Lovastatin inhibits tumor growth and lung metastasis in mouse mammary carcinoma model: a p53-independent mitochondrial-mediated apoptotic mechanism

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The effects of lovastatin, a potent inhibitor of hydroxymethylglutaryl-coenzyme A reductase, were studied in a mouse model of metastatic mammary cancer carrying a p53 mutation. Mice bearing mammary tumors, induced by inoculation of syngeneic BALB/c mice with BJMC:3879 cells, were treated with lovastatin at 0, 25 and 50 mg/kg three times a week. Tumor volumes were significantly reduced in a dose-dependent manner throughout the 6 week study and were associated with both a decrease in DNA synthesis and an increase in apoptosis. The high dose of lovastatin also inhibited lung metastasis. In a corollary in vitro study, flow cytometric analyses of lovastatin-treated mammary cancer cells additionally showed cell cycle arrest at G1 phase and decreases in S and G2/M phases. Laser scanning cytometric analyses further demonstrated that cancer cells in S and G2/M were particularly susceptible to the effects of lovastatin. Transmission electron microscopic evaluation of TUNEL-confirmed apoptotic bodies in lovastatin-treated mammary carcinoma cells revealed many free 3'-OH ends of DNA in condensed chromatin within fragmented nuclei that occasionally assumed a characteristic half-moon shape. Consistent with initiation of apoptosis, cellular caspase-8, caspase-9 and caspase-3 activities were elevated in lovastatin-treated cells. The mitochondrial membrane potential was also decreased, with subsequent release of cytochrome c. However, lovastatin-induced cell death was significantly reduced by the broad spectrum caspase inhibitor z-VAD-fmk, as well as the caspase-9 inhibitor z-LEHD-fmk and the caspase-3 inhibitor z-DEVD-fmk, but not by the specific caspase-8 inhibitor z-IETD-fmk. Since immunoelectron microscopy showed translocation of Bax to the mitochondria in lovastatin-treated cells, lovastatin-induced apoptosis may, therefore, be ultimately dependent on Bax induction of cytochrome c release. These results suggest that lovastatin may be useful as an adjuvant therapy in breast cancers containing p53 mutations due to its ability to both suppress DNA synthesis and induce p53-independent mitochondria-mediated apoptosis.

Introduction

Breast cancer is one of the most frequent female cancers and the incidence has risen dramatically worldwide in the past decade (1); in the USA alone the disease has become the second leading cause of death in women (1). Although breast cancer still ranks fifth as a cause of female mortality in Japan, the number of deaths attributed to breast cancer has risen 2.6-fold between 1975 and 1998 in this country as well (2).

Lovastatin is a competitive inhibitor of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, and is generally regarded as a safe and effective compound used extensively for the treatment of hypercholesterolemia (3). Other properties of lovastatin have been identified, however, that suggest it may also have chemotherapeutic effects. In breast cancer cell cultures lovastatin induces two cell cycle inhibitors, p21Waf1 and p27Kip1, and leads to cell cycle arrest in the G1 phase (4–7). In other in vitro studies apoptosis has been induced by lovastatin in cancer cells derived from various organs, including the breast (7–10).

Statins, including lovastatin itself, have been shown to variously inhibit tumor growth and metastasis in vivo in several different implantable rodent tumorigenesis models, e.g. in mammary carcinoma (7,9–11), fibrosarcoma (12), colon adenocarcinoma (13), melanoma (14) and pancreatic neoplasias (15). However, data specific to the effect of statins on mammary cancers, particularly on their anti-metastatic properties, is limited; only one group, using lovastatin on a murine sarcomammary carcinoma cell line (F3H) established and inoculated into syngeneic mice, has investigated the anti-metastatic effects induced by statins in any depth (9,10). In this study we show that lovastatin suppressed tumor growth and metastasis in vivo in a mouse mammary cancer model and, as a result of data produced in correlated in vitro studies, we believe that we can identify at least part of the suppression mechanism.

Materials and methods

Experimental compound

The sodium salt of lovastatin was used for the in vivo study (Merk & Co., Rahway, NJ). The lovastatin was sonicated into suspension in saline for animal experiments. For the in vitro studies lovastatin was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). For in vivo use the inactive lactone form of lovastatin was converted to the active dihydroxy open acid form by dissolution in ethanol, followed by incubation in 0.4 M NaOH in ethanol at 50°C for 2 h and adjusting the pH to 7.2 with 1 M HCl. Aliquots of this stock solution (10 mM) were stored at –20°C.

In vivo studies

Animals. A total of 42 female 6-week-old BALB/c mice were used in this study (Japan SLC, Hamamatsu, Japan). The animals were housed at 4 per cage in the preliminary dosage-tolerance study and 5 per plastic cage in the tumor growth phase on wood chip bedding with free access to water and food under controlled temperature (21 ± 2°C), humidity (50 ± 10%) and lighting (12:12 h light-dark cycle). All animals were held for a 1 week acclimatization period.
before study commencement. All manipulations of mice were performed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals of Osaka Medical College.

