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In Vitro Mechanisms of Lovastatin on Lung Cancer Cell Lines as a Potential Chemopreventive Agent

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Abstract Lung cancer causes over one million deaths per year worldwide and cigarette smoking, the proximate cause, results in a field cancerization of the respiratory track. Lung cancer cells or premalignant cells may be susceptible to apoptosis or necrosis-inducing agents. Statins inhibit the acetyl coenzyme A pathway reducing L-mevalonate that is a precursor to isoprenoids necessary for post-translational processing, resulting in apoptosis. Lovastatin was added to four lung cancer cell lines and normal human bronchial epithelial cells followed by Western blots to evaluate proteins in the cell cycle, oxidant, and apoptotic pathways. Flow cytometry revealed significant increases in three of four lung cancer cell lines in apoptosis and necrosis after lovastatin treatment at 10 μM for 72 h. Lovastatin adversely affected lung cancer cell survival with increases in cell-cycle checkpoint inhibitors $p21^{WAF}$ and/or $p27^{KIP}$ and a decrease in cyclin D1. All four lung cancer cell lines had a decrease in glutathione after lovastatin treatment consistent with reduced protection against reactive oxidant species. Three of four lung cancer cell lines had increased cytochrome c release with reduced pro-caspase-3 and increases in activated caspase-3. Lovastatin induces apoptosis and necrosis in lung cancer cell lines by causing alterations in the cell cycle, reducing glutathione, and activating p53, Bax protein, and caspases while increasing cytochrome c in apoptosis pathways. Targeting HMG-CoA reductase may represent an approach to lung cancer chemotherapy, e.g., reversing ground glass opacities detected on CT scans or resolving airway preneoplasias detected by bronchoscopy before they progress to malignant transformation.

Keywords Apoptosis · Cancer · Lovastatin

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Introduction

Lung cancer is the leading cause of cancer-related death for men and women, with more than 170,000 newly diagnosed cases each year in the U.S. and one million cases worldwide [1]. Despite the advances made in diagnosis and treatment in the last few decades (surgery, radiation, and chemotherapy), the prognosis of lung cancer has improved minimally, with a 5-year overall survival generally under 15% [1].

Inducing apoptosis or the apoptotic pathway is an interesting approach for both curing cancer and understanding how cancer cells block these pathways [2]. Inhibitors of the 3-hydroxy-3-methylglytaryl-coenzyme A (HMG-CoA-R), or statins, inhibit the biosynthesis of L-mevalonate and may induce apoptosis of certain cells. L-Mevalonate is a precursor for cholesterol, as well as for isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGP). These isoprenoids are common to all eukaryotic cells and are required for protein isoprenylation. The isoprenoids are important lipid moieties added during post-translational modification of a variety of proteins, including G proteins and G-protein subunits Ras, Rho, Ral, and Rab. Due to the depletion of L-mevalonate, post-translational modification of these proteins is unable to occur, e.g., activation of Ras is blocked by interfering with its prenylation rendering it incapable of binding GTP. Ras-GTP stimulates downstream signaling molecules, including phosphoinositide-3 (PI3) kinase, which has been directly implicated in cell

survival, and mitogen-activated protein (MAP) kinase, which is involved in proliferation [3–5]. Inhibition of HMG-CoA reductase suppresses the syntheses of the substrates providing the isoprenoid moieties required for the post-translational modification of the cysteine residue in the conserved carboxyl terminus sequence (generally CAAX) of diverse proteins, some of which have important roles in signal transduction (Ras, nuclear lamins, RhoA and B, Rap) and cytoskeletal organization [6, 7]. As a result, statins have impacts on many cellular functions essential for normal cell homeostasis, including proliferation, differentiation, and cytoskeletal organization.

The statin family is composed of the following members: pravastatin, simvastatin, lovastatin, fluvastatin, atorvastatin, rosuvastatin, and cerivastatin. The first three are derived from fungal fermentation and the latter ones are synthetic. At the pharmokinetic level (absorption, distribution, metabolism, excretion) statins have important differences, including half-life, systemic exposure, maximum plasma concentration, bioavailability, protein binding, lipophilicity, metabolism, and excretion routes. Lovastatin (a fungal metabolite of *Aspergillus terreus*) and related compounds are widely used for treating clinical hypercholesterolemia. Tan et al. [5] have shown that lovastatin causes dose- and time-dependant apoptosis of fibroblasts from patients with idiopathic pulmonary fibrosis or fibroblasts ectopically expressing c-myc. In a guinea pig wound chamber model, lovastatin reduced granulation tissue formation by 65%, and there was ultrastructural evidence of fibroblast apoptosis [5].

The molecular mechanisms underlying antitumor activity of statins have not been fully elucidated. We designed a series of *in vitro* investigations to determine the effect of HMG-CoA reductase inhibition on lung cancer cells and establish a mechanistic basis of statins as a potential chemopreventive agent in treating preneoplasias in the airway or lung parenchyma.

Materials and Methods

Materials

Lovastatin (a lactone form, Sigma, St. Louis, MO) was dissolved in ethanol and activated *in vitro* to its dihydroxy-open acid form as described [8]. DL-Mevalonic acid lactone was purchased from Sigma and converted to the potassium salt as described [9].

