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Pharmacological Control of the Mevalonate Pathway: Effect on Arterial Smooth Muscle Cell Proliferation[1]

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ABSTRACT

The mevalonate (MVA) pathway is involved in cell proliferation. We investigated drugs acting at different enzymatic steps on rat aorta smooth muscle cell (SMC) proliferation. Competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (0.1–10 μM) dose-dependently decreased (up to 90%) SMC proliferation. This effect was prevented by 100 μM MVA, 10 μM all-*trans* farnesol (F-OH) and 5 μM all-*trans* geranylgeraniol (GG-OH), precursors of protein prenyl groups, but not by 2-*cis* GG-OH, precursor of dolichols, squalene and ubiquinone. The same inhibitory effect was obtained with 6-fluoromevalonate (1–50 μM), an inhibitor of MVA-pyrophosphate decarboxylase. Partial recovery of cell proliferation was possible by all-*trans* F-OH and all-*trans* GG-OH, but not MVA. Squalestatin 1 (1–25 μM), a potent squalene synthase inhibitor, blocked cholesterol synthesis and slightly inhibited (21% decrease) SMC proliferation only at the highest tested concentration. NB-598 (1–10 μM), a potent squalene epoxidase inhibitor, blocked cholesterol synthesis without affecting SMC proliferation. Finally, the benzodiazepine peptidomimetic BZA-5B (10–100 μM), a specific inhibitor of protein farnesyltransferase, time- and dose-dependently decreased SMC proliferation (up to 62%) after 9 days. This effect of BZA-5B was prevented by MVA and all-*trans* GG-OH, but not by all-*trans* F-OH. SMC proliferation was not affected by the closely related compound BZA-7B, which does not inhibit protein farnesyltransferase. Altogether, these findings focus the role of the MVA pathway in cell proliferation and call attention to the involvement of specific isoprenoid metabolites, probably through farnesylated and geranylgeranylated proteins, in the control of this cellular event.

Several studies suggest that the MVA pathway plays a role in cell growth (Goldstein and Brown, 1990; Habenicht *et al.*, 1980; Chen, 1984; Fairbanks *et al.*, 1984). MVA is intracellularly synthesized from HMG-CoA, and this process is catalyzed by HMG-CoA reductase, the rate-limiting enzyme in this pathway (Goldstein and Brown, 1990). MVA metabolism yields a series of isoprenoids that are vital for diverse cellular functions, ranging from cholesterol synthesis to growth control (Goldstein and Brown, 1990; Maltese, 1990; Grunler *et al.*, 1994; Glomset *et al.*, 1990). The exposure of cultured cells to competitive inhibitors of HMG-CoA reductase (statins), such as lovastatin, compactin or simvastatin not only blocks the biosynthesis of MVA but, in addition, pleiotropically inhibits DNA replication and cell cycle progression (Habenicht *et al.*, 1980; Fairbanks *et al.*, 1984; Doyle and Kandutsch, 1988; Sepp-Lorenzino *et al.*, 1991). These effects of statins are prevented by the addition of MVA to the culture medium, but not by the addition of cholesterol, the major end-product of the MVA pathway (Habenicht *et al.*, 1980; Fairbanks *et al.*, 1984; Doyle and Kandutsch, 1988; Corsini *et al.*, 1991). These observations have been interpreted to indicate the involvement of nonsterol derivative(s) of MVA for cellular proliferation to proceed. Potential MVA-derived products (Goldstein and Brown, 1990; Maltese, 1990; Grunler *et al.*, 1994; Quesney-Huneeus *et al.*, 1983) candidates for this role include: isopentenyl adenosine, present in some types of transfer RNAs; dolichol phosphates, required for glycoprotein synthesis; polyisoprenoid side chains of ubiquinone and heme-a, involved in electron transport. Studies with the BHK cell line suggest that isopentenyl adenosine is able to overcome the inhibitory effect of statins on DNA synthesis (Quesney-Huneeus *et al.*, 1980). Even though in other cell systems, the

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ABBREVIATIONS: MVA, mevalonate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; statin, HMG-CoA reductase inhibitor; PFTase, protein farnesyltransferase; PGGTase, protein geranylgeranyltransferase; F-OH, farnesol; GG-OH, geranylgeraniol; SMC, smooth muscle cell; MEM, Minimum Essential Medium; FCS, fetal calf serum; 6-Fmev, 6-fluoromevalonolactone; PP, pyrophosphate.

ability of isopentenyl adenosine to rescue proliferation has not been confirmed (Perkins *et al.*, 1982; Sinensky and Logel, 1985; Larsson and Zetteberg, 1986; Munro *et al.*, 1994), Faust and Dice (1991) found that the expression of protein i^6A26, which contains isopentenyl adenosine, decreases during lovastatin treatment and increases after subsequent MVA treatment and restoration of cell proliferation. Wejde *et al.* (1993) found that high concentrations of dolichol cause a slight restoration of DNA synthesis in lovastatin-treated cells; there is also evidence for a critical role of protein glycosylation in the control of cell proliferation (Doyle *et al.*, 1993; Carlberg *et al.*, 1994). None of these compounds, however, is consistently able to restore proliferation when endogenous MVA metabolism is suppressed. The potential importance of early intermediates in the MVA pathway, rather than more distal isoprenoid metabolites, has also been proposed as a critical regulator of cellular proliferation (Cuthbert and Lipsky, 1991).

Recently, several proteins post-translationally modified by the covalent attachment of MVA-derived isoprene groups (prenylation), farnesyl-PP or geranylgeranyl-PP, have been identified (Maltese, 1990; Glomset *et al.*, 1990; Sinensky and Lutz, 1992; Casey, 1992). These proteins must be prenylated as a prerequisite for membrane association, which, in turn, is required for their function (Glomset *et al.*, 1990; Glomset and Farnsworth, 1994; Jakobisiak *et al.*, 1991; Farnsworth *et al.*, 1990). Identification of some of them, nuclear lamin B (Farnsworth *et al.*, 1989), ras proto-oncogene (Casey *et al.*, 1989; Hancock *et al.*, 1989) and the γ subunit of heterotrimeric GTP-binding proteins (Glomset and Farnsworth, 1994; Yamane *et al.*, 1990, 1991; James *et al.*, 1996), raises the possibility that nonsterol products of the MVA pathway necessary for cellular proliferation may be one or more prenylated proteins. For this reason, the two classes of enzymes that catalyze the addition of a prenyl group to proteins, PFTase and PGGTase I and II (Casey and Seabra, 1996; Yokoyama and Gelb, 1993; Chen *et al.*, 1991; Zhang *et al.*, 1994), have gained attention as novel targets (Tamanoi, 1993; Gibbs *et al.*, 1994; Reiss *et al.*, 1990; Hancock, 1993) for the development of agents aimed at controlling abnormal cell growth, such as myocyte proliferation under atherogenic conditions or tumor development (Ross, 1993; Lowy and Willumsen, 1995). Among the prenylated proteins, much attention has been focused on p21 ras for its key role in the pathophysiology of cell proliferation (Lowy and Willumsen, 1993; Boguski and McCormick, 1993). Because farnesylation of p21 ras is required for its mitogenic activity, an intensive search for PFTase inhibitors is underway (Gibbs *et al.*, 1994; James *et al.*, 1993; Bishop *et al.*, 1995; Kohl *et al.*, 1995). The interference of lovastatin with cell cycle progression in carcinoma T24 cell line could be attributed to inhibition of farnesyl-PP biosynthesis and thereby isoprenylation of ras proteins (Jakobisiak *et al.*, 1991). In other cell systems, however, the inhibition of cell growth by lovastatin is independent of ras function (DeClue *et al.*, 1991).

