Ether-linked Phosphoglyceride Content of Human Leukemia Cells

Marie C. Chabot, Dianne G. Greene, Joni K. Brockschmidt, Robert L. Capizzi, and Robert L. Wykle

Departments of Medicine [M. C. C., R. L. C.], Biochemistry [M. C. C., D. G. G., R. L. W.], and Prevention Research [J. K. B.], and the Cancer Center of Wake Forest University, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103

ABSTRACT

The glycerolipids of most cells are characterized by a specific proportion of ether linkages at the sn-1 position of the glycerol backbone. A number of tumors are known to have altered concentrations of ether-linked lipids compared to normal tissues. However, no thorough examination of the ether-lipid content of human leukemia cells has been reported despite the importance of these lipids in normal leukocyte function. In the present study samples were obtained from adults with acute myelogenous leukemia (AML), chronic granulocytic leukemia in blast crisis, and acute lymphocytic leukemia and from healthy human donors. The cellular lipids were extracted, the individual phospholipid classes were isolated, lipid phosphorus content was determined, and the lipids were converted to diglyceride benzoate derivatives for separation and quantitation of the subclasses by high performance liquid chromatography. The data indicate that all the leukemic cells analyzed have an altered phospholipid composition compared to their respective normal leukocytes. Furthermore, among the AML patients both the percentage of the choline-containing phosphoglyceride fraction (PC) which is alkyl linked and the moles alkyl-PC/10⁶ cells differ significantly by FAB subtype. A positive correlation between the levels of alkyl-PC and the degree of cellular differentiation is observed. Although no differences are observed between chronic granulocytic leukemia in blast crisis and AML lipids, the leukemic cells contain dramatically lower levels of alkyl-linked PC than do normal polymorphonuclear leukocytes. In contrast, no differences are observed between the alkyl-PC content of normal and leukemic lymphocytes. In light of the relations among ether-lipids, protein kinase C, and cell differentiation, these data suggest the ether-linked lipids are important in myeloid cell function and differentiation.

INTRODUCTION

The relationship between cellular lipids and cancer has been studied for a number of years. Many investigators have reported that the somewhat characteristic neutral lipid and phospholipid compositions of normal cells are altered in their neoplastic counterparts (1). A number of tumors, including human leukemia cells, are reported to have an increase in the relative content of phosphatidylcholine and decreased levels of phosphatidylethanolamine (2–5). In addition, a reciprocal relation between the levels of sphingomyelin and phosphatidylcholine is often observed (1–3).

Ether-linked lipids occur as neutral lipids or phospholipids which contain an ether linkage at the sn-1 position of the glycerol backbone. Thus, lipid classes may be divided into 3 subclasses, the ester-linked 1,2-diacyl species and the ether-linked 1-O-alkyl-2-acyl and 1-O-alk-1'-enyl-2-acyl species. The role of ether-linked lipids in cancer has been an area of research of importance in myeloid cell function and differentiation. Since that time, a number of studies have been published concerning ether-lipids and cancer. Most studies have focused on the neutral lipid fractions of tumor cells in which 1-O-alkyl-2,3-diacylglycerol levels are often elevated (1). In addition, a few investigators have reported that the levels of ether-linked glycerophospholipids are elevated in some tumors (1, 6, 8).

Although a variety of tumors and normal tissues have been characterized in terms of their ether-lipid content, only recently have human leukocytes and their malignant counterparts received much attention. Human PMNs contain high levels of ether-linked phosphoglycerides. Mueller et al. (9) demonstrated that 44% of the PC fraction is comprised of 1-O-alkyl-linked species. The PE are also enriched in ether-linked species; 66% of the PE is 1-O-alk-1'-enyl-linked (9). An important role in the alkyl-PC in normal cell function is now becoming clear. In stimulated human PMNs, the alkyl-PC serves not only as a precursor for PAF (10–12) and a donor of arachidonic acid (13–16) but also serves as a precursor for alkylacylglycerols and alkyl-linked phosphatidic acid, whereas the diacyl PC yields diacylglycerol and diacyl phosphatidic acid (17, 18). Both subclasses of the PC-derived diglycerides may have unique functions as second messengers and may be important in regulating protein kinase C (19–22). Diacylglycerols stimulate protein kinase C activity (20, 21), while alkylacylglycerols inhibit this enzyme in some systems (19, 22). As such, these lipids may function as on/off signals within the cell and, as a result, may regulate many cellular functions.

