Mitochondrial Cholesterol Contributes to Chemotherapy Resistance in Hepatocellular Carcinoma

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Abstract
Cholesterol metabolism is deregulated in carcinogenesis, and cancer cells exhibit enhanced mitochondrial cholesterol content whose role in cell death susceptibility and cancer therapy has not been investigated. Here, we describe that mitochondria from rat or human hepatocellular carcinoma (HCC) or primary tumors from patients with HCC exhibit increased mitochondrial cholesterol levels. HCC sensitivity to chemotherapy acting via mitochondria is enhanced upon cholesterol depletion by inhibition of hydroxymethylglutaryl-CoA reductase or squalene synthase (SS), which catalyzes the first committed step in cholesterol biosynthesis. HCC transfection with siRNA targeting the steroidogenic acute regulatory protein STAR, a mitochondrial cholesterol–transporting polypeptide which is overexpressed in HCC compared with rat and human liver, sensitized HCC to chemotherapy. Isolated mitochondria from HCC with increased cholesterol levels were resistant to mitochondrial membrane permeabilization and release of cytochrome c or Smac/DIABLO in response to various stimuli including active Bax. Similar behavior was observed in cholesterol-enriched mitochondria or liposomes and reversed by restoring mitochondrial membrane order or cholesterol extraction. Moreover, atorvastatin or the SS inhibitor YM-53601 potentiated doxorubicin-mediated HCC growth arrest and cell death in vivo. Thus, mitochondrial cholesterol contributes to chemotherapy resistance by increasing membrane order, emerging as a novel therapeutic niche in cancer therapy. [Cancer Res 2008;68(13):5246–56]

Introduction
Cholesterol is an integral component of cellular membranes that plays an essential role in maintaining their integrity and function (1). In addition to the regulation of membrane order, cholesterol induces membrane packing in lateral microdomains (rafts) of the plasma membrane, providing a scaffold for a variety of membrane-associated signaling proteins (2, 3). Due to this role in modulating membrane structure and function, cholesterol levels in cell membranes are tightly regulated. The main sources of cellular cholesterol involve either its uptake from cholesterol-rich low-density lipoproteins or its de novo synthesis through the conversion of 3-hydroxy-3-methylglutaryl-CoA into mevalonate by 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR), the rate-limiting step in cholesterol synthesis, which is transcriptionally regulated by endoplasmic reticulum–based transcription factor SREBP-2 (1, 4).

Cholesterol accumulation and enhanced cholesterol-rich lipid rafts have been reported in several solid tumors that modulate tumor cell growth and survival by activating particular signaling pathways such as Akt (5, 6). In addition, cholesterol metabolism is abnormal in many malignancies with loss of cholesterol feedback and HMG-CoAR up-regulation despite enhanced cholesterol levels (7). Moreover, malignant cells exhibit elevated levels of mevalonate, which has been shown to promote tumor growth in vivo and proliferation of breast cancer cells (8). Consistent with this scenario, statins, which block HMG-CoAR by competing with mevalonate for binding to the active site, have been proposed for cancer prevention and/or treatment, although their efficacy as anticancer agents has not always been established (9). Furthermore, even in those cases where statins showed promising results, it remained unclear whether the therapeutic effects were due to the cholesterol-lowering activity or to the down-regulation of isoprenoids, which are known to modulate multiple proteins by posttranslational modifications (9, 10).

Mitochondria are cholesterol-poor organelles with estimates ranging from 0.5% to 3% of the content found in plasma membranes (1, 11). However, unphysiologic mitochondrial cholesterol levels have been described in solid tumors. For instance, mitochondrial cholesterol levels of tumors from Buffalo rats bearing transplanted Morris hepatomas were 2- to 5-fold higher than the content found in mitochondria prepared from host liver, and correlated with the degree of tumor growth and malignancy (12–15). Although the mechanisms underlying the mitochondrial cholesterol accumulation in cancer cells are poorly understood, recent observations have reported the activity of cholesterol-transporting polypeptides, including the sterol regulatory protein (StAR) in human HepG2 cells that contribute to the mitochondrial intermembrane trafficking of cholesterol (16). Although cholesterol enrichment in mitochondria can impair specific mitochondrial components accounting, in part, for the mitochondrial dysfunction described in cancer cells (13, 15, 17–20),
Hepatocellular carcinoma (HC) is one of the main causes of cancer-related deaths that frequently arises on a background of chronic inflammation and exhibits high resistance to current therapy (23). Thus, because mitochondria are known to play a key role in cell death (22) and cholesterol-regulated mitochondrial membrane order has been reported to modulate MMP and the subsequent release of apoptotic proteins (19), the purpose of this study was to examine the role of mitochondrial cholesterol in the susceptibility of HC cells (HCC) to chemotherapy in vitro, the mechanisms involved, and the relevance in an in vivo HC model. Our findings indicate that mitochondrial cholesterol contributes to chemotherapy resistance through altered membrane order, hence emerging as a novel therapeutic target in cancer therapy.

