ABSTRACT

We have studied the effects of lipoxygenase inhibition and metabolite addition on HL-60 cells induced to differentiate. When HL-60 are induced by dimethyl sulfoxide (DMSO) in the presence of an inhibitor of lipoxygenase, caffeic acid, there is a marked change from the expected phenotype of mature granulocytes to a population composed predominantly of mature monocytes. (DMSO alone: 54% granulocytes, 10% monocytes; DMSO + caffeic acid: 23% granulocytes, 53% monocytes.) Addition of leukotriene D4 to DMSO-induced, caffeic acid-inhibited cultures resulted in a dose-dependent recovery of the granulocyte phenotype. Addition of lipoxygenase inhibitors to phorbol ester-treated HL-60 cells did not alter the expected monocytic differentiation. These results support a role for leukotriene D4 in the regulation of granulocyte differentiation of HL-60 cells induced with DMSO.

INTRODUCTION

An intact lipoxygenase pathway of metabolism is necessary for granulocyte-monocyte progenitor cell proliferation and differentiation (1–3). Growth of cells of myeloid leukemia origin have also been shown to be dependent on an intact lipoxygenase metabolic pathway (4–6). Growth of the human myeloid leukemia cell line, HL-60, is inhibited in a dose-dependent manner by lipoxygenase inhibitors (4). Anthes et al. (7) have shown that an 8-fold increase in leukotriene synthesis occurs when HL-60 is induced to differentiate by DMSO, and Goerg et al. (8) reported a rapid upregulation of 5-lipoxygenase metabolites when HL-60 is induced to differentiate. Ziboh et al. (9) have recently demonstrated that when HL-60 is induced to granulocytic differentiation there is a marked increase in LTD4 synthesis, and when induced to monocytic differentiation there is a marked decrease in the synthesis of LTC4 and LTD4, with a significant increase in LTE5. To further examine the role of lipoxygenase metabolites in myeloid differentiation, we have examined the effects of lipoxygenase inhibitors and metabolites on HL-60 cells induced to granulocytic or monocytic differentiation.

MATERIALS AND METHODS

Lipoxygenase Inhibitors and Metabolites. Inhibitors of lipoxygenase used in this study were piriprost (Upjohn Co., Kalamazoo, MI) (10) and caffeic acid (Sigma Chemical Co., St. Louis, MO) (11). Caffeic acid was prepared in ethanol and concentrated so as not to exceed a final ethanol concentration of 2%, a concentration found to have no effect on cell growth. Piriprost was dissolved in double-distilled H2O. Leukotrienes were provided by the Upjohn Co. and were prepared in ethanol.

Leukemic Cells. HL-60 (12, 13) (American Type Culture Collection, Rockville, MD) was maintained in liquid culture in RPMI-1640 medium ( Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). For differentiation studies, cells from log phase cultures were diluted to 2 x 10^5 cells/ml and cultured for 5 days. Inducing agents, lipoxygenase inhibitors, and leukotrienes were added at the time of plating.

Differentiation Induction. Cells were induced to granulocytic differentiation by the addition of DMSO (Sigma) to a final concentration of 1.3% (13). Monocytic differentiation was induced by addition of 10^-8 M TPA (Sigma) (14).

Cell Characterization. Following incubation, 10^6 cells were placed on glass slides utilizing a Shandon cytocentrifuge (Shandon Southern, Sewickley, PA). Slides were stained with Wrights-Giemsa stain for morphology, â-NAE for monocytes, and ACE for granulocytes; all stains were obtained from Sigma.

Adherence. Cells (10^5/ml) were incubated in the presence of appropriate inducers, inhibitors, and leukotrienes. Samples were set up in duplicate pairs. At the end of a 6-day culture period, one plate from each pair was gently aspirated and the nonadherent cells were counted. The other member of each pair was vigorously scraped to remove adherent cells and the total cells were counted. The percentage of adherent cells was calculated from the difference in cell count between the two plates divided by the total count.

RESULTS

Effects of Lipoxygenase Inhibitors and Differentiation Inducers on HL-60 Growth. A concentration of caffeic acid known to have minimal effect on HL-60 growth was selected. Caffeic acid at 17.5 μM inhibited HL-60 growth after 5 days of culture by approximately 25% (Table 1), DMSO alone inhibited by about 40%, and TPA inhibited by 50%. The combination of caffeic acid and DMSO caused additive inhibition (60%), whereas the combination of caffeic acid and TPA was no more inhibitory than TPA alone.

Effects of Lipoxygenase Pathway Manipulation on HL-60 Differentiation. Addition of caffeic acid alone had no effect on HL-60 differentiation as determined by morphological examination of Wrights-Giemsa-stained cytospins. As expected, DMSO treatment resulted in granulocytic differentiation, whereas TPA induced predominantly mature monocytes and macrophage differentiation. Caffeic acid alone did not appear to induce differentiation (Fig. 1).

