

Lovastatin Induces Apoptosis in Malignant Mesothelioma Cells

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Malignant mesothelioma causes profound morbidity and nearly universal mortality that is refractory to conventional treatment with aggressive surgery, radiotherapy, or chemotherapy. We report that pharmacologic concentrations of lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor, induced apoptosis in human malignant mesothelioma cell lines. Mesothelioma cell viability was decreased in a dose-dependent manner by lovastatin (5 to 30 μM). These effects were not reversed by exogenous growth factors or cholesterol, but were reversed by addition of 100 μM mevalonate, confirming that lovastatin affected mesothelioma viability by inhibiting mevalonate synthesis. Lovastatin appeared to decrease mesothelioma viability by inducing apoptosis, as indicated by morphologic changes, histologic evidence of nuclear condensation and degeneration, and flow-cytometric analysis of DNA content. Lovastatin's effects on cell viability were partially reversed in the presence of farnesol, and treatment of mesothelioma cells with a specific farnesyl-protein transferase (FTP) inhibitor decreased cell viability and induced morphologic changes indistinguishable from those caused by lovastatin. In addition, lovastatin-treated cells showed translocation of ras guanosine triphosphate (GTP)-binding proteins from membrane to cytosolic fractions on Western blots, suggesting that lovastatin's effects on mesothelioma were mediated in part by disrupting acylation of GTP-binding proteins. Thus, lovastatin is a commercially available and clinically well-tolerated agent that reduces viability and induces apoptosis of mesothelioma cells, and may provide the basis for adjunctive treatments of patients with mesothelioma. Rubins JB, Greatens T, Kratzke RA, Tan AT, Polunovsky VA, Bitterman P. Lovastatin induces apoptosis in malignant mesothelioma cells.

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Malignant mesothelioma arises from the cells lining the pleural and peritoneal cavities, and is usually associated with previous exposure to asbestos or previous chest irradiation (1, 2). Currently, the incidence of malignant mesothelioma appears to be rising, probably reflecting the long latency between the widespread exposure to asbestos-containing materials in previous decades and the clinical presentation of this cancer (3). Malignant mesothelioma produces profound morbidity through pain and restrictive lung disease, and is associated with a median survival after diagnosis of less than 9 mo (1, 3). Current surgical treatments, including aggressive extrapleural pneumonectomy, are themselves associated with appreciable morbidity and mortality, and are not thought to be curative. In ad-

dition, there is no effective chemotherapy or radiation therapy for mesothelioma (1). Because of the failure of conventional therapies against this lethal malignancy, alternative treatment strategies to control tumor growth have been explored (3-5).

Lovastatin (mevinolin) is a fungal metabolite of *Aspergillus terreus*, and has been reported to inhibit cell growth in a number of malignant tumors (6-12) and normal proliferating cell cultures (9, 13). Lovastatin specifically inhibits synthesis of mevalonate by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the synthesis of cholesterol. Lovastatin and related compounds are widely used and well-tolerated pharmaceuticals for treating clinical hypercholesterolemia (14). However, effects of lovastatin on cultured and malignant cells appear to be mediated by its inhibition of mevalonate and subsequent nonsterol metabolites, rather than by its affecting cholesterol production. Mevalonate and its metabolites have been identified as essential factors for cell-cycle progression (15), and tumor cells appear to have higher demands for these nonsterol metabolites than do normal cells (12). More recently, farnesyl was identified as a critical mevalonate metabolite in the regulation of cell growth. Both nuclear membrane proteins, such as lamin B, and ras and related guanosine triphosphate (GTP)-binding proteins undergo posttranslational addition of a hydrophobic farnesyl moiety, which localizes these proteins to their appropriate membranes