In light of the importance of the ether-linked PC in normal cell function, we have studied the phospholipid subclass composition of human leukemia cells. Until recently, such studies were extremely difficult because they required large quantities of material and the required techniques were cumbersome. Several years ago we developed a normal-phase HPLC procedure for the separation and quantitation of phospholipid subclasses which requires only 10⁶ cells for an analysis. Using this procedure we recently demonstrated that, in contrast to the high levels of ether-lipids observed in human PMNs, two human leukemia cell lines, HL-60 and K 562, contain dramatically lower levels of ether-linked phosphoglycerides (23). Consistent with our findings, Billah et al. (24) and Naito et al. (25) have reported low levels of ether-linked phosphoglycerides in HL-60 cells. We have now expanded our study of human leukocytes and analyzed the phospholipid subclass composition of leukemia cells isolated from adults with AML, CGL-B1, and ALL and compared these profiles to those of normal human leukocytes. Patients with AML are categorized into 1 of 7 subtypes according to the FAB classifications (M-1–M-7) (26). These subtypes are based largely on the degree of cellular differentiation, histochemistry, and the presence of lineage-specific cell
ETHER LIPIDS IN LEUKEMIC CELLS

MATERIALS AND METHODS

Isolation of Normal Human Leukocytes. Heparinized venous blood was collected from healthy donors and processed as described previously (27). The erythrocytes were removed by sedimentation at 1 x g for approximately 45 min with Plasmagel. Leukocytes were pelleted from the Plasmagel by centrifugation (225 x g for 10 min), resuspended in Ca2+-free PBS, layered over Isolymp, and centrifuged at 400 x g for 30 min. The neutrophil pellet was washed with PBS and the residual erythrocytes were removed by hypotonic lysis.

The band containing the lymphocytes and monocytes was collected and washed with PBS, and the two cell types were separated by adherence of the monocytes to a substratum. Lymphocytes were collected and washed, and the lipids were extracted as described below.

Isolation of Leukemic Blasts. Peripheral blood or bone marrow samples were obtained from adults with AML, CGL-BI, or ALL. The samples (1-2 ml bone marrow, 10 ml peripheral blood) were diluted to 35 ml with RPMI-1640 medium and 15 ml Ficoll/Hypaque solution was underlayered. The tubes were centrifuged at 1000 x g for 20 min after which the band at the Ficoll-RPMI interface was collected and washed in fresh RPMI. The leukemic blasts in the samples ranged from 34-100%.

Analysis of Cellular Lipids. After removal of contaminating erythrocytes by hypotonic lysis, the cells were resuspended in deionized H2O and the cellular lipids were extracted according to the method of Bligh and Dyer (28) except the methanol contained 2% acetic acid. The lipids were quantitated by phosphorus determination according to the method of Rouser et al. (29). The lipids were dried under N2, converted to their phospholipid classes, the lipids were reconstituted on silica plates with cyclohexane:2-propanol (99.85:0.15, v/v) at a flow rate of 1 ml/min. The retention times of the alkylacyl-, alk-1-enylacyl-, and diacylglycerolipids were determined using standards synthesized from lipids of known subclass composition. The nmoles of alkyl-PC/106 cells were calculated by multiplying the total nmoles of PC/106 cells based on lipid phosphorus analysis by the percentage of the PC fraction which is alkyl linked.

Statistical Analysis. Various statistics are presented describing the distribution of the percentage of the PC fraction which was alkyl linked and the nmoles of alkyl-PC/106 cells. These distributions were compared for the various groupings defined as “Results” using the Wilcoxon rank sum test, with one exception. Samples from patients with CGL-BI were available for both bone marrow and peripheral blood. These samples were compared in terms of their alkyl-PC content using the Wilcoxon signed rank test.