In conclusion, the identity of the nonsterol derivative(s) of MVA required for cell growth remains to be elucidated, and the role of prenyl-modified protein(s) in DNA synthesis and cellular proliferation needs to be further explored.

Our observations that all-*trans* F-OH or all-*trans* GG-OH can prevent the statin-induced inhibition of SMC growth in the absence of other isoprenoids (Corsini *et al.*, 1993, 1995a), together with recent studies showing that both all-*trans* F-OH and all-*trans* GG-OH are readily incorporated into cellular proteins (Corsini et al., manuscript in preparation; Crick *et al.*, 1994; Danesi *et al.*, 1995), support the potential role of prenylated protein(s) in the control of cell proliferation.

In the present study, we have further addressed this matter by investigating the effect of drugs affecting diverse enzymatic steps of the MVA pathway on arterial SMC proliferation with the final goal of identifying potential MVA products involved in the control of cell proliferation.

Methods

Eagle's MEM, trypsin ethylenediaminetetraacetate, penicillin (10,000 U/ml), streptomycin (10 mg/ml), tricine buffer (1 M, pH 7.4) and nonessential amino acid solution (100×) were purchased from Gibco (Grand Island, NY), and FCS was from Mascia Brunelli (Milan, Italy). Disposable culture flasks and Petri dishes are from Corning Glassworks (Corning, NY), and filters are from Millipore (Bedford, MA). [2-^{14}C]Acetate, sodium salt (58.9 mCi/mmol) and [1α,2α(n)-^{3}H]cholesterol (47.7 Ci/mmol) are from Amersham (Amersham Place (Buckinghamshire), UK). Isoton II was purchased from Coulter Instruments (Milan, Italy). All-*trans* F-OH, ubiquinone and squalene are from Sigma (St. Louis, MO). All-*trans* GG-OH was kindly provided by Prodotti Roche (Milan, Italy). 2-*cis* GG-OH was prepared by oxidation of all-*trans* GG-OH with MnO_2 to yield a mixture of isomeric aldehydes which was purified by preparative thin-layer chromatography on silica gel plate F_{254} (Merck, Milan, Italy) with use of hexane/diethyl ether (7:3). The 2-*cis* aldehyde was converted to 2-*cis* GG-OH by reduction with sodium borohydride in ethanol, and the product was purified as for the aldehyde. Silica Gel G thin-layer chromatography products are from Merck (Milan, Italy). All reagents are analytical grade.

Drugs. Simvastatin in its lactone form, provided by Merck, Sharp & Dohme Research Laboratories (Woodbridge, NJ), was dissolved in 0.1 M NaOH to give the active form, and the pH was adjusted to 7.4 by adding 0.1 M HCl. Pravastatin, provided by Bristol Myers Squibb Pharmaceutical Research Institute (Princeton, NJ) and cerivastatin, provided by Ricerca Bayer Farmacologia (Milan, Italy), were dissolved in 0.15 M NaCl, and their solutions were sterilized by filtration. Racemic fluvastatin, provided by Sandoz Prodotti Farmaceutici (Milan, Italy), and L 645–164, provided by Merck, Sharp & Dohme Research Laboratories (Woodbridge, NJ), were dissolved in ethanol. 6-Fmev was prepared according to Quistad *et al.* (1981) and was dissolved in ethanol. Squalestatin 1, provided by Glaxo (Greenford, UK), was dissolved in water; NB-598, provided by Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan), was dissolved in dimethyl sulfoxide. BZA-5B and BZA-7B, provided by Genentech Inc. (South San Francisco, CA), were dissolved in dimethyl sulfoxide/dithiothreitol according to James *et al.* (1993).

Cell cultures. SMC were cultured, according to Ross (1971), from the intimal-medial layers of aorta of male Sprague-Dawley rats (200–250 g). Cells were grown in monolayers at 37°C in a humidified atmosphere of 5% CO_2 in MEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 mM tricine buffer and 1% (v/v) nonessential amino acid solution (Corsini *et al.*, 1991). The medium was changed every third day. Cells were used between the fourth and tenth passages. SMC were identified for growth behavior, morphology and use of monoclonal antibody specific for α-actin, the actin isoform typical of SMC (Skalli *et al.*, 1986). The cells grew out of explants after 12 to 16 days, piled up after confluence and contained numerous myofilaments and dense bodies, as observed by transmission electron microscopy (Ross, 1971).

Synthesis of total sterols. The synthesis of cholesterol was determined by measuring the incorporation of radioactive acetate into cellular sterols (Corsini *et al.*, 1987; Brown *et al.*, 1978). Cell

monolayers, after incubation with [2-^{14}C]acetate (1 μCi/ml; specific activity, 0.9 μCi/mol) for 72 h (see "Miscellaneous protocols"), were washed with phosphate-buffered saline and digested with 0.1 M NaOH. Aliquots were saponified at 60°C for 1 h in alcoholic NaOH after the addition of [1α,2α(n)-^{3}H]cholesterol as internal standard (0.04 μCi/sample). The nonsaponified material was extracted with low-boiling petroleum ether and counted for radioactivity. To evaluate the incorporation of labeled acetate into cellular sterols, these were separated from the nonsaponified fraction by thin-layer chromatography with use of petroleum ether (boiling point, 40–60°C)/diethyl ether/acetic acid (70:30:1). Radioactivity was measured with Insta-Fluor scintillator cocktail (Packard, Milan, Italy).

