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Potent Suppression of Proliferation of A10 Vascular Smooth Muscle Cells by Combined Treatment with Lovastatin and 3-Allylfarnesol, an Inhibitor of Protein Farnesyltransferase

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ABSTRACT

Statins, which inhibit 3-hydroxy-3-methylglutaryl-CoA reductase and thus the synthesis of cholesterol, are remarkably effective in the treatment of cardiovascular disease. In addition to their favorable effect on lipid profile, these drugs may also prevent the proliferation of vascular smooth muscle that is characteristic of atherosclerosis. We hypothesize that statins prevent the post-translational prenylation, and thus inhibit the function, of critical small GTPases in vascular smooth muscle cells. We have therefore assayed the effect of lovastatin on both the growth of A10 vascular smooth muscle cells and the status of their Ras and RhoB proteins. We find that $\leq$1 μM lovastatin potently inhibits the proliferation of A10 cultures, and higher concentrations ($\geq$3 μM) induce apoptosis. We have also tested the effect of 3-allylfarnesol (3-alFOH), an inhibitor of farnesyl transferase (FTI). The data show that although $\geq$10 μM 3-alFOH is required for a cytostatic effect, the action of 3 μM 3-alFOH can be greatly potentiated by even nanomolar levels of lovastatin. We also find that lovastatin and 3-alFOH exhibit synergism to cause the up-regulation and relocalization of RhoB from the membrane to cytosolic compartments. This relocalization of RhoB, which is presumed to reflect an inhibition of its prenylation, correlates with the proapoptotic activities of combined 3-alFOH and lovastatin treatment. These data suggest that RhoB may be a valuable pharmacological target in cardiovascular disease, and that combinations of statins and certain FTIs may be of value in treatment of disorders that are characterized by excess cell proliferation.

Hyperplasia of vascular smooth muscle is responsible for the intimal thickening that occurs after arterial wall damage. It may also contribute to the atherosclerotic process (Raines and Ross, 1993). Strategies that prevent inappropriate proliferation of vascular smooth muscle cells may therefore be of significant value in the treatment of cardiovascular disease. The drugs termed statins, which inhibit hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and thus reduce the production of cholesterol, are remarkably effective in the management of hypercholesteremia, with proven efficacy to improve lipid profile, to reduce risk of myocardial infarction and stroke, and to decrease mortality. It is becoming clear, however, that the beneficial effects of statins may also be due to effects other than the inhibition of hepatic HMG-CoA reductase. For example, there is poor correlation between improved clinical endpoints and effects on baseline low-density lipoprotein levels in several major clinical trials (for review, see Kolovou, 2001). An additional therapeutic mechanism for the action of statins could be the inhibition of vascular smooth muscle proliferation (Negre-Aminou et al., 1997; Raiteri et al., 1997). Indeed, lovastatin is known to be effective in prevention of the restenosis of coronary arteries after angioplasty (Sahni et al., 1991).

Inhibition of HMG-CoA reductase causes a reduction in supply of mevalonate metabolites that are critical not only

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ABBREVIATIONS:. HMG, 3-hydroxy-3-methylglutaryl; FTI, inhibitor of farnesyl transferase; 3-alFOH, 3-allylfarnesol; DMSO, dimethyl sulfoxide; AMC, 7-amino-4-methyl-coumarin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HLB, hypotonic lysis buffer; PAGE, polyacrylamide gel electrophoresis; HEK, human embryonic kidney; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; FPP, farnesyl pyrophosphate.

for the synthesis of cholesterol but also for the post-translational modification of certain proteins with prenyl groups (Khwaja et al., 2000). Considerable effort has been invested in the development of selective inhibitors of protein prenylation after the recognition that Ras farnesylation is essential for its ability to stimulate cell proliferation even after its oncogenic activation (Gibbs et al., 1994). Several inhibitors of protein farnesylation are now in advanced clinical trial for the therapy of various malignancies (Gibbs, 2000). Current evidence suggests that other members of the Ras superfamily of proteins, particularly RhoB, may be more relevant clinical targets for these farnesyl transferase inhibitors (FTIs) (Lebowitz et al., 1995; Aznar and Lacal, 2001).

There is some evidence that blockade of the function of Rho proteins, through inhibition of their prenylation, may also contribute directly and indirectly to the effects of statins on vascular smooth muscle cells. For example, statins induce an increase in endothelial nitric-oxide synthase activity that is dependent on the inhibition of Rho (Laufs et al., 2000). Statins have been reported to directly affect the prenylation of Rho proteins in vascular smooth muscle cells, although at high drug concentrations (Guijarro et al., 1998). We hypothesize that if the effect of statins on vascular smooth muscle cell proliferation is mediated through blockade of Rho protein prenylation, statins should exhibit synergy with selective inhibitors of prenylation (Fig. 1). Furthermore, if such a mechanism is to be relevant to the clinical use of statins, the effects should occur at drug concentrations that can be pharmacologically achieved. We therefore tested the effects of single and combined treatments of A10 vascular smooth muscle cells with lovastatin and an inhibitor of farnesyltransferase. The results demonstrate a correlation between their synergistic effects to suppress proliferation, to induce apoptosis, and to block the prenylation of RhoB.

Materials and Methods

Drugs

Lovastatin was purchased from Sigma-Aldrich (St. Louis, MO). The 3-allylfarnesol (3-alFOH) was synthesized as described previously (Gibbs et al., 1999). Both drugs were maintained as 100 mM stocks in dimethyl sulfoxide (DMSO) in aliquots under nitrogen at −80°C. AMC and acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin were purchased from BD PharMingen (San Diego, CA).