Antibodies used for Western blot to p21 ^{WAF}, P27 ^{KIP}, cyclin D1, p-53, Bax, pro-caspase-3, actin, and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to Bcl-2, cytochrome c, active caspase-3, and caspase-8 were from BD Pharmingen (San Jose, CA). Peroxidase conjugated goat anti-rabbit IgG and goat anti-mouse IgG from Santa Cruz Biotechnology were used as secondary antibodies. Protease inhibitors cocktail was from Roche Diagnostics (Indianapolis, IN).

Cell Culture and Treatment

Human A549, Calu-1, H-460, and H-441 were obtained from American Type Culture Collection (ATCC), whereas NHBE cells were from Cambrex BioScience (East Rutherford, NJ). All cell lines were maintained in culture with growth supplements in a humidified atmosphere of 5% CO_2 in air at 37°C according to the manufacturer's recommendations.

Prior to treatment cells were grown up to 75–80% of the confluence in 100-mm tissue culture dishes. Fresh medium and 10 μM lovastatin and/or 100 μM mevalonate were added to the cell for 24 and 74 h of treatment. The lovastatin concentration was chosen because this was physiologically relevant to treatment levels of the drug that result in serum concentrations of 1–15 μM. Untreated cells were seeded initially at less density to keep control cells at levels allowing exponential growth at the end of the experiment.

In Vitro Proliferation Assay

The cytostatic/cytotoxic effects of lovastatin on A549, Calu-1, H-460, H-441, and NHBE cells *in vitro* were tested in a standard MTS assay using CellTiter 96^{R} Aq_{ueous} one Solution Cell Proliferation Assay kit (Promega, Madison, WI). The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] tetrazolium compound (Owen's reagent) was bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product, as measured by the absorbance at 490 nm by EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc.), was directly proportional to the number of living cells in culture. The means and standard deviations were determined for triplicate samples.

Western Blot Analysis

Cells were washed twice with phosphate-buffered saline (PBS) and whole-cell lysates were prepared in ice-cold buffer containing 25 mM Tris HCl (pH 7.4), 130 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM NaF, 1 mM sodium orthovanadate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, and protease inhibitors cocktail. Lysates were incubated 30 min on ice and centrifuged at 14,000*g* for 15 min at 4°C prior to SDS-PAGE. Protein concentration was determined using the Bio-Rad protein assay (Hercules, CA). Fifty micrograms of samples

were mixed with 2 × Laemmli buffer, boiled for 5 min, loaded on SDS-PAGE, and separated electrophoretically. Proteins were transferred onto PVDF membrane. The membrane was incubated with blocking buffer (5% [wt/vol] nonfat dried milk in Tris-buffered saline 0.1% [wt/vol] Tween-20) for 1 h and then probed with various antibodies for 1 h at room temperature or overnight at 4°C. Primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies and, after three washes, positive signals were visualized using enhanced chemiluminescence reagents (ECL, Pierce, Holmdel, NJ) on HyBlot CL™ Hyperfilm. In all cases, the results illustrated are from three replicate experiments. Quantification of Western blots was performed using Quantity One (Bio-Rad, Germany) evaluation software.

Glutathione Assay

GSH levels were measured by Calbiochem's Glutathione Assay Kit (San Diego, CA). The method is based on the specific transformation of the substitution product obtained with GSH into a chromophoric thione with a maximal absorbance at 400 nm. Briefly, cells were lysed in ice-cold 5% methaphosphoric acid (1.4 ml/100-mm dish) and homogenized using a Teflon pestle; then after centrifugation at 3000*g* for 10 min at 4°C, the resulting supernatant (200 µl) was used for the assay.

Flow Cytometry

Upon induction of apoptosis, cytoplasmic phosphotidylserine translocates to the external surface of the cell membrane, allowing its *in vitro* detection through interaction with annexin V [10]. Treated and untreated cells were harvested and combined with their media to collect any detached cells. The cells' suspension concentration was adjusted to approximately 1×10^6 cells/ml with PBS and 0.5 ml of each sample was processed according to the manufacturer's protocol (Calbiochem, San Diego, CA). Early apoptotic cells (annexin V positive) and necrotic/late apoptotic cells (annexin V positive, PI positive) as well as living cells (double negative) were immediately analyzed by flow cytometry on a FACScan flow cytometer. The log of annexin V-FITC fluorescence was displayed on the *x* axis and the log of PI fluorescence on the *y* axis.

Statistical Analysis

Statistical comparison of mean values was performed using Student's *t* test. All *p* values are one-tailed. The difference was considered to be statistically significant when $p < 0.01$.

Results

HMG-CoA Reductase Inhibitor Lovastatin Causes a Dose-Dependent Decrease in Proliferation of Lung Cancer Cell Lines

To determine the effect of statins on lung cancer cell growth, we treated four lung cancer cell lines with a common statin drug over 72 h at various doses. The HMG-CoA reductase inhibitor reduced proliferation of lung cancer cell lines Calu-1 and H-460 at 10 µM and A549 at 15–30 µM (Fig. 1). Interestingly, for the lung cancer cell line H-441, survival was reduced to 50% at 1 µM and there was no further reduction despite increasing concentrations of lovastatin. There was no significant effect of lovastatin on NHBE cell proliferation across the dose range tested. Time-course experiments demonstrated maximal effects at 72 h. Cell proliferation was examined by the MTS assay that measured mitochondrial dehydrogenase activity reflecting the proliferation status of the cells.

