Survivin Down-regulation Plays a Crucial Role in 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Inhibitor-induced Apoptosis in Cancer*

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3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HRIs) are widely used to reduce serum cholesterol in patients with hypercholesterolemia. Previous studies have shown that HRIs can induce apoptosis in colon cancer cells. In this study, we investigated the mechanisms underlying the apoptosis-inducing effect of HRIs in greater detail. The HRI lovastatin induced apoptosis in the human colon cancer cell line SW480 by blocking the cholesterol synthesis pathway. Immuno-blot analysis of anti-apoptotic molecules, including survivin, XIAP, cIAP-1, cIAP-2, Bcl-2, and Bcl-XL, revealed that only survivin expression was decreased by lovastatin. Survivin down-regulation by RNA interference induced apoptosis, and survivin overexpression rendered the cells resistant to lovastatin-induced growth inhibition. These results indicate that survivin down-regulation contributes substantially to the proapoptotic properties of lovastatin. Farnesyl pyrophosphate and geranylgeranyl pyrophosphate, downstream intermediates in the cholesterol synthesis pathway, simultaneously reversed survivin down-regulation and the blocking of Ras isoprenylation by lovastatin. Ras isoprenylation is important for the activation of Ras-mediated signaling, including the activation of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway. The PI3-kinase inhibitor down-regulated survivin in SW480 cells. In addition, lovastatin blocked Ras activation and Akt phosphorylation. We conclude that survivin down-regulation is crucial in lovastatin-induced apoptosis in cancer cells and that lovastatin decreases survivin expression by inhibiting Ras-mediated PI3-kinase activation via the blocking of Ras isoprenylation.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)3 reductase inhibitors (HRIs) are widely used to reduce serum cholesterol and are well tolerated by patients with hypercholesterolemia (1). HRIs prevent the formation of mevalonate from HMG-CoA by inhibiting the enzyme HMG-CoA reductase, thereby inhibiting cholesterol synthesis (2). In large clinical trials designed to study the changes in coronary events in coronary heart disease patients receiving HRIs, the number of newly diagnosed colon cancer cases showed a reduction of between 43% (3) and 19% (4) during a 5-year follow-up period. It has been reported that HRIs could induce apoptosis in colon cancer cells (5–7). Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), two downstream intermediates in the cholesterol synthesis pathway, belong to a class of compounds named isoprenoids. They are bound to several cellular proteins, including small GTPases such as Ras, Rho, and Rac, by a post-translational modification known as isoprenylation. This process involves the addition of a 15-carbon farnesyl chain in FPP (farnesylation) or a 20-carbon geranylgeranyl chain in GGPP (geranylgeranylation) to a cysteine sulfhydryl group near the carboxyl terminus. Isoprenylation of these proteins is crucial for membrane attachment (8, 9) and the subsequent acquisition of biological activity (10). In particular, Ras proteins are important regulators of cell survival and proliferation (11, 12). Since FPP and GGPP are downstream of mevalonate in the cholesterol synthesis pathway, depletion of mevalonate by HRI reduces FPP and GGPP availability. Therefore, the growth-inhibitory effect of HRIs appears to be mediated by the inhibition of Ras isoprenylation. However, the dominant target in Ras-mediated signaling for HRI-induced growth inhibition has remained elusive.

Survivin, a member of the inhibitor of apoptosis (IAP) family, is expressed in embryonic and fetal organs but has not been identified in differentiated normal tissues, with the exception of the thymus, basal colonic epithelium, endothelial cells, and neural stem cells. Elevated survivin expression was observed in human cancers of various origins such as the breast (13), esophagus (14), stomach (15, 16), colon (17, 18), pancreas (19), liver (20), uterus (21), and ovaries (22) as well as in leukemias (23). Since survivin overexpression desensitized cancer cells to several anticancer agents (24) and to irradiation (25), which induce apoptosis, it acts as a resistance factor against these cancer treatment modalities.