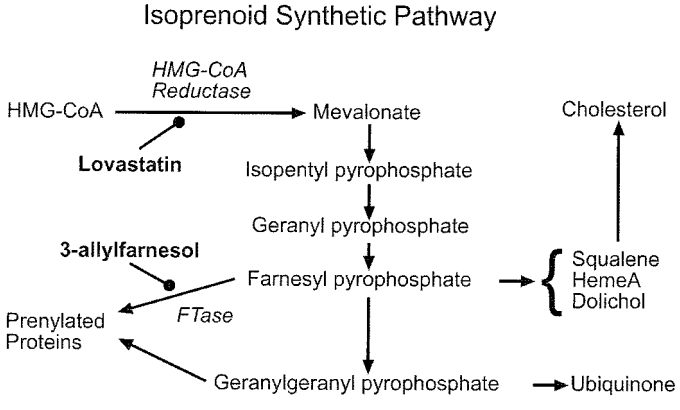

Fig. 1. Isoprenoid synthetic pathway, indicating the sites of action of lovastatin and 3-alFOH through which protein farnesylation may be compromised.

Cells

The A10 rat vascular smooth muscle cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in a humidified incubator with 5% CO_2 and grown on 100-mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Invitrogen, Carlsbad, CA), that was supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The cells were split 1:10 when approaching confluence. Aliquots in DMEM/fetal bovine serum/DMSO (4:5:1) were prepared for storage in liquid nitrogen, and all experiments were performed on cells grown between three and 10 passages from receipt.

Cell Proliferation Assay

A10 cells were plated at 100,000 cells/60-mm culture dish. Cells attached and spread within 2 h of plating. The media were then removed and fresh growth media with drugs were added, as noted in the figure legends. The medium was replaced every 2 days (with fresh drug addition as required) until the number of viable cells on the dish was counted with a hemocytometer after trypsinization. Ability to exclude trypan blue was used as a measurement of viability. Data are presented as mean ± S.E.M. of the number of independent experiments shown in each Figure. Differences from control were assessed by two-way analysis of variance with Bonferroni post tests when multiple comparisons were required (GraphPad Prism 3.0a; GraphPad Software, San Diego, CA).

Cell Cycle Analysis

A10 cells were plated at 250,000 cells/100-mm culture dish and allowed to attach overnight. The media were then removed and fresh growth media with drugs were added. The medium was replaced every 2 days until the time of analysis. The medium was carefully removed and combined with a 1-ml phosphate-buffered saline (PBS) rinse to constitute the "detached sample". The adherent cells were trypsinized and suspended in growth medium. Samples of the adherent and detached suspensions were counted and assessed for viability by exclusion of trypan blue. The remaining cell suspensions were washed 1× with PBS and then suspended in PBS before being fixed and processed for flow cytometric analyses of DNA content as described previously (Reiners et al., 1999). Percentages of apoptotic cells and cells in the G_1, S, and G_2/M stages of the cell cycle were determined with a DNA histogram-fitting program (MODFIT; Verity Software, Topsham, ME). Attempts were made to collect a minimum of 10^4 events/sample for subsequent analyses.

Subcellular Fractionation

A10 cells were plated at approximately 50% confluence and grown for 24 h. The media were then replaced with fresh media plus drugs as required (two plates per condition). Approximately 18 h later, the cultures were rinsed twice with hypotonic lysis buffer (HLB) (10 mM HEPES, pH 7.4, with NaOH; 5 mM $MgCl_2$; 1 mM EDTA·Na; 20 μg/ml aprotinin; 20 μg/ml leupeptin; and 100 μM phenylmethylsulfonyl fluoride) and then scraped with 0.5 ml/plate of HLB into microcentrifuge tubes on ice. The cells were allowed to swell for 10 min and then disrupted by sonication for 5 s and six passages through a 26-gauge syringe needle, combining material from duplicate plates. KCl was added to 150 mM final concentration and the nuclei removed by a 10-min centrifugation at 1,000*g*. The postnuclear supernatant was centrifuged at 10,000*g* for 15 min to prepare an organelle fraction. The postorganellar supernatant was centrifuged at 100,000*g* for 45 min to prepare a microsomal fraction. All pellet fractions were resuspended in 40 μl of HLB. The supernatant from the 100,000*g* spin was used as the soluble/cytosolic fraction, mixed with an equal volume of 300 mM NaCl, 2% Nonidet-40, 1% sodium deoxycholate, 0.2% SDS, and 100 mM Tris pH 7.4, and precipitated by addition of 10% trichloracetic acid on ice for 1 h. The precipitate was washed three times with cold acetone and allowed to dry in a

fume hood. The precipitate was dissolved in 40 μl of 1 M $NaHCO_3$, pH 8.8, at 37°C. All fractions were then mixed with 40 μl of 2× sample loading buffer (Mattingly et al., 2001a) and separated by SDS-polyacrylamide gel electrophoresis (PAGE).

Transfection

Calcium phosphate precipitation-mediated transfection of COS-7 and human embryonic kidney (HEK)293 cells was performed as described previously (Mattingly et al., 1994), using expression vectors pKH3RhoA (Beqaj et al., 2002) and pCMV3HARhoB (Lebowitz et al., 1997a) that express RhoA and RhoB with hemagglutinin (HA) tags at their N termini. A10 cells were transfected using the FuGENE 6 reagent according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Cells were grown for 48 h and then whole cell lysates were prepared in boiling Laemmli sample buffer as described previously (Mattingly et al., 2001a).

