Cholesterol Starvation Induces Differentiation of Human Leukemia HL-60 Cells

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Abstract
Cholesterol metabolism is particularly active in malignant, proliferative cells, whereas cholesterol starvation has been shown to inhibit cell proliferation. Inhibition of enzymes involved in cholesterol biosynthesis at steps before the formation of 7-dehydrocholesterol has been shown to selectively affect cell cycle progression from G2 phase in human promyelocytic HL-60 cells. In the present work, we explored whether cholesterol starvation by culture in cholesterol-free medium and treatment with different distal cholesterol biosynthesis inhibitors induces differentiation of HL-60 cells. Treatment with SKF 104976, an inhibitor of lanosterol 14-α demethylase, or with zaragozic acid, which inhibits squalene synthase, caused morphologic changes alongside respiratory burst activity and expression of cluster of differentiation antigen 11c (CD11c) but not cluster of differentiation antigen 14. These effects were comparable to those produced by all-trans retinoic acid, which induces HL-60 cells to differentiate following a granulocyte lineage. In contrast, they differed from those produced by vitamin D3, which promotes monocye differentiation. The specificity of the response was confirmed by addition of cholesterol to the culture medium. Treatment with PD 98059, an inhibitor of extracellular signal-regulated kinase, abolished both the activation of NADPH oxidase and the expression of the CD11c marker. In sharp contrast, BM 15766, which inhibits sterol Δ7-reductase, failed to induce differentiation or arrest cell proliferation. These results show that changes in the sterol composition may trigger a differentiation response and highlight the potential of cholesterol pathway inhibition as a possible tool for use in cancer therapy. [Cancer Res 2007;67(7):3379–86]

Introduction
Cholesterol homeostasis is abnormal in malignant cells. Freshly isolated acute myeloid leukemia (AML) and chronic myelogenous leukemia cells display much higher specific low-density lipoprotein uptake and degradation (1–3) and hydroxymethylglutaryl (HMG)-CoA reductase activity (2) than leukocytes from healthy subjects. A recent study by Banker et al. (4) reported that the levels of mRNAs for the low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) are increased by daunorubicin or cytarabine treatment in almost all cultured AML samples, an effect which may increase leukemia cell survival and impart relative resistance to therapy.

Statins, which block cholesterol biosynthesis through competitive inhibition of HMG-CoA reductase, the rate-limiting enzyme in the pathway, have been shown to inhibit cell proliferation and to have several effects that are of interest in relation to cancer prevention, including effects on cell differentiation (5, 6). However, the effects of inhibiting cholesterol biosynthesis are dependent on the model used. In both neuroblastoma cells (7) and AML cells (8), lovastatin induces widespread differentiation followed by extensive apoptosis. In human malignant glioma cell lines and untransformed rat astrocytes, lovastatin also causes effective apoptosis but does not induce differentiation (9). Treatment with statins has been also shown to sensitize AML cells to radiochemotherapy, suggesting that cellular cholesterol is critical for cell survival (10). Acute lymphoid leukemias, however, seem to be resistant to statins (11). Phenylacetate, an inhibitor of mevalonate-5-phosphate decarboxylase, has also been reported to inhibit cell growth in a variety of cell lines but data on its effects on cell differentiation are inconclusive (9, 12).

Administration of relatively high doses of statins to rodents has been shown to reduce the growth of various types of tumor (13, 14). Some retrospective studies of patients receiving statins as lipid-lowering drugs for the prevention of cardiac events showed a slight reduction in the incidence of breast cancer (15) and colorectal cancer (16). However, some other clinical studies (17–19) and two recent meta-analyses (20, 21) found no evidence that the use of statins reduces the risk of cancer. A recent phase II trial also failed to reveal any relevant activity of phenylacetate against malignant glioma (22).

