Modulation of Retinoic Acid-induced Differentiation of Human Leukemia (HL-60) Cells by Serum Factors and Sphinganine

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INTRODUCTION

Recent approaches to therapy for various types of cancer have focused on drugs that induce the maturation of the aberrant, differentiation-resistant cells causing the disease (1, 2). One compound currently being tested clinically for its ability to work in this manner is retinoic acid (3). RA is a potent inducer of differentiation in numerous established myeloid cell lines (4, 5) as well as primary cultures of cells isolated from patients with promyelocytic leukemia (6). Clinical trials and case reports thus far show variable success of therapy with RA (7–10). Recent reports indicate that differentiation induced by RA may be facilitated by concurrent treatment with other agents such as DNA synthesis inhibitors, hormones, and other biological modifiers (10–14).

The biochemical mechanism by which RA causes differentiation remains unclear. However, some evidence suggests that the calcium- and phospholipid-dependent protein kinase (protein kinase C) is involved in the response of cells to this drug (15–17). This kinase regulates some serum factor-induced processes (18, 19) as well as the differentiation of myeloid cells induced by phorbol esters (20). In this study, the human leukemia cell line HL-60 was used to investigate the effects of different sera as well as inhibition of protein kinase C on differentiation. HL-60 is a bipotent cell line (21) isolated from a patient with acute myelocytic leukemia (22, 23). These cells undergo granulocytic differentiation when treated with RA (24) and have been used extensively to characterize hematopoietic cell differentiation (24).

The ability of RA to induce differentiation of HL-60 cells was found to vary depending on the serum in which the cells were grown. The possible role of protein kinase C in mediating these serum effects was investigated with the long-chain base sphinganine. Long-chain bases are among the most potent inhibitors of this enzyme reported to date (25) and their effectiveness has been established in numerous cellular systems (26–37) including HL-60 cells (38). The presence of sphinganine was found to increase the percentage of RA-treated HL-60 cells that exhibited mature morphology as well as the functional competence of these cells.

MATERIALS AND METHODS

Materials. Defined bovine calf serum (HyClone) was obtained from HyClone Laboratories (Logan, UT). Wright-Giemsa stain (Camco Quik II) and RPMI 1640 medium were from American Scientific Products (Atlantic, GA). Fetal calf serum was from Gibco (Grand Island, NY). erythro-Dihydrosphingosine (sphinganine), PMA, NBT, and cytochrome c (type VI from horse heart), insulin, transferrin, all-trans-RA, and human serum (type AB from male donors) were purchased from Sigma Chemical Company (St. Louis, MO). Fatty acid-free BSA was from Boehringer Mannheim (Indianapolis, IN). [3H]RA was from DuPont NEN (Wilmington, DE).

Cell Culture. HL-60 cells (ATCC CCL240; obtained from the American Type Culture Collection) were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and either no serum (free) or 10% serum (from calf, fetal calf, or human), at 37°C in an atmosphere of 5% CO2. When serum-free medium was used, 5 µg/ml insulin and 5 µg/ml transferrin were included. The cells were routinely passaged every 3 or 4 days and seeded at a density of 2.5 x 10⁵ cells/ml. Cells used for differentiation experiments were between passages 19 and 35. The cell numbers were measured with a Coulter counter. Viability was assessed by exclusion of 0.2% trypan blue.

Differentiation of HL-60 Cells. Cells (5 x 10⁴/well) were plated in 12-well culture dishes in 2 ml of medium containing the specified serum and were treated with 1 µM RA (added from a 10 mM stock solution in dimethyl sulfoxide) or the appropriate concentration of sphinganine (prepared as the 1:1 molar complex with fatty acid-free BSA). Sphinganine was then added every 24 h for 4 days.

Morphology was judged on slides prepared with a Shandon Southern Cytospin and stained with Wright-Giemsa stain (Camco Quik II). The percentage of cells capable of reducing NBT was determined by counting the number which contained precipitated formazan after a 30-min 37°C incubation with an equal volume of NBT (1 mg/ml in 140 mM NaCl, 9.2 mM Na₂HPO₄, 1.3 mM NaH₂PO₄, pH 7.4) and 200 nm PMA.