Western Blotting

Primary antibodies used were polyclonal and monoclonal anti-RhoB (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100, monoclonal anti-Ras at 1:250 and monoclonal anti-ERK mitogen-activated protein kinase at 1:5000 (Transduction Laboratories, Lexington, KY), polyclonal anti-cathepsin B (Moin et al., 1992) at 1:4000, and 12CA5 monoclonal anti-hemagglutinin (Mattingly, 1999) at 1:5000. Secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology) were diluted to 1:25,000, and detection was performed with Dura enhanced chemiluminescence reagents (Pierce Chemical, Rockford, IL). Data were collected using an LAS 1000 plus imaging system (Fujifilm, Tokyo, Japan) and exposure to film. For sequential probing of the membranes with different primary antibodies, the blots were stripped as described previously (Mattingly et al., 2001b).

To verify the selectivity and sensitivity of the commercial anti-RhoB antibodies they were tested against HA-tagged RhoB and RhoA proteins that were overexpressed in COS-7 and HEK293 cells (Fig. 2). The results show that the polyclonal anti-RhoB antibody shows greater sensitivity for the detection of RhoB than does the monoclonal antibody, with detection of both the overexpressed and endogenous proteins present in whole cell extracts of COS-7 cells (Fig. 2A). The polyclonal antibody to RhoB can selectively recognize RhoB and not RhoA (Fig. 2B). The ability of the antibody to recognize both endogenous and overexpressed RhoB in lysates of A10 cells was then confirmed (Fig. 2C).

Caspase-3 Assay

At the time of harvest the medium was removed and saved. Cultures were washed twice with PBS, and the washings were combined with the medium. After centrifugation, the pelleted detached cells were washed once with PBS and suspended in lysis buffer (10 mM Tris pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, and 10 mM $NaPP_I$). Adherent cells were covered with lysis buffer, dislodged with a cell scraper, and transferred to small tubes. Adherent and detached cell populations were stored at −80°C until the day of assay. On the day of assay, cell lysates were sonicated for 1 s and centrifuged for 5 min at 10,000*g* at 4°C. Caspase-3 activity in the supernatant fluids was assayed by monitoring the release of AMC from the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin. The assay has been described in detail previously (Reiners and Clift, 1999). Fluorescence was monitored on a SpectraMax Gemini plate reader (Molecular Devices, Sunnyvale, CA). Caspase-3-specific activities are reported as nanomoles of AMC released per minute per milligram of protein. The bicinchoninic acid assay was used for the determination of protein content. Bovine serum albumin was used as the standard.

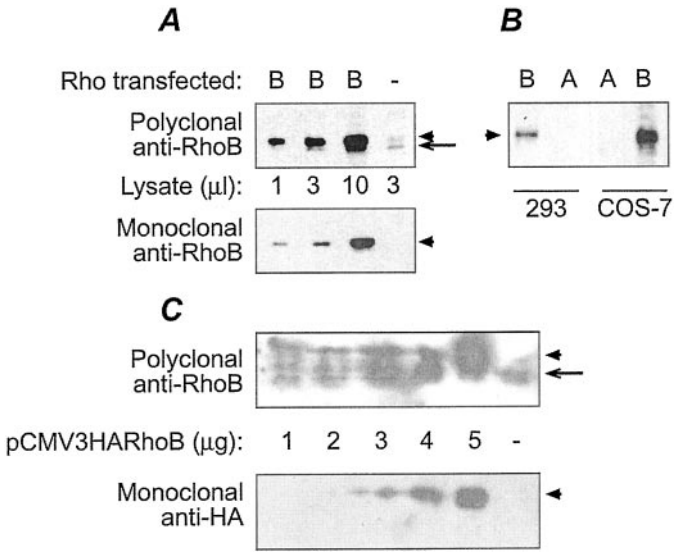

Fig. 2. Verification of the sensitivity and selectivity of antibodies to RhoB. COS-7 (A and B), HEK293 (B), and A10 (C) cells were transfected with expression vectors for HA-tagged RhoA and RhoB as shown and whole cell lysates subjected to Western blotting. The upper band (indicated with the arrowhead) that is seen with the polyclonal anti-RhoB antibody (A and C, top) and the band seen with the monoclonal anti-RhoB antibody (A, bottom) were also recognized by the anti-HA monoclonal antibody (C, bottom) and so represent the transfected, epitope-tagged forms of RhoB. The addition of the HA epitope tag to the N terminus would be expected to decrease the mobility of the protein during SDS-PAGE. The lower band (indicated by the long arrow) that is recognized by the polyclonal anti-RhoB antibody (A and C, top), which is also present in lysates from control transfected (indicated by −) cells, presumably represents endogenous RhoB. Note that the monoclonal anti-RhoB antibody (A, bottom) is less sensitive and only recognizes the overexpressed HA-tagged RhoB, but not the endogenous protein. Polyclonal anti-RhoB antibody recognizes RhoB but not RhoA (B).

Results

Lovastatin Potently Inhibits Proliferation of A10 Rat Vascular Smooth Muscle Cells. To characterize the effects of prenylation inhibitors on the growth of vascular smooth muscle cells, the A10 cell line was selected because it provides a uniform population of cells that exhibit significant phenotypic resemblance to neointimal cells (Rao et al., 1997). Because statins are clinically used in chronic treatment regimens, we investigated the effect of lovastatin on the growth of A10 cells over an 8-day period (Fig. 3A). In agreement with an earlier study that used human aortic smooth muscle cells from the internal mammary artery (Negre-Aminou et al., 1997), concentrations of lovastatin as low as 1 μM were cytostatic to A10 cultures ($p < 0.05$), whereas 3 μM lovastatin was cytotoxic and reduced cell numbers to values below that plated.