Materials. RPMI-1640 medium was purchased from Gibco (Grand Island, NY) and Ficoll/Hypaque was from Pharmacia, Inc. (Piscataway, NJ). All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and were HPLC grade. Thin-layer chromatography plates were from Analtech (Newark, DE), and lipid standards were from Serdary Research Laboratory (London, Ontario, Canada). Phospholipase C (B. cereus, Grad. I) was obtained from Boehringer-Mannheim (Indianapolis, IN). Benzoic anhydride and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Previous studies in our laboratory have shown that two human leukemia cell lines, HL-60 and K562, contain dramatically lower levels of ether-linked phosphoglycerides than do PMNs isolated from the peripheral blood of healthy human donors (23). Therefore, we were interested in expanding our study to include freshly isolated leukemia cells from patients. We were particularly interested in determining (a) whether the

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**Table 1** FAB classification of acute myeloid leukemia

<table>
<thead>
<tr>
<th>Designation</th>
<th>Predominant cell type</th>
<th>Cytomorphology and cytochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1: undifferentiated</td>
<td>Myeloblasts</td>
<td>Minimal evidence of granulocytic differentiation, e.g., Auer rods, azurophilic granules, ≥3% myeloperoxidase-positive blasts; positive Sudan black B; negative nonspecific esterase and block positive PAS stains.</td>
</tr>
<tr>
<td>M-2: myelocytic</td>
<td>Myeloblasts, promyelocytes, myelocytes</td>
<td>Myeloblasts plus signs of differentiation to promyelocyte or beyond, peroxidase, Sudan black B, nonspecific esterase, and PAS stains as with M-1.</td>
</tr>
<tr>
<td>M-3: promyelocytic</td>
<td>Hypergranular promyelocytes</td>
<td>Large peroxidase-positive granules; microgranular variant has been described evident by Romanovsky stain and/or electron microscopy of peroxidase-stained cells, marked dysplasia with multinucleated nuclei, many Auer rods, occasional in bundles, positive Sudan black B, negative nonspecific esterases, and PAS stains.</td>
</tr>
<tr>
<td>M-4: myelomonocytic</td>
<td>Promyelocytes, myelocytes, promonocytes, monocytes</td>
<td>Both granulocytes and monocytic differentiation in varying proportions with a minimum of 20% promonocytes and monocytes in marrow and/or peripheral blood.</td>
</tr>
<tr>
<td>M-5</td>
<td>Monoblasts</td>
<td>≥80% monoblasts; &lt;20% myeloblasts and promyelocytes; positive nonspecific esterase stain.</td>
</tr>
<tr>
<td>M-5a: monoblastic</td>
<td>Monoblasts</td>
<td>&lt;20% monoblasts; promonocytes and monocytes predominate; positive nonspecific esterase stain.</td>
</tr>
<tr>
<td>M-6: erythroleukemia</td>
<td>Erythroblasts</td>
<td>&gt;50% of nucleated cells are erythroblasts, most of which are megablasts and markedly dysplastic; positive PAS; &gt;30% of blasts are myeloblasts and/or promyelocytes. Many immature and dysplastic megakaryocytes distinguished by megakaryocyte-specific stains with numerous immature platelets.</td>
</tr>
<tr>
<td>M-7: megakaryocytic</td>
<td>Megakaryoblasts, megakaryocytes</td>
<td></td>
</tr>
</tbody>
</table>

* Taken from Bennett et al. (26).  *

PAS, periodic acid-Schiff.  

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phospholipid composition of these cells differed from the normal cells and (b) whether the subclass composition within the PC fraction was different in the leukemic and normal cells.

The phospholipid class composition was determined for four of the AML FAB subtypes and normal human PMNs (Table 2). Little difference was observed among the various FAB subtypes of AML. However, there were rather striking differences between the phospholipid composition of the AML cells as a group and the normal PMNs. The AML cells had relatively high levels of PC and PS/PI compared to PMNs, whereas the sphingomyelin and PE levels were lower in AML cells. As a result, the ratios of PC/PE and PC/sphingomyelin were greater in the leukemic cells than in the PMNs.