When caffeic acid was added to HL-60 cell cultures at the time of induction with DMSO, the cells that formed after 5 days of incubation exhibited monocytic morphology on Wrights-Giemsa staining rather than the expected granulocytic morphology, with an increase in monocytes from 10 to 53% (P < 0.001) (Fig. 1). When LTD4 (10^-9 M) was added in addition to the DMSO and caffeic acid, there was less of a shift from granulocytes to monocytes, with only 17% monocytes rather than the 53% with DMSO and caffeic acid (P < 0.001) (Fig. 1). Similar results were obtained using piriprost as an alternate inducer.
lipoxigenase inhibitor in place of caffeic acid (Fig. 1), with an increase in monocytes from 3% to 44% (P < 0.02).

The addition of caffeic acid to TPA-induced HL-60 cells resulted in no change in the expected monocytic morphology (data not shown). In a separate set of experiments, precursors of LTD4, LTA4, and LTC4 were also able to recover granulocyte content, whereas LTB4 at similar concentrations did not (Table 2). The shift back from monocytic to granulocytic phenotype by addition of LTD4 was concentration dependent, with maximal effect at 10^{-5} M LTD4 (Fig. 2).

When retinoic acid was used rather than DMSO to induce granulocyte differentiation, addition of lipoxigenase inhibitors resulted in marked cytotoxicity with only 25% viable by 5 days and bizarre morphology of the remaining cells.

We examined the ability of LTD4 to affect the monocytic differentiation induced by TPA. No effect was seen when LTD4 was added to TPA-induced HL-60 cells (data not shown).

### Table 1: Effect of lipoxigenase pathway manipulation on HL-60 growth

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cell count at 5 days (x 10^6)</th>
<th>% viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>28 ± 3.0</td>
<td>93</td>
</tr>
<tr>
<td>Caffeic acid (17.5 μM)</td>
<td>21 ± 2.3</td>
<td>86</td>
</tr>
<tr>
<td>DMSO (1.3%)</td>
<td>17 ± 3.1</td>
<td>80</td>
</tr>
<tr>
<td>DMSO + caffeic acid</td>
<td>11 ± 0.9</td>
<td>76</td>
</tr>
<tr>
<td>DMSO + caffeic acid + LTD4 (5 × 10^{-4} M)</td>
<td>12 ± 1.1</td>
<td>75</td>
</tr>
</tbody>
</table>

- * Cells are plated at a starting concentration of 1 x 10^5 cells/ml. All additions are made directly to plates at the time of plating.
- Viability determined as percentage of cells excluding trypan blue.
- Means ± 1 SD of 3 independent experiments.

### Table 2: Effect of lipoxigenase metabolites on HL-60 differentiation

<table>
<thead>
<tr>
<th></th>
<th>% bands and mature granulocytes</th>
<th>% monocytes and macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>2.5 ± 2.8</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>DMSO (13%)</td>
<td>63.0 ± 15.4</td>
<td>1.5 ± 1.3</td>
</tr>
<tr>
<td>DMSO + caffeic acid (17.5 μM)</td>
<td>22.2 ± 7.2</td>
<td>52.3 ± 24.4</td>
</tr>
<tr>
<td>DMSO + caffeic acid + 10^{-4} M LTD4</td>
<td>56.7 ± 10.5</td>
<td>11.0 ± 9.6</td>
</tr>
<tr>
<td>DMSO + caffeic acid + 10^{-4} M LTA4</td>
<td>24.3 ± 18.3</td>
<td>58.3 ± 26.8</td>
</tr>
<tr>
<td>DMSO + caffeic acid + 10^{-4} M LTC4</td>
<td>50.8 ± 11.1</td>
<td>12.3 ± 7.0</td>
</tr>
<tr>
<td>DMSO + caffeic acid + 10^{-4} M LTD4</td>
<td>69.2 ± 10.3</td>
<td>14.3 ± 9.8</td>
</tr>
</tbody>
</table>

- Means of 3 individual experiments ± SD.

### DISCUSSION

Changes in their arachidonic acid metabolic products, particularly the leukotrienes, are among the myriad of metabolic and molecular changes that occur as HL-60, a human myeloid...
leukemic cell line, differentiates from proliferating blasts and promyelocytes to cells that express phenotypic and functional characteristic of mature granulocytes and monocytes (7-9). Goerig et al. (8) reported increases in lipoxygenase metabolites within minutes of differentiation induction. Ziboh et al. (9) demonstrated marked differences in the leukotriene profiles of uninduced HL-60 cells and those induced to granulocytic differentiation by DMSO or monocytic differentiation by TPA. These differences may reflect another characteristic of the mature cell function or may perhaps be essential intermediates in the differentiation process. In the current studies, we have attempted to clarify the role of the observed changes in lipoxygenase metabolites by manipulating the pathway during the induction period. Inhibition of lipoxygenase metabolism during TPA-induced monocytic differentiation did not affect the ability of HL-60 cells to differentiate to monocytes. These results suggest that lipoxygenase metabolites are not essential for monocyte differentiation and that the reported increases in LTB4 levels are consistent with the known ability of mature monocytes to produce this metabolite (15).