Both statins and phenylacetate act very early in the cholesterol pathway, inhibiting the synthesis of both sterols and nonsterol mevalonate derivatives. These different mevalonate derivatives exert many distinct biological actions, with important physiologic implications. Cholesterol is used for membrane formation and is a principal component of lipid rafts and caveolae, in which many receptors for extracellular signals reside (23). Nonsterol isoprenoids are precursors of ubiquinone and heme A and are used for protein prenylation, which allows certain proteins to localize to membranes where they exert specific functions (24). Thus, it is not surprising that blockade of their synthesis results in the alteration of multiple processes. Furthermore, differences in the sensitivity to these inhibitors and/or the demand for nonsterol isoprenoid derivatives among cell types may explain the variability of the results obtained. The profound consequences of these various effects are highlighted in the results of previous studies from our laboratory showing that different inhibitors of cholesterol biosynthesis, acting on distinct enzymes, have different effects on cell cycle progression (25–28).
Promyelocytic HL-60 cells represent a useful model in which to study myeloid cell differentiation. Treatment with all-trans retinoic acid (RA) or DMSO has been shown to cause HL-60 to differentiate into granulocytes (29). In contrast, treatment with vitamin D₃ induces differentiation into monocytes (30).

Exposure of HL-60 cells to agents that cause granulocyte differentiation is accompanied by a rapid decrease in sterol synthesis, whereas this effect is not observed with factors that cause monocyte or macrophage differentiation (31, 32). This is consistent with the observation that peripheral blood granulocytes almost entirely lack the ability to synthesize post-squalene products, whereas monocytes possess an active cholesterol biosynthesis pathway (32). However, it is unclear whether cholesterol biosynthesis is directly involved in myeloid differentiation.

In this study, we examined the effects of cholesterol starvation on differentiation of HL-60 cells. We found that distal inhibition of cholesterol biosynthesis by treatment with either SKF 104976, an inhibitor of lanosterol 14α-demethylase, or zaragozic acid, an inhibitor of squalene synthase, specifically induces respiratory burst activity and the expression of cluster of differentiation antigen 11c (CD11c) but not cluster of differentiation antigen 14 (CD14). These effects were mediated by the mitogen-activated protein kinase (MAPK) pathway. In contrast, BM 15766, which inhibits sterol Δ²-reductase, failed to induce differentiation. Our results show that changes in sterol composition may trigger a differentiation response and highlight the potential of cholesterol pathway inhibition as a possible tool in cancer therapy.

Materials and Methods

**Materials.** 2-[2-Amino-3methoxyphenyl]-4f-1-benzopyran-4-one (PD 98059), phorbol 12-myristate 13-acetate (PMA), and all-trans RA were purchased from Sigma Chemical (St. Louis, MO) and 5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) was from Molecular Probes (Eugene, OR). Cholesterol was from Steraloids, Inc. (Newport, RI) and 1α,25-dihydroxyvitamin D₃ (D₃) was from Fluka (Buchs, Aargau, Switzerland). Cholesterol biosynthesis inhibitors were gifts from the following laboratories: SKF 104976 from SmithKline Beecham Pharmaceuticals (Harlow, Essex CM19 5AW, United Kingdom), and BM 15766 from Beecham Pharmaceuticals (Harlow, Essex CM19 5AW, United Kingdom), were gifts from the following laboratories: SKF 104976 from SmithKline Beecham Pharmaceuticals (Harlow, Essex CM19 5AW, United Kingdom), and BM 15766 from Beecham Pharmaceuticals (Harlow, Essex CM19 5AW, United Kingdom).

**Materials and Methods.** The respiratory burst was detected by oxidation of the fluorescent probe CM-H₂DCFDA, as described elsewhere (33). Cells (5 × 10⁵) were harvested by centrifugation at 1,500 rpm for 5 min, washed with PBS, and resuspended in 100 μL of fresh culture medium. Cells were then incubated for 2 h with 10 μmol/L CM-H₂DCFDA and 100 ng/mL PMA (activated) or PBS (resting) at 37°C in the dark. Fresh culture medium (400 μL) was added after acquisition with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed with the WinMDI 2.8 software (Build #13 01-19-2000, "Copyright Joseph Trotter").