Miscellaneous protocols. Cells were seeded at a density of 2 $\times$ 10^5 SMC/Petri dish (35 mm), and incubated with MEM supplemented with 10% FCS. Twenty-four hours later, the medium was changed to one containing 0.4% FCS to stop cell growth, and the cultures were incubated for 72 h. At this time (time 0), the medium was replaced with one containing 10% FCS in the presence or absence of known concentrations of the tested compounds, and the incubation was continued for a further 72 h at 37°C. At time zero, just before the addition of the substances to be tested, three Petri dishes were used for cell counting. Cell proliferation was evaluated by cell count after trypsinization of the monolayers with use of a Coulter Counter model ZM (Corsini *et al.*, 1993). Cell viability was assessed by trypan blue exclusion and found to be higher than 95% with the concentration of drugs used. In a separate set of Petri dishes, cholesterol synthesis was estimated under the same experimental conditions by measuring the incorporation of [^{14}C]acetate into cellular sterols (Corsini *et al.*, 1987, 1993). Proteins were determined according to Lowry *et al.* (1951). In another set of experiments, when extended incubation (9 days) with drugs was required, cells were seeded at low density (1 $\times$ 10^5 SMC/35-mm Petri dish) so that linear growth rates could be maintained throughout the duration of the assay.

The amount of drugs required to inhibit 50% of cholesterol synthesis and of cell proliferation was calculated by linear regression analysis of the logarithm of the concentrations (μM) *versus* logit (Fisher and Yates, 1953).

Statistical data are expressed as mean $\pm$ S.D., except where otherwise specified. The effects of the tested compounds *versus* control on the different parameters were analyzed by two-tailed Student's *t* test for unpaired data and by Duncan's test.

Results

To define the role of nonsterol products of MVA in modulation of cell proliferation, a pharmacological approach was taken. The effect of drugs affecting diverse enzymatic steps of the MVA pathway on arterial SMC proliferation was investigated (fig. 1).

Effect of HMG-CoA reductase inhibitors. We previously demonstrated the ability of simvastatin and fluvastatin to dose-dependently decrease cholesterol synthesis in SMC and their proliferation (Corsini *et al.*, 1993, 1995a). To extend this preliminary observation, we examined the effects of other HMG-CoA reductase inhibitors on growth and on cholesterol synthesis of cultured SMC. The results show that all the tested statins, except pravastatin, inhibited arterial myocyte proliferation and cholesterol synthesis dose-dependently. Figure 2 depicts a typical dose-response curve obtained after exposure of cells to statins for 72 h. When statins were evaluated in human SMC, lower concentrations than those used in rat SMC were required to inhibit both processes (Corsini *et al.*, 1993), in agreement with the low HMG-CoA reductase activity detected in the former cell line (Corsini *et al.*, 1995b). However, the similar IC_{50} proliferation/cholesterol synthesis ratio for all the inhibitory statins (range, 11.7–16.6), regardless of their different potency, supports a causal relationship between the MVA synthetic pathway and cell proliferation (Corsini *et al.*, 1993, 1995a, 1996). Analysis of the data revealed that there was a significant direct correlation between the potency of statins in suppressing cholesterol synthesis and cell proliferation (fig. 3). Pravastatin at the highest nontoxic concentration inhibited cholesterol synthesis by <80% with virtually no detectable effect on myocyte proliferation (Corsini *et al.*, 1991, 1993, 1995a). Because pravastatin was unable to completely inhibit sterol

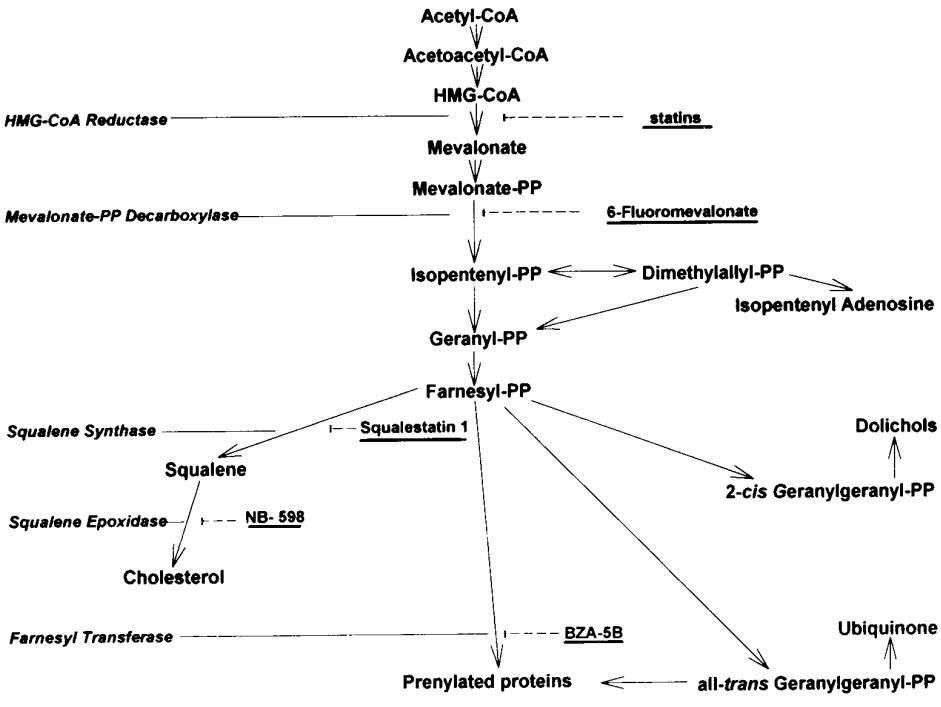

Fig. 1. The MVA pathway. The enzymatic steps inhibited by the various compounds used in this study are shown.

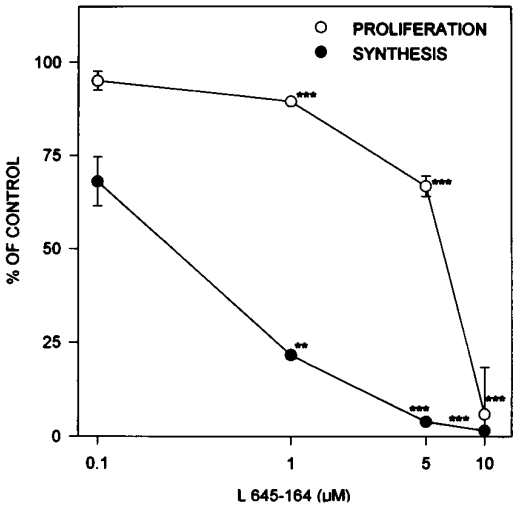

Fig. 2. Effect of L 645–164 on cholesterol synthesis in and proliferation of rat aorta SMC. Cells were seeded (200 × 10^3/dish) and incubated with MEM supplemented with 10% FCS; 24 h later, the medium was changed with one containing 0.4% FCS to stop cell growth, and the cultures were incubated for 72 h. At this time (time 0), the medium was replaced with one containing 10% FCS and the reported concentrations of L 645–164, and the incubation was continued for a further 72 h at 37°C. ^{14}C-Acetate incorporation was used to assay cholesterol synthesis, and the cell number provided an index of the effect on cell replication. Each point represents the mean ± S.D. of triplicate dishes. The mean value of the control experiment (without inhibitor) for cell number was 891 × 10^3 (±4 × 10^3) cells/plate and for cholesterol synthesis the mean value was 22.2 ± 4.5 pmol/mg cell protein per hour; these mean values are designed as 100%. Error bars not shown are within the symbol limits. Inhibitor *versus* control: ** $P < .01$; *** $P < .001$ (Student's *t* test).