Li et al. (26) demonstrated that survivin is expressed in the G2/M phase of the cell cycle in a cycle-regulated manner. Bolton et al. and Chen et al. (27, 28) showed that survivin interacts directly with the mitotic regulator aurora-B kinase and enhances its activity. These results suggest that survivin pro-
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motes mitosis through aurora-B kinase activation and contributes to the aberrant growth of cancer cells.

Recently, we reported that survivin enhances Fas ligand expression in colon cancer cells through the up-regulation of specificity protein-1 (Sp1)-mediated gene transcription. This indicates that survivin enables cancer cells to counterattack immune cells by inducing Fas ligand-triggered apoptosis in the cells of the immune surveillance system (29).

In addition, we observed that survivin up-regulates human telomerase reverse transcriptase, a major determinant of telomerase activity, and maintains telomere length, indicating that survivin prolongs the cellular life span in cancer (30). These findings indicate that survivin is a multifunctional protein that is important for the proliferation of cancer cells in vivo.

Here, we show that the HRI lovastatin induces apoptosis in colon cancer cells and reduces survivin expression. Survivin overexpression desensitized the colon cancer cells to lovastatin. Additionally, survivin down-regulation was found to be linked to the prevention of phosphatidylinositol 3-kinase (PI3-kinase) activation through the blocking of Ras isoprenylation. Taken together, these results suggest that survivin down-regulation may be essential for lovastatin-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Lovastatin, mevalonate, FPP, GGPP, and squaraine were obtained from Sigma-Aldrich. The anti-survivin, anti-Bcl-2, anti-Bcl-X1, anti-Akt, anti-phosphorylated Akt, and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-XIAP, anti-cIAP-1, and anti-cIAP-2 antibodies were obtained from R&D Systems (McKinley Place, NE). The Pi3-kinase inhibitor LY294002 was sourced from Calbiochem.

Tissue Samples—Colon cancer tissues as well as the adjacent normal colonic mucosa counterparts were obtained from patients undergoing surgery at Sapporo Medical University Hospital and Hokkaido Gastroenterology Hospital (Sapporo, Japan). Before the acquisition of these tissues, informed consent was obtained from the patients after explaining the investigational nature of the study. The samples were immediately frozen and stored in liquid nitrogen. The tissues were also stained with hematoxylin/eosin (Merck KGaA, Darmstadt, Germany) and were reviewed by well-experienced gastrointestinal pathologists.

Cell Culture—The human colon cancer cell lines SW480, LS180, and HT-29 were obtained from the American Type Culture Collection (Manassas, VA). These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and grown at 37 °C in a humidified atmosphere of 5% CO2.

Measurement of Cell Viability—The viability of the colon cancer cells treated with lovastatin was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), an inner salt, by the CellTiter 96 AQueous One Solution cell proliferation assay (Promega) according to the manufacturer’s protocol. The amount of soluble formazan produced by viable cells as a result of MTS reduction was assessed by measuring the absorbance at 490 nm using a microtiter plate reader (SpectraFluor, TECAN, Maennedorf, Switzerland). Drug sensitivities were expressed in terms of the IC50 values obtained from the dose-response curves; the values were obtained after fitting the data to the linear quadratic model and corresponded to 50% clonogenic inhibition of the viable-cell fraction.

Detection of Apoptosis—Lovastatin-induced apoptosis was quantified by DNA content analysis. Cells were resuspended in 500 μl of hypotonic fluorescence solution (50 μg/ml propidium iodide in 0.1% sodium citrate and 0.1% Triton X-100) and incubated at room temperature for 30 min in darkness. A flow cytometry (EPICS XL-MCL cytometer, Beckman Coulter, Tokyo, Japan) was used to measure 20,000 events per sample. Cells undergoing apoptosis were also quantified by combined staining with annexin V and propidium iodide using the MEB-CYTO apoptosis kit (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer’s protocol. A total of 5,000 cells was analyzed by flow cytometry. All experiments were performed at least three times under each experimental condition.