**CD marker expression.** To detect cell-surface markers by flow cytometry, 5 × 10⁵ cells were harvested by centrifugation at 1,500 rpm for 5 min, washed with PBS, and resuspended in 100 μL of fresh culture medium. Then, cells were incubated for 15 min in the dark at room temperature with 10 μL of phycoerythrin-labeled CD14 and 5 μL of allophycocyanin-labeled CD11c antibodies. The cells were washed twice with PBS and resuspended in 0.5 mL of 1% paraformaldehyde, then analyzed by flow cytometry. For each sample, separate aliquots of cells were treated with PBS instead of antibodies to determine the fluorescence relative to unlabeled controls.

**Western blotting.** For Western blot analysis, 4 × 10⁵ to 8 × 10⁵ cells were harvested and washed twice with ice-cold PBS, and whole-cell extracts were prepared by mixing the cell pellets with an extraction buffer [50 mmol/L Tris-HCl (pH 7.5), 125 mmol/L NaCl, 1% NP40, 0.225 mg/ml Nao/Na₃/Na₂O₄, 1%glycerol,1mol/LNa₂EDTA, 5 μL/mL Calbiochem Proteases Inhibitor Cocktail Set III].

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then washed with ice-cold PBS and resuspended in 0.5 mL of 10% (w/v) KOH. δH-Cholesterol was added as an internal standard. The samples were treated sequentially with chloroform-methanol (2:1, v/v) and distilled water to obtain the lipid and water-soluble fractions. Then, the nonsaponifiable fractions were obtained and used for sterol separation by reverse-phase high-performance liquid chromatography as described elsewhere (25, 34).

Statistical analysis. All experiments were done at least thrice. Data are shown as mean ± SE. Effects of the different treatments were analyzed by one-way ANOVA and post hoc multiple comparisons were done with Tukey’s test. Calculations were done using Statgraphics Plus v5.0 software (Statistical Graphics, Herndon, VA).

Results

Induction of oxidative burst via inhibition of lanosterol demethylase. To reduce the cell cholesterol content, HL-60 cells were incubated in a defined medium lacking cholesterol and were treated with SKF 104976, a specific inhibitor of lanosterol 14α-demethylase. This treatment led to a marked decrease in cholesterol content (1.18 ± 0.1 versus 6.01 ± 0.4 µg/mg of cell protein in treated and control cells, respectively). NADPH oxidase activity was analyzed as a functional marker of differentiation in human myeloid cells lines. Cells were stimulated with PMA to activate NADPH oxidase and incubated in the presence of CM-H2DCFDA. CM-H2DCFDA passively diffuses into cells and, following cleavage of acetate groups by intracellular esterases, oxidation by superoxide anion/hydrogen peroxide yields a fluorescent adduct that is trapped inside the cell, facilitating its detection by flow cytometry (33, 35). Cells treated for 5 days with 1 µmol/L RA or 1 µmol/L D3, which cause differentiation of HL-60 cells to granulocytes and monocytes, respectively, were used as positive controls. Differentiation induced by any of the agents resulted in intense CM-H2DCFDA oxidation when compared with undifferentiated cells (Fig. 1A). Treatment with SKF 104976 also increased respiratory burst activity in a time- and dose-dependent manner. The maximum effect was achieved with 1.5 µmol/L SKF 104976 at 4 days; under those conditions, the mean fluorescence was even higher than with either RA or D3 (Fig. 1B and C).

Expression of the regulatory subunits of NADPH oxidase, p47phox and p67phox, was analyzed by Western blot. As shown in Fig. 1D, the proteins were barely detected in undifferentiated HL-60 and their expression rapidly increased as a result of treatment with 1.5 µmol/L SKF 104976.