To investigate the effect of lovastatin on the growth of A10 cells in more detail, analyses were performed at 2-day intervals (Fig. 3B). Concentration-dependent, cytostatic effects of lovastatin were obvious within 2 days of treatment. Both 0.3 and 1 μM lovastatin suppressed the proliferation of A10 cultures ($p < 0.05$ for 0.3 μM lovastatin). In contrast, 3 μM lovastatin not only inhibited proliferation but also led to a time-dependent loss of cells. Flow cytometric analyses of DNA contents demonstrated a concentration-dependent loss of S-phase cells in lovastatin-treated cultures (Figs. 4A and 5, C and D). These effects were obvious within 48 h of treatment. This loss of S-phase cells was accompanied by accumu-

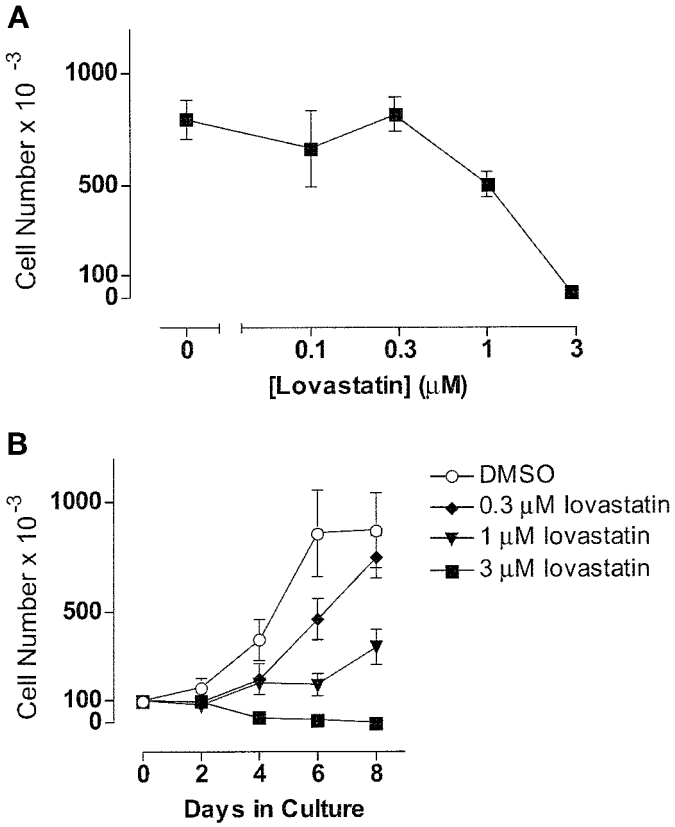

Fig. 3. Lovastatin potently inhibits the growth of A10 vascular smooth muscle cells. A10 cells (100,000 at start of experiment) were grown for 8 days (A) or for the indicated period (B) in the presence of the concentrations of lovastatin shown and then counted. Data are mean ± S.E.M. from at least four independent experiments.

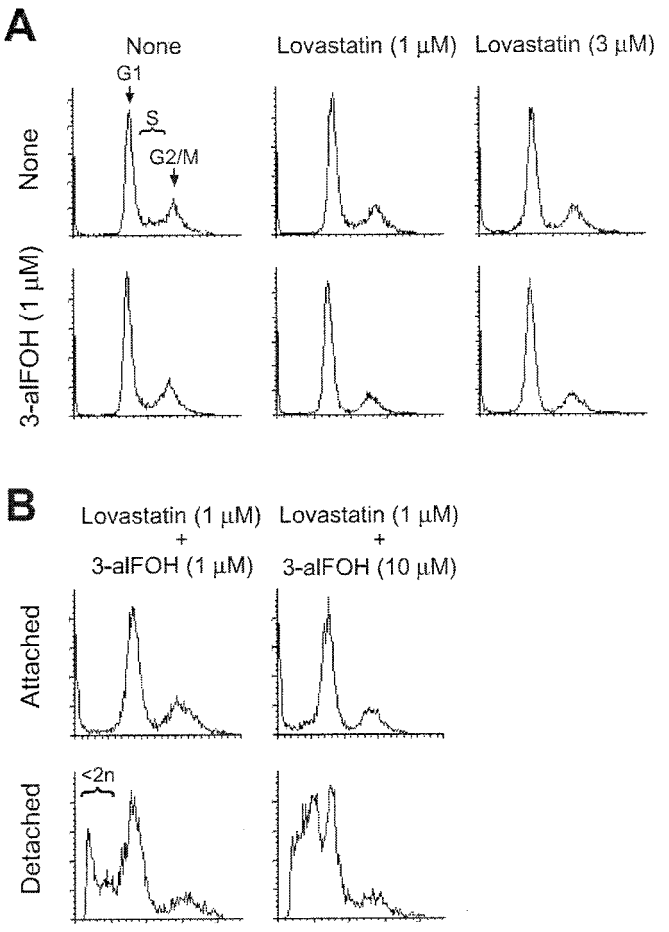

Fig. 4. Flow cytometric analyses of DNA content of A10 cells after treatment with prenylation inhibitors. A10 cultures were treated with lovastatin, 3-alFOH, or combinations of the two drugs for 48 h (A) or 96 h (B) before being harvested for analysis of DNA content. Patterns in A represent analyses of only attached cells (~66–88% of total population). Note the progressive loss of the S-phase population from the cultures with increasing lovastatin treatment. Further note that 1 μM 3-alFOH, although having no effect by itself, greatly reduced the S-phase population when combined with 1 μM lovastatin. Patterns in B represent analyses of both detached and attached populations. Analyses were performed on 10,000 acquired events. The patterns for the detached population in B seem comparable in magnitude to the attached patterns in B because the debris fraction in the detached population was intentionally gated out. If the debris fraction is included the pattern remains the same but is compressed overall. The detached cells show a predominant population with subdiploid DNA content (<2n), which is a characteristic of cells undergoing apoptosis. Similar results were obtained in a second independent study.

lations of G_1 and/or G_2/M cells (Fig. 5). The highest concentration of lovastatin tested (3 μM) also increased the relative percentage of cells that detached from the culture plates (Fig. 5, A and B). A considerable percentage of this detached population had subdiploid DNA content, a characteristic of apoptotic cells (see below).