Materials and methods

Materials and recombiant proteins. Dioleoylphosphatidylcholine, tetraedocoylcardiolipin, and cholesterol were purchased from Avanti Polar Lipids. Dodecyl octaoxetane glycol monooether (C12E6), Methyl-j-cyclodextrin, melittin, Staphylococcus aureus aures α-toxin, tetanolysin, and fluoresceni-sothiocyanate-labeled dextrans of 70 kDa (FD-70) were obtained from Sigma. Recombinant full-length human Bax with an amino-terminal His6 tag (titag), caspase 8–cleaved murine BID with an amino-terminal His6 tag (Bcl2DC) were purified as previously described (24). Oligomeric Bax (oligo-Bax) was obtained by incubating Bax in 100 mmol/L KCl, 10 mmol/L HEPES, 0.1 mmol/L EDTA (pH 7.0) buffer (KHE buffer) containing octylglycoside (2%, w/v) for 1 h at 4°C.

Cell culture, hepatocyte isolation, and mitochondria and mitoplasts preparation. The human hepatoblastoma cell line, HepG2, and the rat hepatoma cell line, Reuber H35, were both obtained from the European Collection of Animal Cell Cultures and grown at 37°C in 5% CO2. Culture medium was supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 1% nonessential amino acids and antibiotics. In some experiments, 10% of delipitated FBS was used. Primary rat hepatocytes were isolated by collagenase perfusion and cultured as described previously (25). Cell viability in response to chemotherapy was determined by trypan blue exclusion. Rat and human liver mitochondria were isolated as described in Supplementary Methods. Mitochondria from HCC were obtained by rapid centrifugation through Percoll density gradient as described in the Supplementary Methods. Mitochondria were isolated from the liver explant at 4°C by differential centrifugation using a sucrose solution followed by homogenization.

Cell Death Detection kit; In Situ terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling assay and tumor progression. After sacrifice, tumors were fixed and paraffin sections (5 μm) from each area were stained with terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling (TUNEL) reagent using a commercial kit (In Situ Cell Death Detection kit; POD from Roche). Immunohistochemical staining of CD34, a specific endothelial cell marker commonly used for microvessel quantification, was performed with rat monoclonal anti-CD34 antibody (Abcam) at a dilution of 1:50 (2 mg/mL). The slices were examined with a Zeiss Axioplan microscope equipped with a Nikon DXM1200F digital camera. Serum α-feto protein levels were measured by the Centro Diagnóstico Médico (Hospital Clinic).

Statistics. Results were expressed as mean ± SD with the number of individual experiments detailed in figure legends. Statistical significance of the mean values was established by the two-tailed distribution Student's t test.

Supplementary methods. This section describes the procedures for Western blot analysis, quantitative real-time PCR, large unilamellar vesicle (LUV) preparation, and fluorimetric assays.
Results

Mitochondrial cholesterol in established HCC. Because previous findings reported enhanced mitochondrial cholesterol content in solid Morris hepatoma tumors with respect to host liver (12–15), we first assessed the mitochondrial cholesterol levels from established HCC. HepG2 and H35 cells were fractionated into mitochondria and analyzed for lipid composition. Electron microscopy and Western blot analysis of PERK, Na⁺/K⁺ ATPase α1, and Rab5A indicated insignificant contamination with endoplasmic reticulum, plasma membrane, and endosomes, respectively, in the final mitochondrial fraction (Fig. 1A). Monitoring Lamp1 levels by Western blot analysis further indicated the lack of lysosomal contamination in the final mitochondrial fraction (Fig. 1A). As shown, the total cholesterol levels in mitochondria from H35 and HepG2 cells were 3- to 10-fold higher with respect to rat and human liver mitochondria without changes in total phospholipids content (Fig. 1B), confirming previous results in implanted hepatoma tumors in vivo (12–15). Interestingly, the levels of mitochondrial cholesterol from primary tumors of patients with HC were higher than the content of mitochondria from nontumor human liver tissue and similar to those found in HepG2 cells (Fig. 1B). Moreover, the enhanced levels of free cholesterol of H35 or HepG2 cells examined by confocal microscopy by filipin staining colocalized with mitochondria, and this increase was further verified by HPLC analyses of isolated mitochondria (Fig. 1C). Finally, these findings on mitochondrial cholesterol up-regulation correlated with enhanced expression of the transcription factor SREBP-2 and HMG-CoAR in H35 and HepG2 cells with respect to rat and human liver samples (Fig. 1D). Together, these findings validate the use of HCC lines to study the role of mitochondrial cholesterol in chemotherapy susceptibility.