We next investigated the subclass composition of the PC fraction isolated from leukemic and normal leukocytes. These subclasses were separated and quantitated as their diglyceride benzoate derivatives by normal-phase HPLC. In the normal PMNs 42% of the PC fraction was alkyl linked (Fig. 1). This value was consistent with that obtained by Mueller et al. (9) who used different analytical techniques. The PC subclass compositions of cells from various FAB subtypes of AML were then determined. The studies focused on 29 patients with AML and were conducted on leukemia cells isolated either from bone marrow or peripheral blood. All AML lipids, regardless of source, contained dramatically lower levels of alkyl-PC than did the normal PMNs. Among the AML patients, both the percentage of the PC fraction which was alkyl linked (P = 0.0102) (Fig. 1) and the nmole of alkyl-PC/10^6 cells (P = 0.019) and as a percentage of the total PC were observed to be lower in the leukemic cells versus those patients who achieved a complete remission (data not shown). Likewise, there was no difference in blasts from newly diagnosed untreated patients versus relapsed AML patients (data not shown). However, comparison of the alkyl-PC content in blasts from AML patients refractory to treatment versus those patients who achieved a complete remission showed marked differences. Blasts from patients with refractory AML had significantly less alkyl-PC both in total quantity (nmole/10^6 cells, P = 0.019) and as a percentage of the total PC (P = 0.016)(Table 3).

The percentage of the PE fraction which was ether linked was also investigated. The PE fraction of normal human PMNs was enriched in alk-1-enyl-linked species. These species make up 66% of the total PE. Similar to our observation of the ether-linked PC, the content of the ether-linked species of the PE was reduced in the leukemic cells, although to a lesser extent (Fig. 3). However, in contrast to alkyl-PC, the levels of alk-1-enyl PE did not appear to be correlated with the degree of cellular differentiation nor did they differ significantly among FAB subtypes.

The ether- lipid content of cells from individuals with chronic granulocytic leukemia was also determined. Peripheral blood and bone marrow samples were available from all 5 CGL-B1 patients. The alkyl-PC content of the bone marrow and peripheral blood blasts did not differ. In addition, no differences were observed in the overall alkyl-PC content of CGL-B1 and AML samples (Figs. 1 and 2). On the other hand, differences were observed between CGL-B1 cells and normal PMNs (Figs. 1 and 2).

The lipids of normal human lymphocytes were compared to blasts from patients with acute lymphocytic leukemia. The phospholipid composition of the leukemic cells varied slightly from normal lymphocytes (Table 4). These changes resulted in an increase in the ratio of PC/sphingomyelin. This was consistent with the observations made in the AML samples. The PC subclass composition of the normal and leukemic cells was then determined. In contrast to the differences observed between normal and leukemic myeloid cells, no differences were observed between the alkyl-PC content of the normal and leukemic lymphocytes (data not shown). In both populations approximately 9% of the PC fraction was alkyl linked.

DISCUSSION

The relationship between cellular lipid composition and cancer has been studied for a number of years. Many investigators have reported alterations in the neutral lipid and phospholipid compositions of neoplastic cells compared to their normal counterparts (1). For example, triacylglycerol levels in Yoshida hepatoma cells are elevated compared to normal liver (31). However, the levels of these lipids are decreased in mammary carcinomas compared to normal breast tissue (32). Among the phospholipids, changes in the distribution of phospholipid subclasses have been noted (1, 2, 4, 5). Several investigators have reported an increase in the ratio of PC/PE in brain tumors (4, 5) compared to normal brain tissues. This finding is consistent with our observations of the PC/PE ratio in leukemic versus normal myeloid cells. In addition, Gottfried (2) has reported an increase in the relative content of PC in human leukemia cells and a reciprocal relationship between the levels of sphingomyelin and PC. It is evident that our findings in the myeloid and lymphoid leukemias are consistent with this earlier finding.

Table 2 Phospholipid composition of human myelogenous leukemia cells

<table>
<thead>
<tr>
<th>Phospholipid class</th>
<th>M-1</th>
<th>M-2</th>
<th>M-4</th>
<th>M-5</th>
<th>CGL-B1</th>
<th>PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>7.7 ± 1.3</td>
<td>7.5 ± 2.0</td>
<td>8.4 ± 2.4</td>
<td>9.6 ± 2.3</td>
<td>8.0 ± 2.5</td>
<td>14.4 ± 2.7</td>
</tr>
<tr>
<td>PC</td>
<td>50.2 ± 5.4</td>
<td>47.5 ± 7.0</td>
<td>47.0 ± 4.2</td>
<td>45.8 ± 2.2</td>
<td>48.2 ± 3.9</td>
<td>40.8 ± 1.8</td>
</tr>
<tr>
<td>PS/PI</td>
<td>13.3 ± 2.8</td>
<td>13.9 ± 4.5</td>
<td>11.8 ± 3.9</td>
<td>14.7 ± 0.1</td>
<td>9.5 ± 4.0</td>
<td>5.4 ± 1.0</td>
</tr>
<tr>
<td>PE</td>
<td>28.8 ± 4.1</td>
<td>31.0 ± 4.8</td>
<td>32.7 ± 3.5</td>
<td>29.9 ± 2.7</td>
<td>34.4 ± 3.8</td>
<td>38.6 ± 2.7</td>
</tr>
</tbody>
</table>

* The total phospholipid phosphorus (nmole/10^6 cells) of the leukemia cells classes was as follows: M-1, 5.6; M-2, 7.9; M-4, 10.3; M-5, 10.7; CGL-B1, 10.9.

