Selective Destruction of Human Leukemic Cells by Alkyl-lysophospholipids


ABSTRACT

The effect of synthetic lysophospholipid analogs on the proliferation of human leukemic cells was studied in vitro. Cells from patients with various forms of leukemia were cultivated in the presence of nine different but chemically closely related lysophospholipids. Alkyl-lysophospholipid analogs were found to cause progressive killing of the leukemic cells over a period of 72 to 96 hr. This cytotoxic effect was dependent on the presence of the ether-linked aliphatic side chain in sn-1 of the glycerol molecule as well as on substitution of the hydroxy group in the sn-2 position. All lysophospholipid analogs that have an acyl bond in sn-1 were ineffective. The viability and replication of normal human cells either were not affected or were affected to a much lesser extent by the same alkyl-lysophospholipids.

Tracer studies with labeled compounds indicate that surface activity is not the cause of the observed cytotoxicity but that the metabolic fate of the substances has to be considered. The data suggest that the absence or relative lack of an alkyl cleavage enzyme in leukemic cells is primarily responsible for the observed cytotoxicity. After the lysophospholipids have been adsorbed from lipoproteins of the serum onto the cellular membrane, the stable alkyl-lysophospholipid analogs interfere apparently with the phospholipid metabolism of the neoplastic cells.

INTRODUCTION

In a study of possible effector mechanisms by which natural lysophosphatidylcholine potentiates the immunological reactivity of the organism (13, 14, 15, 23), it was found that certain synthetic LPA have also a strong antitumor effect on syngeneic transplanted tumors in mice and rats (15, 24).

The concentrations used (10 µg/mouse/day) were considered too low to be responsible for a direct cytotoxic action on the tumor cells in vivo (1, 12). Although the generation of tumor-destructive macrophages seems to play a major role in the antitumor effect of these lysophospholipids in vivo, tumor growth inhibition studies in vitro indicated that a slight but significant direct cytotoxic effect of these compounds on certain murine tumor cells can be observed.

In subsequent experiments human leukemic cells proved to be even more sensitive to the direct cytotoxicity of the different LPA than were cells from established murine tumor lines.

MATERIALS AND METHODS

Cells

Leukemic Cells. Leukemic cells were isolated according to the method of Böyum (5). They were washed 3 times in serum-free DMEM supplemented with 5 × 10^-6 M mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids (10 ml/liter), 50 units penicillin, and 50 µg streptomycin per ml.

Human Bone Marrow Cells. Small fragments of spongy bone substance from the iliac crest were obtained from hematologically healthy patients undergoing plastic surgery on bone. The bone marrow cells were vigorously washed out of the fragments, and the erythrocytes were lysed by repeated exposure to 0.83% ammonium chloride solution. The cells were cultured in a special growth medium consisting of 7 parts supplemented DMEM containing 10% FCS and 3 parts conditioned medium obtained from the supernatant of 10-day-old embryonic lung fibroblast cultures (Flow 2000; Flow Laboratories, 5300 Bonn, Federal Republic of Germany).

Lysophospholipids and LPA. Natural lysophosphatidylcholine was obtained from Sigma Chemical Co., St. Louis, Mo. 1-[14C]Palmitoyl-sn-glycero-3-phosphocholine was purchased from New England Nuclear, Boston, Mass.

[3H]-Labeled racemic 1-octadecyl-2-methyl-sn-glycero-3-phosphocholine was obtained via the synthesis of a 1-octadec-9-ethyl-2-methyl-sn-glycero-3-phosphocholine, which was then catalytically reduced with tritium gas by New England Nuclear and subsequently purified by chromatography. The label thus is located at C-8 and C-9 of the alkyl residue. Specific activity was 28 Ci/mm. 1-Palmitoyl-propanediol-3-phosphol[14C]choline and racemic 1-octadecyl-sn-glycero-3-phospho[14C]choline were synthesized as previously described (30).