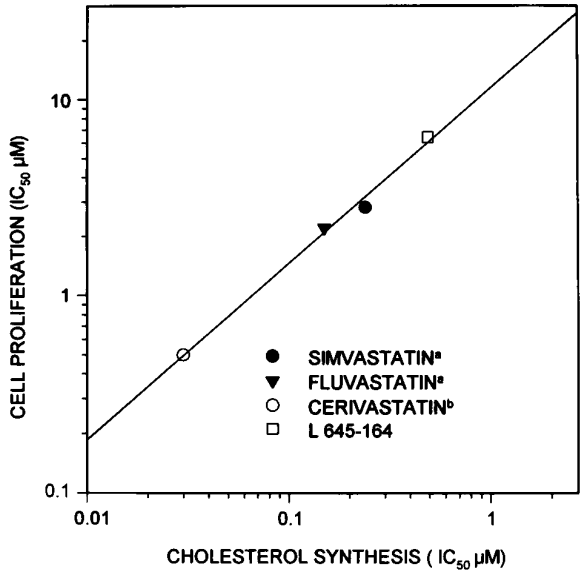

Fig. 3. Linear regression analysis of IC_{50} values for proliferation of and cholesterol synthesis in aorta SMC after incubation with statins (r^2 = .98; $P < .001$). IC_{50} = concentration of drug required to inhibit cholesterol synthesis and cell proliferation by 50%. Data from references Corsini *et al.* (1993)[a] and Corsini *et al.* (1996).[b]

synthesis, shunting MVA away from cholesterol production may have allowed sufficient endogenous MVA-derived products (dolichols, ubiquinone, isoprenoids) to be formed to support cell proliferation (Goldstein and Brown, 1990; Maltese, 1990; Glomset *et al.*, 1990).

We previously showed (Corsini *et al.*, 1993, 1995a) that the addition of MVA, all-*trans* F-OH or all-*trans* GG-OH, but not squalene, restored cell proliferation inhibited by simvastatin and fluvastatin, which suggested a specific role of isoprenoids in regulating cell proliferation. We made use of statin-treated cells as a target cell system to test a variety of known end-products of the MVA pathway for their ability to counteract the inhibitory effect of these drugs on cell proliferation. As shown in figure 4, inhibition of cell growth induced by simvastatin was completely prevented by the addition of MVA and partially prevented by the addition of all-*trans* F-OH and all-*trans* GG-OH. 2-*cis* GG-OH, a potential precursor of dolichols (Grunler *et al.*, 1994), did not prevent statin blockade of cell proliferation (fig. 4). These results suggest that dolichols are not involved in the regulation of cell growth. Squalene and ubiquinone also failed to restore SMC proliferation inhibited by statins. These results support the concept that SMC require specific isoprenoid metabolites derived from farnesyl-PP and geranylgeranyl-PP, along with an exogenous source of cholesterol (FCS), for proliferation. However, MVA, all-*trans* GG-OH, 2-*cis* GG-OH and all-*trans* F-OH did not induce cell proliferation in the absence of statins, which indicated that they were not acting as mitogens (fig. 5).

To investigate the biochemical specificity of statin-induced inhibition of cell growth and to determine the relative importance of the various branches of the MVA pathway in promoting cell growth, SMC proliferation and cholesterol synthesis were examined in the presence or absence of metabolic inhibitors.

Effect of a MVA-PP decarboxylase inhibitor. Because an early step in the synthesis of isoprenoids is catalyzed by MVA-PP decarboxylase (Goldstein and Brown, 1990) (fig. 1), the effect of 6-Fmev, a compound that blocks the conversion of MVA-PP to isopentenyl-PP (Cuthbert and Lipsky, 1990,

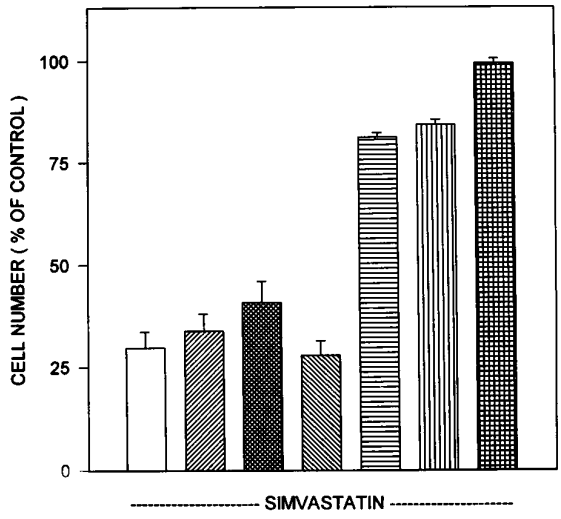

Fig. 4. Ability of MVA and its derivatives to prevent inhibition of rat aorta SMC proliferation by simvastatin. Experimental conditions are as in figure 2. Each bar represents the average of at least triplicate experiments, the results of which differed by <10%. The mean value of control experiments (1) (without inhibitor) was 1243 × 10^3 (±47 × 10^3) cells/plate and is designed as 100%. Duncan's test ($P < .05$): 3 *versus* 4, 5; ($P < .01$): 1 *versus* 2, 3, 4, 5, 6, 7; 2 *versus* 4, 6, 7, 8; 3 *versus* 6, 7, 8; 4 *versus* 5, 6, 7, 8; 5 *versus* 6, 7, 8; 6 *versus* 7; 7 *versus* 8. □ = simvastatin 3.5 µM (2); ▨ = ubiquinone 30 µM (3); ▩ = squalene 10 µM (4); ▧ = 2-*cis* GG-OH 5 µM (5); ▤ = all-*trans* GG-OH5 µM (6); ▥ = all-trans F-OH 10 µM (7); ▦ = MVA 100 µM (8).

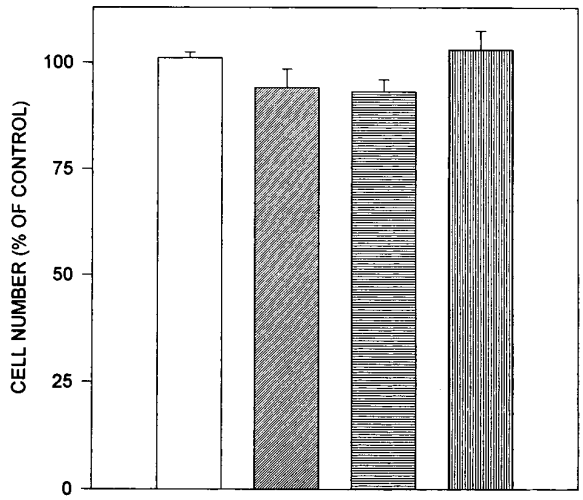

Fig. 5. Effect of MVA, all-*trans* F-OH, all-*trans* GG-OH and 2-*cis* GG-OH on proliferation of rat aorta SMC. Experimental conditions are as in figure 2. Each point represents the mean ± S.D. of triplicate dishes. The mean value of control experiment for cell number was 563×10^3 ($\pm 19 \times 10^3$) cells/plate and is designed as 100%. □ = MVA 100 μM; ▨ = all-*trans* F-OH 10 μM; ▤ = all-*trans* GG-OH 5 μM; = 2-cis GG-OH 5 μM.