Effects of HMG-CoA Reductase Inhibitor Lovastatin on the Cell Cycle

Lovastatin induced cell cycle arrest by increasing checkpoint inhibitors ($p21^{WAF}$, $p27^{KIP}$) and decreasing cyclins and cyclin-dependent kinases (Fig. 2). The checkpoint

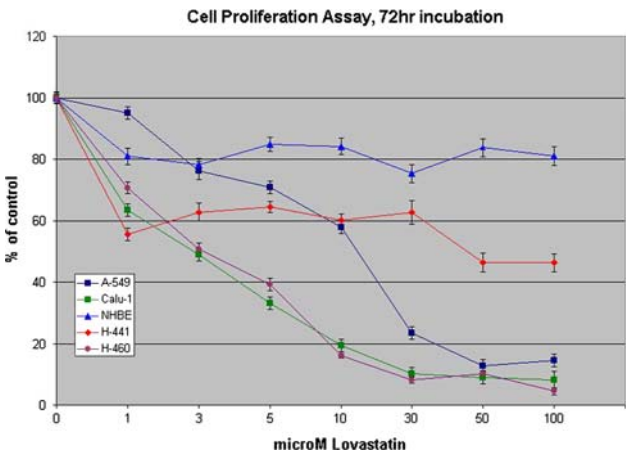


Fig. 1 Inhibition of lung cancer cell proliferation after exposure to lovastatin. Exponentially growing A549, H-441, H-460, Calu-1, and NHBE cells were exposed to an increasing concentration (0–100 µM) of lovastatin for 72 h, and the effects on cell survival were determined using the MTS assay. MTS activity is plotted as a percentage of untreated controls. Each experiment was repeated three times with triplicate samples

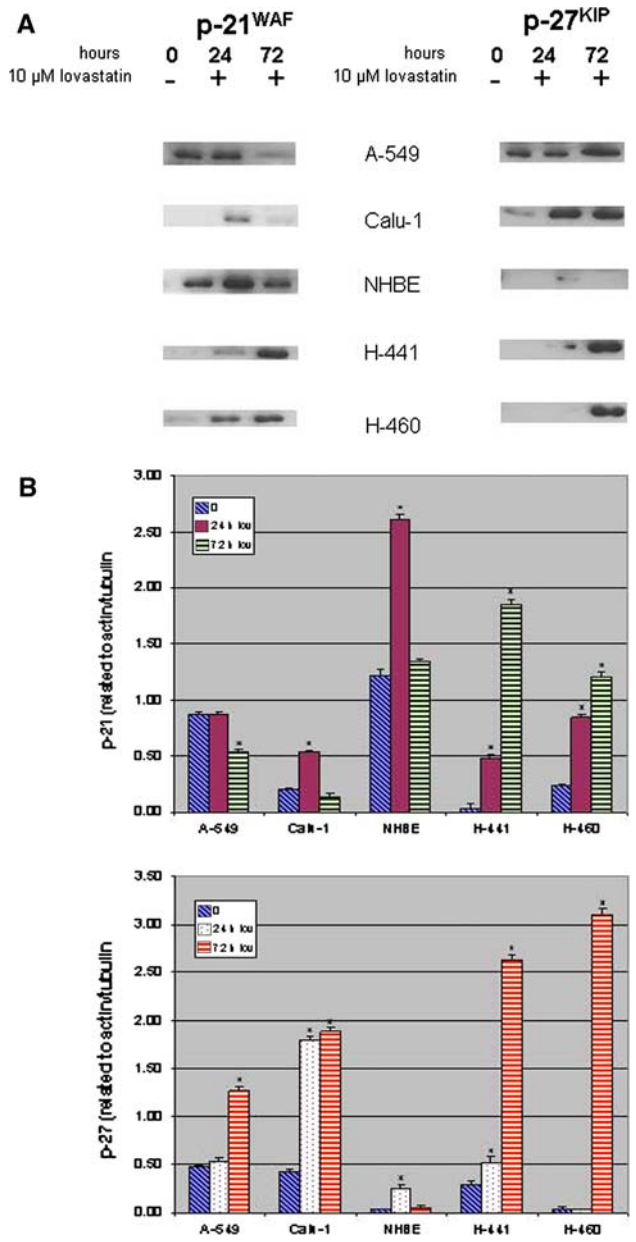


Fig. 2 Expression of p21 ^{WAF} and p27 ^{KIP} proteins in lung cancer cell lines and NHBE after exposure to lovastatin. **A** Exponentially growing cells were left untreated or exposed to 10 μM lovastatin for 24 h and 72 h. Fifty micrograms of total protein was blotted against p21 ^{WAF} and P27 ^{KIP} antibodies (actin as a control for equal loading not shown). Data are representative of three independent experiments. **B** Quantification of p21 ^{WAF} and p27 ^{KIP} Western blots. Data are presented as mean ± SD, $*p < 0.01$

inhibitor p21^{WAF} was significantly increased in Calu-1, H-441, and H-460 lung cancer cell lines (but not A549) at 24 h and further at 72 h in H-441 and H-460 cells undergoing exponential growth (Fig. 2A). In contrast, untreated A549 and NHBE expressed a detectable p21^{WAF} level that declined by 72 h. All four lung cancer cell lines had a significant increase in p27^{KIP} at 72 h, with Calu-1 increasing as early as 24 h; in contrast, there was little effect on NHBE control cells. Laser densitometric analysis of the immunoblots compared to actin controls for equal loading is shown in Figure 2B for both p21 and p27.