Immunoblot Analysis—Cytoplasmic proteins were extracted from the cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) with a protease inhibitor mixture (protease inhibitor cocktail; Sigma-Aldrich). Equal amounts of the cytoplasmic proteins in Laemmli sample buffer were separated on a 4–20% gradient Tris-glycine gel (Invitrogen) under denaturing conditions by using Tris-glycine sodium dodecyl sulfate running buffer (Invitrogen). The proteins were electrophoretically transferred into nitrocellulose membranes (Invitrogen) and detected using the WesternBreeze chemiluminescent immunodetection kit (Invitrogen) according to the manufacturer’s protocol. Band intensity was semiquantified using the PhotoShop Elements software after conversion to a digitized image using an image scanner (GT-9700F, EPSON, Tokyo, Japan).

Quantification of Survivin and HMG-CoA mRNA—The expression of survivin and HMG-CoA mRNA was determined by quantitative reverse transcription PCR (RT-PCR) using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) as described previously (29, 30). The gene-specific primers and fluorescent hybridization probes for survivin and HMG-CoA used in the quantitative RT-PCR were as follows. For survivin, the forward primer was 5’-AAG AAC TGG CCC TTC TTG GA-3’; the reverse primer was 5’-CCG GAC GAA TGC TTG T-3’; and the probe was 5’-(FAM) CCA GAT GAC GAC CCC ATA GAG GAA CA (TMARA)-3’. For HMG-CoA, the forward primer was 5’-GCC GTG CGT CTT C-3’; the reverse primer was 5’-CAC TGC GAA CCC TTC AGA TGT-3’; and the probe was 5’-(FAM) TCT GCA GAA GTG AAA GCC TGG CTG CA (TMARA)-3’. Survivin-2B (retaining a part of intron 2 as a cryptic exon) and survivin-ΔEx3 (lacking exon 3), which are two splice variants of survivin, were not detected by this set of primers and probe (31). The amounts of these two types of mRNAs were normalized to ratios to the amounts of 18S rRNA. PCR products were also confirmed by gel electrophoresis.

Transfection of siRNA for Survivin into Colon Cancer Cells—A small inhibitory RNA (siRNA) was designed to target the coding region of the survivin gene (nucleotides 366–385, relative to the start codon). As the transfection control, we pre-
pared scramble RNA containing the same number of each nucleotide as the siRNA targeting the survivin gene. The following siRNA duplexes were used in this study: survivin, 5′-H11032-GAA UUU GAG GAA ACU GCG A TT-3′/H11032 and 3′/H11032-TT CUU AAA CUC CUU UGA CGC U-5′; scramble RNA, 5′/H11032-GCA UUG GAU AAG ACG UAG A TT-3′/H11032 and 3′/H11032-TT CGU AAC CUA UUC UGC AUC U-5′. Transfections were performed using the Oligofectamine reagent (Invitrogen) according to the manufacturer’s protocol.

**Transfection of Survivin Gene into Colon Cancer Cells**—The plasmid encoding human survivin pcDNA3-Myc-survivin and the vector control pcDNA3-Myc were kindly provided by Dr. John C. Reed (The Burnham Institute, La Jolla, CA). Transfections were performed using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. Genetin-resistant clones were used in this study.

**Ras Activation Assays**—SW480 cells were serum-starved for 48 h in the presence or absence of 20 μM lovastatin and treated...
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with 10% FBS for 10 min. The cells were washed with ice-cold phosphate-buffered saline and lysed in Mg²⁺ lysis/wash buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% w/v Nonidet P-40, 10 mM MgCl₂, 1 mM EDTA, 25 mM NaF, 1 mM sodium orthovanadate, 10% w/v glycerol, and protease inhibitors). Activated Ras was identified using a Ras activation assay kit (Millipore Upstate, Lake Placid, NY) according to the manufacturer’s protocol. Briefly, lysate containing 250 μg of protein was incubated at 4 °C with agarose-conjugated Raf1/Ras-binding domain for 30 min. Agarose beads were washed three times with Mg²⁺ lysis/wash buffer and then resuspended in Laemmli sample buffer. Immunoblotting was performed with the anti-Ras antibody. To determine the amount of total Ras protein and isoprenylated Ras protein, whole-cell lysate was separated on a 6% Tris-glycine gel and a 4–20% gradient Tris-glycine gel, respectively.

