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Decreased Activity of Acetyl-CoA Carboxylase During Chemically Induced Neutrophilic Differentiation of Human Promyelocytic Leukemia Cells

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In order to better understand the mechanism by which changes in the fatty acid composition of cellular lipids occur in leukemia cell lines induced to differentiate, the activity of the first enzyme of fatty acid biosynthesis, acetyl-CoA carboxylase (EC 6.4.1.2) was measured in HL-60 promyelocytic leukemia cells before, during and after treatment with compounds that induce these cells to mature to neutrophillike cells. After 24 h of exposure to dimethylsulfoxide, retinoic acid, or butyric acid, no morphological or biochemical (nitroblue tetrazolium reduction) evidence of differentiation occurred, but acetyl-CoA carboxylase activity decreased 44, 44.5, and 49% respectively, compared to untreated cells. After 7 days of culture in the presence of these agents, 79, 83, and 72% of cells acquired the ability to reduce nitroblue tetrazolium (versus 15% of control cells) and enzyme activity decreased 92.7, 99.7, and 98%, compared to control cultures, with the three compounds respectively. Thus, some of the reported changes in fatty acid composition of leukemia cells with differentiation may arise, in part, from the depression of the de novo fatty acid biosynthetic pathway and the loss of acetyl-CoA carboxylase activity may be a useful marker for neutrophilic differentiation in HL-60 cells.

Key words: acetyl-CoA carboxylase, HL-60, differentiation, leukemia, fatty acid

Several permanent leukemia cells lines have been studied extensively because of their ability to undergo differentiation to mature, functional, nonproliferative forms after treatment with certain chemical agents. A great number of biochemical changes take place during this process with some alterations seen in most major metabolic pathways of the cell. One class of compounds that has received much attention are the fatty acids [1–3]. In Friend erythroleukemia cells induced to mature with DMSO, palmitoleic acid (16:1) was found to decrease and arachidonic acid (20:4) to increase in total cellular lipid [2]. These same fatty acid changes were also found in HL-60 promyelocytic leukemia cells induced to differentiate with DMSO (3). However, when compounds other than DMSO were used to induce maturation of the Friend

Abbreviations used: DMSO, dimethylsulfoxide; NBT, nitroblue tetrazolium.

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cells, different fatty acid alterations occurred [1], suggesting that the alterations are not simply characteristic of the differentiated or nonproliferative state. Clarification of how the changes occur is of importance because fatty acid composition affects a variety of cellular properties such as membrane fluidity [3], cyclic nucleotide levels [4], and the activities of certain enzymes [5] which are related to the cells' ability to react to external stimuli. Conceivably, then, fatty acid alterations could influence various aspects of the expression of the differentiated phenotype of the cell.

To better understand how the described changes in fatty acid composition take place, we studied the activity of the enzyme acetyl-CoA carboxylase (EC 6.4.1.2), the first enzyme of the de novo fatty acid biosynthetic pathway, in the human promyelocytic cell line HL-60 [6]. These cells normally grow resembling promyelocytes but can be induced to cease dividing and differentiate to neutrophils, based on morphological, biochemical, and functional criteria, by compounds as diverse as DMSO, retinoic acid, and butyric acid [7]. Because acetyl-CoA carboxylase is present in leukemia blasts but not mature neutrophils [8], it appeared likely that this enzyme would undergo a change in activity in HL-60 cells during neutrophilic differentiation. It was the purpose of this study to document if a change in acetyl-CoA carboxylase activity occurs in HL-60 cells during neutrophilic differentiation. This change could then serve as a marker for the proliferative or differentiative status of the cells and might help explain how alterations in the cells' fatty acid composition take place as the cells mature.

METHODS

HL-60 promyelocytic leukemia cells were grown in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Sterile Systems, Logan UT; Lot 100273), 50 IU/ml penicillin, and 50 μg/ml streptomycin (GIBCO) in a humidified atmosphere of 5% carbon dioxide at 37°C. The cells were diluted to an approximate cell density of 2.5×10^5 cells/ml once weekly and fed with an equal volume of medium at midweek. Counts of viable cells were performed in a hemocytometer by the exclusion of trypan blue. Enzyme assays were not performed on cultures with fewer than 80% viable cells.

Reagents

$NaH^{14}CO_2$ was obtained from New England Nuclear (Boston, MA; specific activity, 41.0 mCi/mmol, 1 mCi/ml). Ultrafluor (National Diagnostics, Somerville, NJ) was used as a liquid scintillant. All other reagents were from Sigma.