HMG-CoAR or squalene synthase inhibition sensitizes HCC to mitochondria-targeted chemotherapy. We next examined the role of mitochondrial cholesterol in the susceptibility of HCC to chemotherapy targeting mitochondria. Arsenic trioxide or lonidamine, a derivative of indazole-3-carboxylic acid, are both antineoplastic drugs that target mitochondria and induce mitochondrial permeability transition (MPT; refs. 30, 31). Thapsigargin-based prodrugs developed for the treatment of prostate cancer (32) are potent inhibitors of endoplasmic reticulum Ca²⁺ ATPases and induce MPT by Ca²⁺ overload, whereas doxorubicin, an anthracycline antibiotic drug, stimulates mitochondrial reactive oxygen species (ROS) generation (33). Both cell lines displayed a reduced susceptibility to increasing doses of thapsigargin, lonidamine, arsenic trioxide, or doxorubicin compared with primary rat hepatocytes (Fig. 2A). Various statins including lovastatin or atorvastatin have been shown to be effective in reducing cholesterol levels in HepG2 cells (34). Lovastatin pretreatment sensitized H35 cells to mitochondria-targeting drugs (Fig. 2B) with similar results observed with HepG2 cells (Supplementary Fig. S1). Dying cells displayed apoptotic features, such as caspase-3 activation (Supplementary Fig. S2A) and chromatin disruption (Supplementary Fig. S2B). Moreover, the susceptibility of H35 cells to bafilomycin A, an inhibitor of the vacuolar type H^+^-ATPase that impairs lysosomal function and promotes apoptosis independently of MMP (35), was not increased by lovastatin (Supplementary Fig. S1; Fig. 2B) and was not accompanied by enhanced cytochrome c release (Supplementary Fig. S3), suggesting that the sensitizing effect of lovastatin is specific for mitochondria-targeting drugs. To further verify the specific role of cholesterol in chemotherapy resistance, we investigated the effect of SS inhibition, which blocks cholesterol biosynthesis without affecting the isoprenoid metabolism (Supplementary Fig. S4). Cell treatment with YM-53601, a specific SS inhibitor (36), at a dose nontoxic to primary rat hepatocytes (Supplementary Fig. S5), potentiated the susceptibility of H35 cells to thapsigargin, lonidamine, and doxorubicin (Fig. 2C) to a similar extent as seen by lovastatin treatment. Importantly, both lovastatin and YM-53601 reduced the mitochondrial cholesterol levels in both H35 and HepG2 cells (Fig. 2D). Finally, the susceptibility of H35 cells to doxorubicin by lovastatin was prevented by mevalonate, independently of the inhibition of farnesyltransferase, whereas mevalonate failed to restore resistance to doxorubicin after SS inhibition (Supplementary Fig. S6). Moreover, 7-dehydrocholesterol, the immediate precursor of cholesterol synthesis (Supplementary Fig. S4), decreased the susceptibility to doxorubicin by SS inhibition (Supplementary Fig. S6). Collectively, these findings suggest the involvement of mitochondrial cholesterol in the resistance of HCC to drug-induced cell death independently of alterations in isoprenoid metabolism.

siRNA-mediated STAR silencing sensitizes HCC to chemotherapy. To further substantiate that mitochondrial cholesterol enrichment in HCC contributes to mitochondrial-targeting chemotherapy resistance, we investigated the role of silencing STAR. STAR is a cholesterol-transporting polypeptide involved in the intramitochondrial trafficking of cholesterol, which regulates the synthesis of steroids in specialized tissues (11, 16). The levels of STAR were higher in H35 and HepG2 cells compared with rat and human liver samples being more abundant in HepG2 cells than in H35 cells (Fig. 3A), which correlated with the mitochondrial cholesterol levels observed in these cell lines (Fig. 1B). Transfection with siRNA-targeting STAR resulted in a significant reduction of STAR protein and mRNA expression compared with cells transfected with scrambled control siRNA (Fig. 3B), causing a significant reduction of mitochondrial cholesterol levels (Fig. 3C). More importantly, the susceptibility of HepG2 cells to doxorubicin, thapsigargin, or arsenic trioxide was potentiated by transfection with STAR siRNA (Fig. 3D), with similar results observed with H35 cells (Fig. 3D). Thus, these findings validate the observations with HMG-CoAR and SS inhibition, further supporting a key role for mitochondrial cholesterol in the sensitization of HCC to chemotherapy targeting mitochondria.

