ABSTRACT

The human promyelocytic leukemic cell line HL-60 can be induced to mature monocytes and macrophages in vitro by lymphocyte-conditioned medium. We are reporting sequential changes in surface antigenic expressions, which are sensitive markers of the characteristic events in the process of cell differentiation. The promyelocyte membrane antigen, detected by a monoclonal antibody produced using HL-60 cells as an immunogen, was shown to be associated with immature myeloid cells and was used to determine HL-60 cell development. The expression of this membrane antigen, determined to have a molecular weight of 85,000 was lost early in the differentiation period. In the following stage, in which the promyelocytes developed into monocytic cells, a steady increase of cells bearing the OKM1 normal monocyte antigen was observed. When macrophages became predominant in the final culture period, the expression of the OKM1 antigen decreased. The usefulness of these differentiation antigens in studying cellular development is discussed.

INTRODUCTION

HL-60 human leukemic promyelocytes have been used as a model to study the control of myeloid cell maturation. HL-60 cells can develop into either granulocytic or monocytic cells when they are exposed to different inducing agents. Chemicals such as retinoic acid (2) or dimethyl sulfoxide (5) promote granulocytic differentiation, while phorbol diesters (14) or human lymphocyte-conditioned medium (CM) (3) induce monocytic cell maturation. We have shown that CM-induced HL-60 differentiation involves a reduction of promyelocyte proliferation and the consecutive development of mature monocytes and macrophages, as assessed by multiple markers. These include histochemical stains and the development of membrane receptors and mature cell functions. Little is known about the changes in cell surface antigens during the maturation process. We have developed a murine monoclonal antibody to detect a promyelocyte surface antigen, Pro-Im1, which is lost at an early stage of maturation to monocytic cells. The monocyes then acquired the OKM1 surface antigen, Pro-Im1, which is lost at an early stage of maturation to monocytic cells. The gain and loss of antigens have been used to quantify the mature and immature cells and to clarify the relationship between cell proliferation and differentiation.

MATERIALS AND METHODS

Induction of HL-60 Cells. For each experiment, aliquots of HL-60 promyelocytes (4) were cultured in RPMI-1640 with 15% fetal calf serum and 30% CM at 0.25 x 10^6 cells/ml (3). CM was prepared by culturing normal human PBL using the method described elsewhere (3). Pooled PBL at 1 x 10^6/ml were cultured in RPMI-1640 with 0.2% bovine serum albumin and 1% PHA. The medium from 3-day cultures was collected as CM. The maturation inducer activity present in CM for HL-60 cells has been shown to derive from stimulated T-lymphocytes (3). Control CM used in each experiment was prepared by adding PHA to PBL from a single donor that had already been incubated for 3 days or by incubating 0.2 to 1% PHA and medium lacking lymphocytes for 3 days.

Production of Monoclonal Antibody Pro-Im1 against HL-60 Cells. Each BALB/c mouse was immunized i.p. with 10 x 10^6 HL-60 cells once per week for 5 weeks, and spleenic cell hybridization was then performed (10). Cell supernatants from cultures displaying hybrid cell growth were tested for antibodies against HL-60 cells by indirect immunofluorescence. Hybrids showing HL-60 cell antibodies in supernatants after absorbing with Ficol-Hyphae-prepared PBL and monocytes were cloned by a limiting dilution method. One of the clones secreted an antibody of the IgG-2a subclass, reacting with HL-60 cells but not with peripheral blood erythrocytes, lymphocytes, platelets, granulocytes, and adherent monocytic cells, which contained mainly monocytes. This antigen is tentatively called Pro-Im1, since promyelocytes (Pro) were used as immunogen, and it is present mainly on immature (Im) cells.

Cellular Marker Determinations. The examination of cell morphology with May-Grünewald stain, the enumeration of cells with membrane complement receptors, and the determination of phagocytic cells were performed according to methods described previously (3). Surface immunofluorescent staining of cells with murine monoclonal antibodies was performed by indirect immunofluorescence with fluoresceinated goat F(ab')2 antimouse IgG (Cappel Laboratories, Cochranville, PA) and analyzed either with Leitz Ortholux microscope with phase-contrast optics and plasm illuminations or on a cytofluorograph (FACS IV; Becton-Dickinson, Mountainview, CA) as described previously (17). The OKM1 monoclonal antibody reactive with the M, 160,000 membrane protein on normal human monocytes (1, 15) was obtained from Ortho Diagnostic Systems, Inc., Raritan, NJ. Tumor-free mouse serum was used for background staining.

Iodination and Immunoprecipitation. Cell surface iodination with 125I was performed by the lactoperoxidase-catalyzed reaction as described previously (16). The cells were lysed with 0.5% NP40.