Preclinical dosage–tolerance study. To ensure that toxicity would not be a factor, we conducted a preliminary dosage–tolerance study for the proposed 0 (saline), 25 and 50 mg/kg doses of lovastatin using 4 mice for each dose. The animals were injected i.p. with the appropriate dose 3 times a week for 9 consecutive weeks. No deaths occurred in any of the groups and only the 50 mg/kg group showed any weight loss (10%) at week 9 compared with controls, indicating that no dose adjustments were necessary.

Tumor growth study. The BJMC3879 mammary adenocarcinoma cell line was derived from a metastatic focus within a lymph node from a female BALB/c mouse that had been infected with a murine mammary tumor virus (MMTV) injected into the inguinal mammary glands (16). The mammary tumors resulting from MMTV inoculation showed a high metastatic propensity to lungs and lymph nodes (17), a trait retained through culture.

For the current study BJMC3879 cells (5 × 10⁴ cells/ml) were inoculated s.c. into the right inguinal region of 30 female BALB/c mice. Two weeks later, when tumors had developed to ~0.2 cm diameter, groups of 10 mice each were injected i.p. with either vehicle alone or with 25 or 50 mg/kg lovastatin 3 times a week for 6 weeks. Individual body weights were recorded weekly; using calipers, each mammary tumor was also measured weekly and tumor volumes calculated using the formula maximum diameter × (minimum diameter)² × 0.4 (18). Three hours after the last lovastatin or vehicle treatment all animals were injected i.p. with 100 mg/kg 5-bromo-2'-deoxyuridine (BrdU) (Sigma Chemical Co., St Louis, MO). One hour after BrdU injection the mice were killed with diethyl ether and exsanguinated.

Histopathology. At necropsy tumors and lymph nodes, routinely those from the axillary and femoral regions as well as those appearing abnormal, were removed, fixed in 4% formaldehyde solution in phosphate buffer and processed through to paraffin embedding. Lungs were infused with the formaldehyde solution prior to excision and immersion in fixative. The individual lobes were subsequently removed from the bronchial tree, trimmed into seven pieces and examined for metastatic foci before being similarly processed to paraffin embedding. All paraffin-embedded tissues were cut at 4 μm, with sequential sections stained with hematoxylin and eosin (H&E) for histopathological examination and reserved unstained for immunohistochemistry.

p53 immunohistochemistry. The avidin–biotin complex method was used for p53 immunohistochemistry. Unstained sections were immersed in distilled water and heated by microwave irradiation for antigen retrieval prior to incubation with an anti-p53 mouse monoclonal antibody (Clone PC02; Santa Cruz Biotechnology, Santa Cruz, CA) that reacts with the mutant protein in fixed specimens.

DNA synthesis. As previously mentioned, all animals received 100 mg/kg BrdU (Sigma Chemical Co.) i.p. 1 h prior to killing with ether and from the tumors and lymph nodes excised from each animal, 5 μm sections were cut and evaluated for DNA synthesis rate as inferred by BrdU incorporation. Using unstained paraffin-embedded tissue sections, DNA was denatured in situ by incubation in 4 N HCl solution for 20 min at 37°C. The incorporated BrdU was visualized after exposure to an anti-BrdU mouse monoclonal antibody (Clone Bu20a; Dako, Glostrup, Denmark). The numbers of BrdU-positive S phase cells per 5000 cells were counted in five random high power (>400) fields of viable tissue and the BrdU labeling indices were expressed as a percentage of total cells counted. Sections of small intestine served as positive controls.

TUNEL assay for apoptosis. For the quantitative analyses of apoptosis, sections from paraffin-embedded tumors were assayed using the terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling (TUNEL) method in conjunction with an apoptosis in situ detection kit (Wako Pure Chemical Industries) with minor modifications to the manufacturer’s protocol. TUNEL-positive cells were counted in viable regions peripheral to areas of necrosis in tumor sections. The numbers of TUNEL-positive cells per 5000 cells were counted in five random high power (>400) fields and expressed as a percentage of the total cells counted. Sections from testes were used as positive controls.

In vitro studies

Cell lines. BJMC3879 cells, derived from a metastatic focus of murine mammary adenocarcinoma (16,17), were maintained in Dulbecco’s modified Eagle’s medium or RPMI-1640 containing 10% fetal bovine serum with streptomycin/penicillin in an incubator under 5% CO₂. Cell cultures of the human breast cancer line MDA-MB231 were also maintained under the same conditions and served as a corollary test system.

Cell growth. BJMC3879 cells were plated the day before lovastatin treatment at 1 × 10⁴ cells/well in 6-well plates. They were subsequently incubated for 48 h with culture medium containing vehicle alone or with medium containing lovastatin at various concentrations up to 80 μM and counted. In addition, the response to lovastatin over time was determined by examining the effects of 10 and 20 μM lovastatin on cell growth for 24 and 48 h.

Flow cytometric analysis of cell cycle. Flow cytometric analysis was conducted on tryptophanized BJMC3879 cell suspensions harvested after 24 h treatment with 20 μM lovastatin and fixed in cold 70% ethanol. The cells were stained with a 50 μg/ml propidium iodide solution containing 100 μg/ml RNase A for 30 min on ice just prior to analysis with a flow cytometer (EPICS Elite ESP; Coulter Co., Miami, FL) and the percentage of cells in each phase of the cell cycle was determined using a Multicycle Cell Cycle Analysis program (Coulter).

