Cholesterol-modulating agents kill acute myeloid leukemia cells and sensitize them to therapeutics by blocking adaptive cholesterol responses

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The mevalonate pathway produces many critical substances in cells, including sterols essential for membrane structure and isoprenoids vital to the function of many membrane proteins. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a rate-limiting enzyme in the mevalonate pathway. Because cholesterol is a product of this pathway, HMG-CoA reductase inhibitors (statins) are used to treat hypercholesterolemia. Statins are also toxic to several malignancies, including acute myeloid leukemia (AML). Although this toxicity has been attributed to the inhibition of Ras/Rho isoprenylation, we have previously shown that statin toxicity in primary AML cells (AMLs) does not correlate with Ras isoprenylation or with activating Ras mutations. In other studies, we have shown that hypoxic and oxidant injuries induce cholesterol increments in renal tubule cells and that statins sensitize these cells to injury by blocking protective cholesterol responses. We now demonstrate that exposing particular AMLs to radiochemotherapy induces much greater cellular cholesterol increments than those seen in similarly treated normal bone marrow. Treatment of these AMLs with mevastatin or zaragozic acid (which inhibits cholesterol synthesis but not isoprenoid synthesis) attenuates the cholesterol increments and sensitizes cells to radiochemotherapy. The extent of toxicity is affected by the availability of extracellular lipoproteins, further suggesting that cellular cholesterol is critical to cell survival in particular AMLs. Because zaragozic acid does not inhibit isoprenoid synthesis, these data suggest that cholesterol modulation is an important mechanism whereby statins exert toxic effects on some AMLs and that cholesterol modulators may improve therapeutic ratios in AML by impacting cholesterol-dependent cytoreistance. (Blood. 2003;101:3628-3634)

Introduction

Cholesterol is a biosynthetic end product of the mevalonate pathway in eukaryotes, as are many other compounds, including steroid hormones, vitamin D, dolichol, and ubiquinone. In higher animals, cells can also obtain dietary cholesterol from plasma low-density lipoprotein (LDL) through receptor-mediated endocytosis and lysosomal processing. Cells control the importation and biosynthesis of cholesterol by feedback regulation of LDL receptors and of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the mevalonate pathway (reviewed by Goldstein and Brown1). When LDL levels are high, HMG-CoA reductase activity in normal cells is reduced by as much as 90%, and cholesterol synthesis is effectively blocked.1 When cellular sterol levels are high, or when proliferation and the need for cholesterol are low, LDL receptor transcription is repressed. On the other hand, when acutely deprived of cholesterol, normal cells increase sterol synthesis and LDL import. Numerous studies have demonstrated that abnormally elevated levels of serum cholesterol contribute to atherosclerosis and coronary artery disease, and HMG-CoA reductase inhibitors (“statins”), including pravastatin, simvastatin, lovastatin, and atorvastatin, are widely used clinically to treat patients with hypercholesterolemia.2

Cholesterol metabolism is dysregulated in many malignancies, including myeloid leukemias. Freshly isolated acute myeloid leukemia (AML) and chronic myelogenous leukemia cells show more LDL processing than normal blood cells,3,4 and cholesterol levels in leukemia cells often do not exhibit feedback repression in high-sterol media.4 Rapidly proliferating tumor cells presumably require cholesterol for new membrane synthesis. However, high cellular cholesterol may also improve leukemia cell survival and impart relative resistance to therapy. Compared with their parental cell lines, drug-resistant myeloid leukemia cell lines show higher HMG-CoA reductase levels,5 and drug-resistant lymphoid leukemia cell lines have increased cholesterol and phospholipid levels,6 suggesting that acute cholesterol responses may contribute to drug resistance. Consistent with a critical role(s) for the mevalonate pathway in tumor cell growth and survival, and with the rate-limiting role of HMG-CoA reductase in this synthetic pathway, statins have in vivo or in vitro activity against human head and neck, pancreatic, and central nervous system tumors and against human myeloid leukemia cells in xenograft models.7-11 A phase 1 clinical study showed that serum lovastatin concentrations of up to 3.9 μM could be achieved without significant tissue toxicity.12 Dimitroulakos et al showed that similar doses of lovastatin were selectively toxic to AML cell lines and primary AML cell samples but not to acute lymphocytic leukemias.13 We have shown that most AMLs are sensitive to mevastatin as a single

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Supported by a Leukemia and Lymphoma Society Chuck Griffin Memorial Translation Research Award (D.E.B.) and National Institutes of Health grants U01-CA32102 (Southwest Oncology Group/C.L.W.), CA18029 (F.R.A.), DK38432 and DKS4200 (R.A.Z.), U10-CA13539 (Children’s Cancer Group AML Reference Laboratory), and T32-HL07093 (H.Y.L.).