1991; Dhe-Paganon *et al.*, 1994; Nave *et al.*, 1985) and thus acts distal to statins, was examined. 6-Fmev inhibited the proliferation of, and cholesterol synthesis in cultured myocytes in a dose-dependent manner (fig. 6). These results also indicate that the compound displayed similar potency in inhibiting both cellular processes with IC_{50} values of 6.7 and 10.8 μM for proliferation and cholesterol synthesis, respectively. The failure of MVA, but not of all-*trans* F-OH and all-*trans* GG-OH, to partially promote myocyte replication in the presence of 6-Fmol (fig. 7) indicates that its effect is related, at least in part, to the inhibition of isoprenoid biosynthesis. Although the mechanism by which 6-Fmol interferes with cell proliferation is rather complex (Cuthbert and Lipsky, 1991), the present results confirm the specific role of isoprenoids in regulating cellular growth.

Farnesyl-PP plays a central role in the MVA pathway because it is the last common substrate for the synthesis of all the end-products (Grunler *et al.*, 1994) (fig. 1). Therefore, the so-called branch-point enzymes that use farnesyl-PP represent potential pharmacological targets for further addressing the role of the MVA pathway in cell proliferation.

Effect of squalene synthase and epoxidase inhibitors. Selective inhibitors of squalene synthase, the first committed enzyme in the cholesterol biosynthetic pathway (Grunler *et al.*, 1994), and of squalene epoxidase were investigated. Treatment of cells with squalestatin 1, a specific competitive inhibitor of squalene synthase (Baxter *et al.*, 1992), blocked cholesterol synthesis and slightly inhibited (21% decrease) SMC proliferation only at the highest tested concentration (fig. 8). NB-598, a squalene epoxidase inhibitor (Horie *et al.*, 1990), caused complete inhibition of cholesterol synthesis without affecting the proliferation of SMC grown in the presence of exogenous cholesterol (fig. 9). Hence, involvement of the sterol branch of farnesyl-PP metabolism in the control of cell proliferation was obviated.

Effect of a PFTase inhibitor. To directly address the role of all-*trans* F-OH availability for cell proliferation, the effect of PFTase inhibitor on cell growth was examined. Cells were seeded at low density and allowed to grow for 9 days in the absence or presence of various concentrations of the specific inhibitor of PFTase BZA-5B (James *et al.*, 1993). In the absence of the inhibitor, arterial myocytes grew logarithmically (fig. 10). In the presence of BZA-5B, growth of myocytes was inhibited in a time- and dose-dependent manner, reaching ≈60% inhibition after 9 days in the presence of 50 μM

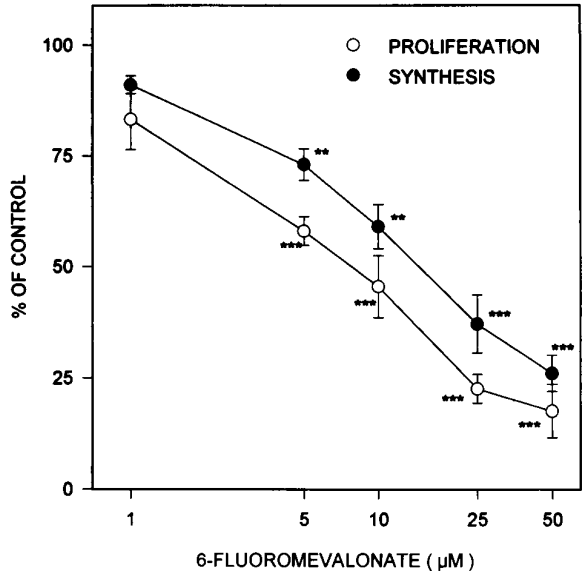

Fig. 6. Effect of 6-Fmev on cholesterol synthesis in and proliferation of rat aorta SMC. Experimental conditions are as in figure 2. Each point represents the mean ± S.D. of triplicate dishes. The mean value of the control experiment (without inhibitor) for cell number was 1399×10^3 ($\pm 104 \times 10^3$) cells/plate and for cholesterol synthesis the mean value was 92.5 ± 4.5 pmol/mg cell protein per h; these mean values are designed as 100%. Inhibitor *versus* control: ** $P < .01$, *** $P < .001$ (Student's *t* test).

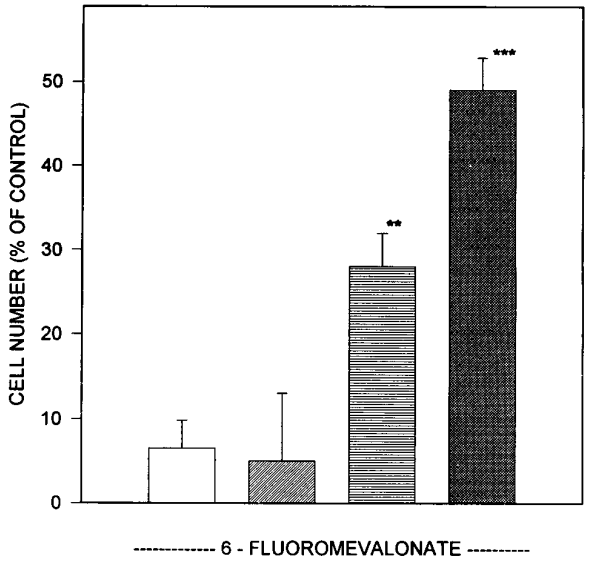

Fig. 7. Ability of MVA, all-*trans* F-OH and all-*trans* GG-OH to prevent inhibition of rat aorta SMC proliferation by 6-Fmev. Experimental conditions are as in figure 2. Each point represents the mean ± S.D. of triplicate dishes. The mean value of control experiment was 1013×10^3 ($\pm 85 \times 10^3$) cells/plate and is designed as 100%. Student's *t* test: ** $P < .01$ all-*trans* F-OH *versus* 6-Fmev; *** $P < .001$ all-*trans* GG-OH *versus* 6-Fmev. □ = 6-Fmev 10 μM; ▨ = 6-Fmev + MVA 100 μM; ▤ = 6-Fmev + all-*trans* F-OH 10 μM; ▩ = 6-Fmev + all-*trans* GG-OH 5 μM.

