Induction of differentiation of human and murine myeloid leukemia cells in culture by tunicamycin

(myeloid differentiation/suspension culture/glycoproteins)

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Communicated by Paul A. Marks, October 5, 1979

ABSTRACT Tunicamycin, an antibiotic that specifically blocks the synthesis of N-acetylglucosamine–lipid intermediates and thereby prevents glycosylation of glycoproteins, induced differentiation of both human (HL-60) and murine (M1) myeloid leukemia cell lines in culture. At 0.1-1.0 μg/ml, it induced differentiation of both HL-60 and M1 cells, characterized by increase in phagocytic cells and changes to resemble mature myeloid cells. Fc receptors were also induced in M1 but not in HL-60 cells; induction of intracellular lysozyme activity was not detected in either HL-60 or M1 cells. With this concentration of tunicamycin, there was marked decrease in rate of incorporation of radioactive glucosamine into macromolecules and a decrease in the rate of DNA synthesis. These data show that glycosylation of cellular proteins has an important role in maintaining these myeloid leukemia cells in an undifferentiated state in culture. The results also indicate that induction of phagocytosis in both HL-60 and M1 myeloid leukemia cells and of Fc receptors in M1 cells does not require continued synthesis of the oligosaccharide portions of cellular proteins by the lipid-linked pathway.

A human myeloid leukemia cell line (HL-60) isolated from peripheral blood leukocytes of a patient with acute promyelocytic leukemia (1, 2), and a murine myeloid leukemia cell line (M1) established from an SL strain mouse with spontaneous leukemia (3, 4) both can be induced to differentiate into mature granulocytes and macrophages by various inducers. These cell lines provide model systems for studies on mechanisms controlling myeloid cell differentiation. The inducers for differentiation include polar compounds such as dimethyl sulfoxide for both human (2) and murine myeloid leukemia cells (5) and glucocorticoids (6) and protein factors from various sources (4, 6–9) for murine myeloid leukemia cells. Lack of information on the precise functional properties of these inducers makes it difficult to understand the molecular mechanisms involved in expression of differentiated myeloid characteristics. For example, the cellular target site of dimethyl sulfoxide and the functional properties of the protein factors are still unknown.

The studies reported here were designed to evaluate whether tunicamycin (10–12), which specifically inhibits synthesis of N-acetylglucosaminyl pyrophosphoryl polysoprenol leading to inhibition of protein glycosylation (13–20), could induce differentiation of HL-60 and M1 cells in culture. At 0.1–1.0 μg/ml of culture, tunicamycin induced differentiation of both HL-60 and M1 cells into mature myeloid cells. The differentiation of HL-60 was characterized by increased phagocytosis as well as morphological changes to the features of more mature myeloid cells. Tunicamycin did not induce Fc receptors or lysozyme activity in HL-60 cells. The differentiation of M1 cells was accompanied by marked increase in phagocytosis and Fc receptors with changes to the morphological characteristics of differentiated cells. Tunicamycin did not induce any detectable lysozyme activity in M1 cells.

MATERIALS AND METHODS

Cells and Culture Conditions. The HL-60 cell line, originally isolated from the peripheral blood of a patient with acute promyelocytic leukemia (1, 2), was provided by Robert C. Gallo (National Institutes of Health) and had been maintained in this laboratory for 6 months. Cells were cultured in RPMI-1640 medium (Flow Laboratories) supplemented with 15% heat-inactivated fetal calf serum and kanamycin at 50 μg/ml. The murine myeloid leukemia cell line M1, clone 34 (3, 4), originally isolated from an SL strain mouse with spontaneous myeloid leukemia by Yasuo Ichikawa (Kyoto University, Japan), had been maintained in this laboratory for 4 years. The culture medium for M1 cells has been described (9, 21). In all experiments, logarithmically growing cells were inoculated into fresh medium at 3 × 10⁶ cells per ml with and without tunicamycin, and the cells were cultured in a humified atmosphere of 5% CO₂ in air at 37°C. Cell growth was determined from the cell number after trypan blue-stained cells were excluded. For morphological assessment of the cells, Cytospin slide preparations of the cells were stained with the May–Grünewald–Giemsa procedure and examined under a light microscope.

Materials. Tunicamycin was isolated from Streptomyces lyosporus fuscus as described (10–12). Polystyrene latex particles (1 μm in diameter) were purchased from Dow Diagnostics. The IgG fraction against sheep erythrocytes was obtained from rabbits as described (21). Micrococcus lysodeikticus cells and hen egg white lysozyme were purchased from Sigma. D-[1-14C]Glucosamine, L-[4,5-3H]leucine, and [2,14C]uridine were obtained from New England Nuclear. D-[6-3H]Glucosamine and [2,14C]thymidine were purchased from the Radiochemical Centre, Amersham. All other reagents were of analytical grade.