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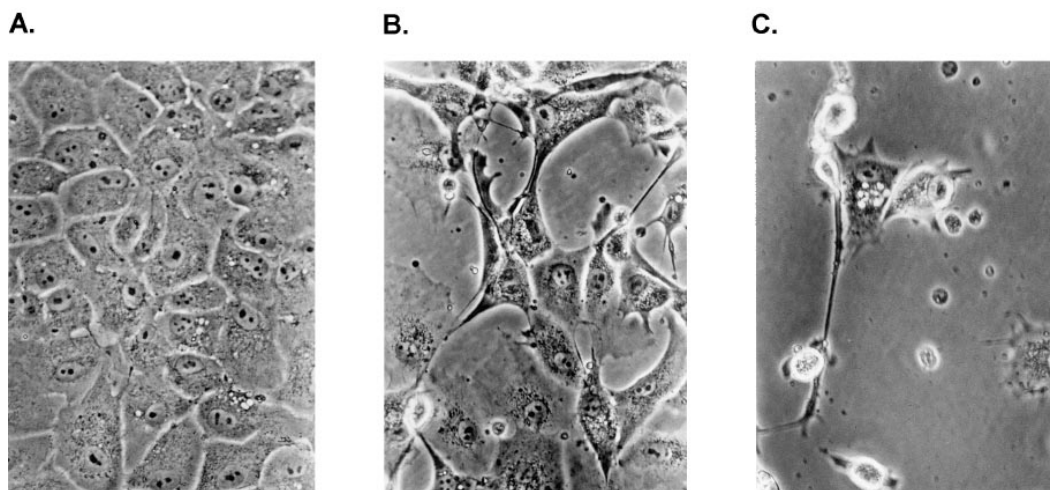


Figure 1. Lovastatin reduced mesothelioma cell viability. Near-confluent cultures of mesothelioma cell line 2691 were treated with control medium (A), or lovastatin at 5 μ M (B) or 20 μ M (C) for 3 d, and were examined with phase-contrast microscopy. Original magnification: $\times 400$.

(16–18). Thus, deprivation of farnesyl by inhibition of HMG-CoA reductase may prevent critical posttranslational processing of key growth-regulating proteins, leading to cell-cycle arrest.

In addition to its antiproliferative activity, lovastatin has been shown to trigger apoptosis in cultured promyelocytic and malignant glioma cells (7, 13). On the basis of these reports, we investigated whether lovastatin would display proapoptotic activity in human malignant mesothelioma. We report that lovastatin induces apoptosis in six human malignant mesothelioma cell lines, and we identify translocation of ras GTP-binding proteins as a possible mechanism of lovastatin's proapoptotic effect.

METHODS

Reagents

Lovastatin, obtained as a lactone (Merck, Sharp & Dohme, Rahway, NJ), was dissolved in ethanol and activated *in vitro* as described (19). Mevalonate, farnesol, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma Chemical Co., St. Louis, MO. N-acetyl-S-farnesyl-L-cysteine and farnesyl-protein transferase, (FTP) inhibitor III ([E,E]-2-[2-Oxo-2-[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic acid, [2,2-dimethyl-1-oxopropoxy]methyl ester, monosodium salt), and anti-lamin B antibody were from Calbiochem (La Jolla, CA). Pan-anti-ras antibody was obtained from New England Nuclear (Boston, MA).

Mesothelioma and Control Fibroblast Cell Lines

Cell lines derived and propagated at the National Cancer Institute (NCI)–Navy Medical Oncology Branch as previously described (20, 21) were cultured in RPMI 1640 (Gibco, Gaithersburg, MD) + 10% fetal bovine serum (FBS) in 5% CO₂ at 37° C. Control normal human lung fibroblasts (CCL 210; American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FBS. Cells were maintained with weekly feeding and subcultivated at a 1:3 split ratio when confluent. For study of cell proliferation and apoptosis, cells were detached with trypsin–ethylenediamine tetraacetic acid (EDTA) and subcultivated at a 1:4 split ratio into culture media. After cells were cultured for 24 h, medium was removed, fresh medium containing lovastatin or other experimental reagents was added, and cultures were continued in 5% CO₂ at 37° C for the times indicated.