Mitochondrial cholesterol modulates membrane order and MPT. Cholesterol, particularly free cholesterol, regulates membrane physical properties (1, 27, 28). Thus, we next determined the membrane order from the steady-state fluorescence anisotropy of DPH-labeled mitochondria. Mitochondria from H35 and HepG2 cells showed higher membrane order (SψPH) compared with mitochondria from rat and human liver (Fig. 4A). Phospholipids can also regulate membrane order. However, the content of phosphatidylcholine, the major phospholipid in membranes, remained unchanged in mitochondria from H35 and HepG2 cells with respect to rat or human liver samples, whereas phosphatidylethanolamine levels in mitochondria from HepG2 cells but not H35 cells were slightly lower than those found in human liver mitochondria (Supplementary Fig. S7). Moreover, mitochondria from H35 and HepG2 cells exposed to the cholesterol-binding agent MCD exhibited a significant depletion of cholesterol levels compared with untreated mitochondria, which translated in reduced membrane order (Fig. 4A). In contrast, incubation of rat liver mitochondria with a cholesterol-albumin complex (19) resulted in significant cholesterol loading, reaching the levels
Figure 1. Increased cholesterol content in mitochondria from rat (H35), human (HepG2) hepatoma cell lines, and human HC samples. A, electron microscopy analysis showing purified mitochondria from H35 and HepG2 cells after cellular subfractionation (scale bars, 1 μm); and Western blot analysis of PERK, Na⁺/K⁺ATPase α1, Rab5A, Lamp1, and cytochrome c expression in homogenates (H) or mitochondrial fraction (Mit) from H35 cells. B, total cholesterol (black bars) and phospholipid levels (white bars) performed by HPLC on lipid extracts from the mitochondrial fraction from H35, HepG2 cells, and human HC samples. C, colocalization of mitochondria and free cholesterol by confocal microscopy using mouse anti–cytochrome c (cyt C) and filipin, respectively. The graphs on the bottom represent the fluorescence intensity profiles plotted from a to b direction for the different cell lines. In addition, mitochondrial-free cholesterol content analyzed by HPLC. Results in B and C are mean ± SD values from at least three independent experiments. * and **, P < 0.01 versus rat and human liver mitochondria, respectively. D, quantitative real-time reverse transcription-PCR mRNA expression of SREBP-2 and HMG-CoA reductase in H35 and HepG2 cells compared with rat and human liver, respectively. Absolute mRNA values were determined, normalized to hypoxanthine phosphoribosyltransferase and reported as relative levels referred to the expression in nontumor counterpart. Prot, protein.
found in mitochondria from H35 cells that increased the membrane order (Fig. 4A). Indeed, the mitochondrial membrane order correlated with the cholesterol content (Fig. 4A). Cholesterol distribution after cholesterol enrichment by the cholesterol-albumin complex was estimated in mitoplasts. Although the bulk of cholesterol (60–70%) was found in the outer membrane, in agreement with previous findings (19), the levels of cholesterol in mitoplasts from cholesterol-enriched mitochondria were higher (2- to 3-fold) than those found in mitoplasts from control mitochondria (data not shown).

We next analyzed the role of cholesterol in the response of mitochondria to MPT triggers. Isolated mitochondria from H35 cells with or without MCD treatment were incubated with the superoxide anion-generating system, xanthine plus xanthine oxidase (X-XO), shown to induce the release of mitochondrial cytochrome c (37). Although rat liver mitochondria released cytochrome c in response to X-XO, mitochondria from H35 were resistant to X-XO–induced cytochrome c release (Fig. 4B); moreover, cholesterol extraction by MCD restored the sensitivity of mitochondria from H35 cells to X-XO–mediated cytochrome c release (Fig. 4B), with similar findings observed in mitochondria from HepG2 cells (Fig. 4B).

Ca2+ induces the transition of the MPT pore causing mitochondrial matrix swelling and release of the proapoptotic proteins (38). Rat liver mitochondria enriched in cholesterol by the cholesterol-albumin complex were resistant to swelling (data not shown), and cytochrome c and Smac/DIABLO release (Fig. 4C) induced by Ca2+. Moreover, mitochondria from H35 cells were insensitive to Ca2+-induced Smac/DIABLO and cytochrome c release (Fig. 4D), whereas cholesterol depletion by MCD restored the response to Ca2+ (Fig. 4D). Thus, mitochondrial cholesterol regulates membrane order and the release of apoptotic proteins by MPT triggers.