Morphology and CD marker expression in HL-60 cells treated with SKF 104976. The morphology of HL-60 cells treated with SKF 104976 compared with control cells and cells induced to differentiate with D3 or RA is shown in Fig. 2A. Undifferentiated HL-60 cells were round and had unsegmented nuclei that occupied >75% of the cross-sectional area of the cell. RA-treated cells had bilobed and segmented nuclei, comparable to those found in metamyelocytes and banded neutrophils, and were relatively regular in shape. D3-treated cells had a reduced nucleus-to-cytoplasm ratio. Moreover, the nuclei assumed the reffrom

Figure 1. NADPH oxidase activity in differentiated HL-60 cells. Flow cytometry histograms showing CM-H2DCFDA (DCF) fluorescence from resting (open) and PMA-stimulated (shaded) HL-60 cells. Numbers indicate the geometric mean fluorescence of a representative experiment. A, effect of treatment with 1 µmol/L all-trans RA (RA) or 1 µmol/L D3 for 5 d. Untreated cells were used as control. B, effect of incubation time in cells treated with 1.5 µmol/L SKF 104976. C, effect of the indicated doses of SKF 104976 (SKF) in cells treated for 3 d. D, Western blot analysis of whole-cell extracts from cells treated with 1.5 µmol/L SKF 104976 for 3 d. p47phox and p67phox expression is shown in the same immunoblot (top) and GAPDH was visualized as a loading control (bottom).
appearance typical of monocytes. Preparations of cells treated with 1.5 μmol/L SKF 104976 for 3 or 5 days were more heterogeneous. A subset of cells were binucleated whereas those cells that were mononucleated were smaller than the undifferentiated, control cells. Moreover, some cells were multinucleated and mitotic aberrations were observed.

Expression of CD11c and CD14 on the cell surface is also shown in Fig. 1. As expected, most of the undifferentiated HL-60 cells did not express any of these antigens. In contrast, most of the cells induced to differentiate to granulocytes with RA expressed CD11c, whereas those that differentiated to monocytes using D3 expressed both CD11c and, characteristically, CD14 (Fig. 2B). These results are consistent with those of previous reports (36, 37). Treatment with 1.5 μmol/L SKF 104976 was accompanied by a progressive increase in the proportion of cells expressing CD11c, which accounted for ~60% of the total at day 4 of treatment (Fig. 2C). This effect of SKF 104976 on CD11c expression was dose dependent (Fig. 2D). In contrast, treatment with SKF 104976 did not produce any detectable change in CD14 expression (Fig. 2C and D).

Prevention of SKF 104976–induced cell differentiation by cholesterol. Having shown that cholesterol starvation induced HL-60 cell differentiation, we analyzed the specificity of this effect by supplementing the medium with free cholesterol (Table 1). In control cells, supplementing the medium with 90 μg/mL cholesterol had no apparent effect on respiratory burst activity or cell-surface CD expression. Simultaneous addition of cholesterol and 1.5 μmol/L SKF 104976 for 3 days practically abrogated the effects of the cholesterol biosynthesis inhibitor alone on both cell-surface expression of CD11c and respiratory burst activity. These results show that cholesterol deficiency specifically induces differentiation of human promyelocytic HL-60 cells.

Effects of other inhibitors of cholesterol biosynthesis on differentiation of HL-60 cells. Zaragozic acid is a potent inhibitor of squalene synthase (38). Treatment of HL-60 cells with 60 μmol/L zaragozic acid for 3 days resulted in a notable reduction of the cell cholesterol content (1.46 ± 0.2 versus 6.01 ± 0.4 μg/mg of cell protein in treated and control cells, respectively). As recently shown in our laboratory, this treatment leads to inhibition of cell proliferation and cell cycle arrest at G2-M phase, similarly to SKF 104976 (27). We therefore assessed whether cell deprivation of any sterol is a stimulus for HL-60 cell differentiation.

Treatment of HL-60 cells with zaragozic acid in medium lacking cholesterol resulted in stimulation of respiratory burst in a time- and dose-dependent fashion (Fig. 3A and B, respectively) and induced cell-surface expression of CD11c but not CD14 (Fig. 3C).
A gradual induction of p47phox and p67phox proteins was observed in cells treated with zaragozic acid (Fig. 3D). The effects closely resembled those of SKF 104976. Moreover, the effects of zaragozic acid on both oxidative activity (Fig. 4A) and expression of CD11c (Fig. 4B) were prevented by simultaneous addition of free cholesterol to the incubation medium. These results indicate that the effects of zaragozic acid are due to the cholesterol deficiency it produces.

