Studies on Some Lipogenic Enzymes of Cultured Myeloid Leukemic Cells

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The microsomal fraction of M1 cells (an established cell line of myeloid leukemia) was capable of catalyzing acylation of *sn*-glycerol 3-phosphate by long-chain fatty acyl-CoA thioesters. The principal lipid product formed was identified as phosphatidic acid. Palmityl-CoA, stearyl-CoA, and oleyl-CoA were more effective acyl donors than linoleyl-CoA and arachidonyl-CoA. M1 cells and macrophages differentiated from them exhibited similar levels of *sn*-glycerol 3-phosphate-acylating activity, which were approximately one-half that in mouse liver and approximately four times that in peritoneal macrophages. The levels of acetyl-CoA carboxylase activity in M1 cells and macrophages differentiated from them were not significantly different from each other and were comparable to those in mouse liver, whereas no activity was detected in peritoneal macrophages. These results indicated that differentiation of the myeloid leukemic cells, which results in loss of leukemogenicity and mitotic activity, is not associated with changes in the activities of these lipogenic enzymes, although the cultured cells exhibited remarkably higher activities than freshly harvested peritoneal macrophages. Furthermore, the present study supports the view that the glycerophosphate pathway makes an essential contribution to the de novo synthesis of phospholipids in M1 cells, as well as in both types of macrophages.

THE LIPID PHASE associated with protein constitutes cell membranes in which cellular phospholipids are mainly located. One of the major functions of phospholipids is to contribute to the structure and function of cell membranes. Lipids constitute about 5% of the wet weight of normal human leukocytes, and their phospholipids comprise about 35% of the total lipids.[1] Thus far, a considerable number of studies have been made on the synthesis of lipids in leukocytes. It has been demonstrated that the rate of incorporation of radioactive acetate into lipids is greater in leukocytes from patients with acute or chronic myeloid leukemia than in normal leukocytes.[2,3] Furthermore, the activity levels of some enzymes involved in lipid metabolism have been shown to be altered in leukocytes derived from patients with leukemia.[4] Most of these studies have been carried out with cell preparations obtained from peritoneal exudates of animals or with mixed cell populations derived from blood. In view of the fact that a high proportion of M1 cells (an established cell line of myeloid leukemia) are induced to differentiate to macrophages by culture with condi-

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tioned medium,[5,6] it appeared of interest to investigate enzymes participating in lipid synthesis in these cells.

In animal tissues, phosphatidic acid is a key intermediate in the synthesis of phospholipids and triglycerides.[7-9] The present study was designed to characterize the enzyme system responsible for the synthesis of phosphatidic acid from *sn*-glycerol 3-phosphate (GP) in M1 cells. The microsomal fraction of these cells was shown to be capable of catalyzing acylation of GP by long-chain fatty acyl-CoA (coenzyme A) thioesters. Some characteristics of this enzyme system were studied. In addition, the levels of GP-acylating activity in M1 cells, and macrophages differentiated from M1 cells, peritoneal macrophages, and mouse liver were compared with one another. Furthermore, the activity of acetyl-CoA carboxylase, the enzyme which plays a critical role in the regulation of long-chain fatty acid synthesis,[10] was determined in these cells.

MATERIALS AND METHODS

Preparation of Cells

The M1 cell line used in the present study was established in vitro from a spontaneous myeloid leukemia of SL strain mice[5] and has been maintained over 5 yr. The cell line consists of a homogeneous population of leukemic myeloblasts that grow in suspension in liquid medium with a doubling time of about 20 hr. Cell cultures were carried out in 6-cm glass or 5-cm plastic petri dishes at 37°C in a continuous stream of water-saturated 5% CO_2 in air. The growth medium consisted of Eagle's minimum essential medium enriched twofold with amino acids and vitamins (modified Eagle's medium), and contained 10% calf serum inactivated by heating at 56°C for 30 min. Unless otherwise stated, cell cultures were conducted for 48 hrs.

Macrophages differentiated from M1 cells were obtained as follows: 5×10^6 M1 cells per petri dish were incubated at 37°C in 5 ml of 50% conditioned medium in modified Eagle's medium supplemented with 10% inactivated calf serum. After decantation of the medium, along with cells in suspension, differentiated macrophages fixed to the petri dishes were resuspended in 5 ml of modified Eagle's medium. Conditioned medium was prepared from a secondary embryo culture as described previously[11] and was stored at −20°C until use.