Suppression of A10 Proliferation by a Farnesyl Transferase Inhibitor. Previous studies have indicated that 3-alFOH acts as a cell-permeable precursor of a farnesyl pyrophosphate (FPP)-competitive inhibitor of farnesyl transferase (Gibbs et al., 1999). Treatment of A10 vascular smooth muscle cells with 3-alFOH required a concentration of 10 μM to produce a significant ($p < 0.05$) suppression of cell proliferation (Fig. 6A). Treatment with 30 μM 3-alFOH reduced cell numbers to a value below that plated (Fig. 6A). Flow cytometric analyses of DNA contents suggested that the cytotoxic effects of this higher concentration reflected the induction of apoptosis (data not shown).

To verify that 3-alFOH was working to inhibit farnesyl transferase in the A10 cells at concentrations ≥10 μM, its effects on the subcellular localization of Ras were determined (Fig. 6B). Treatment of A10 cells with either 30 μM 3-alFOH for 24 h or 10 μM 3-alFOH for 48 h was able to induce the appearance of Ras in the soluble fraction of A10 cells. Cytosolic Ras presumably reflects the precursor form of the protein, before prenylation (Gibbs et al., 1999). Thus, the data in Fig. 6B support 3-alFOH as an inhibitor of farnesylation in this system.

3-alFOH Potentiation of Cytostatic/Cytotoxic Activities of Lovastatin. Combinations of low concentrations of lovastatin and 3-alFOH were very effective at inhibiting A10 proliferation (Fig. 6A). For example, 0.3 μM lovastatin plus 3 μM 3-alFOH (neither of which produced a measurable effect on cell number at 8 days as single agents) reduced cell numbers by one-third ($p < 0.05$). Similarly, the combination of 1 μM lovastatin plus 3 μM 3-alFOH reduced cell numbers to a value below that plated. This latter result was the consequence of at least two activities. First, flow cytometric analyses of DNA contents demonstrated that cotreatment of cultures with lovastatin and 3 μM 3-alFOH potentiated the loss of S-phase cells (Figs. 4A and 5, C and D). Cultures cotreated with 1 μM lovastatin + 1 μM 3-alFOH had S-phase contents comparable with that obtained with 3 μM lovastatin (Fig. 4). Second, the combination treatments resulted in the detachment of cells (Fig. 5B). Flow cytometric analyses of the 96-h

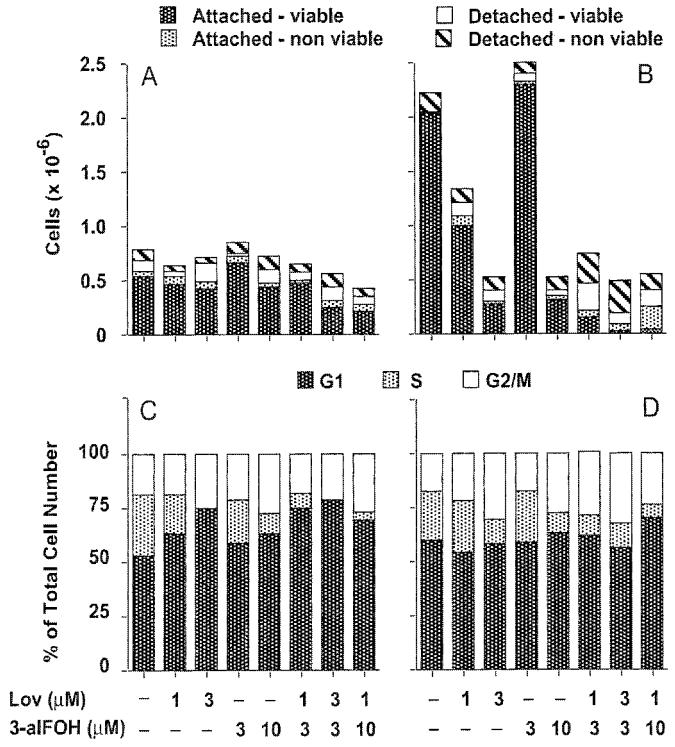

Fig. 5. Cytostatic and cytotoxic effects of lovastatin and 3-alFOH on A10 cultures. A10 cultures were treated with lovastatin, 3-alFOH, or combinations of the two drugs for 48 h (A and C) or 96 h (B and D). The floating/detached and attached cell populations were then harvested separately for analysis of cell numbers and trypan blue permeability (A and B) or DNA contents by flow cytometry (C and D). For the flow cytometry studies, analyses were performed on 10,000 acquired events. Additional independent studies yielded results similar to those presented in the figure.