TUNEL/light microscopy evaluation. BJMC3879 cells, grown in 2-well chamber slides and treated with 20 μM lovastatin for 24 h, were fixed in 4% formaldehyde solution in phosphate buffer and the TUNEL staining procedure performed as described in the in vivo study. The numbers of TUNEL-positive cells per 1000 counted in five random high power (>400) fields by conventional light microscopy were expressed as a percentage of the total cells counted.

TUNEL/transmission electron microscopy evaluation. BJMC3879 cells, grown in 2-well chamber slides and treated with 0 or 20 μM lovastatin for 24 h, were stained in a solution of 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer. These cells were then post-fixed with 1% osmium tetroxide in phosphate buffer, dehydrated, embedded in epoxy resin and cut into thin nickel grids that were then subjected to a staining step using sodium metaperiodate. A sheep anti-digoxigenin antibody conjugated to 10 nm colloidal gold (British BioCell International, Golden Gate, UK) was applied in place of a peroxidase-conjugated antibody (Apop Tag; Intergen Co., Purchase, NY) (19,20); cells previously exposed to the TUNEL procedure were stained with uranyl acetate and lead. All cell preparations were scanned under a model H-7100 electron microscope (Hitachi Co., Tokyo, Japan). Transmission electron microscopic (TEM) images of cell nuclei (control group normal looking nuclei; treated group apoptotic bodies) were captured and image analysis of DNA fragmentation performed using the NIH Image program (21). The labeling density of free 3-OH DNA ends was evaluated based on the number of immunogold particles per nuclear area of 10 randomly selected cells from each of control and lovastatin-treated cultures.

Laser scanning cytometric analysis. Human mammary carcinoma MDA-MB231 cells were grown in 2-well chamber slides (Lab-Tek II; Nalgen Nunc International, Naperville, IL), treated with 20 μM lovastatin for 32 h and fixed in 4% paraformaldehyde solution. The fluorosecin-TUNEL method was then applied for detection of apoptotic cells (Intergen Co., New York, NY). After the TUNEL procedure, nuclear DNA was stained with a 50 μg/ml propidium iodide solution containing 100 μg/ml RNase A for 30 min at 37°C for cell cycle analysis. The numbers of fluorosecin-TUNEL-positive cells were measured and their cell cycle phases determined with a microscope-based multiparameter laser scanning cytometer (LS2; Olympus Optical Co., Tokyo, Japan) and the resulting data analyzed with WinCyte software (Compucyte Co., MA).

BrdU and p53 immunohistochemistry. Mouse BJMC3879 cells treated with lovastatin for 24 h were incubated for 30 min in culture medium containing 50 μM BrdU (Sigma Chemical Co.) and trypsinized. The cells were then counted, fixed with 70% ethanol and distributed at 1×10⁵ cells/well on 96-well opaque plates. The plates were dried using a hair dryer and the dried cells within each well covered with 1 N HCl for 20 min at 37°C, carefully rinsed with PBS and exposed to an anti-BrdU antibody–peroxidase conjugate (Fab fragment; Roche Diagnostics, Indianapolis, IN) for 60 min. Each well was then rinsed and covered with a luminol substrate (Chemiluminescence; Roche Diagnostics) for 5 min. Light emissions of the samples were measured using a Luminoskan Ascent (ThermoElectron Co., Helsinki, Finland). Additional lovastatin-treated BJMC3879 cells grown on 2-well chamber slides were also formalin fixed, microwave irradiated and immunohistochemically stained for p53 as described above.

Caspase activities. The activities of caspase-8, caspase-9 and caspase-3 were measured in cells treated with 20 μM lovastatin for 24 h using a fluorometric protease assay kit (MBL Inc., Nagoya, Japan) in which cells were lysed with 0.1% Triton X-100 lysis buffer and the protein concentration adjusted to 25 μg in each sample. Caspase activity was measured in terms of fluorescence intensity using a VersaFluor fluorometer (Bio-Rad, Hercules, CA).

Specific caspase inhibition. Two hours prior to lovastatin exposure, BJMC3879 cells, plated at 1 × 10⁵ cells/well in 96-well culture plates overnight, were
treated variusly with the pan-caspase inhibitor z-VAD-fmk (fluoromethyl ketone), caspase-8-specific z-IETD-fmk, caspase-9-specific z-LEHD-fmk or caspase-3-specific z-DEVD-fmk; control cells were exposed to the 1% DMSO vehicle in which all inhibitors were dissolved. All caspase inhibitors were obtained from MBL Inc. and applied at concentrations of 10 and 100 μM. After 24 h, cell viability was measured using the MTT assay (Roche Diagnostics) and absorbance was measured spectrophotometrically at a dual wave length of 540 and 680 nm (ImmunoReader; Nippon InterMed Co., Tokyo, Japan).