Figure 2. Inhibition of cholesterol synthesis sensitizes cells to different compounds that act on mitochondria. A, primary rat hepatocytes, H35, and HepG2 cells were incubated with increasing doses of arsenic trioxide (ATO), lonidamine, thapsigargin (Thg), or doxorubicin for 24 h. Cell viability was determined by trypan blue exclusion. At least 100 cells in 4 different fields were counted and expressed as a percentage of total cells. B and C, disruption of cholesterol biosynthesis by lovastain or by the SS inhibitor YM-53601(YM) sensitizes H35 cells to chemotherapeutic agents. H35 hepatoma cells untreated (NT) or incubated with lovastatin (Lov) or YM-53601 for 24 h were exposed to thapsigargin (0.2 μmol/L), lonidamine (0.2 μmol/L), arsenic trioxide (5 μmol/L), doxorubicin (Doxo; 1 μmol/L), batflomycin A (Baf A; 10 μmol/L) for 24 h. Cell death was determined by trypan blue exclusion (n = 3). D, total cholesterol levels of mitochondria from H35 and HepG2 cells 24 h after treatment with lovastatin (2.5 μmol/L) or YM-53601 (1 μmol/L); n = 4; *, P < 0.05 versus untreated cells. Ctrl, control.
Cholesterol impairs Bax-driven mitochondrial release of apoptotic proteins and permeabilization in liposomes. Mitochondrial release of prodeath factors by Bax or Bak can occur by their oligomerization and insertion into the mitochondrial outer membrane independent of MPT (22). Thus, we next examined the effect of cholesterol on Bax-driven mitochondrial permeabilization. Whereas tBid-activated Bax (tBid/Bax) stimulated the release of cytochrome c in control mitochondria, cholesterol-enriched mitochondria were resistant to tBid/Bax-mediated cytochrome c release (Fig. 5A). Similar findings were observed in the release of Smac/Diablo by cholesterol enrichment (data not shown). The incubation of cholesterol-enriched mitochondria with A2C, a fatty acid derivative that intercalates into the lipid bilayer resulting in its fluidization (19), restored the ability of tBid/Bax to release cytochrome c (Fig. 5A), establishing a cause-and-effect relationship between mitochondrial membrane order and release of apoptotic proteins by Bax. Moreover, mitochondria from H35 cells were insensitive to tBid/Bax-induced release of cytochrome c that was restored by lovastatin treatment (Fig. 5A), which caused a significant reduction in the mitochondrial cholesterol levels (Fig. 2D).

To further extend these observations, we next examined the effect of cholesterol on the poration of large unilamellar vesicles (LUVs) by active Bax. LUVs with or without cholesterol were loaded with self-quenching concentrations of FD-70, and the release of LUV-entrapped FD-70 was monitored as an increase in the fluorescence signal due to marker dilution in the external medium (24). As shown, the release of vesicular content by tBid/Bax was impaired in cholesterol-containing LUVs (Fig. 5B). In contrast, cholesterol was required for the release of vesicular contents induced by tetanolysin, a cholesterol-dependent pore-forming toxin known to open proteinaceous channels by inserting a transmembrane α-barrel in the bilayer (Fig. 5B; ref. 39). Treatment of LUVs with MCD reversed the inhibitory effect of cholesterol upon Bax-driven liposome permeabilization and disrupted the cholesterol-dependent channel-forming function of tetanolysin (Fig. 5B). Furthermore, cholesterol inhibited the membrane-permeabilizing activity of tBid/Bax or Bax preoligomerized with octylglucoside (Oligo-Bax).

Figure 3. STAR suppression by siRNA increases the cytotoxicity induced by doxorubicin. A, representative immunoblot showing STAR protein abundance in H35 and HepG2 cellular extracts and in homogenates from rat and human liver. B, STAR mRNA silencing. HepG2 cells were transfected with the STAR siRNA or the control siRNA (Ctrl siRNA) as described in Materials and Methods. Cells were allowed to recover in regular culture medium for 48 h, and the levels of STAR protein were quantified by Western blot in cellular extracts (left; n = 3) and mRNA levels by real-time RT-PCR (right; n = 3). C, mitochondrial cholesterol levels from HepG2 and H35 cells 48 h after STAR mRNA silencing analyzed by HPLC. D, 48 h after transfection with STAR or control siRNA, cells were exposed to doxorubicin (1 μmol/L), thapsigargin (0.2 μmol/L), lonidamine (Lnd; 0.2 mmol/L), and arsenic trioxide (ATO; 5 μmol/L), and cell death was analyzed 48 h later by trypan blue exclusion. At least 100 cells in 4 different fields were counted and expressed as a percentage of total cells; n = 3; * P < 0.05 versus control siRNA–transfected cells treated with doxorubicin.
in a dose-dependent manner (Fig. 5B). Similar to the effect found on Bax, and consistent with previous observations, cholesterol also decreased the permeabilizing activity of melittin, a widely studied antimicrobial peptide thought to breach membrane permeability barrier by forming lipid-containing toroidal pores instead of purely proteinaceous channels (40). In contrast, similar to tetanolysin and consistent with previous findings, cholesterol actually increased the release of vesicular contents induced by the channel-forming protein S. aureus α-toxin (Fig. 5B; ref. 41). Antiapoptotic proteins such as Bcl-2 antagonize the release of mitochondrial prodeath factors during apoptosis. To address whether cholesterol modulates Bcl-2 activity, LUVs with or without cholesterol were incubated with Bcl-2DC before tBid/Bax treatment. As seen, Bax-driven membrane permeabilization of LUVs was inhibited by Bcl-2DC in a dose-dependent manner, despite the presence of membrane cholesterol (Fig. 5C). Consistent with the findings in cholesterol-enriched rat liver mitochondria, the resistance to Bax-induced permeabilization of cholesterol-containing LUVs was reversed by the fluidizing agent A2C (Fig. 5D). Finally, we examined whether cholesterol altered the membrane-inserting capacity of pre oligomerized Bax (Oligo-Bax) using lipid monolayers. Addition of oligo-Bax into cholesterol-containing or cholesterol-devoid lipid monolayers at different initial surface pressures allowed us to determine critical surface pressures values (\( \pi \)) for oligo-Bax, which is a measure of the membrane penetrability of the protein. As seen, \( \pi \) values for oligo-Bax in cholesterol-containing monolayers were notably lower than in monolayers devoid of cholesterol (Fig. 5D). Together, these findings suggest that cholesterol-mediated rigidification of the bilayer directly modulates Bax permeabilizing activity, at least, in part, by reducing the capacity of Bax to insert into the lipid matrix of the membrane.