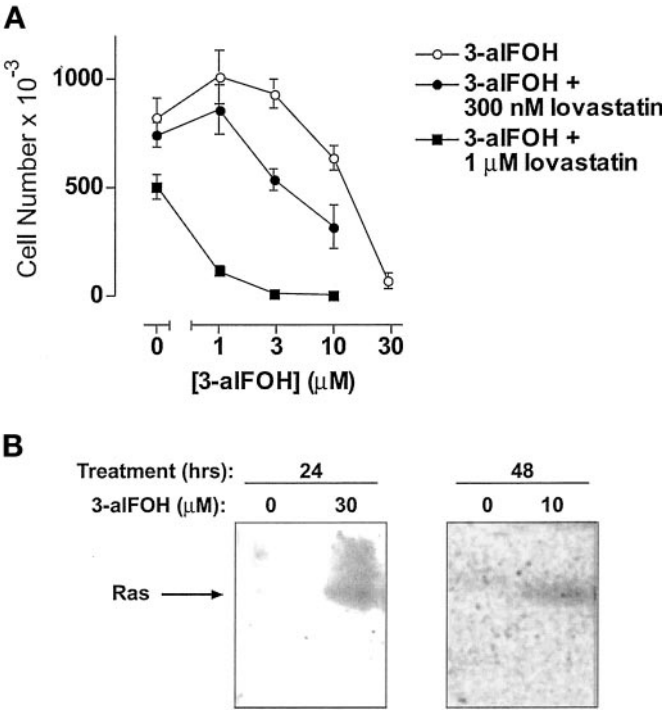

Fig. 6. Inhibition of the growth of A10 cells by 3-alFOH is potentiated by lovastatin. A, A10 cells (100,000 at start of experiment) were grown for 8 days in the presence of the indicated concentrations of 3-alFOH plus lovastatin as shown and then counted. Results are from at least three independent experiments. B, A10 cells were cultured for 24 h in the presence of 30 μM 3-alFOH (left) or for 48 h in the presence of 10 μM 3-alFOH (right), before the preparation of cytosolic extracts that were subjected to Western blotting for Ras content. Appearance of Ras in the soluble fraction of cells is presumed to reflect the inhibition of its prenylation (Gibbs et al., 1999).

detached populations from 1 μM lovastatin + 3 or 10 μM 3-alFOH treatment groups demonstrated a predominant population having subdiploid DNA content, suggestive of an apoptotic population (Fig. 4B). Cotreatment with the higher concentration of 3-alFOH (10 μM) resulted in a higher percentage of cells having subdiploid DNA contents.

The subdiploid DNA contents of apoptotic cells reflect the actions of an endonuclease that is activated by caspase-3. Detached cells generally constituted ~15% of the cellularity of nontreated and DMSO-treated A10 cultures. Of this population ~40% were trypan blue-permeable. In DMSO-treated cultures the caspase-3-specific activities of the detached cell population were ~3- to 4-fold higher than the activity measured in the adherent population (Fig. 7). Exposure to 3 or 10 μM 3-alFOH neither induced the detachment of A10 cells (Fig. 5) nor the activation caspase-3 (Fig. 7). In contrast, concentration-dependent increases in caspase-3 activities were seen in both attached and detached A10 cells after exposure to 1 and 3 μM lovastatin (Fig. 7). The effects of lovastatin were markedly potentiated by cotreatment with concentrations of 3-alFOH (3 or 10 μM) that had no detectable effect by themselves. Specifically, 3-alFOH synergized with lovastatin to increase caspase-3-specific activities in both adherent and detached A10 cells (Fig. 7)

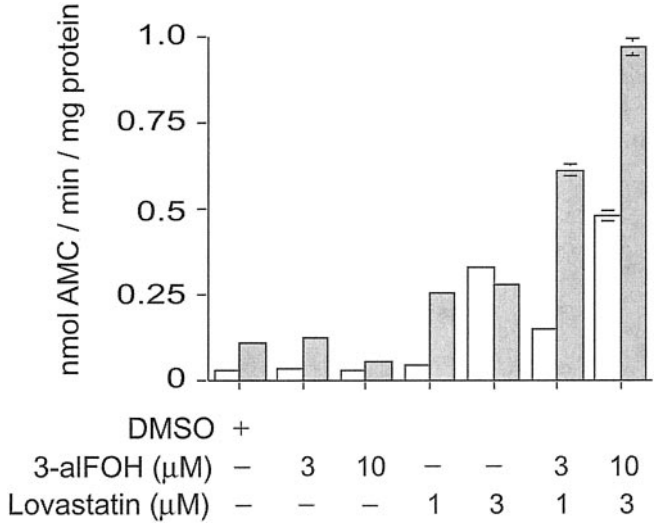

Fig. 7. Activation of procaspase-3 in A10 cultures after combination treatments with lovastatin and 3-alFOH. A10 cells were grown for 72 h in the indicated concentrations of lovastatin and 3-alFOH before the preparation of extracts for analyses of caspase-3 activity. Attached (open columns) and detached (filled columns) cells were independently harvested and assayed. Data represent mean ± S.D. of triplicate analyses. Note that combinations of lovastatin and 3-alFOH exhibit synergy in the activation of procaspase-3 in both the attached and detached cell populations. Similar results were obtained in a second experiment using 24- and 48-h harvest times.

