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 famesol (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

ABBREVIATIONS: MVA, mevalonate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; statin, HMG-CoA reductase inhibitor; PFTase, protein farnesyltransferase; PGGTase, protein geranylgeranyltransferase; F-OH, famesol; GG-OH, geranylgeraniol; SMC, smooth muscle cell; MEM, Minimum Essential Medium; FCS, fetal calf serum; 6-Fmevalonolactone; 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 iA26, 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 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, PPTase and PGGTase I and II (Casey and Seabara, 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 PPTase 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 and 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), suggest that 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). [2-14C]Acetate, sodium salt (58.9 mCi/mmol) and [1a,2a(n)-3H]cholesterol (47.7 Ci/mmol) are from Amersham (Amer- sham Place (Buckinghamshire), UK). Isotin 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 MnO₂ to yield a mixture of isomeric aldehydes which was purified by preparative thin-layer chromatography on silica gel plate F₂₅₄ (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 (Sham Place (Buckinghamshire), UK). Isotin II was purchased from Coulter Instruments (Milan, Italy). All-trans F-OH was prepared according to Quidstad et al. (1981) and was dissolved in ethanol. Squalene, provided by Glaxo (Greenford, UK), was dissolved in water; NB-598, provided by Banyu Pharmaceutical Co., Ltd. (Teukuba, 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% CO₂ 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 confluen- ce 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 [2-14C]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(3)-3H]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 × 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 [14C]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 × 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 ± 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 IC50 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...
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, 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).
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

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**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 × 10³ (± 19 × 10³) 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.

**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 control experiment (without inhibitor) for cell number was 1399 (± 36) cells/plate and is 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 (± 104) 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.
Under parallel experimental conditions, this PPTase 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. 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.
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. Glomsset, 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 se rum 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 SMC 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 sterol 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 mitogen 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 farnesyllation 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 serpine 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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