Respiratory Burst Measurements. After the indicated treatments, cells were collected by centrifugation and washed with modified PBS (0.1 g/liter CaCl₂, 0.2 g/liter KCl, 0.2 g/liter KH₂PO₄, 0.1 g/liter MgCl₂·6 H₂O, 8.0 g/liter NaCl, and 2.16 g/liter Na₂HPO₄·7 H₂O) containing glucose (1 g/liter). The cells were then resuspended in modified PBS plus glucose at a density of 1 x 10⁹ cells/ml. The respiratory burst of each group was quantitated by measuring the reduction of cytochrome c (25 mg/ml), as reflected by the change in absorbance at 549 nm minus 540 nm measured with a SLM Aminco DW-2000 spectrophotometer in the dual wavelength mode. Each measurement was done in triplicate.
urement was done with $2.4 \times 10^6$ cells in a total volume of 2.4 ml. The respiratory burst was initiated with either fMLP (1 $\mu$m) or PMA (100 nM).

Uptake of $^{3}H$RA by HL-60 Cells. $^{3}H$RA (1 $\mu$m; specific activity, 20 mCi/μmol) was added to HL-60 cells (2.5 $\times 10^5$ cells/ml) adapted to growth in the indicated serum. At each time point, 0.5 ml of cells was removed and collected on a Whatman glass fiber filter (Type GF/C) and the excess radiolabeled RA was removed by several washes with PBS. The filters were then dried and radioactivity incorporated by the binding of the radiolabel to the filter was determined with 0.5 ml of medium (supplemented with the various sera) containing 1 $\mu$m $^{3}H$RA in the absence of cells. This quantity was then subtracted from that determined in the presence of cells to give specific binding.

RESULTS

Effect of Different Sera on the RA-induced Differentiation of HL-60 Cells. The maturation of HL-60 cells grown in medium supplemented with sera from different sources was assessed by morphology and NBT reduction (Table 1). Cells grown in serum-free medium showed greater maturation by morphology (only 10% remained promyelocytic) as well as functional (82% were NBT*) criteria after 4 days in RA. Serum had an inhibitory effect on differentiation, which varied with the source. FCS allowed the most differentiation (i.e., only 25% remained promyelocytic and 58% were NBT*), while in calf serum (HyClone) 43% were promyelocytic and 43% were NBT* and in human serum 44% were promyelocytic and only 37% were NBT*. As is typically seen (4), a small percentage of the cells in each serum group exhibit a more differentiated phenotype without addition of RA.

Effect of Different Sera on the Uptake of $^{3}H$RA by HL-60 Cells. Although this is the first documentation of such serum effects for HL-60 cells treated with RA, a similar observation has been made with F9 embryonal carcinoma cells grown in serum-containing versus serum-free media (39). These investigators suggested that serum albumin, which is known to bind RA (40), was responsible for the decreased differentiation. To test this possibility, the uptake of $^{3}H$RA by HL-60 cells was quantitated in serum-free medium, 10% FCS, 10% calf serum, or 10% human serum. The results, shown in Fig. 1, demonstrate that serum decreased the cellular uptake of RA by 40 to 80%, depending on the source. To determine if albumin could be responsible for this decrease, uptake was also measured in serum-free medium supplemented with 100 $\mu$m bovine albumin, which decreased $^{3}H$RA uptake by approximately 70% (data not shown).

Effect of Sphinganine on the RA-induced Differentiation of HL-60 Cells Grown in Different Sera. To determine if other serum factors, such as those that act via protein kinase C, are involved in the observed serum effects, differentiation was assessed in the presence of the long-chain base sphinganine, which is a potent inhibitor of this enzyme. Because it is a normal intermediate of sphingolipid biosynthesis, exogenously supplied sphinganine is metabolized by cells to complex sphingolipids that do not inhibit protein kinase C (27, 38). Therefore, to maintain cellular levels during the 4-day differentiation period, sphinganine was supplied daily. The cytotoxicity of the long-chain base was much greater in serum-free medium and varied somewhat with the different sera; hence, each group was given the highest possible concentration of sphinganine which did not decrease the viability below 70% (after 4 days).