Mitochondrial membrane potential (∆ψm). Changes in cellular ∆ψm were determined using a fluorescent cationic dye, 5,5′,6′,6′-tetrachloro-1,1′,3′,3′-tetraethyl-benzamidazolocarbocyanin iodide (JC-1) (Mit-E-Ψ Mitochondrial Permeability Detection Kit; Biomol Research Laboratories, Plymouth Meeting, PA), which easily penetrates cells and aggregates in negatively charged healthy mitochondria, giving them a red fluorescence. When ∆ψm collapses, the dye can no longer accumulate inside the mitochondria (22) and distributes throughout the cell. The dissipated dye assumes a monomeric form that fluoresces green, rather than red, resulting in an overall decrease in fluorescence intensity. The changes in ∆ψm in lovastatin-treated and control cells measured 24 h after exposure were expressed in terms of relative fluorescence units (RFU) using a VersaFluor fluorometer (Bio-Rad) with a 485 nm excitation filter and a 585−595 nm emission filter.

Release of cytochrome c. After incubation in culture medium with and without 20 μM lovastatin for 12 and 24 h, both floating and attached cells were harvested, rinsed once in PBS, immersed in a cold buffer solution (10 mM Tris–HCl, pH 7.5, 0.3 M sucrose, 1 μM aprotinin, 10 μM pepstatin, 10 μM leupeptin, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), homogenized and centrifuged at 700 g for 10 min at 4°C. The supernatants were centrifuged further at 10 000 g for 30 min at 4°C. Supernatants containing the cytosolic fraction were collected separately and protein concentrations determined by the Bradford assay (Bio-Rad). ELISA was performed using the Cytochrome C ELISA Kit (Chemicon International Inc., Temecula, CA) to determine cytochrome c release into the cytosol.

Western blotting for cytochrome c. The presence of cytosolic cytochrome c was also confirmed by western blots using both 20 μg protein from the cytosolic fraction used for ELISA as well as protein derived from whole cell lysates of cells treated with 20 μM lovastatin for 24 h. Cell lysates were additionally used for analysis of Bcl-2, Bax and Bid expression. Aliquots of 10 and 20 μg protein were fractionated in 10 and 14% Tris–glycine gels under reducing conditions and transferred to nitrocellulose membranes. Primary antibodies used in this study, which included anti-cytochrome c mouse monoclonal antibody (clone 7H8,2C12; Pharmingen, San Diego, CA) and anti-Bax, anti-Bid and anti-Bcl-2 rabbit and anti-actin goat polyclonal antibodies (clones N-20, D-19, N-19 and I-19, respectively; Santa Cruz Biotechnology) were applied to the membranes and the resulting conjugates visualized on X-ray films using enhanced chemiluminescence (Perkin Elmer Life Sciences Inc., Boston, MA).

Immunoelectron microscopy for Bax. BJMC3879 cells, grown in 2-well chamber slides for 24 h in medium with or without 20 μM lovastatin, were fixed in a 0.05% glutaraldehyde/4% paraformaldehyde solution in 0.1 M phosphate buffer. Immunoelectron microscopy was performed as previously described (23). Briefly, fixed cells were sequentially immersed in 0.1 M phosphate buffer containing 5% sucrose for 1 h, in 10% sucrose for 1 h and in 20% sucrose for 1 h. The indirect immunoperoxidase technique was then employed, using an anti-Bax rabbit polyclonal antibody (N-20; Santa Cruz Biotechnology). The cells were finally fixed in 1% osmium tetroxide in 0.1 M PBS for 1 h, stained again with 1% uranyl acetate in 70% ethanol, dehydrated and embedded in epoxy resin. Samples were examined under a model H-7100 electron microscope (Hitachi Co.).

Statistical analysis

Data containing dose-response effects were subjected to analysis of variance (ANOVA), while Scheffe’s test was employed for differences between the means. Data from cytometric studies, ∆ψm analyses, caspase activity and inhibition studies and cytochrome c release evaluations were compiled and compared between control and lovastatin-treated groups using a two-sided Student’s t-test. The two-sided Fisher’s exact probability test was used to evaluate the significance of histopathological findings.

Results

In vivo studies

Body weights and tumor growth. Body weights of control and lovastatin-treated mice bearing mammary tumors are compiled and shown in Figure 1A. As can be seen, mice treated with 50 mg/kg lovastatin were significantly lighter than were controls by experimental week 2 and thereafter, but, even at week 6, the weight difference between high dose and control animals was only 10%. At 25 mg/kg lovastatin, no statistical differences in body weights between control and treated mice were observed. This experiment was terminated at experimental week 6 when the first mortality occurred in the control group due to liver metastasis from a mammary carcinoma.

Tumor volumes are presented in Figure 1B. Tumor growth, as inferred by computed volume, was significantly inhibited in mice receiving both 25 and 50 mg/kg lovastatin from week 2 in a dose-dependent manner when compared with controls. By the end of the experiment the average tumor volume in control animals was 926 ± 414 mm³, while those of mice receiving 25 and 50 mg/kg were 586 ± 211 mm³ and 414 ± 66 mm³, respectively.