RESULTS

Lovastatin Induces Apoptosis in Colon Cancer Cells—To investigate the growth-inhibitory effect of lovastatin on colon cancer cells, we cultured SW480, LS180, and HT-29 cells with various concentrations of lovastatin (5–20 μM) for up to 96 h and determined the viable cell number using the MTS assay (Fig. 1A). The growth of all three cell lines was inhibited by lovastatin in a dose-dependent manner, and the IC₅₀ values of lovastatin for SW480, LS180, and HT-29 cells were 7.1, 25.3, and 46.8 μM, respectively.

To determine whether the growth-inhibitory effect of lovastatin is mediated by the blocking of the cholesterol synthesis pathway, we performed add-back experiments using mevalonate, an intermediate in the cholesterol synthesis pathway (Fig. 1B). SW480 cells were cultured with 20 μM lovastatin and mevalonate at concentrations of 1–100 μM for 48 h. Mevalonate at concentrations greater than 50 μM reversed the lovastatin-induced growth inhibition.

Previous studies demonstrated that lovastatin induced cell cycle arrest and/or apoptosis in colon cancer cells (5–7). We then examined the effect of lovastatin on cell cycle progression. SW480 cells were treated with 20 μM lovastatin for 48 h, and the cell cycle status was assessed by DNA content analysis using flow cytometry (Fig. 1C). When compared with untreated cells, lovastatin-treated cells did not show any tendency toward an increased proportion of cells in the G₀/G₁, S, or G₂/M phases of the cell cycle, indicating that lovastatin did not induce cell cycle arrest in SW480 cells. On the other hand, lovastatin markedly increased the hypodiploid DNA content, indicating SW480 cell apoptosis. This resulted in 20.7% more apoptosis in the lovastatin-treated cells than in the untreated cells, and 100 μM mevalonate antagonized the lovastatin-induced apoptosis.

To confirm this observation, SW480 cells were treated with 20 μM lovastatin, and the proportion of apoptotic cells was determined using annexin V-propidium iodide staining, which is more sensitive for detecting apoptosis than detection based on the hypodiploid DNA content. As shown in Fig. 1D, 24.2% more annexin V-positive cells were observed among the lovastatin-treated cells than among the untreated cells, indicating that lovastatin induced apoptosis in SW480 cells.

Lovastatin down-regulates survivin protein by inhibiting gene transcription. SW480 cells were cultured with 20 μM lovastatin in the presence or absence of 100 μM mevalonate for 24 or 48 h. The expression level of antian apoptotic proteins (A) was examined by immunoblot analysis. Survivin mRNA (B) was quantified using quantitative RT-PCR. Data are represented as the percentage of untreated cells under each experimental condition (mean ± S.D.). Open bars, 24-h treatment; gray bars, 48-h treatment. Significant differences were observed in the expression of survivin and mevalonat in lovastatin-treated cells.

Lovastatin Down-regulates Survivin in Colon Cancer Cells—Many investigators showed that the expression of antiapoptotic molecules, including those from the Bcl-2 and IAP families, was elevated in cancer cells, and reduction in the expression of these proteins by gene-targeting techniques resulted in the induction of apoptosis. To identify the possible molecular mechanism underlying lovastatin-induced apoptosis, we examined the expression level of antian apoptotic proteins, including members of the IAP (survivin, XIAP, cIAP-1, and cIAP-2) and Bcl-2 (Bcl-2 and Bcl-X₁) families, by immunoblot analysis in SW480 cells treated with 20 μM lovastatin. As shown in Fig. 2A, only survivin expression was attenuated by lovastatin; it decreased to undetectable levels at 48 h after treatment. In addition, mevalonate completely reversed the lovastatin-induced survivin down-regulation.