We then analyzed the effects of other more distal inhibitors of the cholesterol biosynthesis pathway. BM 15766 is a competitive inhibitor of sterol D7-reductase, and treatment of HL-60 cells with 25 μmol/L BM 15766 has been shown to block [14C]acetate incorporation into cholesterol, with a reduction in cell cholesterol content but an accumulation of 7-dehydrocholesterol (27). In the present study, treatment of HL-60 cells with BM 15766 for up to 5 days did not result in any appreciable increase in respiratory burst activity or the expression of CD markers (data not shown).

**The ERK pathway is required for differentiation of HL-60 cells induced by cholesterol starvation.** The ERK pathway is known to be involved in myeloid cell differentiation (39). Analysis of ERK protein expression in HL-60 cells revealed that treatment with SKF 104976 was accompanied by a rapid and marked increase in the cell levels of phosphorylated ERK without apparent changes in total ERK (Fig. 5A). To ascertain whether the ERK pathway is involved in cell differentiation induced by cholesterol starvation, HL-60 cells were treated with increasing concentrations of SKF 104976 and 30 μmol/L PD 98059, a specific inhibitor of MAPK/ERK kinase (MEK). As shown in Fig. 5B, PD 98059 totally prevented the expression of CD11c. This finding indicates that active ERK is required for differentiation induced by SKF 104976.

**Discussion**

Induction of a differentiation response in malignant cells can have positive clinical implications, such as the loss of proliferative potential and the induction of apoptosis (8, 11). In previous studies, we showed that inhibition of cholesterol biosynthesis in HL-60 cells resulted in the arrest of cell cycle progression (25–28). In this study, we have shown that inhibition of cholesterol biosynthesis induces differentiation in promyelocytic HL-60 cells, as indicated by morphologic changes, gradual induction of p47phox and p67phox protein expression, increased NADPH oxidase activity, and the expression of specific cell-surface markers.

To deprive cells of cholesterol, they were incubated in a cholesterol-free medium and treated with zaragozic acid, SKF 104976, or BM 15766, which inhibit squalene synthase, lanosterol

### Table 1. Abrogation of SKF 104976–induced HL-60 cell differentiation by cholesterol

<table>
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<tr>
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<th>ΔDCF (n = 4)</th>
<th>%CD11c (n = 3)</th>
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<tbody>
<tr>
<td>Control</td>
<td>25.0 ± 12.4a</td>
<td>8.44 ± 0.90a</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>6.8 ± 7.6a</td>
<td>6.33 ± 0.96a</td>
</tr>
<tr>
<td>SKF 104976</td>
<td>172.5 ± 31.7b</td>
<td>59.9 ± 5.86b</td>
</tr>
<tr>
<td>SKF 104976 + cholesterol</td>
<td>36.6 ± 2.9a</td>
<td>12.7 ± 0.60a</td>
</tr>
<tr>
<td>P (ANOVA)</td>
<td>&lt;0.001</td>
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**NOTE:** Data are shown as mean ± SE. HL-60 cells were treated with vehicle (control), 1.5 μmol/L SKF 104976, or 90 μg/mL cholesterol for 3 d. Effects of the different treatments were analyzed by one-way ANOVA and post hoc multiple comparisons were done with Tukey's test. Groups in the same column that do not share a superscript letter are statistically different (P < 0.05). ΔDCF, difference of the medians of CM-H2DCFDA fluorescence between PMA-stimulated and resting cells. %CD11c, percentage of cells expressing the CD11c antigen.
14-α-demethylase, and sterol Δ⁷-reductase, respectively. These inhibitors caused a reduction in cell cholesterol content that was accompanied by accumulation of the substrates of the respective target enzymes: lanosterol and dihydrolanosterol in cells treated with SKF 104976 and 7-dehydrocholesterol in cells treated with BM 15766; no intermediate sterol accumulated in cells treated with zaragozic acid (27). Treatment with SKF 104976 or zaragozic acid induced extensive differentiation of HL-60 cells. In both cases, this effect was specific because it was prevented by adding cholesterol to the incubation medium. In sharp contrast, cells treated with BM 15766 did not show any signs of differentiation. These findings correlated with the differential action of these cholesterol biosynthesis inhibitors on cell growth: SKF 104976 and zaragozic acid have been reported to arrest cell cycle progression and cell growth, whereas BM 15766 was ineffective (27). In light of the requirement for cholesterol during cytokinesis (40, 41), these results indicate that 7-dehydrocholesterol, which accumulates in BM 15766-treated cells, may substitute cholesterol for cell division, whereas lanosterol and dihydrolanosterol, which accumulate in cells treated with SKF 104976, may not (27). The similarity between the effects of cholesterol biosynthesis inhibition on cell proliferation and cell differentiation is consistent with the suggestion that these two processes are regulated simultaneously (42, 43).
The differentiation program induced by cholesterol starvation differs from that induced by either RA or D3. Cells treated with SKF 104976 were morphologically heterogeneous; binucleated cells were abundant and many of them showed evidence of abnormal mitosis. In addition, the mononucleated cells present in SKF 104976–treated preparations were smaller than control, undifferentiated cells. This complex phenotype contrasted with those 104976–treated preparations were smaller than control, undifferentiated cells. This complex phenotype contrasted with those in control preparations, which were predominantly mononucleated and had a typical phenotype of RA–induced differentiation. In contrast, cells treated with SKF 104976 were morphologically heterogeneous; binucleated cells were abundant and many of them showed evidence of abnormal mitosis. This complex phenotype contrasted with those in control preparations, which were predominantly mononucleated and had a typical phenotype of RA–induced differentiation.