Lovastatin and 3-alFOH Induce an Up-Regulation of RhoB into Cytosolic Compartment of A10 Vascular Smooth Muscle Cells. To investigate whether the effects of lovastatin and 3-alFOH on cell proliferation and apoptosis correlate with alterations in protein prenylation in A10 vascular smooth muscle cells, subcellular fractionation was performed, and the fractions were immunoblotted for RhoB and

Ras proteins (Fig. 8). Properly processed Ras and RhoB would be expected to be in membrane fractions, whereas unprenylated precursors would be soluble in the cytosol (Guijarro et al., 1998; Gibbs et al., 1999). The unprenylated precursors also typically migrate more slowly on SDS-PAGE gels (Gibbs et al., 1999). Lovastatin induced the relocalization of almost all the RhoB and a very small fraction of the Ras from the microsomal fraction to the soluble/cytosolic portion of A10 cells after an overnight treatment. Inhibition of farnesylation by 3-alFOH alone was without any apparent effect on either Ras or RhoB. This lack of effect may be a consequence of RhoB being alternatively geranylgeranylated after FTI treatment, and so it would remain attached to the membrane. The lack of effect on Ras is probably due to both alternative geranylgeranylation, and to the relative stability of the prenylated Ras protein pool that existed before drug addition. Note that this overnight incubation time with 10 μM 3-alFOH is much shorter than the 48-h treatment that did produce an effect on Ras (Fig. 6B). Combinations of lovastatin and 3-alFOH showed a greater ability to cause the relocalization of Ras than did lovastatin alone, although the effect was still less than 50%. However, combination treatment caused a marked increase in the amount of RhoB, with the increased protein being in the soluble fraction.

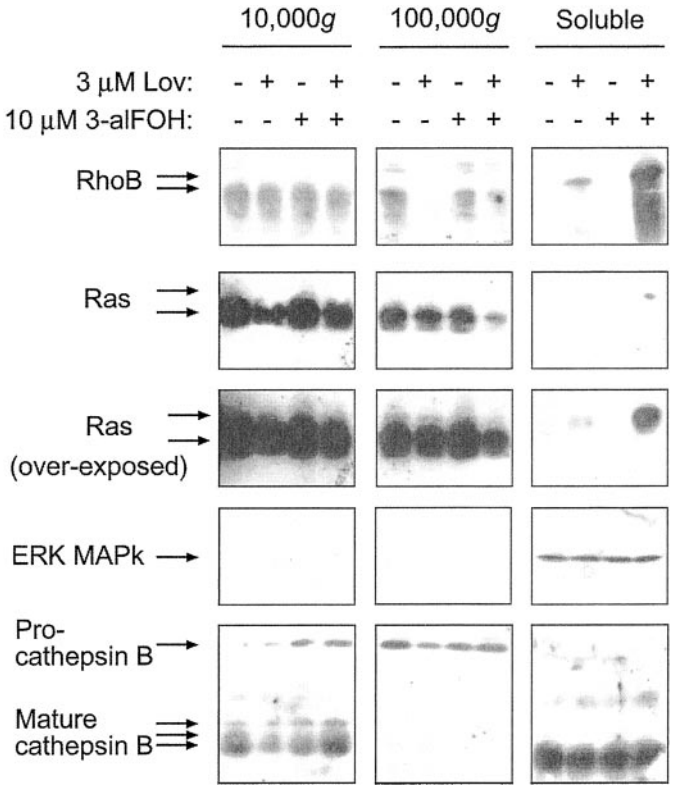

Fig. 8. Lovastatin and 3-alFOH induce relocalization of RhoB and Ras to the soluble compartment of A10 vascular smooth muscle cells. A10 cells were grown for 18 h in the presence of the indicated concentrations of lovastatin and 3-alFOH before preparation of subcellular fractions. Top, Western blot for RhoB. Membrane fractions contain prenylated RhoB (bottom arrow), whereas the unprenylated RhoB precursor (top arrow) is found in the soluble fraction after treatment with lovastatin or lovastatin plus 3-alFOH. Second from top panel, Western blot for Ras. Membrane fractions contain prenylated Ras (bottom arrow), whereas unprenylated Ras (top arrow) is found in the soluble fraction after combination treatment with lovastatin and 3-alFOH. Middle, a long exposure of the Ras Western blot to more clearly reveal the partial relocalization caused by treatment with lovastatin or lovastatin plus 3-alFOH. Next to bottom panel, Western blot for ERK mitogen-activated protein kinase, a cytosolic marker. Bottom, Western blot with an antibody that recognizes both the proform of cathepsin B (top arrow) that is a marker for the particulate/microsomal compartment, and multiple forms of mature cathepsin B (bottom three arrows) that are markers for the large organelle fraction. The origin of the low molecular weight band in the cytosolic fraction that is recognized by the anti-cathepsin B antibody is unclear, but it may represent some disruption of the lysosomes during the fractionation.

To confirm the integrity of the subcellular fractions after drug treatment, the blots were stripped and reprobed for markers characteristic of each fraction. The cytosolic marker ERK mitogen-activated protein kinase showed that there was minimal cytosolic contamination of the other fractions, and that the increases in cytosolic Ras and RhoB in treated cultures reflected selective effects. The microsomal marker procathepsin B showed that the cytosolic fraction had minimal microsomal contamination. Mature cathepsin B, which is expected to be in the lysosomes, consists of the 25- and 26-kDa double-chain form and 31-kDa single-chain form (Moin et al., 1992). Mature cathepsin B was found in the organelle fraction, as expected. However, cathepsin B cross-reacting material was also found in the soluble fraction and may represent lysosome breakage that occurred during the fractionation protocol.