Peritoneal macrophages were obtained by intraperitoneal lavage with 5 ml of 0.12 *M* NaCl containing 13.3 m*M* phosphate buffer, pH 7.0 (phosphate-buffered saline), from 8- to 12-wk-old SL strain mice which had been injected intraperitoneally with 3 ml of thioglycollate medium 3 or 4 days before collecting the cells.[11] The peritoneal cell suspension was centrifuged at 150 *g* for 5 min. The cells were resuspended in modified Eagle's medium, and 4×10^6 cells were seeded per plate. After incubation at 37°C overnight, cells in suspension were decanted, and the petri dishes were rinsed with phosphate-buffered saline. Macrophages, which adhered to the dishes, were scraped off with the use of a rubber policeman.

All the cells thus obtained were collected by centrifugation in 50-ml plastic tubes at 480 *g* and 4°C for 10 min, and the cell pellet was washed twice with chilled 0.135 *M* NaCl containing 50 m*M* Tris-HCl buffer, pH 7.4. The cells were finally suspended in chilled 0.25 *M* sucrose to give a density of $2\text{-}8 \times 10^7$ cells/ml.

Enzyme Preparations

All manipulations were performed at 0°-4°C. Cells suspended in 1.5-3 ml of 0.25 *M* sucrose were disrupted sonically for 30 sec at minimum output with a Branson W185 sonifier. The cell homogenate was centrifuged at 9500 *g* for 10 min. The resulting supernatant was centrifuged further at 105,000 *g* for 60 min. The pellet obtained (microsomal fraction), which was suspended in 0.25 *M* sucrose to yield a protein concentration of 1-4 mg/ml, was used to assay GP-acylating activity. A 0.5- to 1-ml aliquot of the 105,000 *g* supernatant fraction was filtered through a Sephadex G-50 column (1 × 13 cm) equilibrated with 50 m*M* Tris-HCl buffer, pH 7.5, containing 5 m*M* 2-mercaptoethanol and 1 m*M* EDTA, and was used to assay acetyl-CoA carboxylase activity.

Subcellular Fractionation

The cell homogenate prepared by sonication as described above was fractionated at 0°–4°C by sequential centrifugation at 1000 *g* for 20 min, 9500 *g* for 10 min, and 105,000 *g* for 60 min. Each pellet was suspended in 0.25 *M* sucrose to give a protein concentration of 1–4 mg/ml.

Preparation of the Microsomal Fraction of Mouse Liver

Fresh samples of normal mouse liver were homogenized, and the microsomal fraction was prepared as described previously.[12]

Assay of Enzymes

GP-acylating activity was assayed at 20°C by determining the incorporation of GP-1(3)-^{14}C into lipid as described previously,[12] except that 7.5 nmoles of palmityl-CoA and, routinely, 0.2 mg of protein were added to the reaction mixture (0.35 ml). Acetyl-CoA carboxylase activity was determined at 37°C by the $H^{14}CO_3^-$ fixation assay, which measures the rate of acetyl-CoA-dependent incorporation of $H^{14}CO_3^-$ into acid-stable material. The procedure of this assay was as described previously,[13] except that 5 ml of the scintillator solution of Patterson and Greene[14] was used for the determination of radioactivity and that, in some experiments, the specific radioactivity of $KH^{14}CO_3$ was doubled. At least two different protein concentrations (0.03–0.12 mg per assay mixture) were used for this assay to ensure the proportionality between the initial reaction velocity and the amount of enzyme added. The initial reaction velocity was proportional to the amount of enzyme added up to 0.15 mg of protein per assay mixture. The $H^{14}CO_3^-$ incorporation into acid-stable material proceeded linearly with time for at least 30 min, when 0.05 mg of protein was used. Hence, incubation was conducted routinely for 10 min. The radioactive acid-stable reaction product was identified as malonyl-CoA according to the procedure described by Matsuhashi et al.;[15] the thioester was hydrolyzed by mild alkaline treatment, and the resulting radioactive product was shown to be indistinguishable from authentic malonic acid upon paper chromatography with *n*-butanol–acetic acid–water (4:1:5, v/v) and with ethanol–ammonia–water (20:1:4, v/v) as the developing solvent.

Cytological Examinations

Morphological characteristics of cells were examined in May-Grünwald-Giemsa-stained smears of a cell suspension as reported previously.[5] Peroxidase reaction and phagocytosis were also tested as described previously.[6] M1 cells exhibited positive peroxidase reaction and no phagocytosis, while macrophages lacked peroxidase reaction and showed prominent phagocytosis.