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agent and in drug combinations, further suggesting that statins might be effective in anti-AML regimens.

Plasma membrane localization is essential to the function of many proteins that impact cellular proliferation and/or survival. Because the membrane localization of many of these signaling proteins requires mevalonate-derived farnesyl or geranylgeranyl isoprenoid modifications, the antitumor effects of statins have been assumed to be primarily due to their blockade of isoprenylation. For example, Ras proteins are activated by mutation and/or overexpression in a large fraction of human tumors, including myeloid leukemias, thereby contributing to reduced apoptosis, increased cell proliferation, and poor clinical outcomes. Isoprenylation of Ras and other similarly modified membrane proteins is specifically mediated by farnesyl-protein and geranylgeranyl-protein transferases, and farnesyl-protein transferase inhibitors can, like statins, induce tumor regression in mice carrying mammary or lymphoid tumors overexpressing H- or N-Ras. However, we have recently shown that statin toxicity in primary AMLs is variable and not consistently higher in samples with Ras overexpression or with Ras activated by “hotspot” mutations.

Other have argued that non-Ras geranylgeranylated proteins are important statin effectors. However, our cell line data show that dependence on geranylgeranylation as well as farnesylation can be dissociated from statin sensitivity in AMLs. In addition, statin concentrations necessary to inhibit specific protein isoprenylation are 100- to 500-fold higher than those required to inhibit cholesterol synthesis in the same cells and are higher than the concentrations that are toxic to AML cells.

In other studies, we have shown that acute renal injuries caused by ischemia, oxidative damage, and ureteral obstruction increase renal cortical cholesterol content. Cholesterol levels are similarly increased in cultured human HK-2 proximal renal tubule cells subjected to iron-mediated oxidant stress. In these kidney cell models, increased cellular cholesterol makes cells highly resistant to subsequent damage, producing a state known as “acquired cytorresistance.” Reducing cellular cholesterol levels pharmacologically blocks acquisition of cytorresistance and can generate profound mitochondrial dysfunction and death in kidney cells, further demonstrating the critical role of cholesterol in renal cell homeostasis.

Given these observations, we have undertaken the present study to assess the potential role of cellular cholesterol in protecting AMLs against radiochemotherapeutic insults. Toward this end, several AML cell lines and cryopreserved primary AML cell samples were treated with chemotherapy, radiation, or cholesterol modulators, alone or in combination. The impacts of these treatments on cell survival and cellular cholesterol content were then assessed.

Materials and methods

Cell culture

Human AML cells of the NB4, HL60, ML-1, and KG1a lines were grown in RPMI 1640 media supplemented with 5% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT) (NB4, KG1a) or in Iscove media supplemented with 10% heat-inactivated cosmic calf serum (Hyclone) (HL60, ML-1). The doubling times of these cell lines ranged from 20 to 28 hours. Normal bone marrow samples were obtained from the Fred Hutchinson Cancer Research Center Center Cryobiology Laboratory (Seattle, WA). Cryopreserved primary bone marrow cell samples from AML patients with increased myeloblast fractions were obtained from the Southwest Oncology Group (SWOG) cell repository (Albuquerque, NM) and the Children’s Cancer Group (CCG) AML Reference Laboratory (Seattle, WA). Normal and AML primary cell samples contained approximately 10^7 cells per sample and thawed with 33% to 98% viability into Iscove media supplemented with 20% BCS, interleukin-3 at 50 ng/mL (Biosource, Camarillo, CA), and stem cell factor/kit ligand at 100 ng/mL (Amgen, Thousand Oaks, CA). Samples were then cultured for up to 48 hours. Cells were treated for 24 or 48 hours with cytarabine (ARA-C; Sigma, St Louis, MO), daunorubicin (DNr; ICN Biomedicals, Aurora, OH), mevastatin (Sigma), or zaragold acid A (gift from Merck Research Laboratories, Rahway, NJ), alone or in combination. In some assays, cells were cultured in serum-depleted media (RPMI 1640 supplemented with 0.2% BCS) for 24 or 48 hours in the presence or absence of exogenously added low-density lipoproteins (LDLs; more than 95% purity per electrophoretic analysis by the manufacturer) (Calbiochem, San Diego, CA). In other assays, cells were treated with cholesterol esterase (Sigma) or cholesterol oxidase (Sigma) for 24 hours. γ-Irradiation was achieved with a Gammacell-1000 irradiator (Atomic Energy of Canada, Mississauga, ON).