The other synthetic analogs of 2-lysophosphatidylcholine and 2-lysophosphatidylethanolamine (LPA) have been synthesized as described elsewhere (3, 7). Briefly, the following modifications on the molecule have been carried out: The acyl bond has been replaced by an ether bond in sn-1 of the glycerol; the number of carbon atoms on the paraffin side chain has been varied; the hydroxy group in sn-2 has been substituted or replaced by hydrogen; and the polar head group in sn-3 has also been modified, thereby changing the...
charge pattern of the molecule (6). The LPA with changes in positions sn-1, sn-2, and sn-3 were symbolized as: sn-1, ES = ester or ET = ether linkage; aliphatic side chain, X number of carbon atoms; and sn-2, the functional group replacing —OH. Where the phosphocholine in sn-3 has been replaced, the modified polar head group is indicated (see Table 1).

In Table 1 the LPA are listed that have been used thus far. All substances were dissolved in supplemented DMEM containing 10% FCS in a 10-fold concentrated stock solution, were sterilized by filtration, and were stored frozen at −20°.

Lipid Analysis

[3H]-Labeled and [14C]-labeled lipids and phospholipids were extracted essentially as described by Ways and Hanahan (28).

The extracted lipids were separated on silica-coated thin-layer plates by using chloroform:methanol:H2O (60:40:10) as solvent. The separated compounds were detected by iodine vapor, marked, and scraped off into scintillation vials. One ml H2O was added to [3H]-labeled lipids. These vials were shaken for 48 hr to obtain a very fine suspension of silica gel. To this suspension were added 10 ml of the following scintillation cocktail: 8.4 g PPO; 700 ml Triton X-114; 2100 ml xylol; and 130 ml H2O. The probes were then counted after addition of the scintillation cocktail.

[14C]-Labeled compounds were measured immediately after addition of the scintillation cocktail.

Growth Inhibition Assay

The cells were suspended in medium, with or without the various LPA, at a final concentration of 1 x 10⁶ cells/ml unless otherwise stated. Quadruplicate cultures of 0.2 ml were incubated at pH 7.35 for up to 96 hr in microtiter plates (Falcon Plastics, Oxnard, Calif.). Every 24 hr, 0.2 µCi [methyl-3H]thymidine (specific activity, 21 Ci/mmol) (Amer- sham & Buchler, Braunschweig, Federal Republic of Germany) was added to each well. After 24 hr the cells and supernatant were harvested with a multisample harvester (Skatron AS; Lierbyen, Norway) on glass filter paper. The filter discs were exposed in vitro to different LPA at a final concentration of 5 µg/ml. This concentration is more than 10-fold lower than the concentration causing immediate cytolysis. After a 4-hr incubation at 37°, the phospholipids were extracted, separated, and measured. The same assay was used for the other labeled LPA described previously.

1-O-Alkyl Cleavage Enzyme (21). Cells (5 x 10⁵/5 ml DMEM + 10% FCS) were cultivated with 50 nmol [3H]alkyllysophosphocholine (ET-18-OCH₃) in Petri dishes equipped with a gas-permeable membrane for cellular support (Peri- perm; W. C. Heraeus GmbH, 6450 Hanau, Federal Republic of Germany). Every 24 hr the cells and supernatant were separated, and the lipids and phospholipids were measured as described previously. The enzymatic activity was determined as the amount of cleaved substrate.

RESULTS

The first experiments were performed with leukemic cells isolated from a patient with acute myelocytic leukemia. Chart 1 shows the proliferative activity of these cells when exposed in vitro to different LPA at a final concentration of 5 µg/ml. This concentration is more than 10-fold lower than the concentration causing immediate cytolysis.

None of the LPA with an acyl bond in sn-1 affected cellular proliferation (Chart 1B), whereas 2 of the group of
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chemically closely related alkyl-LPA induced a marked depression of cell growth: ET-18-H and ET-18-OCH<sub>3</sub>. Microscopic observation revealed distinct signs of cell injury after 24 hr, followed by complete destruction of the leukemic cells.