Assay for Myeloid Differentiation. Phagocytic activity was measured as described by using polystyrene latex particles (9, 21). The number of phagocytic cells was determined under a light microscope, phagocytic cells being defined as those containing five or more latex particles. Cells with Fc receptors were determined by measuring rosette formation with sheep erythrocytes coated with rabbit IgG, as described (21). The number of rosette-forming cells with three or more erythrocytes per cell was determined under a light microscope. Lysozyme activity was determined in cell lysates and growth medium by a modification of the lyoplate method of Oserman and Lawler as described (21, 22) with heat-killed M. lysodeikticus as a substrate. Hen egg white lysozyme was used in each experiment as a standard, and 1 unit of lysozyme activity was defined as equivalent to 1 μg of hen egg white lysozyme.

Abbreviation: UDP-GlcNAc, UDP-N-acetylglucosamine.
Measurements of Synthesis of Glycoprotein, Protein, DNA, and RNA. HL-60 or M1 cells were cultured in the presence (0.5 μg/ml) and absence of tunicamycin, and an aliquot of culture was removed. The cells were collected by centrifugation at 800 \times g for 10 min and then were resuspended in fresh culture medium. The cell suspension was incubated with \(^{14}C\)glucosamine at 0.5 μCi/ml (55 mCi/mmol; 1 Ci = 3.7 × 10\(^{10}\) becquerels) or \(^{3}H\)glucosamine at 2.5 μCi/ml (250 mCi/mmol) at a final cell concentration of 1–1.5 × 10\(^{6}\) per ml for 90 min for assay of glycoprotein synthesis. For measurements of total protein, DNA, or RNA synthesis, the cell suspension at 2–5 × 10\(^{6}\) cells per ml was incubated with \(^{3}H\)leucine at 10 μCi/ml (51.6 mCi/mmol), \(^{14}C\)thymidine at 0.1 μCi/ml (58 mCi/mmol), or \(^{14}C\)uridine at 1 μCi/ml (57 mCi/mmol), respectively, for 60 min. Incubation was at 37°C under a humidified atmosphere of 5% CO\(_2\) in air. After completion of labeling, ice-cold phosphate-buffered saline (135 mM NaCl/2.7 mM KCl/5.3 mM Na\(_2\)HPO\(_4\)/1.45 mM KH\(_2\)PO\(_4\), pH 7.4) was added to the incubation mixture and the cells were collected by centrifugation. The cells were resuspended in phosphate buffered saline and an aliquot was removed to obtain cell numbers. Trichloroacetic acid was added to the remainder of cell suspension to make final concentration of 10%; 100 μg of bovine serum albumin was added as carrier. The precipitates were collected on Millipore filters and the radioactivity was measured in a toluene-based scintillation fluid.

RESULTS

Effects of Tunicamycin on Cell Growth and Induction of Phagocytosis. Growth of HL-60 or M1 cells was inhibited when the cells were cultured in the presence of various concentrations of tunicamycin (Fig. 1A and C); inhibition of growth of both of these cells was detected with as little as 0.1 μg/ml of tunicamycin per ml and was almost complete with 0.5 μg/ml per ml. With up to 1 μg/ml of tunicamycin per ml, < 2% of HL-60 or M1 cells were dead on the basis of trypan blue exclusion test during culture. Concentrations higher than 5 μg/ml were toxic to the cells, and the percentage of dead cells increased.

When these cells were cultured without inducers, < 2% of the HL-60 cells and < 10% of the M1 cells had phagocytic activity, but phagocytic activity increased when the cells were cultured with increasing concentrations of tunicamycin (Fig. 1B and D). Induction of phagocytosis by tunicamycin was dose-dependent, and maximal induction of phagocytosis in both HL-60 and M1 cells was observed with 0.5 μg/ml of tunicamycin per ml. After culture with 0.5 μg/ml of tunicamycin per ml, 35–40% of the HL-60 cells showed phagocytic activity, representing at least a 10-fold increase in absolute numbers of phagocytic cells. On culture with 0.5 μg/ml of tunicamycin per ml, 70% of the M1 cells had phagocytic activity within 20 hr, representing 20-fold increase in absolute numbers of phagocytic cells.

Induction of differentiation of HL-60 cells by tunicamycin at 0.25 μg/ml was inhibited by the simultaneous presence of excess amounts of UDP-N-acetylgalactosamine (UDP-GalNAc) in the medium (Fig. 2). The inhibition of differentiation was dependent on the concentration of UDP-GalNAc in the medium. In contrast, induction of differentiation of HL-60 cells by tunicamycin at 0.25 μg/ml or of M1 cells by tunicamycin at 0.5 μg/ml was not inhibited by simultaneous culture of the cells with glucosamine or GlcNAc up to 1 mg/ml (data not shown).