Lovastatin treatment for 72 h significantly inhibited cyclin D1 in A549 and Calu-1 lung cancer cell lines (Fig. 3A, B for densitometric analysis compared to actin controls). There was no expression of cyclin D1 in H-460 cells, and H-441 cells had a significant increase. The opposite effect seen in H-441 cells at 72 h was consistent with their greater survival in the MTS assay beyond 1 μM (Fig. 1).

HMG-CoA Reductase Inhibitor Lovastatin Reduces the Level of Glutathione (GSH)

Cellular levels of glutathione (GSH) are important protectors of lung cells against reactive oxygen species (ROS) damage. After 72 h of lovastatin treatment, all four lung cancer cell lines had a reduction in GSH (Table 1); H-460, Calu-1, and A549 had a dramatic reduction, while the H-441 lung cancer cells had a smaller 19% decrease. NHBE cells also had a 50% decrease in GSH levels.

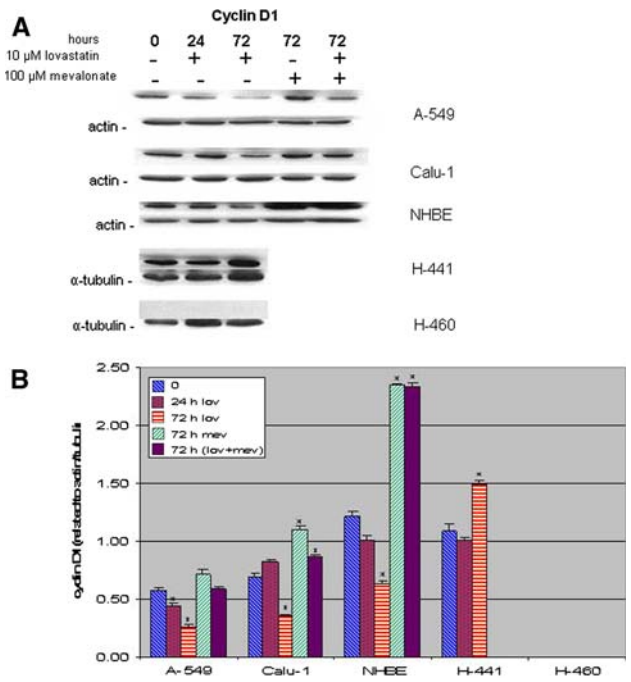


Fig. 3 Modulation of cyclin D1 expression in lovastatin-treated lung cancer cell lines and NHBE cells. **A** Cell lysates (50 μg) derived from cells grown in the absence or the presence of 10 μM lovastatin and 100 μM mevalonate were subjected to immunoblot analysis (actin or α-tubulin as loading controls). Data are representative of three independent experiments. **B** Quantification of cyclin D1 Western blots. Data are presented as mean ± SD, $*p < 0.01$

Table 1 GSH levels following lovastatin treatment

Cell line	μM GSH/mg protein	
	0 h	72 h
H-441	27.4 ± 2.8	22.6 ± 5. 9
H-460	46.0 ± 3.1	3.4 ± 0.1
Calu-1	11.8 ± 1.0	1.5 ± 0.1
A549	29.7 ± 3.5	0
NHBE	27.6 ± 3.0	13.3 ± 1.7

Cellular stress due to DNA damage activates the transcription factor p53 that can activate pathways leading to cell cycle arrest or apoptosis. We postulated that this pathway might be involved in explaining the lovastatin effect on cell proliferation and survival. In addition to the NHBE control cells, A549 and H-460 lung cancer cell lines are p53 wild type. Interestingly, after lovastatin treatment the H-460 lung cancer cell line had a significant induction of p53 at 24 h (Fig. 4A). H-441 lung cancer cells have a mutated p53 and Calu-1 cells are p53 null. There was no change in H-441 or the control NHBE cells. The proapoptotic protein Bax that is p53 inducible was also significantly increased in the H-460 lung cancer cell line at both 24 and 72 h, consistent with the increased apoptosis in these cell lines and the induction of p53 (Fig. 4B). There was no change in NHBE cells in regard to Bax after lovastatin treatment.

HMG-CoA Reductase Inhibitor Lovastatin Increases Apoptosis and Necrosis in Lung Cancer Cell Lines

The decrease in cell proliferation and survival after lovastatin treatment may be due to apoptosis and/or necrosis, particularly after depletion of intracellular GSH levels. Annexin V-based apoptosis detection was performed on the lung cancer cell lines 72 h after lovastatin treatment. All four lung cancer cell lines had an increase in apoptosis: A549, 3.0–8.3%, $p < 0.05$; H-460, 9.3–19.5%, ns (not significant); H-441, 3.1–8.9%, ns; and Calu-1, 5.4–11.2%, $p < 0.05$ (Fig. 5). All lung cancer cell lines also had an increase in necrosis: A549, 0.9–4.6%, $p < 0.05$; H-460, 1.4–30.7%, $p < 0.05$; H-441, 5.8–9.1%, ns; and Calu 1, 0.9-4.4%, $p < 0.05$ (Fig. 5, upper-right quadrant). NHBE control cells had no change. Increases in apoptosis and

