Regulation of Proliferation and Ras Localization in Transformed Cells by Products of Mevalonate Metabolism

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ABSTRACT

Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, and 6-fluoromevalonate (Fmev), an inhibitor of diphosphomevalonate decarboxylase, blocked the synthesis of downstream mevalonate products, including prenyl-derived lipids, and prevented membrane localization of Ras in the myeloid cell line U-937. In contrast to lovastatin, which induced cytosolic localization of Ras in U-937 cells, Fmev failed to increase cytosolic Ras and also completely prevented the proliferation of U-937 cells. Growth of U-937 cells was restored by the addition of lovastatin to Fmev-blocked cells. These results implied that a product of mevalonate metabolism proximal to isopentenyl diphosphate was responsible for the suppression of proliferation. To delineate the action of this endogenous inhibitor of cell proliferation and determine the relationship between its impact on Ras localization and cell proliferation, the effect of Fmev on a variety of leukemia- and lymphoma-derived cells was examined. Whereas Fmev blocked the growth of these cell lines, there were more than 50-fold differences in the concentrations required to inhibit the growth of individual cell lines by 90%. Regardless of its effect on cell proliferation, the biochemical effect of Fmev was similar. Thus, Fmev uniformly prevented the conversion of radiolabeled mevalonate to isopentenyl diphosphate and other downstream products, including synthesis of sterol and nonsterol lipids and prenylation of proteins. A correlation was noted between higher intrinsic rates of mevalonate synthesis by a cell and susceptibility to inhibition by Fmev. Thus, sensitivity of a cell line to inhibition by Fmev was associated with markedly increased rates of HMG CoA reductase activity that were further increased by incubation with Fmev. Whereas Fmev depleted cellular levels of the prenylated protein Ras in the sensitive cell line U-937, there was no depletion of cellular Ras levels in the resistant cell line EL-4, but rather, there was a shift of Ras from membrane to cytosol, as expected for inhibition of prenylation. These results suggest that leukemic cells with increased HMG CoA reductase activity produce increased levels of an endogenous mevalonate-derived inhibitor that leads to Ras depletion and suppression of cell growth. As a result, inhibition of the growth of these transformed cells might be specifically accomplished by Fmev.

INTRODUCTION

Ras, a Mr. 21,000 guanine nucleotide-binding protein, is activated by signal transduction pathways involved in growth and differentiation (1). Mutations that constitutively activate Ras are found in ~20% of all human tumors (1, 2) and are particularly common in pancreatic (3) and colon (4) cancer. Cellular transformation by mutationally activated Ras requires plasma membrane association of the oncoprotein (5–7). This observation has been exploited in the development of potential cancer chemotherapeutic agents that block membrane association of Ras (8–11). Initially, newly synthesized Ras is localized in the cytosol (12–14). A series of posttranslational modifications of the COOH terminus results in plasma membrane targeting (12–14). These include the addition of a 15-carbon farnesyl lipid to the cysteine at amino acid position 186, the removal of the three COOH-terminal amino acids at positions 187–189, and carboxymethylation of the new COOH-terminal cysteine. In addition, either palmitylation of other cysteine residues in the COOH terminus (H-Ras, N-Ras, and K-RasA) or a polybasic domain (K-RasB) is important in enhancing membrane association (7). These processes occur stepwise, and the first step, that of farnesylation of the full-length polypeptide, is thereby essential for plasma membrane localization (12–14). Thus, compounds and mutations that block the process of farnesylation interfere with the transformation and proliferation that are dependent upon mutationally activated Ras.

Lovastatin, a specific inhibitor of HMGCoA reductase, blocks farnesylation of Ras (12, 13) because HMG CoA reductase catalyzes the synthesis of mevalonate, the precursor of the farnesyl lipid moiety (Ref. 15; see Fig. 7 for biochemical pathways). Unfarnesylated Ras remains in the cytosol of lovastatin-blocked cells. In addition, inhibitors of the enzyme farnesyltransferase prevent posttranslational modification and membrane association of Ras (8, 9). Farnesyltransferase inhibitors prevent cell growth that is dependent on mutationally activated Ras (8–11). We have demonstrated that Fmev, a fluorinated mevalonate analogue that blocks the conversion of mevalonate diphosphate to isopentenyl diphosphate (16, 17), also inhibits farnesylation of Ras (18, 19). Like lovastatin, Fmev prevents membrane localization of Ras (19). Unlike lovastatin, however, Fmev does not induce the accumulation of Ras in the cytosol (19). Moreover, Fmev also induces the activity of an endogenous inhibitor of cellular proliferation (19, 20). This inhibitor is a product of mevalonate proximal to isopentenyl diphosphate (19, 20). The current studies were undertaken to examine the potential dysregulation of this inhibitor of cellular growth in various transformed cell lines and determine the relationship of its impact on Ras localization and cellular growth.