Cell-proliferation Assay

Cell proliferation was quantified with the colorimetric MTT assay in 96-well plates as described (7).

Analysis of Apoptosis

Cells were examined morphologically for apoptosis, using inverted phase-contrast microscopy (CK2; Olympus Corporation, Tokyo, Japan) and fluorescence microscopy after acridine orange staining as described (22). DNA integrity was assessed through fluorescence-activated cell sorting (FACS) after propidium iodine staining, and by agarose-gel electrophoresis as previously described (22).

Immunoblotting for ras and Lamin B Proteins

After treatment with lovastatin or control media, crude cellular membranes and cytosol fractions were prepared from mesothelioma cells through previously described methods (23). Protein concentrations of membrane and cytosol fractions were determined with the Lowry method, with bovine serum albumin (BSA) as a standard. Aliquots of lysate, membrane, and cytosol fractions prepared as described earlier were separated on 11% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose, and immunoblotted with pan-anti-ras or anti-lamin B antibody as described (23).

Statistics

Data points in line figures and bar graphs represent the mean and SEM of data from three repeat experiments. Where indicated, means were compared through one-way analysis of variance (ANOVA) using SPSS software for Windows 6.1 (SPSS Inc., Chicago, IL).

TABLE 1
INHIBITION OF MESOTHELIOMA-CELL GROWTH BY LOVASTATIN

Cell Line	50% Effective Dose* (μ M)
2369	1
2691	3
2373	25
2595	30
2461	90
513	100

* Dose of lovastatin required to produce 50% reduction in numbers of viable cells as compared with untreated controls after 72 h, as determined with MTT assay and morphology (see MATERIALS).

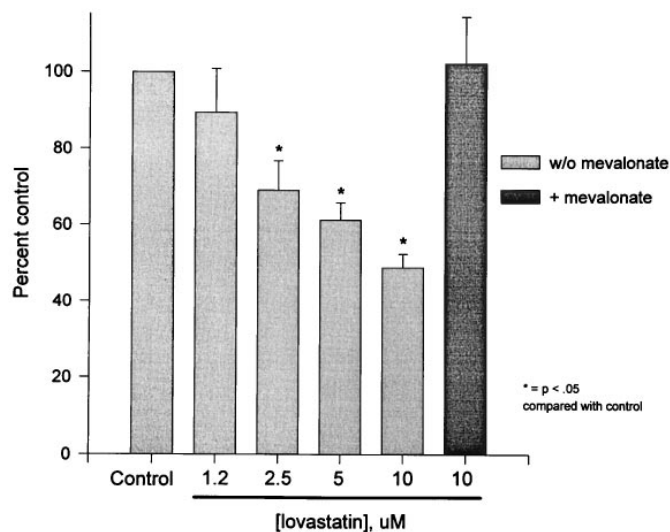


Figure 2. Lovastatin effect on mesothelioma-cell viability was reversed by exogenous mevalonate. Lovastatin was added in the concentrations indicated to nearly confluent cultures of mesothelioma cell line 2691 with (*dark gray bars*) or without (*light gray bars*) exogenous mevalonate. After 3 d, the numbers of viable cells were determined with a colorimetric assay as described in METHODS, and results were expressed relative to number of untreated cells. Data represents means and SE of three experiments. * $p < 0.05$ compared with untreated cells.