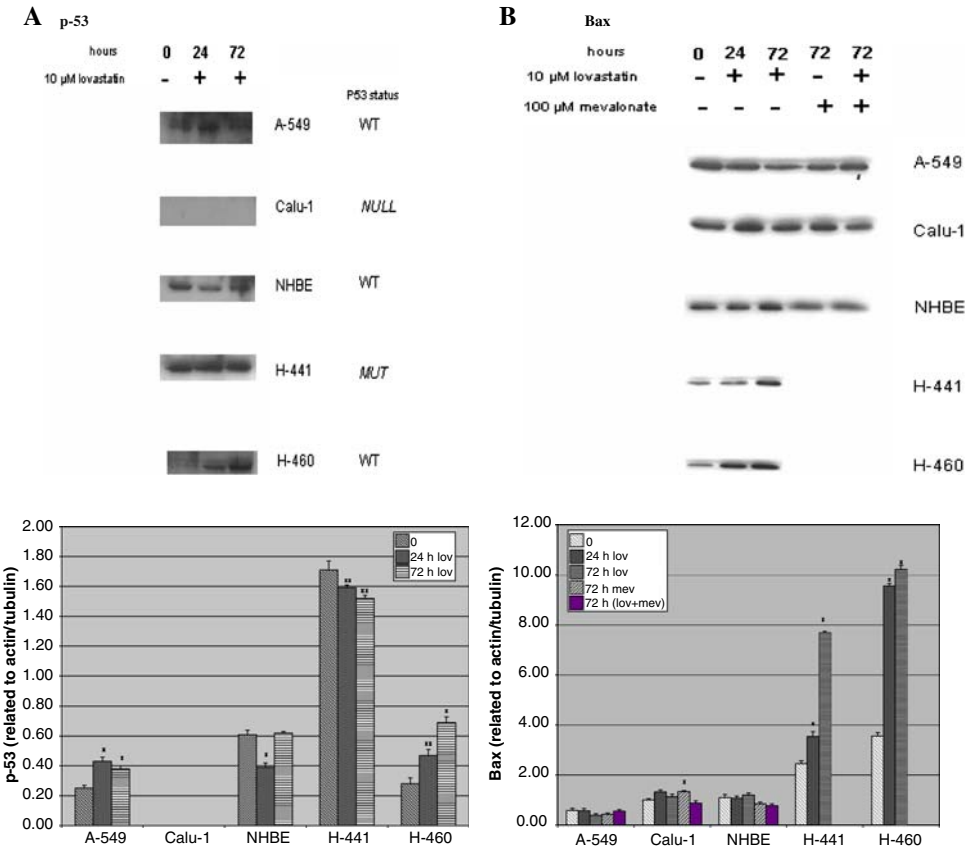


Fig. 4 Role of p53 and Bax in lovastatin-induced apoptosis in NHBE, Calu-1, A549, H-441, and H-460 lung cancer cells. Lung cancer cells were cultured in the absence or the presence of lovastatin and lysates subjected to Western blot analysis at 0, 24, and 72 h. **A** p53 Western blot and quantitation. The p53 status is indicated on the right. **B** Bax Western blot and quantification of Bax. Data are presented as mean ± SD, $*p < 0.01$