MATERIALS AND METHODS

Cell Culture and Measurement of DNA, Sterol, and Mevalonate Synthesis. The following leukemia- and lymphoma-derived cell lines were obtained from the American Type Culture Collection: the B-cell line Daudi (Burkitt lymphoma ATCC CCL 213); the lymphoma cell line EL-4 (ATCC TIB 39); the acute monocytic leukemia cell line THP-1 (ATCC TIB 202); the acute promyelocytic leukemia line HL-60 (ATCC CCL 240); and the histiocytic lymphoma-derived myeloid cell line, U-937 (ATCC CRL 1593). The normal human B-lymphoblastoid cell line, MarB, that had been generated by transformation of peripheral blood mononuclear cells with Epstein-Barr virus using standard techniques (21) was obtained from Dr. S. Zwillitch (University of Texas Southwestern Medical School). For some experiments, the Epstein-Barr virus-transformed human B-lymphoblastoid cell line 526.11 (22) was used. The Jurkat T-cell line was obtained from Dr. L. Davis (University of Texas Southwestern Medical School). All cells were maintained in RPMI-1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) iron-supplemented BCS (HyClone Laboratories, Inc., Logan, UT). For some experiments, cells were adapted to medium supplemented with 10% (v/v) LPP, prepared as detailed (18). Fmev was synthesized by CPM Laboratory (Carrollton, TX) using the procedure of Quistadt et al. (23). Lovastatin (Merck, 5-Fluoromethyl-3-hydroxy-3-methylglutaryl coenzyme A reductase; BCS, bovine calf serum; LPP, lipoprotein-poor plasma.

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3 The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; Fmev, 6-fluoromevalonate; BCS, bovine calf serum; LPP, lipoprotein-poor plasma.
For measurements of DNA synthesis, cells were cultured (±5 × 10⁶ cells per well) in triplicate in microtiter plates, with additions as indicated in the individual experiments; in preliminary experiments, this concentration of cells allowed maximal proliferation during the ensuing 1–4-day incubation. DNA synthesis was measured by the incorporation of [³H]thymidine as described (24). For measurements of sterol synthesis, 1 × 10⁶ cells/ml were cultured in 17- × 100-mm polypropylene tubes, with additions as indicated in the individual experiments. Incorporation of [¹⁴C]acetate into digitonin-precipitable sterols was measured in triplicate incubations as detailed previously (24).

For measurements of mevalonate synthesis (HMG CoA reductase activity), cells cultured in T25 tissue culture flasks in medium supplemented with 10% (v/v) BCS were harvested, washed by centrifugation to remove serum proteins, and resuspended; and the cell pellet was frozen at —90°C until the activity was assayed. HMG CoA reductase activity was measured by a modification of the procedures of Brown et al. (25) and Panini et al. (26) as described (19). Briefly, cell pellets were resuspended in activation buffer containing 200 mM KCl, 50 mM potassium phosphate (pH 7.4), 5 mM DTT, 5 mM EGTA, 5 mM imidazole, 100 μM leupeptin, and 0.25% (v/v) Tergitol nonionic detergent (Union Carbide Chemicals, Danbury, CT), sonicated, and incubated for 30 min at 37°C. After 1 min of centrifugation at 16,000 × g, aliquots of the supernatant were harvested for measurements of HMG CoA reductase activity (50 μl) and protein. To each reaction tube, an equal volume of 2× mix [200 mM potassium phosphate (pH 7.4), 12.5 mM DTT, and 5 mM EGTA], 175 μg of NADPH, and 3.75 nmol of [¹⁴C]HMG CoA (Dupont NEN Research Products, Boston, MA) was added. Following incubation in a shaking water bath for 1 h at 37°C, the reaction was stopped with 5 N HCl, followed by incubation for 30 min at 37°C after addition of 2 μmol of [³H]mevalonolactone (Dupont) as an internal recovery standard. After a 20-min centrifugation at 16,000 × g, 50 μl of supernatant were spotted onto plastic-backed silica gel G plates and chromatographed using a 1:1 mixture of benzene:acetone as the solvent system. Chromatograms were sprayed with EN³HANCE (Dupont), the chromatograms were subjected to fluorography. The fluorographically identified spots were scraped and counted by liquid scintillation spectroscopy.