Induction of Differentiation

HL-60 cells were suspended in fresh medium at a concentration of 5×10^5 cells/ml. DMSO was added neat at a concentration of 1.25% (v/v). All-*trans*-retinoic acid and butyric acid were kept as stock solutions in ethanol and added to the medium such that the final concentration was 1×10^{-6}M for retinoic acid and 5×10^{-4}M for butyric acid and the ethanol concentration was 0.1%.

Assessment of Differentiation

Morphologic differentiation was assessed with cytocentrifuge preparations (Shandon Instruments, Sewickley, PA) stained with Wright-Giemsa. The NBT dye reduction test was performed as described [9]. Because the extents of differentiation

were essentially the same as measured by both techniques, only the NBT results are reported here.

Assay of Acetyl-CoA Carboxylase

The assay is a slight modification of the method described by Shafrir and Bierman [10]. Briefly, 1×10^7 cells were pelleted at 400 g and washed with iced calcium-free phosphate-buffered saline (GIBCO). The pellet was resuspended in 1 ml of a disruption buffer composed of Tris buffer, 50 mM pH 7.5; disodium EDTA, 1 mM; dithiothreitol, 1 mM; sucrose, 250 mM; phenylmethylsulfonyl fluoride, 0.2 mM. The cell suspension was sonicated for 3 min at Power Level 6 in a bath type sonicator (Heat Systems Model W-225R, Plainview, NY) and centrifuged at 100,000 g at 4°C for 1 hr (Beckman Ti 50.1 rotor, 33,000 RPM). One hundred-microliter aliquots of the supernatant were put in 1.5 ml microcentrifuge tubes to which was added 25 μl of an activation mixture composed of Tris buffer, 50 mM, pH 7.5; dithiothreitol, 10 mM; Disodium EDTA, 12.5 mM; magnesium chloride, 100 mM; fatty-acid-poor bovine serum albumin, 10 mg/ml; potassium citrate, 100 mM; and phenylmethylsulfonyl fluoride, 0.2 mM. This was incubated at 37°C for 30 min. Next, 25 μl of a substrate solution was added composed of four parts of the activation mixture and one part of a solution of acetyl CoA, 0.33 mM; equine muscle ATP, 4 mM; and Sodium bicarbonate 1.5 mM in water. Simultaneously, 15 μCi of $NaH^{14}CO_3$ were added and the reaction allowed to proceed at 37° for 30 min. The reaction was terminated by the addition of 100 μl of 5M HCl in ethanol. Unreacted bicarbonate was volatilized by heating the mixture at 85°C until the volume was reduced to approximately 100 μl. This was dissolved in 4 ml of Ultrafluor and counted in a Searle Isocap 300 scintillation counter. A negative control consisting of the reaction mixture without cell extract was subtracted from all values. The protein content of the extracts were assayed with the Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a standard. Results are expressed as picomoles of malonyl-CoA formed per minute per mg protein. Mixing experiments were performed by varying the proportion of the control and treated sample extracts in incremental ratios of volume as protein contents were approximately equal for each extract. All extracts were assayed in duplicate and the results averaged. All inductions were done in duplicate or triplicate.

RESULTS

Induction of Differentiation

As previously reported [7], DMSO, retinoic acid, and butyric acid were all effective inducers of neutrophilic maturation, producing 79, 83, and 72% NBT-reducing cells, respectively, after 7 days of culture (Table I). Untreated cultures contained 15% of cells capable of reducing NBT. Assessment of differentiation by means of differential counts gave essentially the same results (data not shown). No evidence of maturation was seen with these three compounds after 24 hr of incubation.

Acetyl-CoA Carboxylase Activity After Treatment With Differentiation Inducing Agents

Under the assay conditions described, the incorporation of bicarbonate into the acid stable fraction was linear with time for the 30-min incubation period after the addition of the activation mixture. Omission of ATP or acetyl CoA from the activation

TABLE I. Effect of Chemically Induced Neutrophilic Differentiation on Acetyl-CoA Carboxylase Activity*

Compound concentration	Cell count ($\times 10^6$/ml)	NBT positivity (%)	Cell protein (mg/10^7 cells)	Specific activity (pmol/mg/min)	Decrease (%)
None	1.71	15	0.252	442	—
DMSO (1.25% v/v)	0.56	79	0.220	32.3	92.7
Retinoic acid (1×10^{-6}M)	0.41	83	0.150	1.2	99.7
Butyric acid (5×10^{-4}M)	0.88	72	0.207	8.9	98.0

*HL-60 cells were incubated for 7 days with one of several compounds and assayed for NBT dye reduction ability and acetyl-CoA carboxylase activity as described in Methods. Data are averages of duplicate experiments.

mixture reduced isotope incorporation to the level of the negative control. While extracts were analyzed for enzyme activity within days of preparation, the extracts could be stored for several weeks at 4°C or several months at −70°C without loss of enzyme activity.