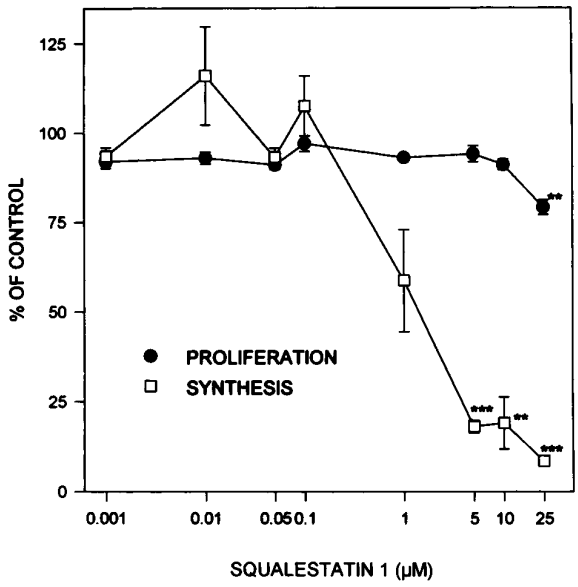

Fig. 8. Effect of squalestatin 1 on cholesterol synthesis in and proliferation of rat aorta SMC. Experimental conditions are as in figure 2; fresh solutions of squalestatin 1 are re-added to the culture medium every 24 h. Each point represents the mean ± S.E. of three experiments, each run in triplicate. The mean value of the control experiments (without inhibitor) for cell number was 1556 × 10^3 (±45 × 10^3) cells/plate and for cholesterol synthesis the mean value was 15.5 ± 1.1 pmol/mg cell protein per h; these mean values are designed as 100%. Error bars not shown are within the symbol limits. Inhibitor *versus* control: ** P < .01, *** P < .001 (Student's *t* test).

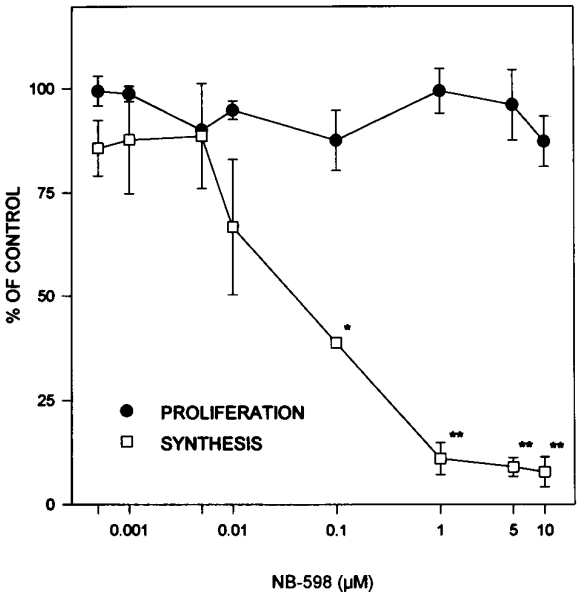

Fig. 9. Effect of NB-598 on cholesterol synthesis in and proliferation of rat aorta SMC. Experimental conditions are as in figure 2. Each point represents the mean ± S.E. of three experiments, each run in triplicate. The mean value of the control experiment (without inhibitor) for cell number was 1057 × 10^3 (±62 × 10^3) cells/plate and for cholesterol synthesis the mean value was 24.2 ± 2.2 pmol/mg cell protein per h; these mean values are designed as 100%. Inhibitor *versus* control: * P < .05, ** P < .01 (Student's *t* test).

BZA-5B (fig.10). Under parallel experimental conditions, this PFTase inhibitor had no effect on cholesterol synthesis (93% of the control value). In experiments of 3 days duration, BZA-5B (100 μM) inhibited the growth of SMC by 60% (fig.

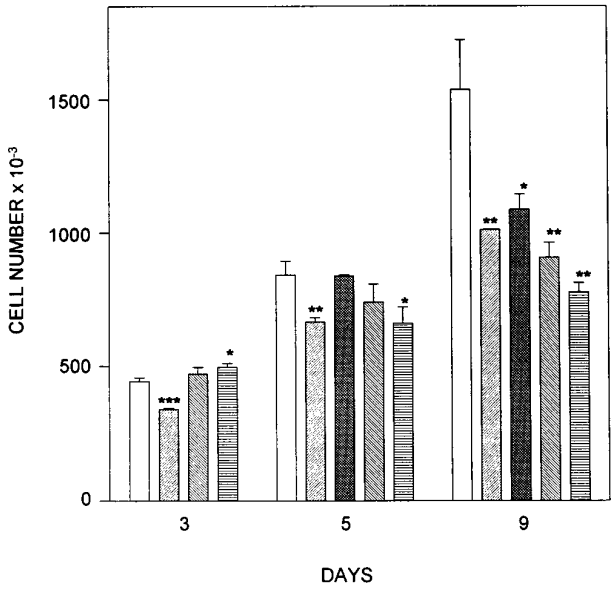

Fig. 10. Effect of BZA-5B on proliferation of rat aorta SMC. Cells were seeded (100 × 10^3) and incubated with Eagle's MEM supplemented with 10% FCS; 24 h later the medium was changed with one containing 0.4% FCS to stop cell growth and the cultures were incubated for 72 h. At this time (time 0, cell number = 322 × 10^3/dish), the medium was replaced with one containing 10% FCS and the reported concentrations of BZA-5B, and the incubation was continued for 3, 5 and 9 days, respectively. On days 3 and 5, cells were re-fed with the same medium. Each point represents the mean ± S.D. of triplicate dishes. Error bars not shown are within the symbol limits. Inhibitor *versus* control: * P < .05, ** P < .01, *** P < .001 (Student's *t* test). □ = control; ▨ = simvastatin 5 μM (3 days); ▨ = simvastatin 1 μM (5, 9 days); ▨ = BZA-5B 10 μM; ▧ = BZA-5B 25 μM; ▤ = BZA-5B 50 μM.

11). Similar concentrations of BZA-5B have been reported to inhibit the growth of H-ras-transformed cells (James *et al.*, 1993). The addition of all-*trans* GG-OH restored cell proliferation to control levels, whereas MVA, but not all-*trans* F-OH, partially prevented the inhibitory effect of BZA-5B (fig. 11). The proliferation was not affected when cells were grown with an inactive inhibitor analog, BZA-7B (fig. 11) (James *et al.*, 1994), which is identical to BZA-5B except for a serine in place of a cysteine. Taken together, these results implicate the involvement of prenylated protein(s) in regulating cell proliferation.

Discussion

A major focus of the current studies is the identification of a nonsterol MVA derivative(s) whose presence in cells correlates with cell proliferation. Four experiments substantiate the relationship between all-*trans* F-OH, all-*trans* GG-OH (probably through one or more prenylated proteins) and rates of cell growth.