Detection of Ras by Immunoblotting. Cells were incubated for varying lengths of time in complete medium with or without Fmev and lovastatin, as indicated in the individual experiments. After harvesting, the number of cells recovered was quantitated. Soluble (cytosolic) and particulate (membrane) fractions were separated by 100,000 × g centrifugation after homogenization in hypotonic buffer and removal of nuclei by low-speed centrifugation (28). Proteins in the soluble fractions were concentrated by centrifugation using Microcon 10 microconcentrators (Amicon, Inc., Beverly, MA). Both cytosol and membrane fractions were solubilized in SDS buffer, and protein content was assayed (DC protein assay; Bio-Rad). Equal amounts of protein were loaded in each lane after the addition of 2-mercaptoethanol and bromphenol blue. Individual proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). Ras proteins were identified by immunoblotting with mouse monoclonal anti-Ras antibody LA045 against amino acid residues 96-118 of H-, K-, and N-Ras (29, 30). Bound antibody was detected with affinity-purified horseradish peroxidase-conjugated goat antimouse IgG and enhanced chemiluminescence (Amersham International, UK) as previously detailed (19).

RESULTS

Ras Localization Is Affected Differently by Fmev and Lovastatin in a Human Transformed Cell Line. The initial experiments examined the effects of Fmev and lovastatin on membrane localization of Ras in the human histiocytic lymphoma-derived cell line U-937. In untreated U-937 cells, the majority of immunoreactive Ras was detected in the 100,000 × g pellet or crude membrane fraction (Fig. la, Lane 1). A small amount of immunodetectable Ras was observed in the 100,000 × g supernatant or cytosol fraction (Fig. 1b, Lane 1). The addition of Fmev was accompanied by a concentration-dependent decrease in membrane-associated Ras (Fig. la, Lanes 2 and 3). There was no concomitant increase in cytosol-localized Ras (Fig. 1b, Lanes 2 and 3). In contrast, incubation with 5 μM lovastatin resulted in a decrease in membrane-localized Ras (Fig. 1a, Lane 4), together with a substantial increase in cytosolic Ras (Fig. 1b, Lane 4). When cells were incubated with both Fmev and lovastatin, the results were similar to those obtained with lovastatin alone. The effects of Fmev and lovastatin on endogenous cholesterol synthesis were similar. Each caused a concentration-dependent inhibition of lipid synthesis. Thus, 50 μM Fmev decreased incorporation of radiolabeled mevalonate into lipid by 80 ± 5% (mean ± SE; n = 3), and this increased to 93 ± 1% inhibition with 500 μM Fmev. Lovastatin (0.5 μM) decreased incorporation of radiolabeled acetate into sterols from 236.3 ± 6.6 pmol/h/10⁶ cells (n = 3) to 24.1 ± 0.7 pmol/h/10⁶ cells (89.8% inhibition) and to 3.0 ± 0.4 pmol/h/10⁶ cells (98.7% inhibition) with 5 μM lovastatin.

Fmev-inhibited Proliferation of U-937 Cells Is Restored by Lovastatin. The effects of Fmev and lovastatin on the proliferation of U-937 cells were examined next. As illustrated in the representative experiment depicted in Fig. 2, the addition of increasing concentrations of Fmev resulted in a dose-dependent inhibition of proliferation. Concentrations of Fmev that decreased membrane-associated Ras (Fig. 1a, Lane 2) completely prevented the continued growth of U-937 cells (Fig. 2). Previous studies had indicated that Fmev-mediated inhibition of cell proliferation was related to the action of an inhibitor that was a product of mevalonate proximal to isopentenyl diphosphate (19, 20). To address this possibility, the effects of lovastatin, an inhibitor of HMG CoA reductase and therefore mevalonate synthesis,
on Fmev-mediated suppression were examined. As shown in Fig. 2, lovastatin (0.5 and 5 μM) had no effect on DNA synthesis in control U-937 cells. When added to Fmev-blocked cells, however, lovastatin restored proliferation. These data indicate that the inhibition of U-937 cell proliferation by Fmev was associated with the accumulation of a mevalonate-derived inhibitor.