Cell extracts were prepared from cultures of HL-60 cells left untreated, and at multiple time points from similar cultures treated with one of the three inducing drugs. In no case was a decrease in acetyl-CoA carboxylase activity detectable at 30 min, 1,2,4,6,8, or 12 hr. At 24 hr, however, decreases in enzyme activity 44,44.5 and 49% with respect to control cultures were seen in DMSO-, retinoic-acid-, and butyric-acid-treated cultures, respectively. The degree of inhibition increased steadily to as high as 99.7% in retinoic-acid-treated cultures after 7 days of treatment (Table I, Fig. 1).

To determine the mechanism by which the enzyme activity decreased, mixtures containing varying proportions of extracts derived from induced and control cells were assayed for acetyl-CoA Carboxylase activity. Enzyme activity was graphed versus increasing proportion of induced cell extract. The presence of an inhibitor of enzyme activity in the induced cell extract would result in a nonlinear, concave plot because mixtures of induced and uninduced extracts would demonstrate less enzyme activity than would be predicted from their individual activities. Linear plots ($r = -0.97$) were obtained for all three inducers at 24 hr (data not shown). However, a minor concavity of the plots was seen at 7 days with cells exposed to retinoic ($r = -0.90$) and butyric ($r = -0.94$) acids whereas that of DMSO-treated cells ($r = -0.97$) remained linear (Fig. 2). These data are compatible with the presence of an inhibitor of enzyme activity that may be formed after 7 days of culture with some inducing drugs.

DISCUSSION

These data demonstrate that acetyl-CoA carboxylase activity decreases to low levels as HL-60 cells terminally differentiate to neutrophillike cells. The small residual activity seen likely represents enzyme activity in the minor undifferentiated population (17–28% NBT-negative cells, as in Table I). As the percentage decrease in enzyme activity exceeds the percentage of cells that reduce NBT, both at 1,3, and 7 days of incubation, it appears that the enzyme activity decreases earlier in the

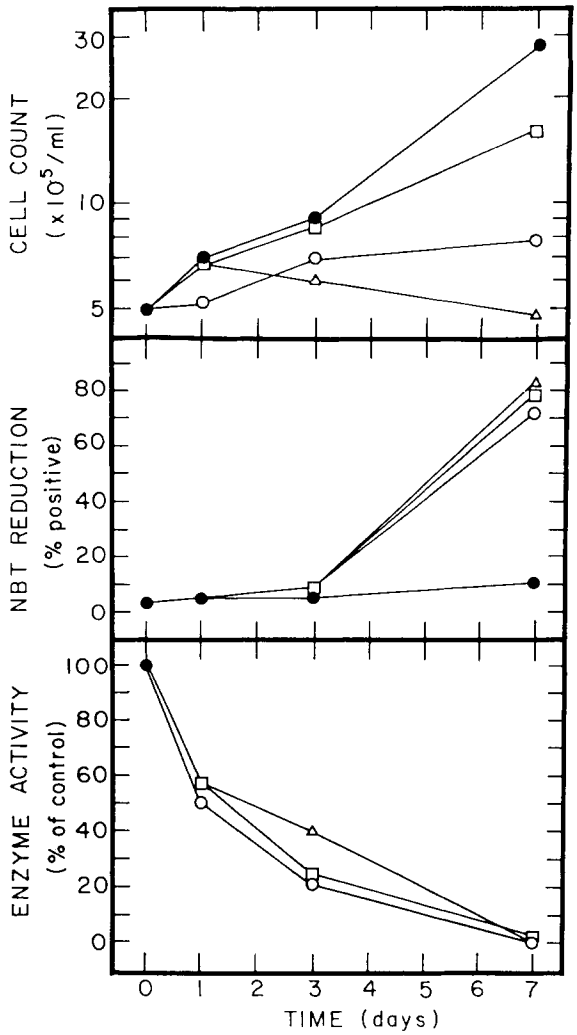

Fig. 1. HL-60 cells were cultured at an initial concentration of 5×10^5 cells/ml in the presence of DMSO 1.25% (squares), butyric acid 5×10^{-5}M (open circles), retinoic acid 1×10^{-6}M (triangles), or no drug (closed circles). Aliquots from duplicate flasks were removed at the beginning and at days 1,3, and of 7 incubation. Viable cell counts, NBT dye reduction test, and acetyl-CoA carboxylase assays were performed and the averages shown. Enzyme activities are expressed as a percentage of the day 0 control (untreated) cell extract; there was no significant change in the absolute enzyme activity of untreated cultures over the 7-day incubation.