For immunoprecipitation, 50 μl of 10% (v/v) Sepharose 4B beads which had been coupled with the IgG fraction of affinity-purified (16) rabbit anti-mouse IgG at 3 mg/ml of gel were incubated with 200 μl of supernatant containing the precipitating monoclonal antibody (10 μg/ml) at room temperature for 30 min. The antibody-coated beads were washed twice with phosphate-buffered saline, followed by incubation with the radiolabeled 0.5% NP40 containing cell lysate for 1 hr. The immunoprecipitates were then washed twice with buffer containing 10 mm Tris-HCl (pH 8.0), 0.5 m NaCl and 0.1% NP40, followed by a third wash sedimenting through a 20% sucrose cushion to remove the nonspecific binding material. Supernatant obtained from mouse myeloma P3 cells secreting IgG1 immunoglobulin was used as a negative control for the antibody. The immunoprecipitates obtained were eluted by boiling.
the beads either with 4 M urea and 2% sodium dodecyl sulfate in 0.1 M Tris-HCl (pH 8.0) buffer and electrophoresed on sodium dodecyl sulfate polyacrylamide gels as described by Laemmli (11), with 1.5-mm-thick slab gels and bovine heart cytochrome c, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, β-lactalbumin, and bovine heart cytochrome c were coelectrophoresed on each SDS-PAGE gel. Gels were then stained, dried on filter paper in a vacuum, and autoradiographed on Kodak X-Omat R film.

Two-Dimensional (Isoelectric Focusing SDS-PAGE) Gel Electrophoresis. Immunoprecipitated material was analyzed on a 2-dimensional gel system, modified from the technique of O'Farrell et al. (12). Essentially, first-dimensional resolution was performed on an isoelectric focusing slab gel, constructed with ampholines, pH 3.5 to 10 (actual gradient, 3.8 to 9.0). The slabs were then sliced into strips for subsequent second-dimensional resolution with SDS-PAGE. Marker proteins of known molecular weight (β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, β-lactalbumin, and bovine heart cytochrome c) were coelectrophoresed on each SDS-PAGE gel. Gels were then stained, dried on filter paper in a vacuum, and autoradiographed on Kodak X-Omat R film.

RESULTS

HL-60 Cell-Differentiation Antigens. CM has been shown to consistently induce HL-60 promyelocytes to develop to cells expressing the properties and features of monocytes and macrophages (3). The Pro-Im1 antibody, detecting a M, 85,000 HL-60 membrane protein, and the OKM1 antibody for monocytes were used to analyze antigenic expressions during the maturation process. Chart 1 depicts the reaction patterns on the cytofluorograph using the anti-Pro-lm1 antibody prior to and after treatment of HL-60 cells with CM. It can be seen that a majority of untreated HL-60 cells expressed the Pro-Im1 antigen, with most cells showing moderate intensity of the stain, while the OKM1 antigen is not expressed. Exposure of HL-60 cells to CM resulted in an initial reduction of the proportion of cells with the Pro-Im1 antigen, followed by an increase of the OKM1-bearing cells. Averaged from 5 experiments, Table 1 shows that the percentage of HL-60 cells expressing the Pro-Im1 antigen decreased from approximately 81.3% of untreated cells to 12% after 2 days of incubation with CM and to less than 4% by Day 4. Cells expressing the OKM1 antigen increased from 0.3% of untreated HL-60 cells to a mean of 97.5% by Day 3 to Day 4. The changes in both surface antigens were observed only in cultures treated with lymphocyte conditioned medium, in line with changes of cell morphology and other membrane markers.

The expression of the OKM1 antigen was associated with other markers of cell maturation. Complement receptor-bearing cells increased from 2% initially to approximately 54% on Day 4, and phagocytic cells increased from 4% initially to 90% of the cultured cells by Day 6. After 5 days, a decrease in the proportion of cells with the OKM1 antigen coincided with a reduction of the number of monocytes and a concomitant increase of macrophages leading to termination of the cultures. These observations indicate that induction of maturation of HL-60 promyelocytes to monocyctic cells by the activity of CM was the cause of the sequential expression of the promyelocyte antigen Pro-Im1 and the monocyte antigen OKM1.