Cell Lines Differ in the Fmev Concentration Required to Inhibit Proliferation. The effectiveness of Fmev at inhibiting the growth of different transformed cell lines was then examined to determine whether other leukemia- and lymphoma-derived cell lines would exhibit the same profound suppression of cell growth observed with U-937 cells. All cells were cultured under conditions imparting optimal proliferation in the absence of inhibitors. Cell lines differed substantially in the impact of a specific concentration of Fmev. Proliferation of some cell lines (illustrated in Fig. 3), was markedly suppressed by very low concentrations of Fmev. Thus, the calculated concentration of Fmev that inhibited proliferation by 50% (IC50) ranged between 4 μM (526.11) and 11 μM (Mar-B), and the concentration causing 90% inhibition (IC90) ranged between 12 μM (526.11) and 31 μM (Daudi) for cells that were classified as “sensitive” to Fmev. In contrast, other cell lines were relatively “resistant” to Fmev-mediated suppression (Fig. 3, bottom). For these cell lines, the IC50 of Fmev ranged between 114.1 (Jurkat) and 304.1 μM (HL-60). Similar results were obtained repeatedly in sensitive U-937 cells (IC50, 7 ± 3 μM, mean ± SE, n = 3; IC90, 14 ± 3 μM; see also Fig. 2) and resistant EL-4 cells (IC50, 225 ± 49 μM, n = 3).

When EL-4 cells were cultured in lipoprotein-deficient medium and, therefore, became dependent on endogenously synthesized cholesterol for ongoing membrane synthesis, the antiproliferative effect of Fmev was greatly enhanced (IC50, 4 μM; IC90, 14 μM), as would be expected if Fmev was effectively inhibiting the production of cholesterol under these conditions. These results imply that Fmev inhibited mevalonate metabolism effectively in the resistant lines when endogenous sterol synthesis was required. However, when endogenous sterol synthesis was not required because the cells were cultured in sterol-containing medium, there was a 50-fold disparity in the concentration needed for comparable inhibition of proliferation in resistant cell lines compared to sensitive ones.

Fmev Suppresses Mevalonate Metabolism Equivalently in Sensitive and Resistant Cell Lines. The simplest explanation for the differences in the effectiveness of Fmev was that Fmev suppressed mevalonate metabolism less efficiently in the relatively resistant cell lines. This possibility was directly addressed by measuring the effect of Fmev on the metabolism of radiolabeled mevalonate in U-937 and EL-4 cells. For these experiments, cells were incubated with trace quantities of [3H]mevalonate in the presence or absence of varying concentrations of Fmev for 24 h, and then cell lysates were separated into lipid, protein, and small molecular weight aqueous fractions. In both U-937 and EL-4 cells, Fmev inhibited the incorporation of [3H]mevalonate into lipid and protein fractions (Fig. 4a) in a concentration-dependent manner (data not shown). There was a reciprocal increase in labeled mevalonate in the aqueous phase in Fmev-blocked U-937 and EL-4 cells (Fig. 4a). This increase represents mevalonate, mevalonate phosphate, and mevalonate diphosphate (data not shown).

The addition of 500 μM Fmev suppressed mevalonate incorporation into lipid by 93 ± 1% and 95 ± 1% (n = 3) and into protein by 43 ± 7% and 83 ± 5% (n = 3) in U-937 and EL-4 cells, respectively. The concentrations of Fmev required to inhibit incorporation of mevalonate into lipid by 50% in U-937 and EL-4 cells were similar (6 and 4 μM for U-937 and EL-4 cells, respectively, calculated from cells incubated with varying concentrations of Fmev). Mevalonate modification of proteins was more readily inhibited in EL-4 cells (IC50, 18 μM) than in U-937 cells (inhibited <50% with 500 μM Fmev).