As illustrated in Fig. 2, sphinganine had little effect on the RA-induced differentiation of HL-60 cells in serum-free medium; however, it increased the percentage of mature cells in all of the serum-containing media. The cells in FCS treated with RA and sphinganine differentiated to levels close to those seen with RA alone in serum-free medium (as judged by morphology). Likewise, the percentage of mature cells after RA and sphinganine treatment in calf serum was similar to those obtained with RA in FCS. The percentage of NBT-positive cells

<table>
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<th>Blast*</th>
<th>Pro</th>
<th>Myel</th>
<th>Meta</th>
<th>Band</th>
<th>Seg</th>
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* Blast myeloblast; Pro, promyelocyte; Myelo, myelocyte; Meta, metamyelocyte; Band, banded neutrophil; Seg, segmented neutrophil.
was also increased with human serum, although to a lesser extent. Treatment with sphinganine alone (i.e., without RA; data not shown) slightly increased the percentage of differentiated cells (usually between 10 and 20%).

Effect of Sphinganine on the Respiratory Burst of HL-60 Cells Differentiated by RA. During granulocytic maturation, cells normally acquire the ability to produce superoxide when activated by physiological stimuli such as the chemotactic peptide fMLP. Whereas phorbol ester-stimulated superoxide production requires only a functional NADPH oxidase, the response to fMLP entails the presence of the fMLP receptor and the coupling mechanisms to the oxidase. HL-60 cells induced to differentiate by dimethyl sulfoxide and dimethyl formamide appear to develop all these components and produce superoxide when stimulated by fMLP (41, 42). However, cells treated with RA show little activation in response to this peptide (42), suggesting that maturation induced by this agent is incomplete. To determine whether sphinganine treatment affects the ability of RA-differentiated HL-60 cells to be activated by fMLP, the rate of superoxide production in response to both this peptide and PMA was quantitated.

For these experiments, the cells were given sphinganine only for the first 2 days, because activation of the NADPH oxidase by either phorbol esters or fMLP is a protein kinase C-dependent event of which sphinganine is a potent inhibitor (27). Treatment for only 2 days allows subsequent metabolism of the long-chain base (38) so that protein kinase C is not inhibited by day 4, when this process was measured. When conducted in this way, the initial rate of superoxide production in response to fMLP was increased in cells treated with RA alone (2.2-fold) relative to the undifferentiated cells (Fig. 3). The rate of the fMLP-initiated respiratory burst was further increased (4.1-fold, relative to control) in HL-60 cells treated with both RA and sphinganine. Some variability in the fMLP-stimulated rate of superoxide production was seen between different groups of cells; averages of results from five separate experiments show that this rate increased 75 ± 30% for RA alone and 299 ± 100% for RA plus sphinganine.

The rate of superoxide production in response to PMA (Fig. 3) was increased in cells differentiated with RA (2.1-fold) or RA and sphinganine (2.3-fold), relative to the control rate, in amounts consistent with the increases in NBT reduction (Fig. 2). The average increase (calculated from five experiments) was 82 ± 24% for RA-treated cells and 123 ± 60% for RA plus sphinganine; hence, the effects of sphinganine are seen with both agonists but are most evident with fMLP.