Cell Surface Antigen Recognized by anti-Pro-Im1 Antibody. To characterize the HL-60 cell surface antigen recognized by anti-Pro-Im1, HL-60 promyelocytes were radiolabeled externally with 125I by the lactoperoxidase-catalyzed reaction. The cells were lysed with detergent, and the resulting solubilized surface membrane proteins were reacted with anti-Pro-Im1. The radio-labeled immune complexes were absorbed onto Sepharose 4B beads that had been coupled with IgG fraction of affinity-purified rabbit anti-mouse IgG reagent. The immunoprecipitates were first analyzed by SDS-PAGE under reduced conditions and subsequently by 2-dimensional gel electrophoresis involving isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. The SDS-PAGE gel patterns of immunoprecipitable

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**Table 1**

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Culture period (days)</th>
<th>% of antigen-bearing cells</th>
<th>% of cell type of total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pro-Im1</td>
<td>OKM1</td>
</tr>
<tr>
<td>Untreated</td>
<td>2</td>
<td>81.3</td>
<td>1</td>
</tr>
<tr>
<td>CM-treated</td>
<td>3</td>
<td>12.5</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>97.5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2</td>
<td>55</td>
</tr>
</tbody>
</table>

* Morphological analysis showed that the remaining cells were myeloblast-like and myelocyte-like cells.

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Chart 1. Reactivity of anti-Pro-lm1 (—) and anti-OKM1 (—) for HL-60 promyelocytes, as determined by cytofluorographic analysis. A. intensity of untreated HL-60 cells reacting with the anti-Pro-lm1 monoclonal antibody. B, an increased number of monocytes reacting with anti-OKM1 after incubation of HL-60 cells with CM for 3 days. Cells treated with a control antibody were used as controls (— — — ).

Fluorescence Intensity (Linear)
materials with anti-Pro-Im1 showed a polypeptide chain with molecular weight of 85,000, whereas the negative IgG control antibody, secreted in the supernatant from myeloma P3 cells, did not precipitate any discernible components. Under nonreduced condition, anti-Pro-Im1 precipitated a similar molecule with a molecular weight of 85,000, indicating the Pro-Im1 is a surface protein of single polypeptide chain.

Antibody Pro-Im1 reacted strongly with the promyelocytes of the HL-60 line and with approximately 80% of the cells from the myelomonoblast line ML-1 (9), but it did not react with the early blast or erythroblast line K562 (9). The antibody was shown not to react with T-cell lines MOLT-3, MOLT-4, and Peer; B-cell lines Daudi and Raji; pre-B-cell lines Nalm 6, Nalm 12, and Josh; and Malemona lines Weinberg and Wolfe. It reacted with approximately 5 to 25% of the cells from the buffey coat cell preparations of normal bone marrow aspirates and not with peripheral blood leukocytes and RBC. These data would suggest that within the hematopoietic system, anti-Pro-Im1 reacts primarily with immature myeloid cells.

**DISCUSSION**

In this paper, we have reported the sequential expressions of 2 cell surface membrane antigens during the process of induced maturation of HL-60 leukemic promyelocytes to monocytic cells. A monoclonal antibody produced in our laboratory was used to identify the Pro-Im1 antigen present on untreated HL-60 cells and was lost during an early stage of interaction of HL-60 cells with the maturation inducer. The subsequent development of monocytic cells was associated with the emergence of the OKM1 normal monocyte antigen. The loss and gain of immature and mature stage cell antigens can be seen to mark the known sequence of major events in induced cell maturation (3) and has made it possible to study differentiation on the molecular level.

The loss of promyelocyte antigen was detected before the reduction of the number of promyelocytes and changes in promyelocytes as determined by morphological criteria. We have shown previously that a reduction of promyelocyte replication precedes the reduction of the number of promyelocytes in the culture and the expression of the mature cell characteristics (3). Changes in the expression of the Pro-Im1 antigen can be used as a sensitive differentiation antigen for HL-60 promyelocytes and for early events in HL-60 differentiation. Cells expressing the OKM1 monocyte antigen were also detected earlier than cells with monocyte morphology or other markers of cell maturation. As many as 100% of the cells in cultures treated with CM were found to have gained the OKM1 antigen during the induction experiments. Later in the culture period, the number of monococytes decreased as they differentiated to macrophages (15), the proportion of OKM1-bearing cells also decreased.

The loss of the promyelocyte antigen Pro-Im1, which is a Mr 85,000 single-polypeptide chain structure, and the gain of the OKM1 Mr 160,000 monococytes, which have 2 chains 155,000 and 95,000 after reduction (15), suggest that changes of membrane proteins are an integral part of the differentiation process. A similar interpretation has been proposed by others (6-8, 13).

In this paper, the differentiation of HL-60 promyelocyte cell surface markers such as Pro-Im1, which is a differentiation marker of the promyelocyte stage, as well as its relationship with differentiated cell markers, have not been described. Biochemical analysis of the pattern of glycoproteins on HL-60 cells by Gahmberg et al. (7) revealed the loss of a major Mr 160,000 surface protein during dimethyl sulfoxide-induced development to granulocytic cells. The synthesis of a new Mr 130,000 glycoprotein which can be detected on normal PBL was also observed.

**REFERENCES**


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Differentiation Antigens of HL-60 Promyelocytes during Induced Maturation

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