Histopathology. Histopathologically, the mammary carcinomas induced by BJMC3879 cell inoculation proved to be moderately differentiated adenocarcinomas that, by immunohistochemistry, contain the same p53 mutation carried by the cell line (Figure 2A). Lung metastasis occurred in 100% of control animals and in 80% of the animals in both lovastatin-treated groups, but the metastatic foci tended to be markedly smaller in the 50 mg/kg group (Figure 2C) than were those observed in both the mice receiving 25 mg/kg and the control animals (Figure 2B). For qualitative analysis, metastatic foci were characterized into two categories: microscopic and macroscopic foci consisting of >30 cells and macroscopic lesions measuring >1 mm. In the high dose (50 mg/kg) group, there was a significant decrease in the numbers of foci per mouse >1 mm (P < 0.05 as compared with control), as well as a tendency toward fewer metastases of any size (Figure 3A). The number of metastases-positive lymph nodes per mouse tended to decrease in a dose-dependent manner as well, but a statistical significance could not be demonstrated (Figure 3B). Liver metastasis was observed in the single control case that died at week 6.

Levels of DNA synthesis and apoptosis. DNA synthesis levels in mammary carcinomas of lovastatin-treated mice, as inferred by BrdU labeling indices, are illustrated in Figure 3C. DNA synthesis in tumors apparently decreased in a dose-dependent manner, reaching statistically significant values in mice receiving 25 mg/kg from week 2 (P = 0.05) and, as inferred by the TUNEL assay, are shown in Figure 3D; apoptosis was significantly increased in tumors from the 50 mg/kg group as compared with levels seen in tumors from control mice.

In vitro studies

Effects of lovastatin on cell growth and cell cycle. Inhibition of cell growth was evident with as little as 5−10 μM lovastatin and occurred in a dose-dependent manner; concentrations of > 20 μM induced a strong inhibitory effect. We therefore chose exposures of 10 and 20 μM for further in vitro study.

As demonstrated in Figure 4A, significant inhibition of cell growth occurred within 24 h of exposure to both 10 and 20 μM
lovastatin. After 48 h treatment, 20 μM induced a 64% inhibition of cell growth (Figure 4B), inducing a rounded cell morphology and reduced plate adherence without apparent cytotoxicity.

As measured by flow cytometry, 24 h exposure to 20 μM lovastatin induced a significant elevation of the numbers of cells in G1 phase as compared with untreated cells (Figure 4C). There were also significant reductions in S and G2/M fractions in lovastatin-treated cell suspensions (Figure 4C). DNA synthesis in treated BJMC3879 cells, as assessed by BrdU incorporation, was also decreased in a dose-dependent manner (Figure 4D).

Effects of lovastatin on apoptosis. Quantitative analysis of TUNEL staining by light microscopy revealed a significant increase in the numbers of TUNEL-positive cells after 24 h lovastatin treatment as compared with control cells (Figure 5A). Laser scanning cytometry of human mammary carcinoma MDA-MB231 cells stained by the fluorescent TUNEL procedure with propidium iodide indicated that lovastatin both increased the number of cells in G1 arrest and suppressed the number entering the S and G2/M phases (Figure 5B and C). The relative intensity of fluorescein TUNEL-positive cells is represented in Figure 5D; only 14% of control cells demonstrated weak (i.e. nearly equivalent to background) TUNEL staining, while 60.6% of lovastatin-treated cells were strongly TUNEL positive. Figure 5E shows the cell cycle distribution of lovastatin-treated cells; ~50% of cells in the S and G2/M phases were TUNEL positive, whereas TUNEL-positive cells accounted for only 10.5% of cells in G1.
Apoptosis was further confirmed by ultrastructural analysis of untreated (Figure 6A) and treated BJMC3879 cells (Figure 6B), the latter demonstrating fragmented nuclei with characteristic half-moons of condensed chromatin. In quantitative analyses of TUNEL by TEM (Figure 6C), a small number (8.3 ± 1.7/μm²) of free 3′-OH DNA ends, as visualized by adhered immunogold particles, were observed in nuclear chromatin of untreated mammary carcinoma cells (Figure 6A, inset), while the number of immunogold particles was significantly elevated (129.5 ± 75.8/μm²) in the chromatin of lovastatin-treated cells (Figure 6B, inset).

Apoptosis signaling pathway. Significantly elevated activities of not only caspase-3 but also of caspase-8 and caspase-9 were observed in BJMC3879 cells treated with 20 μM lovastatin for 24 h (Figure 7A). To determine whether caspase activation is necessary to induce lovastatin-induced apoptosis, BJMC3879 cells were treated with caspase inhibitors. Combination treatment with 20 μM lovastatin and 100 μM z-VAD-fmk (a broad spectrum inhibitor of caspases), z-LEHD-fmk (an inhibitor specific to caspase-9) or z-DEVD-fmk (a specific inhibitor of caspase-3) significantly increased cell viability as compared with lovastatin alone (Figure 7B). However, z-IETD-fmk (a specific inhibitor of caspase-8) did not prevent lovastatin-induced cell death (Figure 7B). This strongly suggests the engagement of the mitochondria-mediated apoptotic pathway.