Use of the human bone marrow samples (normal and AML) was approved by the institutional review boards of the Fred Hutchinson Cancer Research Center, CCG, and SWOG.

Flow cytometry assays

DNA histograms and sub-G1 apoptosis measurements were generated from treated cells by flow cytometric analysis as we previously described. Briefly, cells were fixed for 60 minutes in 80% ethanol at 4°C, permeabilized in 0.25% Triton X-phosphate-buffered saline for 5 minutes at room temperature, and washed in PBS/SER (phosphate-buffered saline containing BCS to 2%, human serum AB [Gemini, Calabasas, CA] to 2%, and sodium azide to 0.1%). Cells were subsequently incubated in the dark for 1 hour with R-phycocerythrin (R-PE)–labeled mouse antihuman proliferating cell nuclear antigen (PCNA; clone PC10) antibody (Dako, Glostrup, Denmark) at 375 ng per sample in PBS/SER to label cycling cells. After further washing, the DNA-specific dye 7-AAD (7-aminocoumarinomycol D; Sigma) was added at 25 μg/mL to stoichiometrically stain DNA, and cells were incubated for 30 minutes before multiparameter flow cytometric analysis.

Cholesterol measurements

Cellular cholesterol levels were measured by 1 of 2 techniques: Gas chromatography. As we previously described, lipids from a suspension of 10^7 cells were extracted in chloroform-methanol (1:2) and dried under nitrogen. After reconstitution in hexane, samples were transferred to glass tubes containing an internal standard solution (stigmastanol, 100 μg/mL in ethyl acetate [EtOAc]; Sigma), dried under nitrogen, and reconstituted in 100 μL bis–(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma) (25% vol/vol EtOAc). These samples were then sealed in an injection vial and heated for 1 hour at 60°C. After completion of BSTFA derivatization, samples were applied to a Hewlett Packard 5890 Series II gas chromatograph fitted with a flame ionization detector and a 30 m x 0.32 mm DB-5 (0.32 μm) column (J&W Scientific, Folsom, CA). The initial temperature (100°C) was maintained for 3 minutes, after which it was increased by 40°C per minute to 290°C and thereafter by 5°C per minute to 300°C for 5 minutes. Cholesterol ethers were quantitated after elution from the gas chromatograph at 12.5 minutes.

Amplex Red assay (Molecular Probes, Eugene, OR). This is a fluorometric technique that relies on the oxidation of cholesterol into a ketone and hydrogen peroxide. The hydrogen peroxide reacts stoichiometrically with the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazone) in the presence of horseradish peroxidase to form the fluorescent compound resorufin. To perform this assay, cell samples were initially counted by microscopy via the trypan blue exclusion method and then washed once in PBS. The cells were then resuspended in the Amplex Red reaction buffer (0.1 M potassium phosphate, 0.05 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, pH 7.4) at a concentration of 2000 μM and vortexed; both viable and nonviable cells were included. After 50-μL aliquots of each of the resulting cell lysates were pipetted into a 96-well tissue culture plate (Falcon/Becton Dickinson, Franklin Lakes, NJ), a 50-μL aliquot of an Amplex Red working solution (300 μM Amplex Red reagent, 2 U/mL horseradish peroxidase,
2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase) was added to each well. The plate was then incubated for 90 minutes at 37°C, protected from light. Fluorescence was subsequently measured on a CytoFluor II fluorescent plate reader (PerSeptive Biosystems, Framingham, MA) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. A cholesterol standard curve was also determined for each plate using a cholesterol calibrator (Sigma) diluted at various concentrations in Amplex Red reaction buffer in lieu of cell lysates.

**Calculations and statistics**

All values are presented as the mean ± 1 standard error of the mean. Statistical comparisons were performed with either the paired or unpaired Student t test, as appropriate.