Discussion

Inhibition of Farnesyl Transferase. Prenylation of a cysteine residue in a subset of mammalian proteins, including almost all members of the Ras superfamily of small GTPases, is now known to be an essential step in the functional maturation of these proteins (Khwaja et al., 2000). The discovery that farnesylation remains necessary for the growth-promotion functions of Ras proteins, even after their prevalent oncogenic activation in human cancer progression (Gibbs et al., 1994), has provided the stimulus for the development of selective inhibitors of farnesyl transferase, the enzyme responsible for this post-translational modification (Gibbs, 2000). As these agents have moved through preclinical and now clinical trials, significant questions have arisen as to whether Ras proteins are the most relevant target for the cytostatic action of FTIs. Prendergast and colleagues have argued that the lack of correlation between the cytostatic action of FTIs and their effects on the prenylation of Ras proteins suggests that another farnesylated protein in cells, in particular RhoB, may be the most relevant target for FTIs (Lebowitz et al., 1995). In support of this hypothesis are the facts that RhoB has a short half-life in cells (as opposed to the highly stable Ras proteins) and thus is more vulnerable to drugs that inhibit its synthesis (Lebowitz et al., 1995), and that fibroblasts from mice that have had their RhoB gene deleted are resistant to the effects of FTIs (Liu et al., 2001). A prenylation-independent form of RhoB can also confer resistance to FTI treatment (Lebowitz et al., 1997b). Transformation of RhoB-deficient fibroblasts with oncogenic H-Ras generates a model that is resistant to both FTIs and DNA-damaging agents (Liu et al., 2001). A connection between RhoB and apoptosis has also been made in other systems, including central nervous system neurons (Trapp et al., 2001), NIH-3T3 fibroblasts (Fritz and Kaina, 2000), and rat thoracic aorta smooth muscle cells (Guijarro et al., 1998).

Synergism between FTIs and Lovastatin. RhoB is distinct within Ras-like small GTPase superfamily in that it can

be found in two forms in cells, one modified with the farnesyl prenyl group, and one modified with geranylgeranyl (Adamson et al., 1992). The shift to predominantly the geranylgeranylated form that occurs in cells treated with FTIs (Lebowitz et al., 1997a) has been proposed to be a critical event as geranylgeranylated-RhoB may be proapoptotic (Du and Prendergast, 1999; but also see Chen et al., 2000). The failure of 3-alFOH, as a single agent, to cause relocalization of RhoB to the soluble fraction in the current study would be consistent with a shift of RhoB to the geranylgeranylated form under this treatment condition. Exposure to 3-alFOH did result in cytotoxicity and apoptosis at concentrations sufficient to inhibit farnesyl transferase. However, the apoptotic effects of 3-alFOH could be markedly potentiated by combined treatment with lovastatin. Note that 3-alFOH is unusual among FTIs, in that its active form is an FPP-competitive, rather than a peptide-competitive, inhibitor of protein farnesyltransferase (Gibbs, 2000). It would be expected that an FPP-competitive FTI such as 3-alFOH would exhibit a more synergistic effect with lovastatin, which blocks endogenous FPP synthesis, than other peptide-competitive FTIs (Yonemoto et al., 1998).

Subtoxic concentrations of 3-alFOH and lovastatin exhibited synergism to induce the apoptosis of A10 cells, and up-regulated the expression of unprenylated RhoB. This up-regulated expression of soluble RhoB preceded the development of apoptosis. Important issues are whether unprenylated RhoB is responsible for the apoptosis noted in our studies, and what the potential mechanistic link between RhoB and apoptosis may be. RhoB is an endosomal protein that may play a role in the endocytotic process (Mellor et al., 1998; Gampel et al., 1999), but any link between this function and cell death is unclear. RhoB is also known to be a product of an immediate-early gene that is induced by DNA damage (Fritz et al., 1995) and that may play a role in stress responses through modulation of signal transduction pathways (Fritz and Kaina, 2001). Inhibition of Ras prenylation may also be significant because Ras is known to play an important antiapoptotic role in vascular smooth muscle cells (Miyamoto and Fox, 2000).

Inhibition of Vascular Smooth Muscle Proliferation by Statins. The beneficial effect of statins on cardiovascular morbidity and mortality may reflect activities in addition to their reversal of hypercholesteremia via inhibition of hepatic HMG-CoA reductase (Kolovou, 2001). An additional mechanism of action might be inhibition of the proliferation of neointimal vascular smooth muscle cells either directly (Negre-Aminou et al., 1997) or indirectly via their ability to cause sensitization to proapoptotic signals (Knapp et al., 2000). Such effects of statins in smooth muscle systems may be associated with their ability to inhibit the prenylation of critical cellular proteins, such as RhoB (Guijarro et al., 1998) or RhoA (Laufs et al., 1999). Our investigation of the effects of lovastatin on the proliferation of the A10 neointimal-like cell line demonstrates that lovastatin can potently inhibit both the growth of these cells and the prenylation of RhoB, with pronounced effects occurring at 1 to 3 μM lovastatin, a concentration much closer to what can be achieved therapeutically (Duggan et al., 1989) than the 100 μM used previously (Guijarro et al., 1998). Exposure of A10 cells to 1 to 3 μM lovastatin resulted in the loss of S-phase cells, and an accumulation of G_1 and/or G_2/M phase cells. Such accumulations require the activation of both G_1 and G_2/M checkpoints. The cytostatic effect of simvastatin in human aortic smooth muscle cells has been ascribed to a blockade of ρ-dependent down-regulation of the cyclin-dependent kinase inhibitor p27kip1 (Laufs et al., 1999), a potent inducer of the G_1 checkpoint. Inhibition of the prenylation of CENP-E may be responsible for the accumulation of G_2/M cells observed in the current study. CENP-E is a kinetochore motor that is required for completion of mitosis. It is only functional when farnesylated (Ashar et al., 2000).

There is remarkable synergy between lovastatin and 3-alFOH to induce cytostatic and proapoptotic effects in A10 cells that seems to be associated with their effects on RhoB. RhoB may be a useful biomarker to test the molecular effects of such drug regimens in more physiological systems. This study also supports the consideration of combination statin and FTI drug treatments in cardiovascular disease models. Furthermore, the addition of statins to current and potential FTI trials for cancer and other proliferative disorders, such as neurofibromatosis, may afford greater efficacy at lower dose levels.

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