It was possible that the presence of unlabeled, endogenously synthesized mevalonate was confounding the analysis of the inhibitory effect of Fmev. To examine this possibility, endogenous mevalonate synthesis was prevented by blocking the activity of HMG CoA reductase with 5 μM lovastatin. This concentration of lovastatin decreased incorporation of radiolabeled acetate into digitonin-precipitable sterols by ≳98% (n = 2). The effect of Fmev on the metabolism of radiolabeled mevalonate was qualitatively unchanged by the presence of lovastatin. Thus, Fmev blocked incorporation of labeled
mevalonate into lipid and protein in a similar, concentration-dependent manner (Fig. 4b). There were quantitative differences in the distribution of labeled mevalonate within cells with and without suppression of endogenous mevalonate synthesis (compare Fig. 4, a and b), but there was no effect on the differential capacity of Fmev to suppress mevalonate metabolism in U-937 and EL-4 cells. The major impact of lovastatin was that the incorporation of radiolabeled mevalonate into protein was increased by ~3-fold (EL-4) to >10-fold (U-937). The aqueous (small molecular weight) fraction was unchanged by lovastatin, whereas less radiolabeled mevalonate was incorporated into lipid. In Fmev-blocked cells (Fig. 4b), the inclusion of lovastatin marginally increased the residual incorporation of mevalonate into protein, somewhat more in U-937 cells than in EL-4 cells (P > 0.05). However, the accumulation of radiolabeled mevalonate in the aqueous (small molecular weight) fraction of Fmev-blocked cells was unaltered by lovastatin. These results clearly demonstrate the efficacy of Fmev at blocking the conversion of mevalonate to downstream products in both U-937 and EL-4 cells but do not provide an explanation for the differential resistance of these transformed cells to the antiproliferative effect of Fmev.

**Differences in HMG CoA Reductase Activity and Induction Correlate with the Degree of Fmev Inhibition.** The possibility that sensitive and resistant cells might differ in the accumulation of a mevalonate-derived inhibitor was addressed by determining HMG CoA reductase activity in the cell lines in the presence or absence of Fmev. In control U-937 cells, levels of HMG CoA reductase activity were almost 6-fold higher in U-937 cells than in comparably cultured EL-4 cells (Table 1). This difference was additionally amplified by incubation with Fmev. Thus, Fmev increased the already elevated levels of HMG CoA reductase activity in U-937 cells by an additional >2-fold. In contrast, Fmev caused only a 0.4-fold increase in HMG CoA reductase activity in EL-4 cells.

**Cell Lines Also Differ in the Effects of Fmev on Ras Localization and Depletion.** Similar to the findings in U-937 cells (Fig. 1), Fmev (500 μM) alone decreased membrane-localized Ras in EL-4 cells (Fig. 5, Lane 2). Low concentrations ofLovastatin (0.5 μM) also modestly decreased membrane-localized Ras in EL-4 cells (Lane 3). In contrast to U-937 cells, however, Fmev increased cytosolic Ras in EL-4 cells (Lane 6). Thus, the effect of Fmev on localization of Ras in EL-4 cells was similar to the effect of lovastatin (Lane 7).

**Lovastatin Inhibits Proliferation of EL-4 Cells and Does Not Restore Fmev-blocked Growth.** Low concentrations oflovastatin (0.5 μM) did not alter growth of unblocked EL-4 cells (Fig. 6). However, the addition of higher concentrations (5 μM lovastatin) inhibited proliferation substantially. Whereas the low concentration oflovastatin (0.5 μM) restored the growth of U-937 cells that was suppressed by Fmev (Fig. 2), there was no effect on the proliferation of Fmev-blocked EL-4 cells, despite less inhibition by Fmev (Fig. 6). Furthermore, the addition of a higher concentration of lovastatin (5 μM) to EL-4 cells resulted in increased inhibition of cell growth and a complete blockade of proliferation with concentrations of Fmev.