First, MVA, all-*trans* F-OH and all-*trans* GG-OH are all able to prevent the inhibition of SMC proliferation induced by statins (Corsini *et al.*, 1993). The observation that the antiproliferative effect of statins occurs when cholesterol synthesis is suppressed by more than 80%, supports a causal relationship between the MVA pathway and cell proliferation (Corsini *et al.*, 1993, 1995b; Parker *et al.*, 1990). These findings suggest that strong inhibition of MVA production elicited by statins might impede sufficient formation of endogenously derived products (fig. 1) to support cell proliferation.

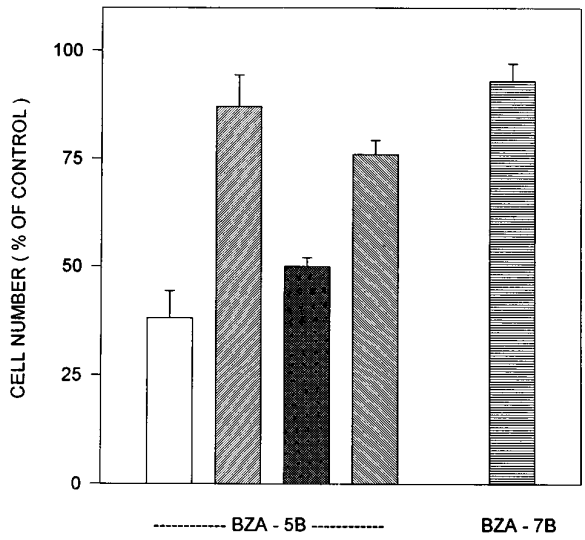

Fig. 11. Effect of BZA-5B, BZA-7B, MVA and its derivatives on proliferation of rat aorta SMC. Experimental conditions are as in figure 10. Cells were incubated with the tested compounds for 72 h. Each point represents the mean ± S.D. of triplicate dishes. The mean value of the control experiment (without inhibitor) for cell number was 416×10^3 ($\pm 20 \times 10^3$) cells/plate and is designed as 100%. Duncan's test: $P < .05$, MVA *versus* control; $P < .01$, BZA-5B *versus* control, MVA, all-*trans* GG-OH; $P < .01$, all-*trans* F-OH *versus* control, MVA, all-*trans* GG-OH. □ = BZA-5B 100 μM; ▨ = BZA-5B + all-*trans* GG-OH 2.5 μM; ▩ = BZA-5B + all-*trans* F-OH 10 μM; ▧ = BZA-5B + MVA 100 μM; ▤ = BZA-7B 100 μM.

One critical end-product of MVA is cholesterol that is required for cell membrane formation in proliferating cells (Goldstein and Brown, 1990; Chen, 1984; Grunler *et al.*, 1994). It is unlikely, however, that inhibition of cholesterol synthesis explains the action of statins on SMC proliferation. In fact, cells are stimulated to grow by exposure to a medium containing 10% FCS, which provides an exogenous source of cholesterol, and under such conditions, statins still inhibit cell proliferation. Recently, several proteins that are involved in growth factor signal transduction have been shown to be lipid-modified by the covalent attachment of farnesyl-PP and geranylgeranyl-PP groups, which are derived from MVA (Goldstein and Brown, 1990; Fairbanks *et al.*, 1984; Farnsworth *et al.*, 1990; Casey *et al.*, 1989). Statins inhibit the biosynthesis of these two isoprenoids, and one possible mechanism by which they affect cell growth may be the interference with signaling pathways that require prenylated proteins. The fact that all-*trans* GG-OH and all-*trans* F-OH can, under these experimental conditions, partially prevent statin-induced inhibition of cell growth in the absence of other prenyl intermediates, suggests that proteins modified by these isoprenoids are necessary for cell proliferation. In addition, it was shown that both labeled all-*trans* F-OH and labeled all-*trans* GG-OH are readily incorporated into cellular proteins and other metabolites made from prenyl-PP, which thus supports our experimental approach (A. Corsini, C. C. Farnsworth, P. McGeady, M. H. Gelb and J. A. Glomset, manuscript in preparation; Crick *et al.*, 1994, 1995; Danesi *et al.*, 1995).

Ubiquinone, 2-*cis* GG-OH (a potential precursor of dolichols) and squalene failed to overcome the inhibitory effect of statins; this rules out the possibility that the putative regulator(s) of cell proliferation is one of these products of the MVA pathway. Another MVA-derived isoprenoid, isopentenyl adenosine, was also investigated. Unfortunately, this product, like many adenosine derivatives, is often toxic for mammalian cells (Mittelman *et al.*, 1975) and causes cell death. However, when assayed in several cultured cells, isopentenyl adenosine was unable to prevent the antiproliferative effect of statins (Perkins *et al.*, 1982; Sinensky and Logel, 1985; Larsson and Zetteberg, 1986; Munro *et al.*, 1994).

The failure of ubiquinone, dolichols, isopentenyl adenosine and squalene to prevent the inhibitory effect of statins on cell proliferation has been reported (Faust and Dice, 1991; Wejde *et al.*, 1993; Corsini *et al.*, 1993; O'Donnell *et al.*, 1993). The present data do not exclude the possibility that other isoprenoid products may also be involved in cell proliferation. Wejde *et al.* (1993) found that an unidentified MVA-derived lipid component, which coeluted with dolichol, completely restored DNA synthesis in lovastatin-treated cells.

Because several different products of the MVA pathway are essential elements in several cellular processes (Goldstein and Brown, 1990; Grunler *et al.*, 1994), it seems likely that different isoprenoid requirements may be revealed under different experimental conditions. For example, Doyle *et al.* (1993) have shown that cells arrested in growth by MVA depletion behave differently from cells synchronized by serum depletion. Under their experimental conditions, dolichol phosphates and its glycosylated derivatives were the MVA-derived compounds limiting cellular growth. Further understanding of the roles of different isoprenoids in cell proliferation requires a deeper knowledge of the cellular processes regulating the levels of isoprenoids and their functional diversity.

The second approach for understanding the relationship between isoprenoid metabolism and cell proliferation makes use of 6-Fmev. This compound (Nave *et al.*, 1985; Reardon and Abeles, 1987) is a useful pharmacological tool for examining the role played in cell proliferation by MVA derivatives just downstream of MVA-PP decarboxylase. When the decarboxylation of MVA-PP to isopentenyl-PP was blocked by 6-Fmev, both cholesterol synthesis and SMC growth were inhibited in a dose-dependent manner. Unlike statins, this drug inhibits cholesterol synthesis and cell growth with similar potency, which implies different antiproliferative mechanisms. It has been proposed recently that 6-Fmev inhibits the proliferation of cells by two distinct but related mechanisms (Cuthbert and Lipsky, 1990, 1991; Sawamura *et al.*, 1993). By inhibition of isoprenoid synthesis, 6-Fmev suppresses SMC proliferation, and this effect was partially prevented by providing an exogenous source of all-*trans* F-OH and all-*trans* GG-OH. The other mode of action appears to result from the accumulation of an inhibitory group of metabolites derived from MVA or one of its phosphates. Our results, together with these previous observations (Cuthbert and Lipsky, 1990, 1991; Sawamura *et al.*, 1993), indicate that MVA is a critical determinant of cellular proliferation because it is a source of both positive and negative regulatory influences.