HMG-CoAR or SS inhibition potentiates chemotherapy in tumor xenografts. We further evaluated whether cholesterol regulates cancer therapy in vivo using tumor xenografts. Nude mice were s.c. injected with human hepatoma HepG2 cells, and tumor-bearing animals were then subjected to lipid-lowering treatments.

**Figure 4.** Mitochondrial cholesterol modulation regulates the release of cytochrome c and Smac/Diablo by ROS and Ca\(^{2+}\). **A,** total cholesterol levels determined by HPLC of untreated mitochondria or after cholesterol enrichment by a cholesterol-albumin complex (CHOL) and Me-\( \beta \)-cyclodextrine treatment (MCD; top). Middle, membrane order (S\(_{2\text{ph}}\)) determined from the fluorescence anisotropy monitored at 366 nm (emission, 440 nm) of mitochondria labeled with DPH, using polarizing filters in both excitation and emission planes, and normalized as per milligram of mitochondrial protein; \( n = 3 \); * \( P < 0.05 \) versus untreated mitochondria; ** \( P < 0.05 \) versus rat liver mitochondria. Bottom, correlation between mitochondrial cholesterol content and membrane order (S\(_{2\text{ph}}\)). **B,** control and cholesterol-depleted (MCD) mitochondria from H35 and HepG2 cells were incubated with xanthine (0.1 mmol/L) plus xanthine oxidase (40 mU/mL; X-XO), and the effect on the release of cytochrome c was analyzed by Western blot in the supernatants at the indicated times. Rat liver mitochondria were exposed to X-XO, and the release of cytochrome c was analyzed by Western blot in the supernatants. C, release of apoptogenic proteins by Ca\(^{2+}\) in control and cholesterol-enriched mitochondria from rat liver. Supernatants and pellets from mitochondria with or without CaCl\(_{2}\) (100 \( \mu \)mol/L) treatment for 30 min were analyzed by Western blot and immunoblotting. D, representative immunoblots of cytochrome c and Smac/DIABLO in supernatants and pellets of control and Me-\( \beta \)-cyclodextrin–treated mitochondria from H35 cells, with or without CaCl\(_{2}\) (100 \( \mu \)mol/L) exposure for 30 min. B to D, the blots shown are representative of 3 to 4 independent experiments showing similar results. The mitochondrial hsp60 protein was visualized as a loading control.
Atorvastatin was chosen for in vivo studies as it has been shown to be more effective than other statins both in humans and experimental animals (42, 43). We observed that both atorvastatin and YM-53601 administration significantly decreased the intratumor cholesterol levels (Fig. 6A). No differences in the pattern or in the amount of microvessel formation were observed in any of the experimental groups (Supplementary Fig. S8), suggesting that cholesterol content plays a minor role in tumor vascularization in this xenograft model. However, although atorvastatin notably reduced the size of the tumors or the increase in \(\alpha\)-feto protein levels in serum (data not shown) after 2 weeks of administration, such effect was not observed with YM-53601 treatment (Fig. 6B), indicating that inhibition of tumor progression by statins may be in part caused by isoprenoids down-regulation, in agreement with previous studies (9, 10). Interestingly, however, mice treated with atorvastatin or the SS inhibitor displayed a greater reduction in tumor growth after doxorubicin administration compared with untreated mice (Fig. 6C). Furthermore, a higher number of apoptotic cells assessed by TUNEL staining was observed in tumors from atorvastatin or YM-53601 treated mice after treatment with doxorubicin.
doxorubicin administration compared with doxorubicin alone (Fig. 6D), indicating an increased susceptibility of HCC to the chemotherapeutic agent. Together, these data highlight the potential relevance of the increased mitochondrial cholesterol in modulating the response of HC to chemotherapy in vivo.