RESULTS

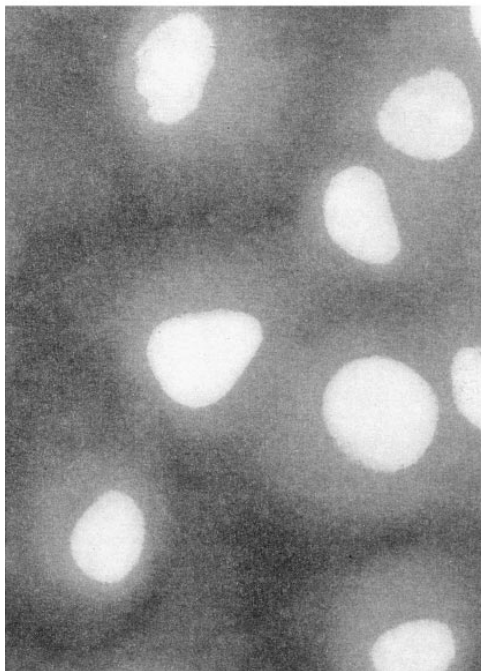
Lovastatin Reduced Malignant Mesothelioma Cell Viability *In Vitro*

In initial experiments, the susceptibility of the human malignant mesothelioma cell lines to agents previously reported to inhibit growth of this cancer was examined (5). Mesothelioma cells proliferated normally despite serum deprivation (0.3% FBS) and despite addition of 1 $\mu\text{g}/\text{ml}$ of interferon- γ (IFN- γ) or tumor necrosis factor- α (TNF- α), either singly or in combination (data not shown).

In contrast, after incubation for 3 d with pharmacologic doses of activated lovastatin, all six mesothelioma cell lines showed varying degrees of reduced viability as compared with untreated cells (Figure 1 and Table 1). The sensitivity of the different mesothelioma cell lines did not strictly correlate with their specific growth rates (although faster-growing cell lines were generally more sensitive to lovastatin) nor with their morphology (epithelial versus sarcomatous). Data shown are for mesothelioma cells treated when nearly confluent; however, less dense cell cultures (approximately 50% confluent) were equally sensitive to lovastatin.

Lovastatin reduced mesothelioma-cell viability despite the supplementation of culture media with cholesterol and growth factors present in 10% FBS, indicating that lovastatin did not inhibit viability by depriving cells of cholesterol. Also, lovastatin-induced mesothelioma cytotoxicity was reversible, with cell cultures returning to normal morphology and confluence approximately 3 to 5 d after lovastatin-containing medium was removed and replaced with fresh medium. Furthermore, lovastatin's inhibition of mesothelioma-cell viability was fully reversed by addition of 100 μM exogenous mevalonate (Figure

A.



B.

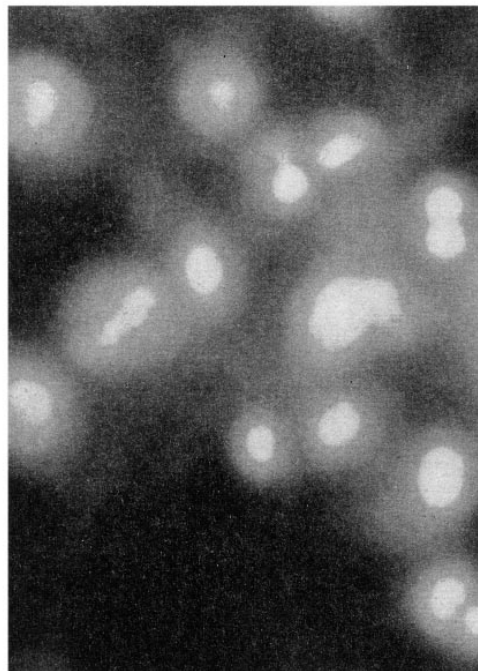


Figure 3. Lovastatin induced morphologic changes of DNA fragmentation in mesothelioma cells. Mesothelioma cells (2691) cultured to near-confluence Lab-Tek slide wells were treated with control medium (A) or 10 μM lovastatin (B) as described for Figure 1. After 3 d, cells were fixed with 70% ethanol, stained with acridine orange, and examined with fluorescence microscopy.

2), suggesting that lovastatin reduced malignant-mesothelioma-cell viability by depriving cells of nonsterol mevalonate metabolites.