**Results**

**Cholesterol levels can be measured in AML cells**

To study cellular cholesterol as a drug-resistance effector and cholesterol modulation as a rational antileukemia strategy, we needed the ability to determine cholesterol levels in different AML cell lines and in primary AML cell samples. To that end, we first measured cholesterol using gas chromatography, as we previously described.22,28 NB4 cells grown in serum-containing media showed somewhat higher cholesterol levels (78 ± 12 nmol/10 million cells, equivalent to 7.8 nmol/10⁶ cells) than HL60 cells (57 ± 10 nmol/10 million cells, or 5.7 nmol per 10⁶ cells) when cholesterol was quantitated in equal numbers of viable cells. However, because these gas chromatographic assays require 10 million cells for each measurement, they are not well suited for measuring the impact of different experimental variables on cellular cholesterol levels, especially in size-limited primary AML cell samples. Therefore, we adapted the Amplex Red assay, which measures the release of hydrogen peroxide upon cholesterol oxidation and can be used with much smaller cell numbers in a 96-well plate format. Having demonstrated the feasibility and reproducibility of the Amplex Red assay (Figure 1A-B), we used it to measure total cholesterol levels in AML cell lines. Consistent with data derived from gas chromatography assays, Amplex Red assays of the cholesterol content of 10⁶ cells in 100-μL volumes were consistently higher in NB4 cells (4.4 ± 0.2 μM in 100 μL assay, equivalent to 4.4 ± 0.2 nmol/10⁶ cells) than in HL60 cells (3.6 ± 0.4 nmol/10⁶ cells, P = .12 relative to NB4) or KG1a cells (3.3 ± 0.2 nmol/10⁶ cells, P = .001) (Figure 1C). Constitutive cholesterol levels in 29 primary AML cell samples were also determined, ranging from 1.0 to 9.9 nmol/10⁶ cells (mean 2.7 ± 0.3 nmol/10⁶ cells) (Table 1). Therefore, cellular cholesterol levels can be measured using a relatively small number of cells per assay, and these measures show that constitutive levels in primary AML cell samples are quite variable.

**Cholesterol levels are increased in certain AMLs by treatment with therapeutic agents**

We previously showed that sublethal hypoxia and oxidant injuries in kidney cells induce cellular cholesterol increments and that mevastatin cotreatments both block cholesterol increments and increase the toxicity of these injuries.28 To determine whether AML cells also acutely increase cellular cholesterol after exposure to cytotoxic substances, we examined the effects of 3 leukemia-relevant therapeutic agents on cellular cholesterol levels. We treated NB4, HL60, KG1a, and ML-1 cells for 24 hours with equitoxic doses of ARA-C, DNR, or γ-irradiation that produced no more than 10% cell death as measured by trypan blue uptake. Cellular cholesterol levels in ML-1 were not affected by any of these treatments (data not shown). However, we found significantly increased cholesterol levels in treated NB4 cells (treated with radiation, P = .002 compared with untreated cells; DNR P = .03; ARA-C P = .003) and in treated HL60 cells (radiation P = .0004; DNR P = .05; ARA-C P = .03) (Figure 2). Treated KG1a cells showed cholesterol increments that were marginally significant for radiation (P = .045) but not for DNR (P = .3) or ARA-C (P = .25).