Another possible mechanism underlying lovastatin-induced apoptosis is up-regulation of the proapoptotic molecule Bax. However, lovastatin did not affect Bax expression (data not shown).
To clarify whether lovastatin-induced survivin down-regulation occurs at the transcriptional level, we used quantitative RT-PCR to quantify the survivin mRNA in SW480 cells treated with 20 μM lovastatin (Fig. 2B). Survivin mRNA expression in the lovastatin-treated cells was decreased by 60% when compared with that in the untreated cells at 48 h after treatment, and this effect was blocked by the addition of mevalonate.

**Figure 3.** Survivin down-regulation by survivin-specific siRNA induced apoptosis in colon cancer cells. A, survivin protein expression in colon cancer cells transfected with survivin-specific siRNA. SW480 cells were transfected with siRNA that targeted the survivin gene (survivin siRNA), and survivin protein expression was assessed by immunoblot analysis at 3 and 5 days after transfection. Scramble RNA containing the same number of each nucleotide as that in the survivin siRNA was used as the transfection control. B and C, cell viability and proportion of apoptotic cells in survivin-specific siRNA-transfected cells treated with lovastatin. Cell viability (B) and the proportion of apoptotic cells (C) were examined using the MTS assay and DNA content analysis, respectively, at 5 days after transfection. The percentages of hypodiploid DNA content (C) are indicated for each test condition.

**Down-regulation of Survivin Induces Apoptosis in Colon Cancer Cells—**To examine the contribution of survivin down-regulation to lovastatin-induced apoptosis, we introduced siRNA targeting the survivin gene (survivin siRNA) into SW480 cells. Fig. 3A shows survivin protein expression examined by immunoblot analysis in survivin siRNA-transfected cells. The introduction of survivin siRNA completely abrogated survivin protein expression at 3 days after transfection, and this reduction continued until 5 days after transfection. No reduction in survivin protein was observed in cells transfected with the scramble RNA, which contained an equal number of individual nucleotides as those in survivin siRNA.

We then determined the viability of survivin siRNA-transfected cells at 5 days after transfection by using the MTS assay (Fig. 3B). When compared with untreated cells, the scramble RNA-transfected cells did not show a significant change in the viable cell number. However, the introduction of survivin siRNA decreased the viable cell number by 24%. In addition, DNA content analysis revealed that the hypodiploid DNA content had increased in the survivin siRNA transfectants, indicating that survivin down-regulation resulted in the induction of apoptosis (Fig. 3C).

**Survivin Up-regulation Protects Colon Cancer Cells from Lovastatin-induced Growth Inhibition—**Next, we examined whether survivin up-regulation could confer protection against lovastatin-induced growth inhibition. We introduced the survivin gene expression vector pcDNA3-Myc-survivin into LS180 cells. As shown in Fig. 4A, the survivin gene transfectants expressed Myc-tagged survivin protein in addition to endogenous survivin protein. Lovastatin attenuated the expression of endogenous survivin in the survivin gene transfectants as well as in the parent LS180 cells and control vector transfectants (vector control). However, no remarkable change was observed in the expression level of the Myc-tagged survivin protein.

Using the MTS assay, we determined the cell viability of the survivin gene transfectants treated with 10 or 20 μM lovastatin for 72 h (Fig. 4B). Survivin overexpression completely abrogated the growth-inhibitory effect of lovastatin, whereas the growth of the parent LS180 cells and control vector transfectants was inhibited by lovastatin in a dose-dependent manner.
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These results suggest that survivin down-regulation by lovastatin strongly contributes to its proapoptotic properties.

**FPP and GGPP Depletion by Lovastatin Contributes to Survivin Down-regulation**—We next examined the effect of intermediates in the cholesterol synthesis pathway on lovastatin-induced survivin down-regulation. SW480 cells were incubated with 20 \( \mu M \) lovastatin for 48 h in the presence or absence of intermediates such as mevalonate, FPP, GGPP, and squalene. As shown in Fig. 5A, not only mevalonate but also FPP and GGPP reversed survivin down-regulation, whereas squalene did not. Since GGPP biosynthesis requires both FPP and isopentenyl pyrophosphate (another intermediate in the cholesterol synthesis pathway), the addition of only FPP could not produce GGPP. Therefore, FPP and GGPP depletion by lovastatin may contribute to survivin down-regulation individually.