Lovastatin Induced Apoptosis in Malignant Mesothelioma Cells

Although mesothelioma-cell cultures exposed to 5 to 30 μM lovastatin for several days contained many detached cells and scant adherent cells with rounded cell bodies, examination of mesothelioma cells after briefer incubation times with lower lovastatin concentrations revealed reproducible morphologic changes of membrane ruffling and cytoplasmic condensation, consistent with the changes of apoptosis (24) (Figure 1). Ex-

amination of mesothelioma-cell nuclear morphology after staining with acridine orange confirmed marked chromatin condensation and fragmentation characteristic of apoptosis in cells treated with lovastatin (Figure 3), but not in those incubated in medium alone, or in lovastatin-treated cultures supplemented with melavonate (not shown). Nuclear DNA fragmentation of cells was confirmed by agarose-gel electrophoresis, which revealed the characteristic pattern of internucleosomal cleavage in DNA from lovastatin-treated cells but not in that from control cells (not shown).

Mesothelioma-cell apoptosis was quantified with flow cytometry after propidium iodide staining to determine proportions of cells with hypodiploid DNA content (Figure 4). Lovastatin-induced mesothelioma-cell apoptosis was dose-dependent, with susceptible cells showing appreciable increases in the proportion of hypodiploid DNA after incubation with 10 μM lovastatin for 72h. Control normal human lung fibroblast were appreciably less susceptible to lovastatin-induced apoptosis (Figure 4). For the different mesothelioma cell lines, sensitivity to lovastatin-induced apoptosis generally correlated well with lovastatin-induced cytotoxicity (compare Table 1 and Figure 4).

Lovastatin-induced ras Translocation in Mesothelioma Cells

Because one mechanism of growth inhibition by lovastatin in other cells appears to be loss of isoprenylation of GTP-bind-