In further studies, cells of 6 patients with leukemias were investigated, and an analog (ET-12-H) with a short aliphatic side chain was included (Chart 2). The latter molecule is less lipophilic than are the other LPA used and has therefore a lower binding affinity (29). Thus, this compound was tested at a concentration of 50 μg/ml. In these experiments, again, 2-lysophosphatidylcholine and 2-lysophosphatidylethanolamine analogs did not inhibit the growth of leukemic cells. If, however, alkyl-LPA were used, all leukemic cells proved to be sensitive, with the exception of ET-18-OH. For investigation of the role of the surface activity of the analogs in causing cellular damage, other surface-active substances were included in a comparative study, the results of which are shown in Table 2. Two facts emerge from these data. (a) Under the culture conditions described, surface activity alone of the added compounds is not the cause of destruction of human leukemic cells. (b) The most obvious difference exists again between the lysophosphatidylcholine (ES-18-OCH<sub>3</sub>) and the alkyl-lysophosphocholine (ET-18-OCH<sub>3</sub>) analogs, only the latter one being cytotoxic.

As cells from chronic myelocytic leukemia patients exhibited the highest sensitivity to certain alkyl-LPA, these cells were used to establish dose-response curves (Chart 3). ET-18-OCH<sub>3</sub> was effective in concentrations as low as 1 μg/ml. The lowest effective concentration of ET-12-H was about 10 μg/ml. ET-18-OH was not cytotoxic, even in a concentration as high as 20 μg/ml. In further studies the influence of LPA on the proliferation of normal human cells was investigated.

Embryonic human lung fibroblasts were cultured with ET-12-H and ET-18-OCH<sub>3</sub>. [3H]Thymidine incorporation was decreased by about 30%. However, this effect was not
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Table 2

Influence of various surface-active substances on the growth of human leukemic cells in vitro

Ten nmol of each compound were added in the presence of 10% FCS to freshly collected human leukemic cells (chronic myelocytic leukemia) at pH 7.3, pO2 135 torr, and 37°. Quadruplicate cultures were used.

<table>
<thead>
<tr>
<th>Surface-active substance (10 nmol/10⁶ cells/ml)</th>
<th>[³H]Thymidine incorporation (% of controls)</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Control</td>
<td>100 (20,323 ± 768)⁺</td>
</tr>
<tr>
<td>ET-18-OCH₆</td>
<td>58 ± 4⁺</td>
</tr>
<tr>
<td>ES-18-OCH₆</td>
<td>100 ± 14⁺</td>
</tr>
<tr>
<td>ES-18-H</td>
<td>107 ± 13⁺</td>
</tr>
<tr>
<td>Hexadecylglycerol</td>
<td>103 ± 6⁺</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>102 ± 3⁺</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>101 ± 11⁺</td>
</tr>
</tbody>
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⁺ Numbers in parentheses, mean cpm/culture ± S.D.
⁺⁺ For definitions of abbreviations see Table 1.
⁺⁺⁺ Mean ± S.D.

accompanied by cellular destruction, although the cells did not grow as a confluent monolayer (data not shown).

The effect of alkyl-lysophospholipids on human bone marrow cells in vitro is shown in Chart 4. Under the described tissue culture conditions, only ET-18-OCH₆ (and ET-12-H to a lesser extent) inhibited [³H]thymidine incorporation at a later stage of the tissue culture. By comparing the effect of these alkyl-LPA on neoplastic cells, a striking difference is obvious.

Biochemical Studies. The remarkable difference between the lysophosphatidyl- and alkyl-LPA in their effect on proliferation and survival of human leukemic cells suggested a different biochemical fate of these substances in neoplastic cells. Only some representative labeled compounds have been traced thus far in normal and leukemic cells in vitro. Three enzymatic reactions have been studied: lysophospholipase (26); 1-O-alkyl cleavage enzyme, which degrades alkyl-lysophosphocholine (21); and lysophosphatidylcholine and 1-O-alkyl-3-glycerophosphocholine acyltransferase, which acylate the corresponding compounds (27).