Discussion

Since its description decades ago in heterotopic Morris hepatoma xenografts in Buffalo rats, the mitochondrial enrichment in cholesterol has been viewed mainly as a key factor underlying, in part, the mitochondrial dysfunction characteristic of cancer cells (7). In this study, however, we investigated the role of cholesterol accumulation in mitochondria from HCC in response to chemotherapy in vitro and in vivo and the mechanisms involved. First, we confirmed that established human and rat HCC exhibit increased mitochondrial cholesterol levels with respect to nontumor mitochondria of human and rat liver, as reported in solid hepatoma mitochondria compared with host liver (12–15), which paralleled those found in mitochondria from primary tumors of patients with HC. Close to 70% of the cholesterol found in mitochondria from HCC was unsterified, which, in contrast to esterified cholesterol, is known to regulate membrane dynamics and order (1, 2, 19, 27, 28). Moreover, the up-regulation of mitochondrial cholesterol content in HCC correlates with increased expression of SREBP-2 and HMG-CoAR, thus validating the cholesterol deregulation of cancer cells (7–9) and their use to examine the susceptibility to chemotherapy.

Our data uncover the resistance of HCC to chemotherapy-induced cell death that was reversed when cholesterol levels were reduced by inhibition of HMG-CoAR or SS. Although this strategy depletes cholesterol in different types of membranes other than mitochondria such as in specific domains of the plasma membrane, which has been described to modulate cancer therapy by regulating survival pathways such as Akt (5, 6), we provide evidence supporting a specific role for the mitochondrial cholesterol in modulating the susceptibility of HCC to chemotherapy. First, we show that the sensitivity of HepG2 and H35 cells to arsenic trioxide and lonidamine is potentiated by cholesterol depletion by HMG-CoAR or SS inhibition. Arsenic trioxide and lonidamine both induced MPT-mediated apoptosis by targeting the adenine nucleotide translocator (ANT)1 isoform (30, 31). Four ANT isoforms have been described, which are encoded by closely related genes that belong to the mitochondrial carrier family, and ANT is a major component of the MPT that mediate MMP and cell death (44). Mitochondria from murine cells lacking ANT 1 and ANT2 can

Figure 6. Reduction of cholesterol synthesis potentiates doxorubicin therapy in an in vivo murine model. HepG2 cells (2.5 × 10⁶ cells per animal) were implanted s.c. in the flanks of athymic mice as described in Materials and Methods. When tumors averaged 50 mm³ in size (2–3 wk), mice were randomly divided into the experimental groups and treated by a daily p.o. gavage with atorvastatin (10 mg/kg body weight) or YM-53601 (15 mg/kg body weight) for 2 wk. A, cholesterol levels from control (vehicle treated), atorvastatin, and YM-53601–treated tumors. (n = 5). *, P < 0.05 versus control. B, tumor growth measured periodically at the indicated days over the treatment. The volume was calculated as length × width² × 0.5. The experiment was performed twice with similar results. *, P < 0.05 versus vehicle-treated tumor. C, after 2 wk of treatment, some animals received an i.p. injection of doxorubicin (10 mg/kg). Fold increase of tumor growth was evaluated in each experimental group (n = 5) by calculating the changes in tumor size observed after administration of the chemotherapy agent. The experiment was performed twice with similar results. *, P < 0.05 versus doxorubicin-treated control mice. D, apoptosis induced by the chemotherapy agent in tumors from animals treated either with statins or SS inhibitor was analyzed by TUNEL-positive staining areas. Representative images of three samples per group performed showing similar results.
still undergo Ca\(^{2+}\)-induced swelling and MPT, although at a higher threshold (45), which have been viewed as evidence against a role for ANT in MPT and hence in MMP. However, the ability of ANT1/ANT2-deficient cells to undergo MMP might be due to the functional compensation by a novel ANT isofrom identified recently (46) or by other mitochondrial carriers able to form pores in the inner membrane, such as the ornithine/citrulline transporters or the phosphate carrier (47).