Analysis of the effects of inhibiting the mevalonate pathway on differentiation has shown that treatment with statins induces a granulocyte-type differentiation in AML and not ALL cells, as indicated by morphologic changes and increased expression of CD11b or cluster of differentiation antigen 18 (CD18; ref. 8). In keratinocytes, cholesterol depletion by treatment with a combination of lovastatin and methyl-β-cyclodextrin produced a strong upregulation of mRNA for involucrin, a marker of epidermal differentiation (44). On the other hand, patients with acute myelogenous leukemia, especially those with monocytic or myelomonocytic leukemia, frequently display hypocholesteroolemia in association with increased low-density lipoprotein receptor activity in malignant cells (45). This effect is presumably due to the increased demand for cholesterol associated with cell proliferation (46). Taken together, these results suggest that blocking cholesterol provision by inhibition of cholesterol biosynthesis may be of interest as a cancer therapy due to its effects on both cell proliferation and differentiation.

The MEK/ERK/MAPK signaling pathway has been shown to play a critical role during both monocyte and granulocyte differentiation of HL-60 cells stimulated with PMA and RA, respectively (39). In this study, we have shown that treatment of HL-60 cells with SKF 104976 or zaragocic acid produces sustained activation of ERK1/2 and that coinubcation with PD 98059 abrogated differentiation. This pathway is also known to be required for neuronal differentiation in rat PC12 cells (47), maturation of thymocytes from CD4+ CD8− to CD4+CD8+ cells (48), and adipogenesis (49). Taken together, these results firmly indicate the universal role of the MEK/ERK/MAPK signaling pathway in cell differentiation.

In summary, the results of this study indicate that cholesterol starvation may lead to myeloid differentiation. Furthermore, our observation of distinct responses to different cholesterol biosynthesis inhibitors that reduce the cell cholesterol content to a similar extent suggests that differentiation is triggered by specific changes in the sterol composition of the cells. Thus, modulation of the expression of the different enzymes involved in cholesterol biosynthesis may have a role in the differentiation process.

Acknowledgments

Received 11/7/2006; revised 1/4/2007; accepted 1/24/2007.

Grant support: Ministerio de Educación y Ciencia, Spain, grants AGL2004-07075-C02-01 and SAF2005-7308 (M.A. Lasuncio); Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Spain, CIBER CB06/0021 (M.A. Lasuncio).

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We thank Dr. Beatriz Ledo for her assistance in the laboratory and K. Patton for editorial advice.

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