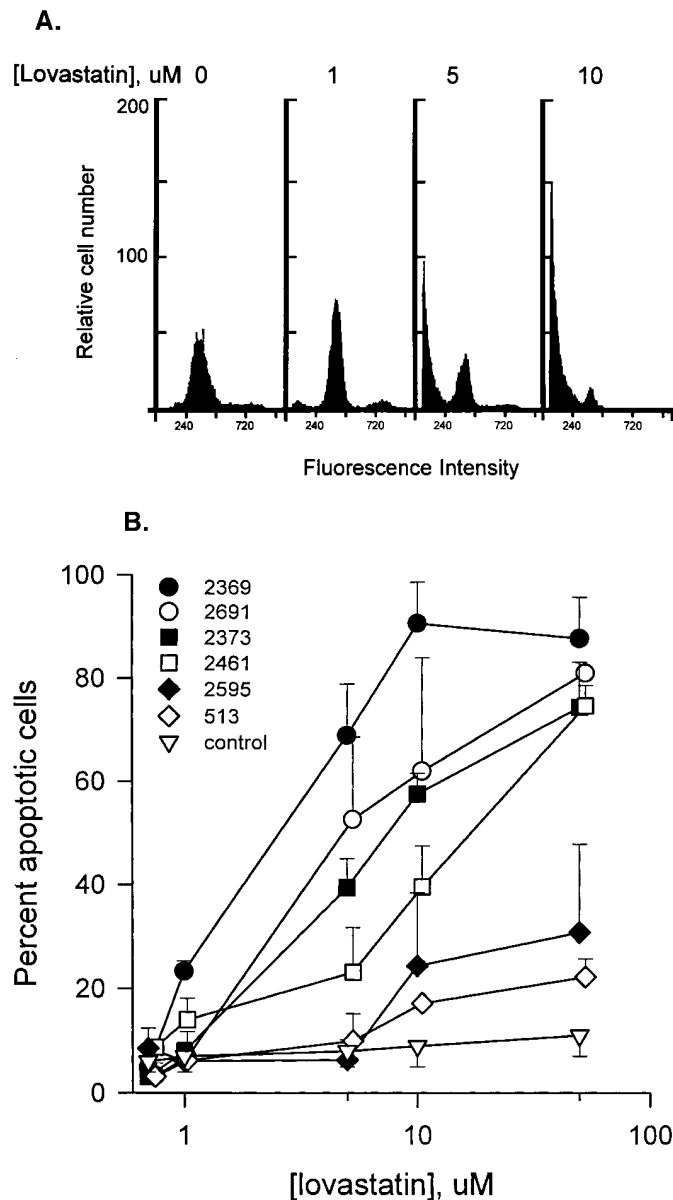


Figure 4. Dose dependence of lovastatin-induced mesothelioma-cell apoptosis. (A) Near-confluent monolayers of mesothelioma cell line 2691 were cultured with the indicated concentrations of lovastatin for 72 h, and the percentage of apoptotic cells was determined by flow cytometry after propidium iodide staining. (B) Percentages of apoptotic cells were calculated for six different mesothelioma cell lines and control normal lung fibroblasts, as described for (A).

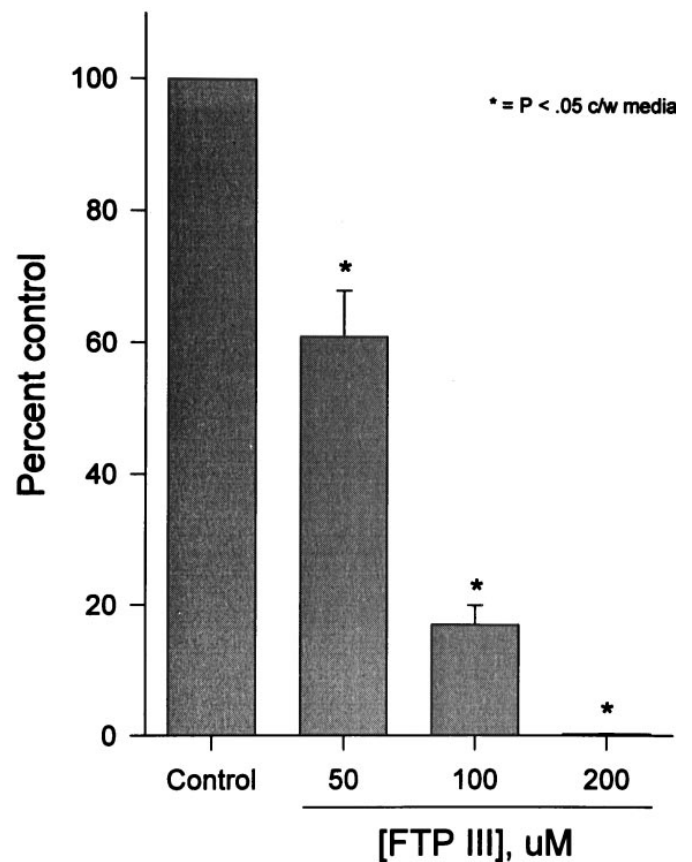


Figure 5. Inhibition of mesothelioma-cell growth by inhibition of farnesyl-protein transferase. Near-confluent cultures of mesothelioma cells (2373) were cultured for 3 d with the indicated concentrations of farnesyl-protein transferase inhibitor (FTP III) or with control medium, and the numbers of viable cells were quantified with the colorimetric assay described under METHODS. Data and statistics are as described for Figure 2.

ing protein (13, 25), we examined whether lovastatin might exert its apoptotic effects in mesothelioma cells through a similar mechanism. Addition of excess farnesol abrogated lovastatin cytotoxicity to mesothelioma cells (data not shown). Furthermore, specific inhibition of FTP, the essential step in GTP-binding protein and nuclear-lamin-protein isoprenylation, significantly inhibited mesothelioma-cell growth (Figure 5) and reproduced the morphologic changes observed with lovastatin treatment (not shown).