**Mechanisms Underlying Lovastatin-induced Survivin Down-regulation Involve Inhibition of PI3-kinase Activation via Blocking of Ras Isoprenylation**—Small GTPase Ras proteins (H-Ras, N-Ras, and K-Ras) play a central role in cell survival and proliferation (11, 12). To play these roles, Ras proteins have to be active, and this localization is achieved by isoprenylation (8–10). To confirm that lovastatin inhibits Ras isoprenylation under the present experimental condition, we performed immunoblot analysis using anti-pan Ras antibodies that recognize H-Ras, N-Ras, and K-Ras (Fig. 5B). In SW480 cells treated with 20 \( \mu M \) lovastatin for 48 h, the band intensity of the fast-migrating band that indicates isoprenylated Ras was decreased when compared with that in untreated cells. The addition of mevalonate, FPP, and GGPP reversed the inhibition of Ras isoprenylation by lovastatin, whereas squalene did not. Since the change in the amount of isoprenylated Ras correlated with that in survivin expression (Fig. 5, A and B), the inhibition of Ras isoprenylation may be linked to survivin down-regulation.

Attempts were made to understand the relationship between Ras isoprenylation and survivin expression. Since activated Ras-mediates signaling involves the PI3-kinase/Akt pathway (11, 12), we treated SW480 cells with 20 \( \mu M \) LY294002 (a PI3-kinase/Akt pathway inhibitor) for 24 h and examined survivin protein expression by immunoblot analysis. As shown in Fig. 5C, LY294002 down-regulated survivin expression, indicating that the PI3-kinase/Akt pathway regulates survivin expression. We then determined whether lovastatin inhibited the Ras-mediated PI3-kinase activation. First, the effect of lovastatin on Ras activity was assessed by detecting the GTP-bound Ras protein (Fig. 5D). SW480 cells were serum-starved for 48 h in the presence or absence of 20 \( \mu M \) lovastatin and treated with 10% FBS for 10 min. FBS stimulation increased the amount of GTP-bound Ras protein in the untreated cells. However, this increase was not observed in the lovastatin-treated cells. Further, mevalonate reverted the Ras activation induced by FBS stimulation, and the change in the amount of GTP-bound Ras protein correlated with that in isoprenylated Ras expression.

Next, the effect of lovastatin on PI3-kinase activity was determined under the same experimental conditions. Since PI3-kinase phosphorylates Akt, its activity was assessed by immunoblot analysis using antibodies against phosphorylated Akt. As shown in Fig. 5E, lovastatin inhibited the FBS-induced increase of phosphorylated Akt protein that was observed in untreated cells. These results indicate that the mechanisms by whichLovastatin down-regulates survivin involve the inhibition of Ras-mediated PI3-kinase activation via the blocking of Ras isoprenylation.

**Survivin mRNA Expression Correlates with HMG-CoA Reductase mRNA Expression in Colon Cancer Tissues**—Many previous studies showed that elevated survivin expression was observed in human cancers of various origins (13–23). Our data indicate that the intermediates in the cholesterol synthesis pathway, particularly FPP and GGPP, regulate survivin expression. Based on these observations, we hypothesized that since the cholesterol synthesis pathway is accelerated in cancer cells, survivin expression is elevated. We then examined survivin and HMG-CoA reductase mRNA expression in colon cancer tissues using quantitative RT-PCR. As shown in Fig. 6, A and B, the expression ratios of both survivin and HMG-CoA reductase mRNAs were significantly greater in cancerous mucosa than in the normal mucosa (survivin: 1.00 ± 2.04 (mean ± S.D.) for normal mucosa, 10.00 ± 8.22 for cancerous mucosa, \( p < 0.001 \); HMG-CoA reductase: 0.48 ± 0.90 for normal mucosa, 0.59 ± 0.44 for cancerous mucosa, \( p < 0.005 \)). In addition, a correlation was identified between survivin mRNA expression and HMG-CoA reductase mRNA expression in colon cancer tissues (\( r = 0.51 \); Fig. 6C). These results suggest that HMG-CoA reductase up-regulation induces survivin expression through the promotion of FPP and GGPP production.
DISCUSSION

