/mL in medium containing 0.25 mmol/L propidium iodide.

Transfected cells were analyzed and sorted with an EPICS 753 flow cytometer (Coulter Corporation, Hialeah, FL) as previously described. Briefly, the brightest 2% to 4% of cells were sorted, grown to confluence, and resorted until a population of cells positive for antibody binding was isolated. The brightest 10% to 15% of fluorescence-positive cells were sorted several more times to enrich for transformed cells exhibiting the highest density of antigen expression. Subpopulations of peripheral blood leukocytes were analyzed by flow cytometry following simultaneous gating on both forward-angle and orthogonal light scatter from the cells. Fluorescence profiles were obtained from monocytes within the adherent cell fraction and from lymphocytes within the nonadherent cell fraction.

Southern blot analysis. Cellular DNAs were digested with the restriction endonucleases BamHI, EcoRI, HindIII and Sacl. Digested DNA was subjected to electrophoresis in 0.8% agarose gels and transferred to nitrocellulose. A probe specific for human repetitive DNA sequences was prepared by self-annealing of boiled, sonicated human lymphoblastoid cell DNA to a C₅ of 2 (where C₅ is the concentration of DNA in moles of nucleotide per liter and t is the time in seconds). Hybridization was performed at 60°C at a monovalent cation concentration of 1.0 mol/L, and the double-stranded DNA was recovered by fractionation on hydroxylapatite. Human repetitive DNA was labeled to a specific activity of 1 x 10⁹ dpm/µg by nick translation and used in blot hybridization under stringent annealing conditions.

Immunoprecipitation studies. Confluent flasks of transformed NIH-3T3 cells or adherent peripheral blood mononuclear cells were washed twice with cold PBS and incubated for 20 minutes at room temperature in 1 mL of PBS containing 5 mmol/L glucose, 3 µg lactoperoxidase, 5 mmol/L glucose oxidase (Sigma) and 1.0 mCi Na¹²⁵I (Amersham Corp, Arlington Heights, IL). Labeled cells were washed twice with PBS and lysed in 100 mmol/L Tris-HCl buffer (pH 7.6) containing 150 mmol/L NaCl, 20 mmol/L EDTA.
RESULTS

DNA-mediated gene transfer. High-molecular weight cellular DNA from normal human peripheral blood monocytes was cotransfected into mouse NIH-3T3 cells together with a plasmid containing the molecularly cloned v-fms gene from the McDonough strain of feline sarcoma virus. Cells expressing the v-fms–transforming gene were allowed to overgrow contact-inhibited NIH-3T3 cells, typically forming 250 to 750 discrete foci of morphologically transformed cells per 100 nanograms of cloned v-fms DNA. The transformed NIH-3T3 cells were subjected to sequential rounds of fluorescence-activated cell sorting to select those cells that bound the monoclonal antibodies MY4, B44.1, or LeuM3. The cells with the brightest fluorescence (top 2% to 4%) were sorted and grown to confluence; this procedure was repeated three to six times to isolate rare cells expressing human monocyte antigens (Fig 1). Positive cells showed equivalent binding of each of the three monoclonal antibodies, regardless of which antibody was used to select the positive cell population. As a control, one of the original populations of transfected cells was sorted with the monoclonal antibody W6/32, which recognizes human histocompatibility class I antigens. Five successive rounds of sorting produced a cell population positive for W6/32 binding that did not bind any of the other monoclonal antibodies tested.

DNA was extracted from primary transformants selected for binding of B44.1 and again cotransfected with v-fms into NIH-3T3 cells. After three successive rounds of sorting with the monoclonal antibody B44.1, fluorescence-positive secondary transformants were isolated. DNA from secondary transformants was cotransfected with v-fms in three independent tertiary rounds of transfection. Pooled cells from each tertiary transfection were sorted after staining with either MY4 or B44.1. Three independently derived fluorescence-positive tertiary cell populations were isolated and plated in soft agar. Subclones derived from the resulting colonies were tested with an extensive panel of monocyte-specific monoclonal antibodies (Table 1) and demonstrated positive binding with ten reagents in addition to MY4, B44.1, and LeuM3 but not with 13 others (Fig 2). Concordant binding of these 13 monoclonal antibodies by independently derived, tertiary NIH-3T3 subclones indicated that these antibodies recognize epitopes on the product of a single human gene.

Southern blot analysis of human DNA in transformed mouse cells. Cellular DNAs from a primary transformant and two independently derived tertiary subclones were analyzed for the presence of human repetitive DNA sequences. The DNAs were digested with BamHI, EcoRI, HindIII, and SacI and analyzed by Southern blotting. As shown in Fig 3, mouse NIH-3T3 DNA did not hybridize to the human repetitive sequence probe, whereas DNA from a primary transformant (lane 1) showed a diffuse hybridization pattern. In contrast, discrete human restriction fragments were shared by the two independently derived tertiary transfor-
mants. Analysis of the sizes of the shared human restriction fragments indicated that the gene encoding gp55 must be equal to or less than 50 kb.