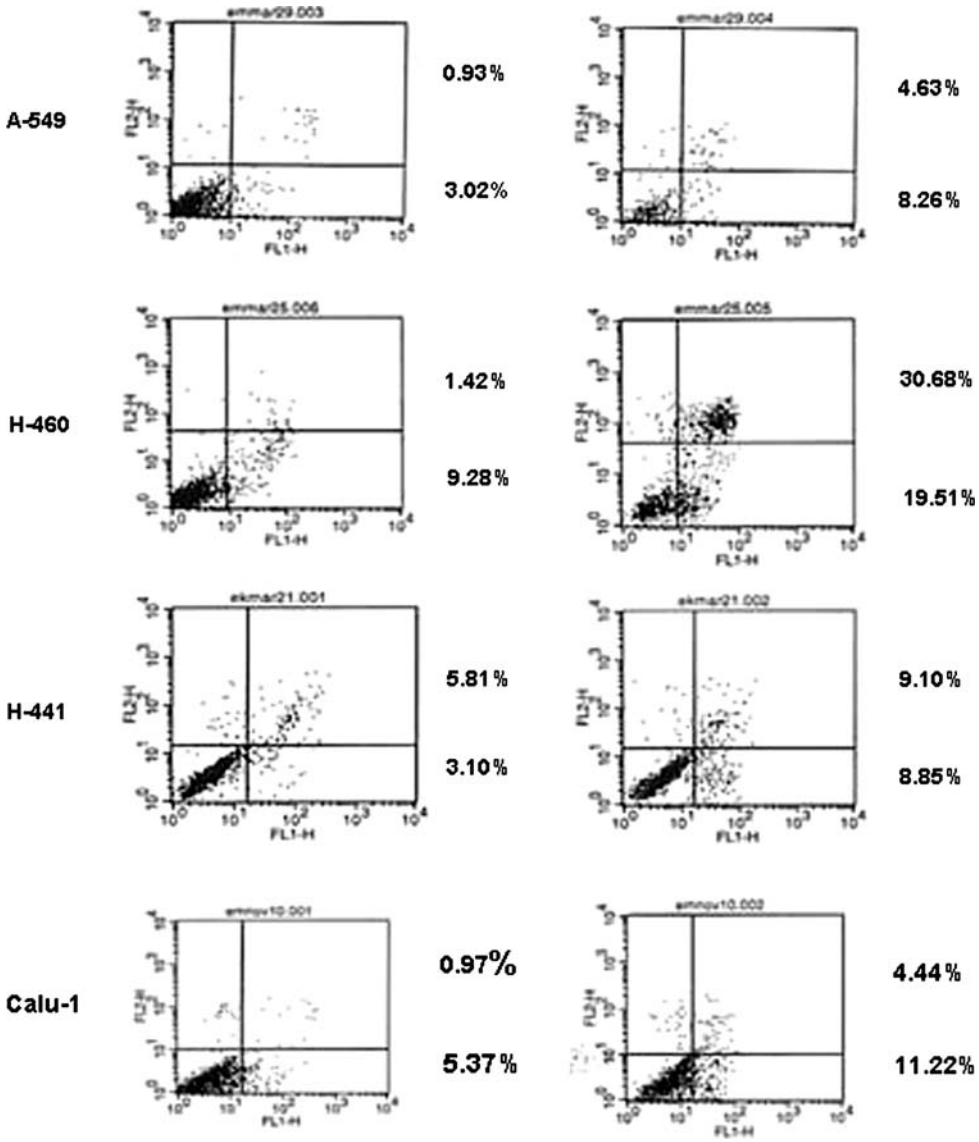
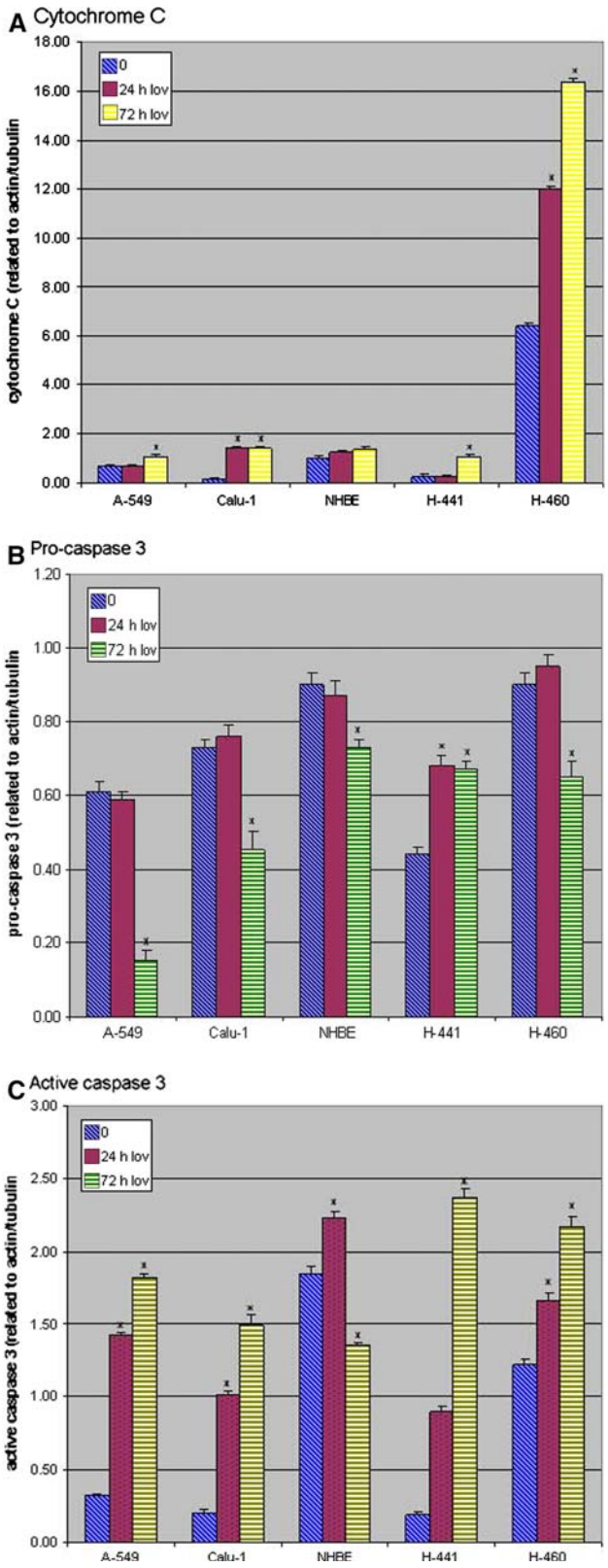


Fig. 5 Detection of cell necrosis/apoptosis by flow cytometry. Lung cells were untreated (left column) or incubated with 10 μM lovastatin for 72 h (right column) and harvested for quantification of apoptosis using annexin V binding assay. Values represent percentage of early apoptotic cells (annexin V positive, as reflected in the lower-right-hand quadrant) and necrotic/late apoptotic cells (annexin V positive and PI positive, depicted in the upper-right-hand quadrant)

necrosis may explain, in part, the reduced lung cancer cell lines' survival after lovastatin treatment.