**Table 1** HMG CoA reductase activity is significantly greater in U-937 cells than in EL-4 cells and is further induced by Fmev

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (μM)</th>
<th>U-937 cells</th>
<th>EL-4 cells</th>
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<tr>
<td>Control</td>
<td></td>
<td>418 ± 28</td>
<td>73 ± 1</td>
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<tr>
<td>Fmev</td>
<td>5</td>
<td>456 ± 34</td>
<td>99 ± 7</td>
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<td>Fmev</td>
<td>50</td>
<td>1040 ± 21</td>
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<td>Fmev</td>
<td>500</td>
<td>764 ± 31</td>
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Fig. 4. Mevalonate metabolism in sensitive and resistant cell lines is comparably inhibited by Fmev. a, U-937 and EL-4 cells were cultured in 10% BCS, with or without Fmev (500 μM) as indicated and incubated with [5-3H]mevalonolactone for 24 h. Cells were separated into lipid, protein, and aqueous fractions, and then the individual components in the various fractions were separated by TLC or SDS-PAGE, identified by comparison with authentic standards, visualized by fluorography, and quantitated by liquid scintillation spectroscopy. b, cells were cultured and processed as in a, except that 5 μM lovastatin was added to all cultures. Columns, means of three separate experiments; bars, SE.

DISCUSSION

The current studies explored the relationship of Ras expression and inhibition of proliferation by Fmev, a fluorinated mevalonate analogue. The results demonstrate that there is a range of effects of Fmev on different cell types, with the concentration required for 90% inhibition (IC₉₀) varying over a >-50-fold range. This variability could not be explained by differences in the capacity of Fmev to block the conversion of mevalonate phosphate(s) to isopentenyl diphosphate. Rather, sensitivity to Fmev-mediated inhibition was associated with an elevated rate of HMG CoA reductase activity in the highly sensitive cell line, U-937, with a further induction of activity following incubation with Fmev. In contrast, the Fmev-resistant cell line, EL-4, had a significantly lower rate of HMG CoA reductase activity that was not substantially altered by Fmev. In addition, U-937 cells manifested substantial depletion of cellular Ras as a result of the Fmev block, whereas Ras was not diminished but rather relocated from the membrane to the cytosol, as would be expected from a decrease in prenylation in the Fmev-resistant cell line, EL-4. Thus, as shown in Fig. 7, the findings are consistent with the conclusion that Fmev-blocked U-937 cells accumulate a mevalonate-derived inhibitor that greater than 100 μM. These data suggest that, in EL-4 cells, unlike U-937 cells, the inhibitory effect of lovastatin on cell growth is dominant.

DISCUSSION

The current studies explored the relationship of Ras expression and inhibition of proliferation by Fmev, a fluorinated mevalonate analogue. The results demonstrate that there is a range of effects of Fmev on different cell types, with the concentration required for 90% inhibition ($IC_{90}$) varying over a $>50$-fold range. This variability could not be explained by differences in the capacity of Fmev to block the conversion of mevalonate phosphate(s) to isopentenyl diphosphate. Rather, sensitivity to Fmev-mediated inhibition was associated with an elevated rate of HMG CoA reductase activity in the highly sensitive cell line, U-937, with a further induction of activity following incubation with Fmev. In contrast, the Fmev-resistant cell line, EL-4, had a significantly lower rate of HMG CoA reductase activity that was not substantially altered by Fmev. In addition, U-937 cells manifested substantial depletion of cellular Ras as a result of the Fmev block, whereas Ras was not diminished but rather relocated from the membrane to the cytosol, as would be expected from a decrease in prenylation in the Fmev-resistant cell line, EL-4. Thus, as shown in Fig. 7, the findings are consistent with the conclusion that Fmev-blocked U-937 cells accumulate a mevalonate-derived inhibitor that greater than 100 μM. These data suggest that, in EL-4 cells, unlike U-937 cells, the inhibitory effect of lovastatin on cell growth is dominant.
demonstrated that mevalonate metabolism in EL-4 cells was a very high-capacity pathway (15). Therefore, it remained possible that a low-affinity, high-capacity pathway might not have been as common in many cell lines. The trivial explanation that Fmev was not blocking mevalonate synthesis or the mevalonate phosphates. The data presented herein therefore demonstrate that intracellular levels of mevalonate and the mevalonate phosphate(s) determine the effectiveness of Fmev in blocking proliferation of many cell lines.