Analysis of the gp55 gene product. Figure 4 demonstrates that a representative monoclonal antibody, 26if, specifically immunoprecipitated a polypeptide common to lysates of either normal human adherent peripheral blood mononuclear cells or one of the tertiary transformants. A second, weakly labeled polypeptide with a slightly lower molecular weight was also precipitated from lysates of tertiary transformants. The electrophoretic mobility of the major polypeptide was greater on nonreducing gels (63 kd, Fig 4) than on reducing gels (55 kd, analysis not shown). NIH-3T3 cells that were transformed by v-fms alone did not yield any specifically immunoprecipitated products (analysis not shown).

Monoclonal antibodies that reacted with transformed mouse cells expressing gp55 were also tested for reactivity with subpopulations of normal human peripheral blood leukocytes. Although these antibodies all recognize gp55 epitopes on transfected cells and human monocytes, their binding patterns with granulocytes and myeloid precursors are reportedly different. The six antibodies in our panel that detected gp55 on our transformed cells indicates that all of these antibodies binds an epitope of gp55.

The presence of bona fide gp55 on transformed mouse cells was verified by immunoprecipitation with monoclonal antibody 26if, which precipitated proteins of identical size from radiolabeled plasma membranes of normal adherent mononuclear cells or the tertiary transformants. The presence of an additional, less efficiently labeled band of slightly lower molecular weight in lysates of monocytes and tertiary transformants suggests that a small fraction of cell surface gp55 molecules may have altered or incomplete posttranslational modification, the exact nature of which is unknown. The electrophoretic mobility of the major precipitated polypeptide on reducing gels (55 kd) agrees with published reports of a 55-kd glycoprotein immunoprecipitated by My4, a 50- to 66-kd glycoprotein by AML-2-23, and a 55-kd glycoprotein by 26if, but conflicts with accounts of a 200-kd protein precipitated by 63D3. In our experiments, the antibodies LeuM3, 63D3 and FCM17 failed to yield specific immunoprecipitable products.

The binding specificities observed in this study are also strikingly similar to those reported for Mo2, which specifically immunoprecipitates a 55-kd glycoprotein from monocytes under reducing conditions. Additionally, Todd has shown that antibodies 26if, 77of, 60bd, and 49a, 23b, and 121nl all competitively block binding of directly fluoresceinated Mo2 to monocytes. The lack of Mo2 binding to our tertiary transformants suggests that Mo2 might recognize an epitope that results from a posttranslational modification of monocyte gp55, which is altered in mouse cell transformants. Alternatively, Mo2 and 121nl might react with the product of another gene that assembles noncovalently with gp55 at the cell surface.

Reported experience with the 13 monoclonal antibodies that detected gp55 on our transformed cells indicates that all of the reagents bind to monocytes, with variable binding to granulocytes and none to lymphocytes. Accordingly, six gp55-specific antibodies that we tested (MY4, LeuM3, 63D3, 26if, B44.1, and AML-2-23) displayed strong specific affinity for peripheral blood monocytes as well as background levels of binding to lymphocytes and uniform but weak binding to granulocytes. These antibodies were previously reported to stain myeloid leukemia cells with monocytic morphology, consistent with their specificity for monocytes in populations of circulating leukocytes.

The expression of gp55 by mature monocytes and macrophages suggests the possibility that it may mediate one of the diverse functional roles of these cells. Sorted 63D3-positive or 26if-positive cells from peripheral blood have been asso-
Fig 3. Southern blot analysis of human DNA sequences in transformed mouse cells. DNA samples were digested with the indicated enzymes before electrophoresis and transfer to nitrocellulose. Hybridization was performed by the use of radiolabeled human DNA consisting of highly reiterated sequence families. The lanes shown were composed from the same blot. DNA samples were obtained from a primary transformant (lane 1), control NIH-3T3 cells (lane 2), and two independently derived tertiary subclones (lane 3, 5, 7, 9 and lanes 4, 6, 8, 10, respectively). Major restriction fragments present in both tertiary subclones are indicated by triangles. The positions of bacteriophage lambda HindIII fragments of known length (noted in kilobase pairs) are indicated to the left of the figure.

Fig 4. Immunoprecipitation of gp55 from normal human monocytes and from mouse NIH-3T3 tertiary transformants. Cells were enzymatically surface radioiodinated with $^{125}$I, detergent lysates were precipitated with antibody, and the radiolabeled immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Lysates from normal human monocytes (lanes 1 and 2) or a tertiary NIH-3T3 subclone (lanes 3 and 4) were precipitated with monoclonal antibody 26if (lanes 1 and 3) or an isotype-matched control mouse myeloma protein (lanes 2 and 4). Positions of molecular weight standards are indicated.

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Fig 5. Flow cytometric analysis of cell surface binding of six human monocyte-specific antibodies that recognize epitopes of gp55. The fluorescence profiles of human peripheral blood cells are displayed after staining of the cells with monocyte-specific monoclonal antibodies MY4 (--), LeuM3 (---), B44.1 (------), AML-2-23 (-----), 83D3 (-----), or 26tif (-----). Fluorescence profiles are shown after staining of normal human peripheral blood monocytes (A), lymphocytes (B), or granulocytes (C). In each panel, the fluorescence profiles are compared with that obtained with control mouse myeloma protein (-----).

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