Materials, Preparations, and Determinations

Thioglycollate medium was purchased from Difco, Detroit, Mich. GP-1(3)-^{14}C, long-chain acyl-CoA thioesters, and lysophosphatidic acid were prepared as described previously.[12] The preparation and determination of acetyl-CoA were as reported earlier.[16] Protein was determined by the method of Lowry et al.[17] with bovine serum albumin (Sigma, St. Louis, Mo.) as the standard; particulate protein was solubilized with 2% deoxycholate prior to the determination. Other reagents including fatty acids, phospholipids, and nucleotides were as described previously.[12]

RESULTS

Preliminary Studies on GP Acylation

The incorporation of GP-1(3)-^{14}C into lipid catalyzed by the microsomal fraction of M1 cells (0.2 mg of protein per assay mixture) increased linearly for the initial 10 min. Therefore, a 5-min incubation period was chosen for the routine assay of GP-acylating activity. As shown in Fig. 1, the rate of GP acylation was not completely linear with the amount of protein added. On the basis of this experiment, the assay was routinely carried out with 0.2 mg of protein per assay mixture. A broad pH optimum around 7.2–8.4 was observed

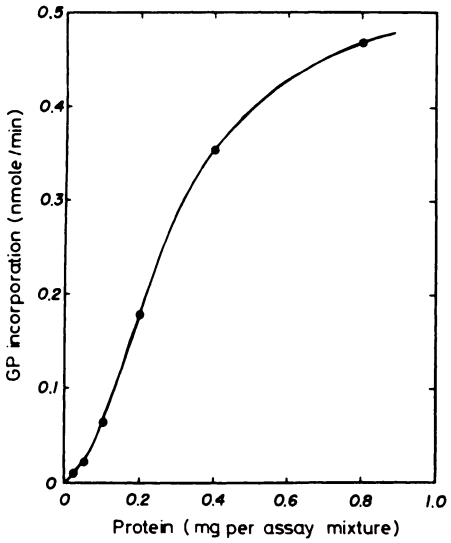

Fig. 1. Dependence of the rate of GP acylation on the enzyme amount. Enzyme activity was assayed with the microsomal fraction of M1 cells as described in Materials and Methods, except that the amount of enzyme added was varied as indicated.

for the GP-acylating activity of the microsomal fraction of M1 cells. Thus, GP-acylating activity was assayed routinely at pH 7.6.

Products of GP Acylation

The lipid product formed by the action of the microsomal fraction of M1 cells was examined by thin-layer chromatography. Figure 2 shows the results of a typical experiment, with the use of chloroform-methanol-10 *N* HCl (87:13:0.5, v/v)[18] as the developing solvent, demonstrating that 89.2% and 7.5%

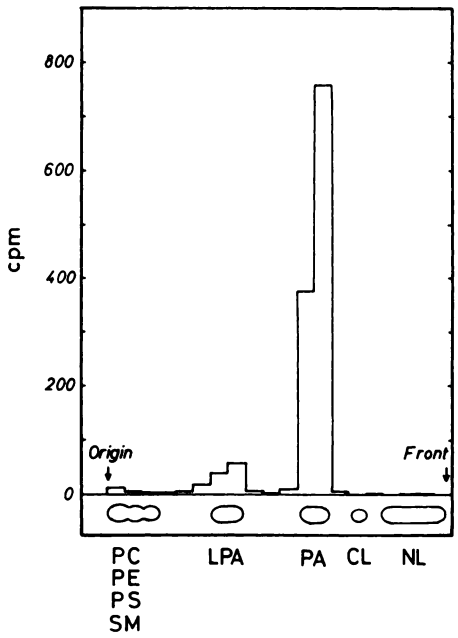

Fig. 2. Thin-layer chromatography of radioactive lipid products. The routine reaction mixture containing the microsomal fraction of M1 cells (0.2 mg of protein) was incubated at 20°C for 5 min. After lipids were extracted and separated by thin-layer chromatography, the radioactivity was determined; the procedures were as described previously.[12] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; LPA, lysophosphatidic acid; PA, phosphatidic acid; CL, cardiolipin; NL, neutral lipids.