In contrast, when these cells are induced with DMSO in the presence of a lipoxygenase inhibitor, there is a change from the expected granulocytic phenotype to a predominance of cells that are monocytic by morphological, histochemical, and functional criteria. This shift does not represent a survival advantage of monocytes over granulocytes because there is a net 300% increase in the absolute number of monocytes in the DMSO plus caffeic acid group as compared to the DMSO alone. These data suggest that an intact lipoygenase pathway is essential for the differentiation of HL-60 cells to granulocytes when induced by DMSO. Peptidoleukotrienes, particularly LTD4, were increased in the previous reports on lipoxygenase metabolism in DMSO-induced HL-60 cells (6, 8). In the current studies we have shown that the addition of LTD4 or its precursors to the cells exposed to both DMSO and a lipoxygenase inhibitor resulted in a population that was predominantly granulocytic. In contrast, LTB4 had no affect under the same conditions.

These studies suggest that differentiation of HL-60 involves activation of multiple pathways. If the events that occur in differentiation include lipoxygenation of arachidonic acid to LTD4, the cells express a granulocytic phenotype. If the same events are triggered with the exception of the lipoxygenase pathway, they become monocytes. A common pathway that is activated in both DMSO- and TPA-induced HL-60 cells is the activation of PKC (16, 17). DMSO may induce PKC activity indirectly by stimulating the hydrolysis of polyphosphoinositide (phosphatidylinositol 4,5-bisphosphate), which releases diacylglycerol, which can activate PKC (17, 18). TPA is reported to either work directly on PKC, substituting for diacylglycerol, or alternatively through phospholipase C (18, 19). It appears that DMSO must stimulate additional events that involve the synthesis of LTD4 and perhaps other lipoxygenase metabolites. LTD4 has been shown to have several effects on DMSO-induced HL-60s that may play a role in differentiation. These effects include increased cytoplasmic alkalization and increases in cytosolic calcium levels (20, 21). Although LTD4 receptors have not been reported on HL-60 cells, it is possible that DMSO, which changes membrane permeability, may facilitate entry of LTD4 into the cells. Preliminary experiments with radiolabeled LTD4 support this mechanism (data not shown). Bonser et al. (22) reported that DMSO-induced HL-60 expresses phospholipase activity that releases arachidonic acid. In those studies, lipoxygenase activity was not studied; however, inhibition of cyclooxygenase by indomethacin had no effect on differentiation. In contrast, inhibition of lipoxygenase does effect differentiation. Addition of LTD4 alone in the absence of DMSO or in TPA-induced populations is not sufficient to cause granulocytic differentiation, and therefore, additional events must be triggered by DMSO that are not initiated by TPA. Other enzyme systems in addition to lipoxygenase must be stimulated. Included among these would be the previously reported activation of a phospholipid and calcium-dependent protein kinase (23) and activation of an Na+/H+ exchanger (24). The cell death seen with retinoic acid in combination with lipoxygenase inhibitors may represent toxicity of the combination. Alternatively, it may indicate that a different sequence of events is involved when HL-60 is induced to granulocyte differentiation by retinoic acid that does not include the necessary signals for monocytic differentiation. Distinct differences have been reported in the pathways to granulocytic differentiation induced by DMSO and retinoic acid (25). Of interest is a recent report (26) that acicpin, a glutamine antagonist, induces monocytic differentiation of HL-60. Among the many actions of acicpin is inhibition of γ-glutamyl transferase, the enzyme necessary for the synthesis of LTD4 from LTC4.

If the availability of LTD4 in addition to protein kinase C activation were all that is necessary for granulocytic differentiation, one would expect that the addition of LTD4 to TPA-induced cultures would be sufficient to induce granulocytic differentiation. We were unable to alter the TPA-induced monocytic differentiation of HL-60 by addition of LTD4. One possibility for this inability to affect differentiation is that DMSO induces other events not involving the lipoxygenase pathway that are essential for granulocytic differentiation. Alternatively, it is possible that the lack of altered differentiation reflects the inability of LTD4 to get into cells and that entry into cells may be facilitated by DMSO.

In summary, an active lipoxygenase pathway and LTD4 synthesis are necessary but not sufficient for the DMSO-induced granulocytic differentiation of HL-60 cells. Differentiation of these cells to granulocytic and monocytic phenotypes share some common pathways, and these may be combined with other unique pathways that lead to the final differentiated process. Recent studies demonstrate that the final phenotypic determination may occur late in the differentiation pathway (27). The current findings have been obtained with a single cell line and inducing agent. However, they may provide insight into the processes that occur in determining the differentiated expression of a normal progenitor with multiple potentials, such as the granulocyte-monocyte colony-forming unit.

REFERENCES


7259


Regulation of HL-60 Differentiation by Lipoxygenase Pathway Metabolites in Vitro

Alan M. Miller, Steven M. Kobb and Raquel McTiernan


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/22/7257

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.