Δψm decreased upon exposure to lovastatin, as shown in Figure 7C, and ELISA further determined that cytochrome c protein levels in cytosolic fractions were significantly increased over levels detected in control cells after 12 and 24 h treatment with 20 μM lovastatin (Figure 7D). The western blots shown in Figure 7E also demonstrate cytochrome c release into the cytosol of cells treated with 20 μM lovastatin for 24 h. Expression of Bax and Bid was very weak both in cytosolic fraction and in whole cell lysates of lovastatin-treated cells (Figure 7E). Expression of Bcl-2 was not detected in either control or lovastatin-treated cells (data not shown) nor was cleavage of Bid observed.

Immunoelectron microscopy for Bax. Since the translocation of Bax to mitochondria is an important step in the induction of apoptosis (24), immunoelectron microscopy was employed to detect Bax protein in the mitochondria of treated and control cells. Immunoelectron microscopy demonstrated that Bax protein was present in and along the outer membrane of mitochondria in lovastatin-treated cells (Figure 7G), but not in control cells (Figure 7F).

Discussion
Several large clinical trials have demonstrated that statins, in general, have the ability to greatly reduce cardiovascular-related morbidity and mortality in patients with and without...
coronary disease. In two additional clinical trials evaluating coronary events in patients, there was a 43 and 19% decrease in the number of new cases of colon cancer diagnosed over a 5 year follow-up period in cohorts taking pravastatin and simvastatin, respectively (25,26). Lovastatin, an inhibitor of HMG-CoA reductase, is one of the statins in therapeutic use today and is widely prescribed to reduce hypercholesterolemia. Additional data from one 5 year clinical safety and efficacy study of lovastatin also revealed a reduction in cancer deaths within the treated group (27).

In several animal studies specifically of mammary disease, chemopreventive effects of lovastatin and simvastatin on radiation-induced mammary tumorigenesis have been demonstrated in rats (11), in syngeneic mice inoculated with mammary carcinoma cells (9,10) and in a transgenic mouse mammary cancer model (7). There are further reports of efficacy against cancers in other organ systems as well (28–31).

The lethal metastatic proclivity of mammary cancer is possibly the characteristic that makes it of such clinical importance. Patients presenting with metastatic disease are frequently incurable. It is reported that once breast cancers reach ≥4 cm, the chances of tumor recurrence or metastases increase dramatically (32), therefore, treatments that offer suppression of both tumor growth and metastasis have significant clinical implications. There is limited information with respect to the anti-metastatic properties of statins in mammary cancers. In fact, it appears that only one group reported any anti-metastatic effects, specifically in studies of lovastatin on syngeneic mice inoculated with cells from a proprietary murine F3II sarcomoid mammary carcinoma culture (9,10).

Lovastatin induces apoptosis (7,8,33). Lovastatin has also been shown to inhibit cell growth with arrest at G1 and reduce transition to the S and G2/M phases of the cell cycle (4–7). This may be related to elevation of two key cell cycle inhibitors, p21Waf1 and p27Kip1 (5–7), that have been reported to suppress cell cycle progression, resulting in G1 arrest in both normal and tumor cell lines derived from mammary glands (6). However, it was recently reported that, at least in prostate cancer cell lines, transcriptional regulation and proteosomal degradation of E2F-1 may be critical regulatory events in growth inhibition and that p21Waf1/Cip1 induction and G1 arrest are not general mechanisms in lovastatin-mediated cell death (34).

The reduction in DNA synthesis and induction of apoptosis are of particular interest in neoplastic diseases. Bearing in mind that lovastatin is a potent HMG-CoA antagonist, it has been reported that HMG-CoA reductase activity is elevated just before DNA synthesis (35). Elevated DNA synthesis and increased cell proliferation are generally regarded as hallmarks of aggressive cancers and current radiation therapies are most effective against the more susceptible proliferating cell. Generally, cells located in the late G1 and G2/M phases of the cell cycle are most sensitive to ionizing radiation-induced cell death, whereas cells in S phase are most resistant (36). The G1 arrest effect of statins potentially sensitizes cells to radiation (36). In fact, it was previously reported that a Ras-associated increase in radiation resistance in osteosarcoma