differentiative process than the acquisition of the ability to reduce NBT or the occurrence of morphologic changes typical of the mature neutrophil.

At the 24-hr time point, the treated cells have lost approximately half of their enzyme activity and a linear plot of enzyme activity versus the ratio of extract derived from control and treated cells was seen. This is consistent with either a simple decrease in enzyme protein or a regulatory modification of the enzyme such as phosphorylation [11]. At 7 days of incubation, the attenuation of enzyme activity is virtually complete, especially with retinoic acid. Curvilinear mixing plots were

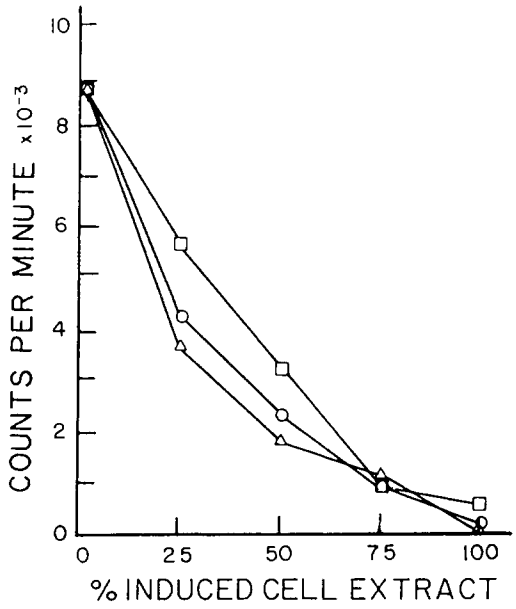

Fig. 2. Extracts of cells treated for 7 days with DMSO (squares), butyric acid (circles), or retinoic acid (triangles) were mixed with extracts of untreated cells in various volume proportions. The acetyl-CoA carboxylase activity of these mixtures (expressed as counts per minute) is plotted versus the proportion of treated cell extract in the mixture (100% = extract from treated cells only).

demonstrated for retinoic-acid- and butyric-acid-treated cells, but not for DMSO-treated ones. While this is consistent with the formation of an inhibitor such as a long chain fatty acid-CoA ester [12] or a protein [13] in the retinoic-acid- and butyric-acid-induced cells, the small magnitude of the deviation from linearity suggests that the predominant mechanism for decrease in enzyme activity is further loss of enzyme protein and/or regulatory modification of the enzyme.

Thus, the loss of acetyl-CoA carboxylase appears to be a marker for terminal differentiation of HL-60 cells when the cells are induced to mature to neutrophillike cells. As inducing agents of three different chemical classes gave similar results, it is unlikely that the decrease is due to a peculiar chemical effect of one of the drugs. The measurement of acetyl-CoA carboxylase levels should be useful in future studies of the biochemical events associated with chemically induced differentiation in leukemia cell lines, especially as the decrease in enzyme activity occurs quite early, even before morphologic evidence of maturation has occurred. This suggests that the loss of acetyl-CoA carboxylase activity may be linked to an early biochemical event related to the cell's decision to differentiate.

In addition, studies that have shown alterations in cellular fatty acid composition occurring with differentiation of leukemia cell lines need to be interpreted with care, as mature HL-60 cells, as opposed to undifferentiated ones, can synthesize new lipids only by chain elongation or by the direct incorporation of precursors from the medium. Both of these pathways have also been shown to be active in mature polymorphonuclear neutrophils [14,15]. The fatty acid content of differentiated HL-60 cells is thus likely to be influenced by the lipids of the fetal calf serum in the culture medium. Furthermore, the agents used to induce maturation appear to have

additional specific biochemical effects which can influence fatty acid incorporation. Therefore, the changes previously reported in the fatty acid composition of leukemia cell lines induced to mature are likely to have resulted from a variety of processes, particularly the suppression of the de novo fatty acid synthetic pathway resulting from the differentiative process itself.

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