Table 1. Distribution of GP-Acylating Activity in the Subcellular Fractions of M1 Cells

Fraction	Total Activity (%)	Specific Activity*	Protein (%)
Homogenate	100	0.460	100
1,000 *g* pellet	2.5	0.135	8.6
9,500 *g* pellet	5.8	0.571	4.7
105,000 *g* pellet	64.8	1.070	28.1
105,000 *g* supernatant	2.0	0.022	42.2

*nmoles of GP incorporated per minute per milligram of protein at 20°C.

of the total radioactivity present on the chromatogram were recovered in the areas corresponding to phosphatidic acid and lysophosphatidic acid, respectively. No significant radioactivity was found in the areas corresponding to neutral lipids and other phospholipids. These results were confirmed by thin-layer chromatography with the use of chloroform-methanol-acetone-acetic acid-water (10:2:4:2:1, v/v)[19] as the developing solvent.

Subcellular Distribution of GP-Acylating Activity

As shown in Table 1, the 105,000 *g* pellet (microsomal fraction) of M1 cells exhibited the highest specific activity of GP acylation, while the 105,000 *g* supernatant fraction was least active. Moreover, the major portion of the enzyme activity was associated with the microsomal fraction. The recovery of the enzyme activity after fractionation of the whole homogenate was 75%, while that of protein was 84%.

Effects of Substrates on GP-Acylating Activity

When the rate of GP acylation catalyzed by the microsomal fraction of M1 cells was plotted against the concentration of GP, a typical saturation curve was obtained. From the Lineweaver-Burk plots,[20] the apparent Michaelis constant for GP at a fixed palmityl-CoA concentration of 21.4 μM was calculated to be approximately 0.4 mM.

In the experiment shown in Fig. 3, the rate of GP acylation was determined in the presence of different acyl-CoA thioesters in varying concentrations. Palmityl-CoA, stearyl-CoA, and oleyl-CoA were more effective than linoleyl-CoA and arachidonyl-CoA. At concentrations higher than optimum, all the acyl donors exhibited an inhibitory effect. The microsomal fraction of M1 cells did not contain a sufficient amount of endogenous acyl-CoA to acylate GP because no significant esterification of GP was observed in the absence of added acyl-CoA.

GP-Acylating Activity of M1 Cells and Macrophages

Table 2 compares the level of GP-acylating activity in the microsomal fraction of M1 cells with those in the microsomal fractions of macrophages and mouse liver. Macrophages differentiated from M1 cells by incubation with conditioned medium as well as relatively fresh peritoneal macrophages were used. More than 70% of the macrophages derived from M1 cells showed phago-

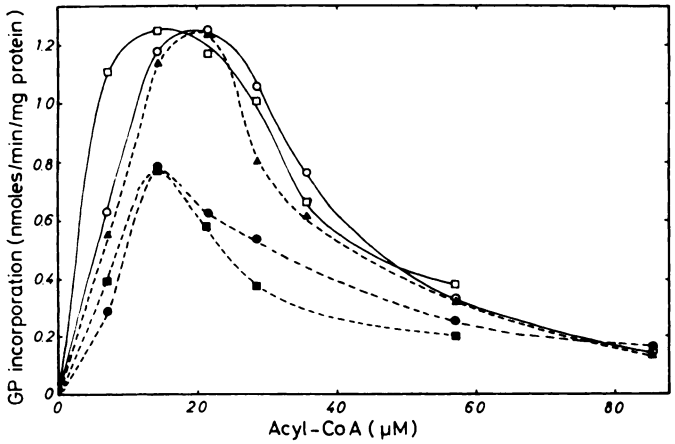

Fig. 3. Acyl donor specificity. Enzyme activity was assayed with the microsomal fraction of M1 cells as described in Materials and Methods, except that palmityl-CoA (open circles), stearyl-CoA (open squares), oleyl-CoA (black triangles), linoleyl-CoA (black circles), or arachidonyl-CoA (black squares) was added in varying concentrations as indicated.

cytosis, whereas less than 5% of the M1 cells did. M1 cells and macrophages differentiated from them exhibited similar levels of GP-acylating activity, which were approximately four times the activity level in peritoneal macrophages and approximately one-half that in the liver.