Cytochrome c Release and Caspase-3 and −8 Activation

We next investigated whether GSH depletion was associated with mitochondrial dysfunction and cytochrome c release, followed by activation of caspases that are involved in apoptosis. After 72 h of lovastatin treatment, all four lung cancer cell lines released cytochrome c (Fig. 6A). H-441 cells had the lowest cytochrome c release (these cells had the highest GSH level and only 50% reduction in cell survival, Fig. 1). Calu-1 and H-460 cells released significant amounts of cytochrome c at 24 h, and A-549, Calu-1, H-441, and H-460 cells released significant amounts of cytochrome c at 72 h. NHBE cells did not release increased amounts of cytochrome c after treatment with lovastatin. To further clarify the GSH decline in lung cancer cell lines treated with lovastatin, we performed immunoblot analysis of the pro- and active forms of caspase-3 that could be activated through cytochrome c. As shown in Figure 6B, [A549, Calu-1, H441, and H-460 lung cancer cell lines] the pro-caspase-3 was significantly downregulated in three out of four lung cancer cell lines and NHBE control cells at 72 h. As shown in Figure 6C, there was a subsequent significant increase and activation of caspase-3 in all four lung cancer cell lines and NHBE control cells at 24 h. At 72 h there was a further significant increase in the four lung cancer cell lines, but the NHBE controls decreased below the control value. These



◄ **Fig. 6** Lovastatin-induced release of cytochrome c and effect on caspases. **A** Cytochrome c: Lung cancer cell lines and NHBE cells were cultured with or without lovastatin and lysates were analyzed by Western blot. There was an increase in cytochrome c release by each of the lung cancer cell lines compared to NHBE cells. Data are presented as mean ± SD, $*p < 0.01$. **B** Pro-caspase-3 expression: Data are presented as mean ± SD, $*p < 0.01$. **C** Active caspase-3: Active caspase-3 increases in all lung cancer cell lines over 72 h with lovastatin treatment. Data are presented as mean ± SD, $*p < 0.01$

observations indicated that activation of the caspase cascade may play a crucial role in lovastatin-induced apoptotic death in lung cancer cells.

Discussion

Although the beneficial effects of HMG-CoA reductase inhibitors in lowering cholesterol is well established, their importance in the area of cancer therapeutics has recently gained recognition [11, 12]. In this report we evaluated lovastatin sensitivity in four lung cancer cell lines and NHBEs, observing that lovastatin inhibited lung cancer cells' proliferation *in vitro*. It appears to be dependent on the depletion of critical mevalonate metabolites. Our results demonstrated that lovastatin inhibited cell growth by triggering a cell cycle checkpoint through an induction of $p21^{WAF}$, $p27^{KIP}$, or both, which in concert with lovastatin inhibits cyclin D1 expression in the G_1 phase of the cell cycle. The ability of lovastatin to block cells in G_1 appears to be due to its ability to inhibit protein geranylgeranylation rather than farnesylation [13].

Hawk et al. [14] reported that lovastatin inhibited human lung cancer cell lines NCI-H-125, H-292, H-441, H-460, and H-661 regardless of a K-Ras mutation. Furthermore, they fed lovastatin in the diet to mice treated with a tobacco-specific nitrosamine and found no effect on lung adenoma incidence or size but found reduced tumor multiplicity in a dose-dependent manner [14]. Apoptosis in H-460 cells is dramatically increased in G_0/G_1 of the cell cycle in the presence of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) at 100 ng/ml over 4 h compared to unsynchronized cells or cells in other phases of the cell cycle [15]. Activation of pro-caspase-3, −8, and −9 occurred demonstrated by decreasing levels with concomitant increases in the cleaved forms. Simvastatin enhanced the TRAIL-induced apoptosis of lung carcinoma H-460 cells. Normal keratinocytes were not susceptible to the TRAIL-induced apoptosis. Lovastatin in breast cancer cell cultures has been reported to increase p21 and p27, leading to cell cycle arrest in the G_1 phase [16]. Shibata et al. [17] showed that lovastatin suppressed tumor growth and metastases to the lung in a mouse mammary cancer model; *in vitro* studies demonstrated cell cycle arrest in G_1 with increased apoptosis in all phases of the cell cycle and activation of caspase-3, −8, and −9 [17]. The mitochondrial membrane potential was also decreased, and electron microscopy detected migration of Bax to the mitochondria with subsequent release of cytochrome

c. Since the breast cancer cell lines used in these experiments contained a p53 mutation, these results suggested a p53-independent mitochondrial apoptotic mechanism. Lovastatin inhibits the epidermal growth factor receptor pathway in five NSCLC cell lines and this effect is amplified with the addition of gefitinib [18]. The combined apoptotic and cytotoxic effects were synergistic, involved the phosphoinositide-3 kinase/AKT pathway, and were independent of activating ATP binding site mutations. Lovastatin and the PPARγ agonist troglitazone synergistically upregulated p27 and dephosphorylated Rb at clinically achievable plasma levels ranging from 0.1–3 μM to 5–15 μM, respectively, in patients [19]. Downregulation of E2F-1 in apoptotic prostate cancer cells *in vitro* after lovastatin treatment may be the key target of this HMG-CoA inhibitor [20]. Chan et al. [21] reviewed the statins as anticancer agents noting that early phase II clinical trials in advanced hepatocellular cancers and metastatic head and neck cancers demonstrated disease stabilization [21].