DISCUSSION

In this study, the differentiation of HL-60 cells induced by RA was shown to be sensitive to the medium in which the cells were maintained. The presence of serum decreased the percentage of cells that were morphologically mature and capable of reducing NBT. Uozumi et al. (43) have also reported that the presence of serum or BSA decreases the differentiation of HL-60 cells induced by RA as well as the cellular uptake of [3H]-RA. The reduced uptake of RA by cells in serum-containing medium may be partially responsible for the lesser differentiation. However, this does not fully account for the variation in differentiation between the serum groups, because the level of RA uptake (serum-free > human serum > calf serum = FCS) does not correspond to the degree to which either morphological or functional maturation was acquired (serum-free > FCS > calf serum = human serum). Other studies, including one in which uptake of RA is shown not to be necessary for induction of differentiation (44) and one in which the increase in transglutaminase expression caused by RA in HL-60 cells was unaffected by albumin (45), suggest that many factors other than the level of RA uptake regulate the cellular effects of this compound. Another factor to be considered is the growth rate of the cells in the various sera, since it has been suggested that this parameter influences differentiation (11). The cells that differentiated more (those grown in serum-free medium) grew somewhat more slowly than those in the serum-supplemented medium, consistent with the hypothesis that serum components influenced both of these processes.

Since sera from different sources vary significantly in many components including growth and differentiation factors (46), these differences in maturation may arise from other signals that affect this process. In fact, the presence of serum has been shown to inhibit the differentiation of HL-60 cells caused by phorbol esters (compared to that in serum-free medium), with calf serum being more inhibitory than fetal calf serum (47).

Since protein kinase C is believed to be the primary regulator of phorbol ester-induced differentiation (20) and participates in the regulation of other serum factor-induced processes (18, 19), a serum component could be modulating differentiation through the activity of this enzyme. To probe the role of protein kinase C in these serum effects on RA-induced differentiation of HL-60 cells, the long-chain base sphinganine, a potent inhibitor of this enzyme in vitro (26) and in intact cells (26–38), was used. Earlier studies using sphinganine showed that the phorbol ester-induced differentiation of HL-60 cells into monocyte/macrophage-like cells is inhibited by this long-chain base (38, 48). Conversely, the maturation of these cells along the same pathway caused by 1α,25-dihydroxyvitamin D3, for which a regulatory role for this enzyme is unclear, is not blocked by this long-chain base (48). While these results indicate that
the involvement of protein kinase C differs in these two pathways, they also demonstrate that treatment of HL-60 cells with sphinganine does not nonspecifically block maturation or cause cell death.

The percentage of cells displaying the mature morphology after 4 days of RA treatment was increased by sphinganine in the groups grown in serum-supplemented medium, demonstrating that the regulation of this differentiation program is different than that involved in either phorbol ester- or 1α,25-dihydroxyvitamin D3-induced differentiation. Furthermore, it suggests that protein kinase C participates in the serum-dependent inhibition of RA-induced development. Whether this enzyme regulates the differentiation stimulated by RA is unknown. As with many other compounds that cause maturation of these cells, the activity of protein kinase C increases as cells mature in response to RA (15). This agent also increases the expression of a phosphoprotein believed to be a substrate of this enzyme in these cells (16). While these findings suggest that this vitamin A analogue stimulates protein kinase C, RA inhibition of this enzyme has also been demonstrated (17).

As expected from the increased percentage of NBT+ cells, the production of Superoxide in response to PMA stimulation is greater when stimulated by fMLP than by PMA, it appears that the long-chain base aids the HL-60 cells to differentiate more completely, so that they become responsive to physiological agonists. A similar increased responsiveness to fMLP has between reported when HL-60 cells were induced to differentiate with the combination of RA and a T-cell-derived lymphokine differentiation-inducing activity (13).

The success of RA therapy is usually predicted by differentiating cells isolated from a patient’s bone marrow and cultured in medium supplemented with FCS with RA (49, 50). While this method has proven effective in some studies (9), the decrease in differentiation in human serum relative to that in FCS observed here suggests these conditions may not be comparable. The finding that treatment of HL-60 cells with sphinganine, an inhibitor of protein kinase C, increases the percentage of cells differentiated by RA as well as the ability of the cells to be activated by a physiological stimulus suggests that RA therapy may be improved by concurrent treatment with a modulator of protein kinase C activity.

ACKNOWLEDGMENTS

We thank Anni Sereni for assisting in the initial experiments of this study and Georgiana Guzman for maintaining the HL-60 cells.

REFERENCES

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