Fig. 4. (A) The dose–response characteristics of 0–80 μM lovastatin on mouse mammary carcinoma-derived BJMC3879 cells. The dosages selected for the in vitro experiments (10 and 20 μM lovastatin) were those that elicited significant cell death without cytotoxicity. (B) Inhibition of cell growth was sequentially examined after treatment with 10 and 20 μM lovastatin, which induced significant cell death 24 and 48 h after treatment. (C) Cell cycle distribution (percentage of cells in a specified phase) was measured in mammary carcinoma cells treated with 20 μM lovastatin using flow cytometry. Lovastatin induced arrest in G1 phase and inhibition of both the S and G2/M phases. (D) DNA synthesis, assessed by BrdU incorporation, was measured in cells treated with 10 and 20 μM lovastatin. DNA synthesis was significantly decreased in lovastatin-treated cells in a dose-dependent manner. Data were calculated from three repeated experiments in control and lovastatin-treated cells. Data presented are means ± SD values. *Statistical significance determined at \( P < 0.01. \)
Fig. 5. (A) BJMC3879 cells (mouse mammary carcinoma cell line), treated or not with 20 μM lovastatin for 24 h, were analyzed for apoptotic cells using the TUNEL procedure with light microscopy. The numbers of TUNEL-positive (apoptotic) cells significantly increased with lovastatin treatment. Four samples from both control and lovastatin-treated cells were examined. Data presented are means ± SD values. **Statistical significance determined at P < 0.01. (B-E) MDA-MB231 cells (human mammary carcinoma cell line) treated or not with 20 μM lovastatin for 32 h, stained with fluorescein-TUNEL and propidium iodide and analyzed for cell cycle phase using laser scanning cytometry. (B) A DNA histogram of treated and control cells shows decreases in S and G2/M fractions induced by lovastatin. (C) Cell distribution (percentage of cells in each phase) demonstrates significant increases in G1 fractions and decreases in G2/M fractions induced by lovastatin (**P < 0.01). (D) This histogram shows increased fluorescein-TUNEL-positive cells (apoptotic cells) with lovastatin exposure as compared with controls. (E) With lovastatin treatment ~50% of S and G2/M phase cells were TUNEL-positive (apoptotic), suggesting that cancer cells in these phases are highly susceptible to lovastatin. Four samples each of control and lovastatin-treated cells were examined. Data presented are means ± SD values. **Statistical significance determined at P < 0.01.
cells can be reversed by lovastatin treatment (37). In contrast, our laser scanning cytometric analysis demonstrated that human mammary carcinoma MDA-MB231 cells cycling through both the S and G2/M phases were highly susceptible to lovastatin-induced apoptosis. This may be a significant clinical benefit if lovastatin induces apoptotic cell death in the typically radiation-resistant S phase.

There are two pathways currently proposed to play major roles in regulating apoptosis in mammalian cells: an extrinsic pathway mediated by one or more death receptors and an intrinsic pathway mediated by mitochondria (38). In the extrinsic death receptor/ligands pathway, caspase activation occurs as a direct consequence of death receptor ligation, with upstream caspase-8 cleaving and activating downstream proteases such as caspase-9 and caspase-3. In the intrinsic mitochondrial pathway, Bax, a member of the Bcl-2 family, plays the leading role. Bax normally resides in the cytosol in a quiescent state. After an apoptotic stimulus, Bax is translocated into the mitochondria and promotes the release of cytochrome c (24), possibly by forming a pore (39) or a voltage-dependent anion channel (40) in the outer mitochondrial membrane. Both the death receptor and the mitochondrial pathways are linked by cleavage of Bid by caspase-8. It is known that cleaved Bid also translocates to mitochondria and induces Bax-dependent release of cytochrome c (41). Once in the cytosol, cytochrome c activates Apaf-1, which then activates procaspase-9, which, in turn, activates caspase-3, triggering apoptosis. We initially confirmed that lovastatin-induced cell death is by apoptosis rather than necrosis using both TUNEL/light microscopy and TUNEL/TEM techniques. Although elevated caspase-8, caspase-9 and caspase-3 activities were observed in lovastatin-treated cells, the fact that lovastatin-induced cell death was reduced by both the broad spectrum caspase inhibitor z-VAD-fmk and inhibitors specific for caspase-9 and caspase-3 (z-LEHD-fmk and z-DEVD-fmk, respectively), but that cell death was not reduced by exposure to z-IETD-fmk, an inhibitor specific for caspase-8, strongly suggests that the intrinsic mitochondrial pathway is engaged in lovastatin-induced apoptosis. This is further indicated by the decrease in Δψm and release of cytochrome c into the cytosol and correlates well with a recent report that in human promyelocytic leukemia HL-60 cells lovastatin also induces activation of caspase-3, release of cytochrome c into the cytosol and a decrease in Δψm (42).

As previously discussed, mitochondria-mediated apoptosis is regulated by Bcl-2 family proteins (43,44), with Bcl-2 protecting against apoptosis by forming heterodimers with pro-apoptotic Bax, thus preventing the collapse of Δψm and release of cytochrome c from mitochondria. We were unable, however, to detect expression of Bcl-2 in either control or lovastatin-treated BJMC3879 cells under our test conditions. We also saw no increase in Bax expression inlovastatin-treated carcinoma cells, a finding in agreement with a recent paper (45). Because Bax translocation is a critical step for reduction of Δψm, we decided to look at the location of Bax in relation to mitochondria using immunoelectron microscopy. We found that Bax protein aggregated along the outer mitochondrial membrane only in cells exposed to lovastatin, indicating that lovastatin-induced apoptosis is, in all likelihood, due to Bax-dependent release of cytochrome c, despite a lack of increased protein expression. Further, caspase-8 activity was elevated in lovastatin-treated mammary carcinoma cells in vitro without apparent Fas ligand stimulation. It could be argued that, since caspase-8 has been shown to be
capable of autoactivation by ligand and is DISC independent (46), lovastatin itself may directly or indirectly activate caspase-8 and initiate apoptosis. However, because we did not find evidence of Bid cleavage and because the caspase-8-specific inhibitor z-IETD-fmk did not prevent lovastatin-induced cell death, it appears that within the parameters of this investigation neither caspase-8 activation nor involvement are factors in lovastatin-induced apoptosis. The precise mechanism by which lovastatin decreases $\Delta\psi_{m}$ is not known. Cholesterol is a major component of the lipoproteins comprising both the cellular and mitochondrial membranes. It is possible that inhibition of cholesterol biosynthesis by lovastatin.