In a third approach, the contribution of *de novo* sterol synthesis in the control of cell proliferation was studied with squalene synthase and squalene epoxidase inhibitors. These enzymes catalyze steps committed to the sterol synthetic pathway (Goldstein and Brown, 1990; Grunler *et al.*, 1994) and thus represent ideal sites for selectively inhibiting sterol

formation, hence addressing the involvement of the sterol branch in cell growth. The administration of squalestatin 1 (Baxter *et al.*, 1992) dose-dependently inhibited cholesterol synthesis and slightly inhibited (25 μM) SMC growth. It is noteworthy to mention that squalestatin inhibits ras PFTase, although with much less potency (Dufresne *et al.*, 1993; Gibbs *et al.*, 1993), and this could explain its effect on SMC growth. Unlike statins, concentrations of squalestatin 1 that completely blocked cholesterol synthesis (5–10 μM) did not affect cell proliferation. These findings are in agreement with a previous study by Bradfute *et al.* (1992), which showed a normal rate of growth of a squalene synthase-deficient mutant CHO cell line in a medium supplemented with serum, and with the inability of squalene to prevent the inhibitory effect of statins on cell proliferation (Wejde *et al.*, 1993; Corsini *et al.*, 1993, 1995a; Keyomarsi *et al.*, 1991). NB-598 (Horie *et al.*, 1990) specifically inhibited cholesterol synthesis without affecting SMC growth, thus ruling out a role of squalene or any distal intermediate of the cholesterol synthetic pathway in cell proliferation.

Finally, a direct link between the inhibition of cell proliferation and protein prenylation is apparent. The involvement of prenylated proteins in cell growth (Maltese, 1990; Glomset *et al.*, 1990; Jakobisiak *et al.*, 1991; Casey *et al.*, 1994) is given further credence by results obtained with BZA-5B. This drug is a potent and specific inhibitor of PFTase (James *et al.*, 1993) and a selective antagonist of H-ras transformed cells (James *et al.*, 1994, 1995), probably through inhibition of ras-dependent cell growth (James *et al.*, 1993, 1994, 1995). The requirement for prolonged incubation of cultured SMC with BZA-5B to induce an antiproliferative effect is consistent with the specificity of the compound for ras (James *et al.*, 1994, 1995), a protein with a very long half-life (20–56 h) (Ulsh and Shih, 1984). Probably because of inefficient cell penetration (Gibbs *et al.*, 1994), high concentrations (10–100 μM) of BZA-5B are required to detect any significant inhibitory effect on cell growth (James *et al.*, 1993). Two sets of control experiments address the issue of the specificity of the effect of BZA-5B in SMC cells. First, the effect of the PFTase inhibitor on SMC proliferation was not observed by the structurally related compound BZA-7B, which does not inhibit PFTase (James *et al.*, 1994). Second, MVA and all-*trans* GG-OH, but not all-*trans* F-OH, partially prevent the inhibitory effect of BZA-5B on cell proliferation. This result may be caused by the fact that PGGTase I is able to geranylgeranylate but not farnesylate proteins that normally serve as farnesyl acceptors (Armstrong *et al.*, 1995; Yokoyama *et al.*, 1995). It is tempting to speculate that PGGTase I can geranylgeranylate mitogenic proteins that are normally modified by the all-*trans* F-OH moiety, such as K-ras, in a reaction that is relatively resistant to inhibition by BZA-5B (Armstrong *et al.*, 1995). Previous studies have shown that geranylgeranylated ras proteins support transformation of animal cells (Cox *et al.*, 1992) and growth of yeast cells (Trueblood *et al.*, 1993). Testing of this hypothesis will require antibodies that recognize K-ras at the low concentrations that are present in normal animal cells; these antibodies are not yet available. The possibility also exists that geranylgeranylated proteins of the rho-family (Vincent *et al.*, 1992; Olson *et al.*, 1995; Vojtek and Cooper, 1995) and rap (Quarck *et al.*, 1994) may compensate for ras in growth factor signal transduction pathway and in the control of cell proliferation. One surprising result is the ability of BZA-5B to inhibit cell growth of untransformed SMC. It has been reported that normal fibroblasts continue to grow in the presence of the PFTase inhibitor at concentrations that markedly slow the growth of H-ras-transformed cells (Lowy and Willumsen, 1995; James *et al.*, 1993). Several aspects of ras function may contribute to this resistance of untransformed cells to the effect of PFTase inhibitors (Lowy and Willumsen, 1995; Marshall, 1995). It seems likely that untransformed fibroblasts may produce forms of ras (K-ras or N-ras) whose farnesylation is relatively resistant to BZA-5B, or that are geranylgeranylated (James *et al.*, 1994, 1996; Mumby *et al.*, 1990). The possibility also exists that fibroblasts can respond to mitogenic stimuli through pathways that bypass ras. This hypothesis is consistent with recent evidence showing that growth factors can activate transcription factors *via* a ras-independent mechanism (Sepp-Lorenzino *et al.*, 1995; Heim, 1996; Pumiglia *et al.*, 1995; Inglese *et al.*, 1995). On the other hand, the observation of an induction of H-ras protooncogene expression in proliferating rat SMC (Sadhu and Ramos, 1993), together with the recent demonstration that the local delivery of transdominant negative H-ras mutants inhibits proliferation after balloon injury of rat carotid artery (Indolfi *et al.*, 1995), indicates a role of H-ras as a key transducer of mitogenic signals in vascular myocytes. The abundance of available data demonstrates that mitogenic signaling in SMC is complex and an overlapping of signaling elements can be induced by hormones and growth factors through G protein-coupled serpentine receptors as well as receptor tyrosine kinases (Ludwig and Rapp, 1995).

The ability of BZA-5B to inhibit SMC proliferation highlights the potential usefulness of compounds that block the action of key components of the mitogenic signaling cascade for therapy of vascular proliferative disorders, such as atherosclerosis and restenosis after angioplasty (Corsini *et al.*, 1995a; Ross, 1993). PGGTase I specific inhibitors (Lerner *et al.*, 1995) have not yet been investigated, and the role of geranylgeranylated proteins in controlling cell proliferation still remains to be directly addressed.

In conclusion, the results presented in this paper demonstrate the importance of the MVA pathway in cell proliferation. These studies reveal the involvement of the specific isoprenoid metabolites farnesyl-PP and geranylgeranyl-PP, which are necessary for the prenylation of one or more proteins that are involved in regulation of cell proliferation.

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