We also examined the effects of 24-hour chemotherapy exposures on the cellular cholesterol content of primary AML cell samples (Table 1). Overall, treatment with daunorubicin resulted in significantly higher levels of cellular cholesterol in primary AML cell samples (120% ± 5% relative to constitutive cholesterol levels in the corresponding untreated cells) compared with similarly treated normal bone marrow cell samples (93% ± 3% relative to constitutive cholesterol levels in the untreated cells, P < .006). A trend toward higher levels of cellular cholesterol after cytarabine treatment was also seen for primary AML cell samples compared with similarly treated normal bone marrow cell samples (123% ± 6% versus 102% ± 9%, P = .09). Because cell line data (Figure 2) indicated that only some AML cell lines substantially increase cellular cholesterol after treatment with particular therapeutic agents, we also analyzed separately the data from primary AML cell samples that increased cellular cholesterol content after chemotherapy (18 of 28 daunorubicin-treated samples; 16 of 27 cytarabine-treated samples). This analysis demonstrated a significant effect in relative cholesterol change in responsive AMLs compared with that seen in similarly treated normal bone marrow cell samples (132% ± 6% versus 93% ± 3% for daunorubicin-treated cells, P < .0002; 139% ± 8% versus 102% ± 9% for cytarabine-treated cells, P < .008). In summary, the data suggest that some primary AML cell samples, as well as some AML cell lines, do acutely (ie, within 24 hours) mount substantial increases in cellular cholesterol content after exposure to relevant therapeutic modalities.
in apoptosis assays that measure the accumulation of cells with less than a 2N/G1 (ie, sub-G1) DNA content. These mevastatin-sensitive NB4 cells also demonstrated increased apoptosis after treatment with $0.1 \mu M$ CE and $0.1 \mu M$ CO ($P = 0.004$ and $P = 0.055$, respectively) (Figure 3B). HL60 cells showed intermediate sensitivity to $50 \mu g/mL$ mevastatin ($P = 0.006$) and showed increased apoptosis after CE and CO treatments (Figure 3B), although these increases were not statistically significant. On the other hand, KG1a cells were relatively insensitive to CE, CO, and mevastatin (Figure 3B). Therefore, statin sensitivity in AML cell lines is associated with sensitivity to other cholesterol-modulating agents.

**Cholesterol-reducing agents are toxic as single agents and can sensitize particular AMLs to treatments with therapeutic modalities**

To further address whether blocking adaptive cholesterol responses to cytotoxic agents results in the sensitization of individual AMLs that can mount such responses, we examined the effects of zaragoric acid (ZGA), which inhibits squalene synthase at the final branch point of the cholesterol synthesis pathway, on NB4 cell survival and cholesterol levels (Figure 4). Twenty-four-hour treatments with ZGA as a single agent at $64 \mu M$ or $256 \mu M$ minimally affected cell death (assessed by trypan blue uptake [TB+]) and cholesterol levels in NB4 cells (Figure 4A-B). ZGA at $256 \mu M$, however, induced more than $80\%$ toxicity within 60 hours, while ZGA at $64 \mu M$ induced more than $80\%$ toxicity after 120-hour treatments (Figure 4C). ZGA (256 $\mu M$) significantly increased the toxicities of cytarabine (0.5 $\mu M$) or $\gamma$-radiation (390 cGy) in NB4 cells after 24-hour cotreatments, relative to the toxicities of cytarabine ($P < 0.001$) or radiation ($P = 0.032$) alone (Figure 4A). Increased toxicities were associated with significant decreases in the cellular cholesterol content of cells cotreated for 24 hours with both ZGA (256 $\mu M$) and either cytarabine or radiation, as compared with levels seen in cells receiving either cytarabine ($P < 0.001$) or radiation ($P = 0.002$) alone (Figure 4B). These data, in conjunction with the data presented in Figure 2, suggest that some AML cell lines, including NB4 cells, acutely augment cellular cholesterol after exposure to cytotoxic agents such as chemotherapy and radiation and that attenuation of these adaptive cholesterol responses results in decreased AML cell survival.

**Figure 2. AML cell lines show cholesterol increments after therapeutic agent treatments.** AML cell lines (NB4, HL60, KG1a) were treated with equitoxic doses of chemotherapy or radiation, with cholesterol levels subsequently determined by the Amplex Red assay. NB4 and HL60 cells showed significantly higher cholesterol levels 24 hours after $\gamma$-irradiation (RAD; 390 cGy) or after 24 hours of treatments with daunorubicin (DNR; 0.05 $\mu M$ for NB4, 0.01 $\mu M$ for HL60) or cytarabine (ARA-C; 0.5 $\mu M$ for NB4, 0.25 $\mu M$ for HL60). KG1a cells yielded marginally significant cholesterol increments 24 hours after RAD (390 cGy) but not after DNR (0.05 $\mu M$) or ARA-C (0.075 $\mu M$). Lysates from 10^5 NB4, HL60, or KG1a cells were used to perform Amplex Red cholesterol assays (100 $\mu L$ assay volumes). Cellular cholesterol measures are expressed relative to that of untreated cells (UN) from the same lineage. Data are presented with standard errors and represent the mean of 3 to 6 independent replicates.