Furthermore, additional evidence for a role of mitochondrial cholesterol in chemotherapeuticsusceptibility derives from the outcome observed by StAR silencing. StAR is a polypeptide responsible for the intramitochondrial transport of cholesterol (11, 16) and both HepG2 and H35 cells overexpress StAR levels. StAR silencing by siRNA sensitizes these cell lines to chemotherapyproduced cell death. Consistent with its role in the mitochondrial trafficking of cholesterol, the sensitization to chemotherapeuticsy by StAR down-regulation could reflect impaired cholesterol transport from the outer to the inner mitochondrial membranes. However, our data indicate that StAR depletion by siRNA resulted in the net decrease of mitochondrial cholesterol levels, implying lower delivery of cholesterol to mitochondrion from extramitochondrial sources by other proteins including (StAR)-related lipid transfer (StART) family members. Although the molecular mechanisms of StAR are poorly understood, recent findings have shown that StAR interacts with specific outer mitochondrial membrane proteins such as VDAC1 and the phosphate carrier protein (48). Whether StAR works in concert with other START members via interaction with specific proteins of the outer mitochondrial membrane to deliver cholesterol to mitochondria remains to be investigated. Thus, collectively, these data strongly suggest that the enrichment of mitochondria in cholesterol plays a role in the resistance of chemotherapy acting via mitochondria.

To explore the mechanism of the mitochondrial cholesterol-mediated resistance to chemotherapy, we focused on the membrane properties of isolated mitochondria from HCC. We observed that mitochondria from HepG2 and H35 cells exhibit higher order which is reversed by cholesterol extraction with MCD or fluidization by A2C, translating in increased MMP and release of cytochrome c and Smac/Diablo in response to Ca\(^{2+}\), superoxide anion, and active Bax. An important piece of evidence supporting the specificity of cholesterol in the regulation of MMP is the fact that the in vitro enrichment of rat liver mitochondria in cholesterol to about the same levels seen in H35 cells reproduces the resistance to MMP and release of intermembrane apoptotic proteins induced by active Bax. Consistent with these observations, cholesterol-containing LUVs are also resistant to the poration induced by Bax, and cholesterol dose-dependently inhibited the vesicular release induced by melittin, which has been proposed to permeabilize membranes by forming toroidal lipidic pores (40). Because positive monolayer curvature stress contributes to MMP by Bax (24), it is conceivable that cholesterol may stabilize mitochondrial membranes against Bax permeabilizing action through induction of negative curvature, consistent with the accumulation of cholesterol in high-curvature regions of membranes (49). Remarkably, cholesterol decreased the capacity of Bax to penetrate into the membrane, which is considered to be a critical step in Bax activation. Thus, through modulation of membrane order and curvature stress, cholesterol may regulate Bax activity and the formation of lipidic pores in mitochondrial membranes and, hence, cell death susceptibility.

We also observed that tumor growth and chemotherapy susceptibility in heterotopic murine tumors xenografts were modulated by HMG-CoAR or SS inhibition, with both strategies decreasing tumor mitochondrial cholesterol levels. The generation of farnesyl diphosphate from mevalonate branches into isoprenoids and squalene, which is then committed for cholesterol synthesis (Supplementary Fig. S4). Isoprenoids are known to modify the function of proteins through posttranslational modifications such as the Rho family of small GTPases that coordinates many aspects of cell motility through the reorganization of actin cytoskeleton and changes in gene transcription (9, 10). The isoprenylation is essential for Rho-mediated invasion of various tumors, including melanoma, pancreatic, and breast cancer cells (50). In the in vivo xenograft model, we observed that HMG-CoAR but not SS reduced tumor growth, suggesting that inhibition of tumor progression by statins may be in part caused by isoprenoids down-regulation. However, the susceptibility of HC xenografts to chemotherapy are observed upon statins or SS inhibition, supporting the relevance of cholesterol rather than isoprenoids generation in the resistance of HCC to chemotherapy. Thus, our findings regarding the role of SS inhibition on chemotherapy along with recent reports in different types of cancer cells such as human prostate carcinoma cell lines (51) highlight the relevance of cholesterol modulation in cancer therapy.

Interestingly in line with our data, recent findings in liposomes confirmed that mitochondrial Bax activation is inhibited by cholesterol (52). Moreover, mitochondria from HeLa cells treated with U18666A, which caused mitochondrial cholesterol up-regulation, showed a delay in the release of Smac/Diablo and cytochrome c, as well as in Bax oligomerization and partial protection against stress-induced apoptosis (52).

In summary, although enhanced cholesterol levels in cell membranes including mitochondria from cancer cells have been long known, we show here for the first time the relevance of the cholesterol-mediated regulation of mitochondrial membrane dynamics in the response of HCC to mitochondrial-targeting chemotherapy in vitro and in vivo. Moreover, the potentiation of HC chemotherapy by SS inhibition, which reduces cholesterol levels including in mitochondria, without perturbing isoprenoid metabolism, validates the specificity of cholesterol in chemotherapy resistance, and revitalizes the potential benefit of cholesterol down-regulation in cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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