* Subtypes of AML (M1-M7) based on the French-American-British classification system.

* Contain diacyl- and ether-linked species.

* Mean ± SD of at least 4 determinations.
A common finding in the early studies concerning the ether-lipid content of animal and human neoplasms was that the alkyl ether analogues of triacylglycerols, 1-O-alkyl-2,3-diacylglycerols, are often elevated. The malignancies studied include Ehrlich ascites cells, mouse preputial gland tumors, lymphomas, and a number of brain tumors (see Ref. 1 for review). In addition, human brain tumors (8) and a number of rat and mouse tumors (7) have been reported to contain elevated levels of ether-linked phosphoglycerides. However, the levels of the ether-containing phosphoglycerides were not different from that of normal cells as were the levels of 1-O-alkyl-2,3-diacylglycerols.

In light of these previous findings, we were somewhat surprised to find that normal PMNs, which are particularly rich in alkyl-PC (9), contained higher concentrations of ether-linked phosphoglycerides than did myeloid leukemic cells. Examination of additional leukemia patients may reveal further differences than those reported here. The variations in ether-lipid content are of particular interest because alkyl-PC serves as a precursor of PAF (10-12) as well as other bioactive compounds. 1-O-Alkyl-2-arachidonoyl-PC is known to serve as a donor of arachidonic acid which may be converted to the active 5-hydroxyeicosatetraenoic acid and leukotriene B4 species (13-16). Furthermore, we have recently shown that, upon stimulation of PMNs, the PC is hydrolyzed to yield diglycerides and phosphatidic acid consisting of both diacyl and alkylacyl species (17). Diacyl- and alkylacylglycerides have been shown to prime phospholipase A2 activation (33) and the rate of the respiratory burst in human PMNs (19); however, whereas diacyl diglyceride primed for extended duration of the respiratory burst and primed lipoxygenase activity, the alkyl diglyceride did not and, in fact, appeared to inhibit these diacyl-priming responses.

It is obvious that alkyl-PC is important in the functioning of mature PMNs. The possibility also exists that this phospholipid plays a key role during leukocyte differentiation. Many growth hormones and the ras oncogene products are now known to stimulate PC turnover (34, 35). In addition, studies with HL-60 cells have yielded interesting findings. Many investigators have used the human promyelocytic leukemia cell line HL-60 as a model system to study leukocyte differentiation. The HL-60 cell line is particularly useful in such studies because it is a differentially bipotent cell capable of undergoing either granu-
locytic or monocytic differentiation in response to chemical inducers. Among these inducers are two compounds for which alkyl-PC could serve as a precursor. Hexadecyl-acetyl-glycerol, an analogue of alkylacylglycerol, has been reported to induce the differentiation of HL-60 cells into cells resembling monocytic phagocytes (36). Alkylacylglycerols are formed in stimulated PMNs (17, 18) and could arise from alkyl-PC through the action of a phospholipase C- and/or D-mediated reaction. Phospholipase A₂ cleaves alkyl-PC to yield alkyl-lyso-PC which can be acetylated to form PAF. HL-60 cells incubated with 1-O-alkyl-2-O-methyl-sn-glycero-3-phosphocholine (an analogue of PAF) were induced to differentiate morphologically into mature granulocytes (37). Thus, alkyl-PC may give rise to different compounds which can induce the differentiation of HL-60 cells. One may speculate that these compounds may also be involved in the in vivo differentiation of myeloid cells. As such, there may be a relation between the low levels of ether-linked phosphoglycerides in myelogenous leukaemia cells and their inability to undergo normal differentiation. The basis for their deficiency may reside anywhere from the genetic basis for their inability to undergo normal differentiation. The basis for the regulation of their synthesis.

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