As shown in Table 3, human leukemic cells degrade 2-lysophosphatidylcholine and its analogs, proving the presence of a lysophospholipase. Table 3 demonstrates also the presence of a lysophosphatidylcholine acyltransferase as well as 1-O-alkyl-3-glycerophosphocholine acyltransferase. 2-Lysophosphatidylcholine as well as alkyl-lysophosphocholine are acylated. The lysophosphatidylcholine analog ES-18-H cannot be acylated as the —OH radical is replaced by —H, but it is effectively metabolized by lysophospholipase.

These findings support the assumption that the alkyl-LPA, which cannot be acylated to alkyl-3-sn-phosphatidylcholine-like ET-18-H or ET-18-OCH₆, can only be degraded, if at all, by a 1-O-alkyl cleavage enzyme, which has been shown to be present in many normal tissues (21) but absent or present with much lower activity in neoplastic cells (22).

The data given in Chart 5 support these previous findings.

Table 3

<table>
<thead>
<tr>
<th>Metabolism of labeled lysophospholipids in human leukemic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemiaᵃ</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>ALL</td>
</tr>
<tr>
<td>ALL</td>
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<tr>
<td>CML</td>
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<td>CML</td>
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</tbody>
</table>

ᵃ Protein content: 10⁶ ALL cells, 5.7 µg; 10⁶ CML cells, 6.8 µg.
ᵇ The position of the label is the same as that described in "Materials and Methods."
ᶜ nmol hydrolyzed LPA.
ᵈ ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia.
ᵉ nmol acylated LPA.
In our studies with 8 different human leukemic cells, we never found a more than 5 to 10% turnover of the adsorbed alkyl-lysophospholipid within 24 hr, whereas normal proliferating human bone marrow cells metabolized the alkyl-lysophospholipid ET-18-OCH₃ with an increasing rate as shown in Chart 5. During these studies no evidence for the enzymatic activity of a lysophospholipase C or D (32) in leukemic cells has been found thus far.

**DISCUSSION**

As an explanation of the antitumor activity of various synthetic alkyl-LPA on transplantable syngeneic tumors in mice, it has been suggested that these compounds might activate tumoricidal macrophages (1, 15). Studies on the interaction of alkyl-lysophospholipid-activated macrophages with murine tumor cells in *vitro* indicated, however, that there might also be a direct cytotoxic effect of very low concentrations of these compounds on tumor cells themselves. By extending these studies to human leukemic cells in *vitro*, a striking direct cytotoxicity of certain alkyl-LPA has been observed. Very low doses (1 to 5 μg/ml) of these substances in the medium cause progressive cell destruction, which develops slowly and apparently depends on a distinct chemical structure of the LPA.

As 2-lysophosphatidylcholine and its synthetic analogs are highly surface-active substances (2, 12, 24) and are therefore potentially cytotoxic for mammalian cells, one could argue that the results presented here are simply due to their surface activity. Three facts argue against this notion. (a) Other surface active substances have no direct cytolytic effect when used in identical concentration and in the presence of serum (Table 2). (b) Cytolysis due to the surface activity of a given substance is usually completed within 1 to 2 hr, depending on the amount added (12, 17, 29). In contrast, the apparent tumor cell destruction in our studies did not start before 24 hr. (c) Several of the LPA used have almost identical surface activity, membrane affinity, and critical micellar concentration, yet they can be divided into substances that kill tumor cells and those that do not (Chart 1).

The essential difference between the studies on the hemolytic activity of 2-lysophosphatidylcholine stated previously and the present investigation is the fact that in our system the synthetic lysophospholipids are added to the neoplastic cells bound onto serum. Under these conditions the LPA are forming lipid:protein complexes (4, 19, 25) that are exchanged with the plasma membranes of the cultured cells. After the exchange from the lipoprotein of the plasma to the cell membrane, these compounds enter the cellular phospholipid pool (8, 9, 25). 2-Lysophosphatidylcholine or analogs, which have an acyl bond in the sn-1 position of the glycerol, can be degraded to glycerol-3-phosphocholine by lysophospholipase (25) or reacylated in the sn-2 position by 2-lysophosphatidylcholine transferase to 3-sn-lysophosphatidylcholine (Table 2). Of the synthetic analogs that are substituted in the sn-2 position, this transfer reaction will be blocked, but the deacylating reaction at the sn-1 position is apparently still sufficient to metabolize these lysophospholipids and to prevent any cellular damage (Table 2).