The levels of cellular glutathione in lung cancer cells were dramatically reduced by lovastatin, which may have a role in causing mitochondrial dysfunction and apoptosis. This condition has often been associated with the opening of existing pores such as mitochondrial permeability transition pores, which could be responsible for cytochrome c release [22]. In physiologic conditions, the intracellular redox status of thiols is highly reductive. GSH, for example, is present in high concentrations in lung epithelial lining fluid [23] and has been reported to maintain the integrity of the air space epithelium *in vitro* and *in vivo*. In contrast, GSH depletion has been linked to the pathophysiology of idiopathic pulmonary fibrosis [24], adult respiratory distress syndrome, bronchopulmonary dysplasia, cystic fibrosis, and toluene isocyanate-induced asthma, thus highlighting its central role in maintaining the functional integrity of a physiologically competent epithelium.

The present report investigated the importance of p53 in the cellular response to lovastatin. Our results show that wild-type p53 is required for the lung cancer cellular apoptotic response and p53-null cancer cells may use different pathways to transmit the damage signals that induce cell death. Apoptosis, induced by statins, alters the expression of several proteins from this family. Bcl-2 promotes cell survival and lovastatin inhibits bcl-2 expression [25]. Proteins of the bcl-2 family play a key role in the control of apoptosis and carry out both proapoptotic and antiapoptotic functions. Regulation of bcl-2 expression is controlled by oncogenic Ras protein [26]. It was also found that the ability to survive in the presence of lovastatin was related to the amount of Bax; H-460 cells had significantly increased apoptosis with significant increases in both p53 and Bax.

We found that low GSH levels and increased sensitivity to lovastatin correlated with cytochrome c release, followed by caspase-3 activation. Mevastatin induced apoptosis in U266 myeloma cells by increasing caspase activity, depolarizing the mitochondrial membrane, and downregulating bcl-2 mRNA and protein [27]. Lovastatin can synchronize lung cancer cell lines (H-460) in G_0/G_1 and significantly enhance sensitivity to TNF-α-related apoptosis-inducing ligand [28]. We have also demonstrated that lovastatin triggered both types of cell death—apoptosis and necrosis.

The apoptosis process, which is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and formation of apoptotic bodies, is triggered by two major pathways. The extrinsic pathway is induced by activation of death receptors on the cell surface, such as Fas-Fas ligand, where the intrinsic pathway involves an imbalance of p53, Bax, and bcl-2, leading to an increase of mitochondrial permeability and cytochrome c release. Both of these pathways lead to caspase activation and cleavage of specific cellular substrates. Reports of mechanisms underlying statins' action have revealed that lovastatin treatment increases levels of caspase-7 as well as caspase-3, caspase-8, and caspase-9 [29]. Once in the cytosol, cytochrome c activates Apaf-1, which then activates pro-caspase-9, which, in turn, activates caspase-3, triggering apoptosis.

Recent studies have suggested that in addition to the cholesterol-lowering effect, statins also inhibit the growth and proliferation of both tumor and normal cells: breast cancer, acute myeloid leukemia (AML), prostate cancer, multiple myeloma, squamous cell carcinoma, lung microvascular endothelial cells, and umbilical vein endothelial cells. As a consequence of mevalonate starvation, cells incubated with statins accumulate in the G_1 phase of the cell cycle or undergo apoptotic death. The antiproliferative effects of HMG-CoA reductase inhibition on cell-cycle arrest are thought to be attributable to an increase in $p21^{WAF/CIP1}$ and $p27^{KIP1}$, suppression of cyclin D1, cyclin E, CDK2, CDK4 expression, and pRb phosphorylation. Lovastatin also inhibits platelet-derived growth factor-induced association of PI3 kinase with PDGF receptor and subsequent inhibition of PI3 kinase activity [30]. Statins also abrogate the proliferative effects of insulin-like growth factor 1, a progression growth stimulus acting synergistically with PDGF [31].

Statins, including lovastatin, have been shown to variously inhibit tumor growth and metastasis *in vivo* in several different implantable rodent tumorigenesis models: in mammary carcinoma, fibrosarcoma, colon adenocarcinoma, melanoma, pancreatic neoplasias, and lung cancer [32]. Farnesyltransferase inhibition can induce tumor regression in transgenic mice with ras, myc, and other

oncogenic mutations by altering both cell cycle and apoptosis. Simvastatin can inhibit small-cell lung cancer growth via the Erk and PI3 kinase pathways and post-transcriptional processing of H-Ras [33].

Two clinical trials evaluating coronary events in patients reported a 43% and 19% decrease in the number of new cases of colon cancer diagnosed over a 5-year follow-up in cohorts taking pravastatin and simvastatin, respectively [34, 35]. A 5-year follow-up study of the safety of lovastatin recorded a 33% lower than predicted incidence of cancer [36]. A nested control study comparing cancer incidence in a hypercholesterolemic population treated with bile-acid-binding resins with that of a population treated with statins (lovastatin, pravastatin, simvastatin) recorded a 28% lower diagnosis of any cancer among patients in the latter group [37]. However, a meta-analysis of randomized control trials with more than 100 patients and over a 1-year follow-up, including the Blais [37] study, reported statin therapy to be neutral on cancer outcome and death [38]. Encouraging data recently reported from the Veterans Affairs Medical Centers that included almost 500,000 individuals taking statins showed a risk reduction of 55% for lung cancer using multiple logistic regression analysis adjusting for covariates such as smoking, diabetes, race, age, sex, and body mass index [39]. Our work suggests that lovastatin induced a specific apoptotic response in lung cancer cells within an achievable therapeutic range. Targeting HMG-CoA reductase may represent an approach for multimodal therapy or it may be a possible agent to reverse premalignant lesions in lung cancer progression.

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