The precise molecular nature of the mevalonate-derived inhibitor and the actual process whereby cellular proliferation is blocked remain unknown. However, one intriguing possibility, suggested by the studies presented herein, is that the amount of total cellular Ras in transformed cells is an important determinant of growth. In contrast, the specific intracellular localization of Ras may be less critical. The effect of cytosolic localization of Ras may be dependent on whether Ras is oncogenically activated or normal. Thus, recent reports indicate that complexes of oncogenic Ras with Raf accumulate in the cytoplasm when farnesylation is blocked, for example, by farnesyltrans-

Fig. 5. Effect of Fmev and lovastatin on immunodetectable Ras in EL-4 cells. EL-4 cells were cultured for 48 h in medium supplemented with 10% BCS, with or without Fmev (500 μM) and lovastatin (0.5 μM), as indicated, after which they were separated into cytosol (soluble) and membrane (particulate) fractions, and Ras was identified by immunoblots as detailed in "Materials and Methods."

Data points, means of triplicate determinations; bars, SE.

depletes Ras. These results suggest that inhibitors of diphosphomevalonate decarboxylase, such as Fmev, may be effective as chemotherapeutic agents for cancer cells exhibiting elevated levels of HMG CoA reductase activity. The findings also imply that mevalonate, a mevalonate phosphate, or a product derived from these metabolic intermediates is an important regulator of Ras metabolism.

The proliferation of many cell lines was suppressed in a concentration-dependent manner by Fmev, even though cells were cultured in medium containing sufficient plasma lipoproteins to provide cholesterol for membrane synthesis. However, the concentration of Fmev that decreased proliferation by 90% varied greatly (>50-fold) between cell lines of both human and murine origin (this report and Ref. 19). The trivial explanation that Fmev was not blocking mevalonate metabolism in resistant cell lines was clearly eliminated. However, synthesis of cholesterol from mevalonate represents a low-affinity, high-capacity pathway (15). Therefore, it remained possible that a high-affinity, low-capacity pathway might not have been as completely suppressed. This possibility was addressed directly by measurements of the capacity of Fmev to prevent incorporation of radio-labeled mevalonate into lipids and proteins. These experiments demonstrated that mevalonate metabolism in EL-4 cells was very effectively blocked by Fmev, although these cells resisted inhibition of proliferation. Furthermore, when the intracellular pool of mevalonate was depleted with lovastatin, the capacity of Fmev to inhibit the incorporation of radio-labeled mevalonate was unchanged or enhanced in both cell lines, making it unlikely that fluctuations in the endogenous pool size confounded the analysis.

In contrast to the similarity of Fmev-blocked mevalonate metabolism in sensitive and resistant cells, mevalonate synthesis was disparate between the cell lines. Not only was mevalonate synthesis (HMG CoA reductase activity) substantially greater in U-937 cells, but it was also further induced by Fmev. The induction of HMG CoA reductase activity was observed with low concentrations of Fmev, consistent with the pattern of inhibition of proliferation by low concentrations of Fmev in these cells. The additional increase in HMG CoA reductase activity in Fmev-blocked cells is compatible with the activity of the proposed nonsterol mevalonate product(s), distal to isopentenyl diphosphate (31), that regulate(s) HMG CoA reductase by altering degradation and/or translation (15). The diminished impact of Fmev on HMG CoA reductase activity of EL-4 cells is in accordance with the diminished flux of mevalonate metabolites in this cell line due to the decreased HMG CoA reductase activity.

Labeled mevalonate and mevalonate phosphates accumulated in the Fmev-blocked cells. Although the accumulation of radioactivity was similar in EL-4 cells and U-937 cells, the actual intracellular concentration will vary with the relative HMG CoA reductase activity of the cell line. Lovastatin increased proliferation of Fmev-blocked U-937 cells, rather than additionally decreasing proliferation, as would be predicted if a compound downstream of mevalonate diphosphate were essential for cell growth. The most reasonable explanation for this observation is that lovastatin decreased mevalonate synthesis and thereby decreased the accumulation of an inhibitor derived from mevalonate or the mevalonate phosphates. The data presented herein therefore demonstrate that intracellular levels of mevalonate and the mevalonate phosphate(s) determine the effectiveness of Fmev in blocking proliferation of many cell lines.