![Graphs and images]

**Fig. 7.** Investigation of the apoptotic signaling pathway in BJMC3879 cells with or without 20 $\mu$M lovastatin for 24 h. We evaluated four or five samples each of control and lovastatin-treated cells. Data presented are mean ± SD values. (A) Caspase activities were evaluated according to fluorometric assays. Activities of caspase-8, caspase-9 and caspase-3 were significantly elevated in the mammary carcinoma cells treated with lovastatin ($**P < 0.01$). (B) Cell viability was determined by the MTT assay in mammary carcinoma cells treated with 20 $\mu$M lovastatin with or without exposure to either 10 or 100 $\mu$M caspase inhibitors. Cell viability (%) was significantly increased by the broad spectrum caspase inhibitor z-VAD-fmk ($**P < 0.01$), by the caspase-9 inhibitor z-LEHD-fmk ($*P < 0.05$) and by the caspase-3 inhibitor z-DEVD-fmk ($*P < 0.05$) at the 100 $\mu$M concentration only. However, cell viability was unaffected by addition of any concentration of the caspase-8 inhibitor z-IETD-fmk. Data are expressed as percentages of the vehicle control. (C) The mitochondrial membrane potential ($\Delta\psi_{m}$) was measured with a fluorescent cationic dye and expressed in terms of intensity. Lovastatin induced a significant decrease in $\Delta\psi_{m}$ ($P < 0.05$). RFU, relative fluorescence unit. (D) The levels of cytosolic cytochrome c, as determined by ELISA, were significantly elevated in cells after both 12 and 24 h lovastatin treatment ($**P < 0.01$). (E) Comparison of western blots of cytosol fractions versus whole cell lysates of BJMC3879 cells treated with 20 $\mu$M lovastatin for 24 h. Elevated levels of cytochrome c were confirmed in cytosolic fractions of lovastatin-treated cells (upper). Lovastatin did not increase the expression of either Bax or Bid in whole cell lysates (lower). $\beta$-actin served as an internal control. (F and G) Bax immunoelectron microscopy of BJMC3879 cells treated or not with 20 $\mu$M lovastatin. (F) Ultrastructural study of an untreated cell, showing weak detection of Bax protein in mitochondria (arrow heads). (G) Bax protein can clearly be seen along the mitochondrial membrane (arrow heads) in a lovastatin-treated BJMC3879 cell. ×39 000. Scale bar = 0.5 $\mu$m.

Lovastatin suppresses cancer growth and metastasis.
may alter the integrity of these membranes (47) or may inhibit
the formation of new membranes in proliferating tumor cells
that require high levels of cholesterol.

It was obvious to us that lovastatin-induced apoptosis in
BJMC3879 cells, which carry a p53 mutation, must occur
through a p53-independent mechanism or, perhaps, the p53
homolog p73 substitutes in inducing apoptosis induction in
cells with non-functional p53. Whatever the case, lovastatin
has been shown to induce apoptosis in a wide variety of
cancer cells, irrespective of their p53 or pRb status. Since
50% of human cancers have mutations in p53 (48), the
fact that lovastatin induces a p53-independent apoptotic
response may be highly relevant to treating many human
neoplasms.

Preclinical studies in different animal species have indicated
that dosages up to 200 mg/kg/day yield drug concentrations in
the range 2–20 μM (49) and that circulating concentrations of
2–4 μM were well tolerated for months in all animal models
tested. In a human phase I study, 25–45 mg/kg/day were used in
cancer treatment (50). These dosages produced drug concen-
trations up to 3.9 μM and are associated with anti-proliferative
effects in vitro (50). We similarly saw anti-proliferative activity
in vitro with ~5 μM and more in our present study series. Thus,
the dosages used in our in vivo study are considered to be close
to the clinical dosages used for cancer treatment.

In conclusion, we report that lovastatin significantly induced
apoptosis and inhibited cell proliferation in vivo in p53-mutated
mouse mammary tumors and in vitro in our study of both
murine and human mammary cancer cells. Possibly of more
importance, we also saw a reduction in the number and size of
lung metastases in the mammary model. Although the inhibi-
tion of metastasis may simply be a reflection of suppression of
tumor growth and decreased DNA synthesis, the therapeutic
benefit remains. This and other studies suggest the usefulness
of lovastatin as an adjuvant to current radio- and chemothera-
pies for breast cancer, particularly those carrying p53
mutations, and possibly as a chemotherapeutic/chemopreven-
tative in and of itself.

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