**Statin sensitivity in AML cell lines is associated with sensitivity to other cholesterol-modulating treatments**

In light of prior studies documenting the sensitivity of some AMLs to statins and our current studies showing that certain AML primary cell samples and cell lines increase cellular cholesterol after exposure to therapeutic agents, we hypothesized that particular AMLs depend critically on cholesterol for their survival. To test this prediction, we evaluated the potential toxicity of several cholesterol-modulating agents: (1) mevastatin, which inhibits HMG-CoA reductase; (2) cholesterol oxidase (CO), which oxidizes free cholesterol; and (3) cholesterol esterase (CE), which cleaves cholesterol esters. As shown in Figure 3A, NB4 cells were significantly sensitive to mevastatin at 5 $\mu g/mL$ ($P = 0.008$ versus untreated cells), 25 $\mu g/mL$ ($P = 0.005$), and 50 $\mu g/mL$ ($P < 0.001$)
To further address whether cellular cholesterol levels can be critical to AML cell survival, we tested whether the effects of cholesterol synthesis inhibition on cell death and cellular cholesterol can be modulated by the availability of exogenous cholesterol in the cell culture environment. Because intracellular cholesterol levels are established not only by HMG-CoA reductase– and squalene synthase–regulated de novo synthesis, but also by receptor-mediated import of ambient cholesterol contained in LDL particles, we examined the effects of mevastatin and ZGA on NB4 cells cultured in a serum-depleted (and therefore cholesterol-depleted) medium. We found that both mevastatin (data not shown) and ZGA (Figure 5A) produced larger cellular cholesterol decrements in NB4 cells cultured for 24 hours under serum-depleted conditions compared with NB4 cells cultured in a cholesterol-containing, serum-replete medium. ZGA at 256 μM was also substantially more toxic to NB4 cells (Figure 5B versus 4A) and HL60 cells (data not shown) cultured under serum-depleted conditions compared with those cultured in a serum-replete medium. In addition, growth in serum-depleted culture medium sensitized NB4 and HL60 cells to mevastatin toxicity (data not shown).

Because serum contains various growth-regulating substances in addition to cholesterol, the enhanced toxicity of ZGA and mevastatin in serum-deprived NB4 cells might have resulted from the depletion of one or more of these other substances rather than from depletion of cholesterol. To demonstrate that the additional toxicity of cholesterol synthesis blockade under serum-depleted conditions arose from critical cellular cholesterol reductions rather than depletion of serum growth factors, for instance, we examined the impact of adding cholesterol, in the form of commercially available LDL, to cells in serum-depleted...
culture media. LDL addition measurably increased cellular cholesterol in serum-depleted NB4 cells (Figure 5A) and reversed the profound toxicity of 256 µM ZGA (P = .003; Figure 5B). HL60 cells cultured in serum-depleted medium also showed high ZGA sensitivity (67% TB + death in serum-depleted medium with ZGA versus 9% in serum-depleted medium without ZGA) that was partially reversed by the addition of LDL (to 40% TB +). KG1a cells, however, were insensitive to ZGA even when cultured in serum-depleted medium (6% TB + with ZGA versus 4% TB + without ZGA). These data indicate that the toxicities of cholesterol synthesis inhibitors such as ZGA and mevastatin in NB4 and HL60 cells are reduced by cholesterol available from the ambient environment and that certain AMLs (eg, KG1a) may be less dependent on cholesterol for their survival.

Discussion

Although a majority of adult patients with AML initially achieve complete remission with conventional chemotherapy, most eventually relapse and succumb to drug-resistant disease. Given this prevalence of drug-resistant AMLs, a number of workers have examined the potential antineoplastic effects of the cholesterol-lowering agents commonly known as “statins,” either alone or as chemotherapy sensitizers. Two groups have shown that lovastatin and simvastatin can sensitize AML cell lines to cytarabine. Another group has demonstrated that lovastatin is toxic to AML cell lines and to primary AML cell samples but not to lymphocytic leukemias. We have previously shown that a subset of primary AML cell samples, but not normal myeloid cells, can be sensitized to cytarabine and/or daunorubicin by mevastatin. Because the mevalonate pathway inhibited by statins produces several by-products in addition to cholesterol, including the farnesyl pyrophosphate and geranylgeranyl pyrophosphate moieties necessary to isoprenylate Ras and other similarly modified membrane signaling proteins, many investigators have assumed that statin efficacy in AML and other malignancies is related to the inhibition of membrane protein isoprenylation. In this report, however, we present data to indicate that direct cholesterol modulation may also play a role in the efficacy of statins against AML. It has long been known that cholesterol metabolism is dysregulated in many malignancies, including AML, in which high rates of cholesterol import and/or synthesis have been documented. Leukemic cell lines selected for resistance to chemotherapy have also been found to contain elevated lipid levels. These findings suggest that increased cholesterol levels may serve to protect leukemic cells. We have previously demonstrated a similar concept in renal tubule cells that protect themselves from various ischemic and oxidative insults by dramatically increasing their cellular cholesterol content, thereby achieving a state of “acquired cytoreresistance.” We now document that some AML cell lines also respond to radiochemotherapeutic insults by significantly increasing cellular cholesterol content above constitutive levels. Certain primary AML cell samples manifest similar behavior after exposure to cytarabine and daunorubicin, with significantly greater increases in cellular cholesterol than that seen in similarly treated normal bone marrow cell samples.