Induction of Fc Receptors. When HL-60 cells were cultured without inducers, 5–15% of the cells had Fc receptors. On culture with tunicamycin at 0.5 μg/ml, the percentage of cells with Fc receptors decreased to < 2% during cell differentiation.

In contrast, when M1 cells were cultured with tunicamycin at 0.25–1.0 μg/ml, 40–50% of the cells had Fc receptors after 40 hr, representing more than a 6-fold increase in absolute numbers of cells with Fc-receptors (Fig. 3).

Induction of Lysozyme Activity. HL-60 cells contained an average of 10–15 units of lysozyme activity per 5 × 10\(^{6}\) cells. When the cells were induced to differentiate by culture for 41, 87, and 160 hr with 0.5 μg/ml of tunicamycin per ml, their intracellular lysozyme activity decreased (Table 1). M1 cells did not have any detectable lysozyme activity, and induction of differentiation of M1 cells by the antibiotic was not accompanied by any increase in the activity. Moreover, there was no significant increase of lysozyme activity in the growth medium of HL-60 and no detectable activity appeared in the culture medium of M1 cells.

Morphological Changes. Examination of HL-60 cell stained by the May–Grünwald–Giemsa technique showed that most of the cells were promyelocytes, characterized by large immature nuclei with a few nucleoli and cytoplasm containing azurophilic granules (Table 2). Less than 10% of the HL-60 cells were more mature myeloid cells. Culture of HL-60 cells with tunicamycin at 0.5 μg/ml resulted in marked morphological changes, characteristic of terminal myeloid differentiation, in the majority of the cells: the cells became smaller, with eccentric pyknotic nuclei and marked reduction or loss of nucleoli, a decreased nucleus/cytoplasm ratio, and less-prominent cytoplasmic granules. Banded or segmented granulocytes were also observed.

Cytological examination showed that 95% of the untreated M1 cells were myeloblasts with round or oval nuclei, two or
three nucleoli, and narrow basophilic cytoplasm with no granules. Differentiation of M1 cells induced by tunicamycin was accompanied by a marked increase in the number of mature cells, characterized by pyknotic nuclei, loss of nucleoli, and decrease in the nucleus/cytoplasm ratio, resembling the morphological appearance characteristic of differentiating M1 cells induced by other inducers (8, 9, 21). After incubation of the cells with tunicamycin at 0.5 µg/ml for 3 days, 55% of the cells were mature cells; the percentage of mature cells increased to 95% after 5 days.

Effects of Tunicamycin on Synthesis of Glycoprotein, Protein, DNA, and RNA. The rates of synthesis of glycoprotein, total protein, DNA, and RNA were measured during culture of HL-60 cells with and without tunicamycin at 0.25 µg/ml (Fig. 4 A and B). A decrease in the rate of glycoprotein synthesis, measured by incorporation of radioactive glucosamine into trichloroacetic acid-insoluble materials was first detected 6 hr after beginning of culture with tunicamycin. At 12 hr, the rate of glycoprotein synthesis was decreased to <20% of that in cells cultured without tunicamycin. It remained low for the following 36 hr. There was less than 25% inhibition of the rate of total protein synthesis during culture of the cells with tunicamycin. Concomitant with the decrease in glycoprotein synthesis, there were decreases in the rates of DNA and RNA synthesis, which were also first detected after 6 hr of culture with tunicamycin.

In M1 cells cultured without and with tunicamycin at 0.5 µg/ml the rate of glycoprotein synthesis was decreased within 8 hr of culture of tunicamycin and continued to decrease (Fig. 4 C and D). By 28 hr, it was decreased to 33% of the control values. In contrast, total protein synthesis measured by incorporation of radioactive leucine into acid insoluble material did not change until after 48 hr of culture with tunicamycin. The decrease in rate of glycoprotein synthesis was followed by inhibition of the rates of DNA and RNA synthesis. By 28 hr, the rate of DNA synthesis decreased to 35% and RNA synthesis was decreased to 70%.

**DISCUSSION**

Tunicamycin selectively inhibits the transfer of GlcNAc from UDP-GlcNAc to dolichol phosphate, thereby preventing the formation of GlcNAc pyrophosphoryl dolichol (14–19). Because GlcNAc–lipid has been shown to be the first intermediate involved in the assembly of the core regions of the oligosaccharides of many glycoproteins (11), inhibition of synthesis of this lipid prevents synthesis of the entire oligosaccharide, leading to inhibition of protein glycosylation. Because of its rather specific mode of action, tunicamycin has been used in studies on the biological roles of the oligosaccharide portions of glycoproteins (14–19).