In this study, we showed that lovastatin inhibits survivin expression and that survivin down-regulation plays an important role in lovastatin-induced apoptosis in colon cancer cells. Induction of apoptosis and survivin down-regulation by lovastatin was also observed in the pancreatic cancer cells, MIAPaCa-2 and PANC-1, and in the acute myelogenous leukemia cells, U937 (data not shown). Therefore, the lovastatin-mediated down-regulation of survivin in cancer cells may be a generalized event in cancer cells.

We also demonstrated that FPP and GGPP depletions via the blocking of mevalonate synthesis are important for lovastatin-induced survivin down-regulation. In this study, HT-29 cells showed more resistance against lovastatin-induced growth inhibition than SW480 cells. Interestingly, HMG-CoA reductase mRNA expression in HT-29 cells is two times greater than that in cancer cells. Induction of apoptosis and survivin down-regulation by lovastatin was also observed in the pancreatic cancer cells, MIAPaCa-2 and PANC-1, and in the acute myelogenous leukemia cells, U937 (data not shown). Therefore, the lovastatin-mediated down-regulation of survivin in cancer cells may be a generalized event in cancer cells.

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SW480 cells (data not shown). Therefore, it is plausible that the lovastatin concentration used in this study may be insufficient for HT-29 to block mevalonate synthesis by HMG-CoA reductase.

Wachtershuser et al. (7) showed that the HRI mevastatin-induced cell cycle arrest occurred at the G2/M phase in human colon cancer cells. However, lovastatin did not result in cell cycle arrest in SW480 cells (Fig. 1C). Since survivin enhanced the activity of aurora-B kinase that is required for progression to the M phase (27, 28, 32), lovastatin may be expected to induce cell cycle arrest at the G2/M phase. The reason for these differences remains unclear. Interestingly, Kappler et al. (33) demonstrated that five human sarcoma cell lines transfected with a survivin-specific siRNA showed G2/M arrest, with fewer than 10% of these cells undergoing apoptosis. In contrast, when we introduced survivin siRNA into SW480 cells, apoptosis was observed rather than cell cycle arrest. Blockade of apoptotic signaling by survivin involves the inhibition of the activation of caspase-9, which subsequently activates effector caspases such as caspase-3 and -7 (34, 35). JANICKE et al. (36) reported that procaspase-3 was absent in MCF-7 human breast cancer cells because of the partial deletion of the CASP-3 gene. Therefore, one possible explanation might be that cancer cells with low caspase expression undergo cell cycle arrest after lovastatin treatment.

As shown in Fig. 2B, lovastatin attenuated survivin expression at the transcriptional level. Results obtained from the experiments with survivin gene transfectants convinced us that the inhibition of survivin gene transcription is the main mechanism underlying lovastatin-induced survivin down-regulation. The reasons are as follows. The expression of Myc-tagged survivin, which was derived from the survivin gene expression vector pcDNA3-Myc-survivin, was not influenced by lovastatin treatment, whereas that of endogenous survivin was decreased in the survivin gene transfectants. Since pcDNA3-Myc-survivin carries the human cytomegalovirus immediate-early enhancer/promoter region that promotes the constitutive expression of cloned Myc-tagged survivin, the transcription of the Myc-tagged survivin gene might escape the effects of lovastatin. Second, if lovastatin promoted the degradation of the survivin protein, both Myc-tagged survivin and endogenous survivin protein would be decreased by lovastatin treatment.

In this study, we demonstrated that the PI3-kinase inhibitor LY294002 attenuates survivin expression. We also showed that lovastatin induced a decrease of Ras activity with a simultaneous decrease in the amount of isoprenylated Ras. Further, the change in the Ras activity correlated with that in the amount of PI3-kinase activity. Based on these correlative results, we suggest that the lovastatin-induced survivin down-regulation is mediated by the inhibition of Ras-induced PI3-kinase activation.