Fig. 6. Lovastatin fails to restore growth of Fmev-blocked EL-4 cells. EL-4 cells were cultured in medium supplemented with 10% BCS and incubated with varying concentrations of Fmev (5–500 μM) alone (○) or with 0.5 μM lovastatin (●) or 5 μM lovastatin (■) for 4 days before quantification of DNA synthesis by the incorporation of [3H]thymidine.
ferase inhibitors (32). The complexes cannot signal to the mitogen-activated protein kinase cascade downstream of Ras and are thus inactive (32). Similarly, inactive cytosolic Ras-Raf complexes were detected when oncogenic Ras was mutated to prevent farnesylation (cysteine 186 → serine; Ref. 33). In contrast, Ras-Raf complexes were not observed when normal Ras was mutated to block farnesylation, and signaling downstream of Raf was not blocked (33). These observations suggest that cytosolic localization of oncogenic Ras may affect proliferation by complexing the effectors Raf and preventing downstream signaling via the mitogen-activated protein kinase cascade. Cytosolic localization of normal Ras, as in U-937 and EL-4 cells, is predicted to be without effect on proliferation because signaling downstream of Raf is not blocked if Ras is normal (33).

The depletion of total Ras in Fmev-blocked U-937 cells implies that mevalonate, a mevalonate phosphate, or a derivative of these metabolic intermediates is involved in Ras metabolism, either directly or indirectly. Because the effects of Fmev and lovastatin are disparate, the change in immunodetectable Ras levels cannot be directly attributed to the failure of lipid modification by farnesylation. Rather, Ras levels may vary inversely with the intracellular abundance of mevalonates. The current studies are consistent with the conclusion that Ras is depleted in Fmev-blocked cells because a mevalonate-derived product accumulates when HMG CoA reductase activity is increased and proliferation is inhibited because Ras is depleted.

In yeast, mevalonates regulate Ras levels by controlling RAS mRNA abundance (34). However, similar studies have not been reported in higher eukaryotes. Therefore, in preliminary experiments, we analyzed the effects of Fmev on cellular levels of ras genes and the stability of ras mRNAs. Fmev had no effect on steady-state levels of H-ras, N-ras, and K-ras mRNAs, quantified by nuclease protection assay in U-937 and EL-4 cells (data not shown). This finding demonstrates that the mechanism of Ras depletion is unlikely to be either transcriptional or related to mRNA stability. Thus, Fmev depletion of Ras is more likely to be the result of either translational or posttranslational regulation of Ras levels. Future studies will define this mechanism precisely.

Lovastatin restored the proliferation of Fmev-blocked U-937 cells but not EL-4 cells. Indeed, lovastatin increased the inhibitory effect of Fmev on the growth of EL-4 cells. One possible explanation for this synergic inhibition is that the proliferation of EL-4 cells requires a mevalonate-derived product distal to isopentenyl diphosphate that is limiting in these cells with decreased HMG CoA reductase activity. Both Fmev and lovastatin inhibit the synthesis of all products distal to and including isopentenyl diphosphate (see Fig. 7). Therefore, this product could be isopentenyl tRNA, farnesylated or geranyl geranylated protein, ubiquinone, dolichol, or another prenyl-derived compound. Alternatively, Fmev may decrease the synthesis of the previously described mevalonate-derived growth promoter even further than does lovastatin alone. This mevalonate product has not been identified. However, our previous studies indicate that it is synthesized directly from mevalonates (18). Subtle differences in the profile of mevalonates between U-937 cells and EL-4 cells may account for the synergistic inhibition caused by Fmev and lovastatin.

In summary, the current studies demonstrate that Fmev inhibits the proliferation of various leukemia- and lymphoma-derived cell lines. The inhibition of proliferation correlates with depletion of cellular Ras, and reversal of Ras depletion is associated with restoration of growth. Ras depletion is achieved by inhibitory compound(s) that accumulate(s) in Fmev-blocked cells and are identical to or synthesized from either mevalonate or the mevalonate phosphates. These data suggest that targeting chemotherapy to take advantage of malignancy-associated perturbations in normal regulation of mevalonate metabolism may be possible, regardless of the presence or absence of oncogenic Ras. HMG CoA reductase activity is tightly regulated in normal cells, and this control would protect nonmalignant cells from accumulating inhibitors of proliferation. Criteria that predict Fmev-mediated inhibition of proliferation in vitro may also predict in vivo responsiveness. Thus, the ability to determine HMG CoA reductase activity in circulating leukemic cells might permit selection of those patients with a greater likelihood of responding to Fmev as an anticancer agent.

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