Tunicamycin at 0.1–1.0 µg/ml of culture medium induced functional and morphological differentiation of both HL-60 and M1 cells in culture. After 20–48 hr of culture of HL-60 and M1 cells with tunicamycin, the percentage and the absolute numbers of phagocytic cells were greatly increased, and this increase was followed by the morphological changes characteristic of maturing myeloid cells. These results show that increased percentage of cells showing phagocytosis was not due to selective killing of undifferentiated cells by the antibiotic. The induction of phagocytosis by tunicamycin was effectively inhibited by simultaneous presence of UDP-GlcNAc, indicating that the antibiotic induces phagocytosis by blocking synthesis.
of GlcNAc–lipid from UDP-GlcNAc. Concomitant with the increase in the number of the phagocytic cells, the number of M1 cells with Fc receptors increased whereas the number of HL-60 cells with Fc receptors decreased. The latter change was not unique to differentiation induced by tunicamycin: differentiation of HL-60 cells by dimethyl sulfoxide also decreased the number of cells with Fc receptors (unpublished observations).

The rate of glycoprotein synthesis in HL-60 or M1 cells measured by incorporation of radioactive glucosamine was markedly decreased early after the start of culture with tunicamycin, together with inhibition of rate of DNA synthesis; the rates of total protein and RNA synthesis did not decrease significantly. These results indicate that induction of phagocytosis in both HL-60 cells and M1 cells and Fc receptor formation in M1 cells does not require continued glycosylation of cellular glycoproteins by the lipid linked pathway.

Tunicamycin did not induce lysozyme activity in either HL-60 or M1 cells. Lysozyme activity increased in the growth medium of HL-60 cells cultured with 1.25% dimethyl sulfoxide (unpublished observation). Lysozyme activity in M1 cells increases when the cells are cultured with various other inducers (5, 21, 23). Thus, it is possible that induction of lysozyme might require glycosylation of cellular proteins.

The early reduction of rate of glycoprotein synthesis was followed or accompanied by a decrease in DNA synthesis, with concomitant inhibition of cell growth. It is possible that inhibition of DNA synthesis was the result of induction of differentiation, because differentiated myeloid cells no longer have the capacity to proliferate. Alternatively, the inhibition of glycosylation of glycoprotein carriers (18) might lead to decreased transfer of metabolites across cell membranes, resulting in inhibition of DNA synthesis, and this inhibition of DNA synthesis might lead to expression of differentiated properties of myeloid cells. The present experiments did not distinguish between these two possibilities.

Myeloid leukemia cells in culture have been studied extensively to obtain information on the molecular mechanisms regulating myeloid differentiation (1, 8), but so far the mechanisms involved in inducing differentiation of these cells in culture by various inducers for differentiation of M1 cells, especially macromolecular inducers, are suggested to be on the plasma membranes (4, 6–8, 23). The present study clearly demonstrates that glycosylation of cellular proteins plays an

<table>
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<th>Inducer</th>
<th>Myeloblasts (%)</th>
<th>Promyelocytes (%)</th>
<th>Myelocytes (%)</th>
<th>Metamyelocytes (%)</th>
<th>Neutrophils</th>
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Table 2. Differential counts of HL-60 cells after 6-day incubation with tunicamycin at 0.5 μg/ml.


**Fig. 4.** Effects of tunicamycin on incorporation of radioactive glucosamine, leucine, thymidine and uridine. Cells were cultured with (—) or without (—···) tunicamycin. At the time indicated, an aliquot was removed for measurement of incorporation of radioactive precursors into trichloroacetic acid-insoluble material. (A and B) HL-60 cells; tunicamycin at 0.25 μg/ml. (A) [3H]Glucosamine (●, ○) and [3H]leucine (▲, △); (B) [14C]thymidine (■, □) and [14C]uridine (▲, △). (C and D) M1 cells; tunicamycin at 0.5 μg/ml. (C) [14C]Glucosamine (●, ○) and [3H]leucine (▲, △); (D) [14C]thymidine (■, □) and [14C]uridine (▲, △).
important role in maintaining these myeloid leukemic cells in a transformed state. There are several reports of changes in the glycosylation of glycoproteins on the plasma membrane before and after transformation of cells (24), but their significance in the process of transformation requires further study. Tunicamycin should be useful for further studies on the molecular mechanisms involved in differentiation of transformed myeloid leukemic cells in culture.

We are grateful to Dr. Robert C. Gallo for kindly providing the HL-60 cell line and to Dr. Yasuo Ichikawa for the M1 cell line. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.