Because farnesylation of GTP-binding proteins is required for proper localization of these proteins to cellular membranes, we determined whether lovastatin treatment would translocate ras, a 21-kD GTP-binding protein that regulates cell proliferation, from cellular membranes to cytosol. Immunoblots of mesothelioma-cell membrane and cytosol proteins with a pan-anti-ras antibody showed decreased membrane-associated ras and increased cytosolic ras protein in lovastatin-treated mesothelioma cells as compared with untreated cells (Figure 6). Compared with the membrane-associated ras proteins, the cytosolic ras proteins showed approximately 3-kD slower migration, which is typical of the unprocessed primary *ras* product. Mesothelioma-cell lines that were more susceptible to lovastatin-induced apoptosis showed greater translocations of ras proteins from membrane to cytosol fractions as compared with less sensitive cell lines (not shown). Thus, lovastatin inhibition of malignant-mesothelioma-cell proliferation and induction of apoptosis was accompanied by a reduced synthesis of farnesyl, which in turn interferes with membrane localization of ras and possibly also of other low-molecular-weight GTP-binding proteins.

By preventing farnesylation and subsequent association of lamins A and B with the nuclear envelope, lovastatin might weaken the integrity of the nuclear membrane and allow cytosolic endonucleases to fragment cellular DNA, producing the characteristic findings of apoptosis (13, 26, 27). To determine whether lovastatin caused translocation of lamin B proteins from nuclear membranes to cytosol, we immunoblotted mesothelioma-cell nuclear membrane and cytosol proteins with anti-lamin B antibody. In contrast to our findings for ras proteins, we could not detect decreases in nuclear-membrane lamin B or increases in cytosolic lamin-B proteins in lovastatin-treated mesothelioma cells (data not shown).

DISCUSSION

Malignant mesothelioma causes profound morbidity and mortality that is not significantly altered by the conventional treat-

ment modalities of aggressive surgery, radiotherapy, or chemotherapy. In this report we have shown that pharmacologic concentrations of lovastatin inhibit the *in vitro* viability of mesothelioma-cell lines that are refractory to manipulations previously reported to inhibit mesothelioma-cell growth, such as serum depletion and cytokines (5). Furthermore, we report that lovastatin induced condensation of cell nuclei and fragmentation of cellular DNA, suggesting that it inhibited mesothelioma-cell viability by inducing apoptosis, rather than by direct cytotoxicity. We also demonstrated that lovastatin's antiproliferative and proapoptotic effects appeared to be mediated by reduction of farnesyl substrate and were associated with translocation of ras, which requires farnesylation for proper localization to cell membranes. Thus, we speculate that lovastatin induces apoptosis and inhibits malignant mesothelioma-cell proliferation *in vitro* by preventing transacylation of key GTP-binding proteins that regulate cell growth.

Lovastatin was originally identified as a cytostatic fungal toxin that blocked cell-cycle progression from the G₁ to the S phase (14). Studies using lovastatin characterized the critical importance of mevalonate and its nonsterol metabolites (isoprenyl and farnesyl) in regulating cell-cycle transit (15, 28). Subsequent studies suggested that lovastatin's cytostatic effects resulted from its blocking the isoprenylation of critical growth-regulatory proteins. A number of regulatory cellular proteins, such as GTP-binding proteins and nuclear-membrane proteins, require posttranslational farnesylation and isoprenylation to assure their proper membrane localization and function (17, 18, 27). Thus, lovastatin presumably interrupts normal growth signaling by interfering with the processing and function of these regulatory proteins.

Lovastatin may induce apoptosis in malignant cells by shifting the balance of intracellular "survival" and "suicide" signals. Apoptosis has been proposed as a default pathway for any cell that does not receive the appropriate balance of "survival signals" communicated by soluble messengers or by adherence to matrix components (29). According to concept, somatic DNA mutations that cannot be adequately repaired trigger apoptosis, and malignancy may result from impaired apoptosis. Accordingly, a number of proto-oncogene products, such as those of p53, c-myc, and bcl-2, have been identified as regulators of normal cellular apoptosis, and defective function of these gene products prevents appropriate apoptosis and permits malignant transformation (29). Regardless of the underlying genetic defect producing the malignancy, induction of apoptosis in tumor cells is increasingly recognized as the un-