To further examine the potential role of cholesterol modulation in protecting certain AMLs against radiation or chemotherapy, we inhibited cholesterol synthesis at the level of squalene synthase, which is the rate-limiting enzyme at the final branch point in the mevalonate pathway leading to cholesterol synthesis. In this manner, we were able to disrupt cholesterol synthesis while minimizing interference with upstream portions of the mevalonate pathway that are responsible for the production of moieties such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. We demonstrated that the squalene synthase inhibitor zaragozic acid A (ZGA) produced cell death only in the cell lines (ie, NB4 and HL60) that mounted cholesterol increments in response to radiotherapeutic or chemotherapeutic insults and that exhibited sensitivity to mevastatin. We subsequently showed that cotreatment of NB4 cells with ZGA and either cytarabine or radiation resulted in a marked attenuation of the cholesterol increment that would otherwise have occurred in these cells after exposure to cytarabine or radiation alone. At the same time, the addition of ZGA to cytarabine or radiation induced at least an additive toxicity in NB4 cells. This association of increased toxicity with the attenuation of cholesterol increments provides further credence for the concept of adaptive cholesterol responses as a cytoprotective mechanism in particular AMLs.

One of the arguments that has been raised in the literature against a role for cholesterol modulation in the antineoplastic effects of statins has been the inability of exogenous squalene and free cholesterol to decrease lovastatin toxicity in NB4 cells. However, cells do not always efficiently incorporate free sterols, as was shown for K562 cells exposed to exogenous squalene and lanosterol. On the other hand, cells can efficiently obtain cholesterol from the ambient environment by receptor-mediated import of LDL. We have shown that treating NB4 cells with ZGA in a serum-depleted (and hence cholesterol-depleted) medium resulted in more than 70% toxicity within 24 hours, while the addition of LDL cholesterol (more than 95% purity) to these cultures reversed the toxicity of ZGA, further supporting the vital cytoprotective role of cellular cholesterol in certain AMLs.

Our findings do not exclude the possibility that reduced isoprenylation may contribute to statin toxicity in particular AMLs but do highlight the possible importance of direct cholesterol modulation in new antileukemia therapies. Nonstatin drugs that reduce protective cellular cholesterol may be more desirable for eradicating cholesterol-dependent leukemias for several reasons. Some AMLs are apparently able to further up-regulate LDL receptor activity after treatment with statins, which could limit the ability of statins to decrease protective cellular cholesterol. In addition, statins apparently block the production of a mevalonate-derived Ras inhibitor that accumulates when the mevalonate pathway is instead blocked at a downstream step by a diphosphomevalonate decarboxylase inhibitor. Therefore, statin treatments could potentially favor the growth of tumor cells that are regulated by this inhibitor, whereas inhibition of cholesterol synthesis distal to diphosphomevalonate decarboxylase activity would not have this effect. Consistent with this idea, Hohl and his coworkers have reported that lovastatin can increase proliferation in a large number of primary leukemia cell samples. Squalene synthase inhibitors that block cholesterol biosynthesis at the final committed step of the mevalonate pathway are being tested for hypercholesterolemia indications, and our findings with ZGA suggest that squalene synthase inhibitors and other direct cholesterol-modulating agents should be rigorously tested as chemotherapy sensitizers in acute myeloid leukemia.

Acknowledgments

The authors thank Sasha Bidwell and Ali C. M. Johnson for providing excellent technical assistance and Irwin Bernstein and Shelly Heimfeld for providing cryopreserved marrow specimens.
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