Acetyl-CoA Carboxylase Activity of M1 Cells and Macrophages

In Table 3 are presented the levels of acetyl-CoA carboxylase activity in the 105,000 *g* supernatant fraction of M1 cells, macrophages differentiated from them, peritoneal macrophages and mouse liver. M1 cells and macrophages derived from them exhibited no significant difference in acetyl-CoA carboxylase activity ($0.1 < p < 0.2$; assessed by the t test), although the former had a ten-

Table 2. GP-Acylating Activity in M1 Cells as Compared With That in Macrophages and Liver

Cell	No. of Experiments	Specific Activity*
M1 cells	12	1.050 ± 0.064
Macrophages derived from M1 cells	5	1.254 ± 0.107
Peritoneal macrophages	2	0.284, 0.316
Liver	4	2.273 ± 0.120

*nmoles of GP incorporated per minute per milligram of protein at 20°C; mean ± SEM.

Table 3. Acetyl-CoA Carboxylase Activity in M1 Cells as Compared With That in Macrophages and Liver

Cell	No. of Experiments	Specific Activity*
M1 cells	7	2.942 ± 0.536
Macrophages derived from M1 cells	6	2.011 ± 0.251
Peritoneal macrophages	3	0
Liver	4	1.836 ± 0.103

*nmoles of $H^{14}CO_3^-$ incorporated per minute per milligram of protein at 37°C; mean ± SEM.

dency to increased activity as compared with the latter. The activity levels in these cells were comparable to that in the liver. On the other hand, peritoneal macrophages showed no detectable acetyl-CoA carboxylase activity.

DISCUSSION

The present investigation has clearly demonstrated that M1 cells exhibit the enzyme activity responsible for the conversion of GP to phosphatidic acid. It has been shown earlier that polymorphonuclear leukocytes obtained from the peritoneal cavity of guinea pigs possess the enzymic apparatus of phosphatidic acid synthesis by GP acylation.[21] Recent work from our laboratory has demonstrated that the phosphatidate-synthesizing system of rat liver microsomes can be resolved into two distinct enzymes, i.e., GP acyltransferase, which catalyzes acylation at position 1 of GP to form lysophosphatidic acid, and 1-acyl-GP acyltransferase, which mediates acylation of lysophosphatidic acid to produce phosphatidic acid.[22,23] No attempt was made, however, to resolve the phosphatidate-synthesizing system of M1 cells.

Studies on the subcellular distribution of GP-acylating activity in M1 cells have revealed that the 105,000 *g* pellet (microsomal fraction) exhibits the highest specific activity. This finding is in agreement with the previous report indicating the localization of this enzyme activity in the microsomal fraction of various mammalian cells[10] including human platelets.[12] Some properties of the enzyme system from M1 cells have been shown to be similar to those of the enzyme system from other animal tissues. The apparent Michaelis constant of the M1 cell enzyme for GP (0.4 m*M*) approximates that of the enzyme from rat liver[24] (0.5 m*M*), rat brain[25] (0.4 m*M*), and human platelets[12] (0.48 m*M*). Higher concentrations of acyl-CoA thioesters inhibit the enzyme activity of M1 cells, as is the case with those of rat liver,[24] rat brain,[25] and human platelets.[12] The enzyme system from M1 cells has a broad pH optimum as reported also with those from rat liver[24] and human platelets.[12] The principal lipid product produced by the microsomal fraction of M1 cells is phosphatidic acid, but a small amount of lysophosphatidic acid is also formed. The microsomal fraction of other mammalian cells including human platelets was likewise shown to yield phosphatidic acid as the main product.[7,12,24,25]

The level of GP-acylating activity in M1 cells and that in macrophages differentiated from them have been found to be approximately one-half that in mouse liver, while the cultured cells possess approximately activity four times as high as peritoneal macrophages. These results support the view that the glycerophosphate pathway makes an essential contribution to the de novo synthesis of phospholipids in M1 cells as well as in these macrophages.

It has been reported that acetyl-CoA carboxylase is present in leukemic blast cells but not in mature granulocytes.[4] Our studies have clearly demonstrated the presence of acetyl-CoA carboxylase, not only in M1 cells, but also in macrophages differentiated from them. However, peritoneal macrophages exhibit no measurable acetyl-CoA carboxylase activity.

The levels of GP-acylating activity and acetyl-CoA carboxylase activity in M1 cells, i.e., cultured myeloid leukemic cells that grow with a doubling time of about 20 hr, have been shown to be similar to those in macrophages differ-

entiated from them, which have lost both leukemogenicity and mitotic activity.[6] This finding indicates that differentiation of the myeloid leukemic cells is not associated with changes in the activities of these enzymes which are essential to the de novo synthesis of lipids, although the cultured cells exhibit remarkably higher activities than freshly harvested peritoneal macrophages.

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