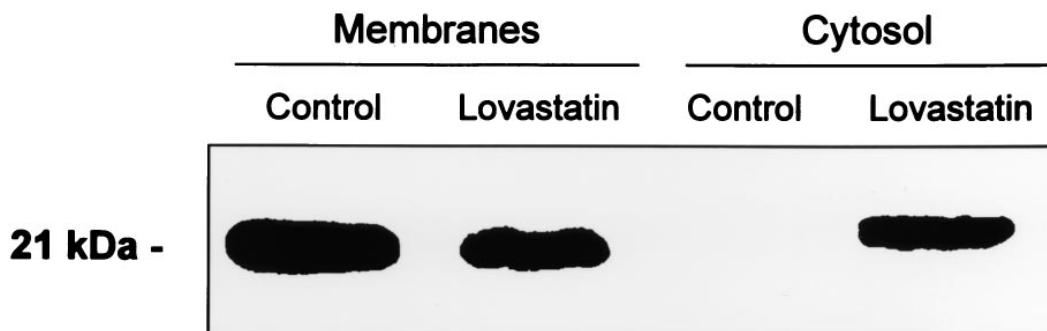


Figure 6. Lovastatin induced translocation of ras from mesothelioma membranes to cytosol. Near-confluent monolayers of mesothelioma cells were treated with control medium or with 10 μ M lovastatin for 3 d, after which cells were lysed and separated into membrane and cytosol fractions by centrifugation, as described in METHODS. Proteins from membrane and cytosol fraction were separated on 11% SDS polyacrylamide gels, transferred electrophoretically to nitrocellulose, and blotted with a pan-anti-ras antibody.

derlying mechanism of effective cancer treatments, such as chemotherapy or radiotherapy (30). Presumably, agents that exert such an effect generate additional cellular signals that counter the inappropriate oncogene-derived survival signals and trigger apoptosis. In a similar fashion, by inhibiting appropriate membrane localization of critical intracellular signaling elements, lovastatin can induce apoptosis and inhibit growth of transformed and malignant cells (6–11, 13).

We have demonstrated apparent translocation of ras from cellular membranes to the cytosol of lovastatin-treated mesothelioma cells, in agreement with studies of lovastatin in other cultured cells (13, 25). However, we recognize that lovastatin may mediate induction of apoptosis and inhibition of tumor-cell growth by blocking acylation of other cellular proteins. Lovastatin was previously reported to inhibit the growth of NIH 3T3 fibroblasts transformed with myristylated ras protein that was independent of isoprenylation, indicating that the antiproliferative effects of lovastatin did not depend specifically on blocking ras farnesylation (19). Although these findings do not preclude the possibility that lovastatin might decrease tumor-cell viability by preventing the processing of ras proteins, other farnesylated proteins certainly may mediate lovastatin-induced apoptosis in malignant cells, including the many ras-related GTP-binding proteins that are involved in a variety of critical cellular processes (31, 32), and nuclear-membrane lamin proteins (27). Although we were not able to detect translocation of lamin B from nuclear membranes to cytosol in lovastatin-treated cells, our assay may have lacked the sensitivity needed to detect small but biologically significant effects of lovastatin on nuclear lamin B, or alternatively, other nuclear lamin proteins may be the relevant targets of lovastatin's effects.

In summary, malignant mesothelioma produces profound morbidity and mortality that is largely refractory to conventional treatment modalities. Our studies suggest that lovastatin, a widely used and well-tolerated pharmaceutical, can substantially inhibit mesothelioma growth and induce tumor-cell apoptosis. Importantly, lovastatin appears to have synergistic activity with antimetabolites used against other malignant cells (10, 33, 34). Taken together with previous reports, our data support further investigation of lovastatin as a potential adjunctive therapy for treatment of this lethal and refractory cancer.

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