It has been demonstrated that survivin expression is induced by the nuclear factor-κB (NF-κB) pathway (37). OZES et al. (38) demonstrated that Akt activates the NF-κB pathway through interaction and phosphorylation of IκB kinase α. Therefore, the attenuation of survivin gene transcription by lovastatin could be mediated by inactivation of the NF-κB pathway through blocking of PI3-kinase/Akt signaling. However, NF-κB has been known to promote the transcription of other antiapoptotic genes, including those encoding XIAP, cIAP-1, cIAP-2, Bcl-2, and Bcl-XL (39–42), and we did not observe the down-regulation of these molecules by lovastatin in colon cancer cells. These results indicate that suppression of NF-κB activation does not appear to fully account for the down-regulation of survivin mRNA by lovastatin.

Recently, LI et al. (43) showed that the survivin promoter contains an Sp1-binding site, and Sp1 promotes survivin transcription. On the other hand, BADRICHANI et al. (44) demonstrated that expression of neither Bcl-2 nor Bcl-XL is influenced by Sp1. Our literature search did not identify any study that indicates that Sp1 promotes the transcription of the XIAP, cIAP-1, and cIAP-2 genes. Phosphorylation of Sp1 at serine and threonine residues has been reported to enhance its DNA binding activity (45, 46), and PI3-kinase is involved in this phosphorylation (47). Based on these observations, the inhibition of Sp1-mediated gene transcription through the blocking of PI3-kinase/Akt signaling may be another mechanism underlying lovastatin-induced attenuation of survivin gene transcription.

Since interference of Ras isoprenylation has a relationship with survivin down-regulation, inhibitors of farnesytransf erase, the enzyme that catalyzes Ras farnesylation, may become another pharmacological approach for targeting survivin. Farnesyltransferase inhibitors (FTIs) effectively inhibit H-Ras signaling (48). However, they do not block the function of K-Ras (49, 50), the Ras isoform that is most frequently mutated in human tumors. In the presence of FTIs, K-Ras is alternatively isoprenylated by the FTase-related enzyme geranylgeranyltransferase I. Consequently, K-Ras retains its membrane association and function (51). Therefore, the inhibition of K-Ras isoprenylation requires co-treatment with FTIs and geranylgeranyltransferase I inhibitors. In our study, the lovastatin-induced decrease in Ras isoprenylation was reversed by GGPP, similar to that by FPP (Fig. 5B); this indicates that alternative geranylgeranylation of K-Ras might have occurred. In addition, both FPP and GGPP could prevent lovastatin-induced survivin down-regulation (Fig. 5A). These results indicate that geranylgeranylated K-Ras as well as farnesylated K-Ras may play an important role in the regulation of survivin expression, and co-treatment with FTIs and geranylgeranyltransferase I inhibitors is essential for survivin down-regulation. Therefore, HRIs have an advantage over FTIs in the targeting of survivin because HRIs can block both farnesylation and geranylgeranylation of K-Ras simultaneously.

We demonstrated that the expression level of HMG-CoA mRNA in colon cancer tissues is greater than that in the uninvoluted mucosa. Similar observations have been reported in leukemia cells (52) and lung cancer cells (53). In addition, our study shows that there is a correlation between HMG-CoA mRNA expression and survivin mRNA expression in colon cancer tissues. These results indicate that acceleration of the cholesterol synthesis pathway induced by the elevated expression of HMG-CoA reductase in cancer cells may promote Ras isoprenylation. Consequently, cancer cells harbor elevated levels of survivin. From this viewpoint, it may be reasonable to use agents that target HMG-CoA reductase for effectively down-regulating the survivin in cancer cells.
Administering lovastatin orally at the concentration that induces apoptosis in colon cancer cells in our study is not possible in vivo (54). However, we believe that if lovastatin could be administered intravenously or into tumor-feeding arteries and high lovastatin concentrations could be achieved in vivo, new avenues for cancer treatment would be opened.

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