In contrast, the metabolism of alkyl-LPA depends partially on the activity of a specific oxygen-requiring O-alkyl cleavage enzyme (18, 20-22). This enzyme has been shown to be part of the multifunctional oxygenase system (10) that is present in normal tissues and is especially active in normal liver (16). Tumor cells apparently lack these alkyl cleavage enzymes (22) or have it only with low activity (Chart 4) and are known to have high levels of various alkyl-lysophospholipids (31).

When alkyl-LPA not modified in sn-2 were added, no cytotoxicity could be observed with leukemic cells. This molecule is still a substrate for the 1-0-alkyllysophosphocholine acyltransferase (27) (Table 3). The 1-alkyl-3-sn-phosphatidylcholine formed is apparently compatible with the survival of tumor cells. The fact, however, that racemic 1-octadecyl-sn-glycerol-3-phosphocholine (ET-18-OH) is inactive is still surprising, as the compound is a racemate. One would expect that the dextrorotatory enantiomer should have some effect on neoplastic cells, as acylation seems unlikely. This question needs further clarification. If, however, sn-2 is substituted, the adsorbed alkyl-lysophospholipid can hardly be metabolized. Even a lysophospholipase D activity could not be observed (32). Thus, these compounds are quite stable in tumor cells. The actual cause of the destruction of the tumor cell by nonmetabolizable alkyl-lysophospholipids remains to be clarified.

Three possibilities might be considered that are not mutually exclusive. (a) The simplest explanation would be that the cellular membranes are disrupted as more and more molecules are adsorbed that cannot be acylated to 1-alkyl-3-sn-phosphatidylcholine. The permeability barrier breaks down. (b) The alkyl-lysophospholipids could interfere with the acylating-deacylating cycle of membrane phospholipid metabolism. Thus, an analog like 1-octadecylpropanediol-3-phosphocholine could compete for both the...
lyso phospholipase and the 2-lysophosphatidylcholine transferase, interfering thereby with the renewal of the phospholipid bilayer (25). This possibility is currently under investigation. (c) Furthermore, assuming that a cellular phospholipase A₂ is normally active, endogenous lysophospholipids could also accumulate, as the "safety enzymes" (26) are partially blocked. Together with the adsorbed lysophospholipids from the serum or plasma (11), a cytolytic concentration might be reached.

As indication that the whole process of cellular destruction is not, however, only due to membrane damage comes from our observation that alkyl-LPA frequently induce multinucleated tumor cells before they are destroyed (P. G. Munder and R. Andreassen, unpublished observation).

Concerning the action of these alkyl-LPA on normal human cells, our experiments do not rule out possible effects on these cells in vitro, as the decrease of [³H]thymidine incorporation into human bone marrow cells was slight and varied from experiment to experiment (Chart 4); but they are able to metabolize alkyl-lysophospholipids with increasing rates, compared to leukemic cells (Chart 5). In parallel studies with murine bone marrow cells, for which optimal culture conditions are known, we found, however, that alkyl-LPA affected cell growth only during the initial culture period. In their later stages the treated cultures had a higher and more prolonged rate of proliferation (data not shown).

In addition, human macrophages derived in vitro from blood-born monocytes* as well as murine peritoneal macrophages* can be activated by alkyl-LPA to kill tumor cells in vitro much more efficiently. This positive activating effect of alkyl-lyso phospholipids on host defense cells is the other important aspect of the biological activity of these compounds, which is at present under investigation. Direct cytotoxicity as well as activation of host defense cells might therefore act synergistically in the host defense against tumors in vivo.

ACKNOWLEDGMENTS

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