Suppression of the Proliferation of Ras-transformed Cells by Fluoromevalonate, an Inhibitor of Mevalonate Metabolism

Jennifer A. Cuthbert and Peter E. Lipsky

ABSTRACT

Mevalonate is the precursor of a number of different products potentially required for the growth of cells, including the prenylated oncoprotein Ras. To determine whether inhibition of mevalonate metabolism would selectively block proliferation of Ras-transformed cells, 6-fluoromevalonate (Fmev), an inhibitor of diphosphomevalonate decarboxylase, was used to block the synthesis of prenyl-derived lipids and prenylated proteins in interleukin-3 (IL-3)-dependent FDC-P1 cells (control FDC-P1 cells) and FDC-P1 cells transformed with oncogenic Ras (RasDC cells) that proliferated in the absence of IL-3. Fmev completely inhibited synthesis of prenyl-derived lipids and prenylated proteins and blocked proliferation of FDC-P1 and RasDC cells. Restoration of the proliferation of Fmev-blocked FDC-P1 cells required both an exogenous source of cholesterol and prevention of the accumulation of mevalonate and the mevalonate phosphates with lovastatin. In contrast, ongoing IL-3-independent proliferation of Fmev-blocked RasDC cells was not completely restored by providing exogenous cholesterol and preventing the inhibition of mevalonate metabolism. However, these cells proliferated when cultures were supplemented with IL-3 together with exogenous cholesterol and lovastatin, implying that Fmev had prevented Ras-dependent, IL-3-independent growth. Fmev markedly diminished total cellular Ras in RasDC cells. In contrast, lovastatin depleted membrane-associated Ras and increased cytosolic Ras but did not diminish total cellular Ras. These data indicate that Fmev depletes total cellular Ras and specifically inhibits the autonomous growth of Ras-transformed cells.

INTRODUCTION

In normal cells, Ras, a plasma membrane-associated 21 kDa guanine nucleotide-binding protein, is involved in signal transduction associated with growth and differentiation (1). Interference with the normal function of Ras in cultured cells suppresses cell growth (2–4), whereas introduction of activated Ras can lead to transformation (1). Mutations that constitutively activate a cellular Ras oncoprotein have been found in ~20% of all human tumors (1, 5) and are particularly prevalent in adenocarcinomas of the colon (6) and pancreas (7). Disruption of an activated Ras gene in human colon cancer cell lines slows proliferation in vitro and suppresses anchorage-independent growth (8), suggesting that therapeutic approaches targeting Ras may be beneficial.

Membrane localization of Ras, which is important for in vitro transforming activity, is normally dependent on posttranslational modification of the carboxy terminus by sequential farnesylation, proteolysis, and carboxymethylation (9–19). Since blocking posttranslational farnesylation of Ras precludes subsequent membrane localization (9, 11, 12, 14, 16), it could prevent the transforming potential of oncogenic Ras. Inhibition of the synthesis of farnesyl 1-diphosphate, by blocking the formation of the precursor mevalonate with lovastatin, effectively prevents posttranslational processing of Ras and subsequent membrane localization (11, 12). In addition, selective inhibition of the growth of Ras-transformed cells by inhibitors of farnesyltransferase, the enzyme that modifies Ras by the addition of the farnesyl group, has been reported (20, 21). However, the efficacy of these compounds at inhibiting the growth of malignant cells, where multiple genetic alterations often contribute to the cancer phenotype, is less certain. In contrast, Fmev,3 a potent inhibitor of the activity of diphosphomevalonate decarboxylase (22, 23), completely blocks protein prenylation (24–26) and inhibits the proliferation of various transformed cells (27) but does not block proliferation of normal cells if cholesterol is provided (26). In these transformed cells, suppression of proliferation is caused by the accumulation of mevalonate or mevalonate phosphate(s) rather than by the inhibition of cholesterol biosynthesis (27). The current studies were carried out to determine whether Fmev would inhibit the growth of Ras-transformed cells by these mechanisms as well as by suppressing the prenylation and membrane association of the mutant cellular oncoprotein.

MATERIALS AND METHODS

Cell Culture and Measurement of DNA, Sterol, and Mevalonate Synthesis and Cell Growth. FDC-P1 (Paterson Laboratories) cells and the ras-transformed FDC-P1 cell line F1 NR G4 (28) that has been transfected with a mutant human H-ras genomic clone derived from the T24 bladder carcinoma (Ref. 29; RasDC) were generously provided by Dr. H. Scott Boswell (Indiana University School of Medicine, Indianapolis, IN). Proliferation of FDC-P1 cells is dependent on the exogenous growth factor IL-3 (30). However, when transformed with oncogenic Ras, cell growth becomes IL-3 independent (28), thus permitting direct evaluation of the effects of experimental manipulations on Ras-dependent proliferation. All cells were maintained in complete medium and RPMI 1640 (Whittaker Bioproducts Inc., Walkersville, MD) supplemented with 10% v/v iron-supplemented BCS (Sigma Chemical Co., St. Louis, MO). In some experiments, medium was supplemented with 10% v/v LPP, prepared as detailed (26). Concentrated culture supernatant from WEHI-3 cells (final concentration, 2–5% v/v) was used as the source of IL-3 for maintaining FDC-P1 cells. Recombinant IL-3 (DNAx Research Institute, Inc., Palo Alto, CA) was used for some experiments, as indicated. Fmev was synthesized by CPM Laboratory (Carrollton, TX) using the procedure of Quistad et al. (31). Lovastatin (Merck, Sharp and Dohme, Rahway, NJ) and the sodium salt of mevalonate were prepared as reported previously (32) and added where indicated. Preliminary experiments demonstrated that lovastatin inhibited endogenous sterol synthesis, measured by the incorporation of radiolabeled acetate into digitoxigenin-precipitable sterols (32), in a concentration-dependent manner (control, 274 ± 12.0 pmol/h/10⁶ cells [mean ± SEM, n = 3], 0.5 μM lovastatin, 56.3 ± 0.4 pmol/h/10⁶ cells [80% inhibition]; 5 μM lovastatin, 12.1 ± 1.1 pmol/h/10⁶ cells [96% inhibition]; 50 μM lovastatin, 1.8 ± 0.3 pmol/h/10⁶ cells [99% inhibition]).

For measurements of DNA synthesis, cells were cultured (~5 × 10⁵ cells/well) in triplicate in microtiter plates with additions as indicated in the individual experiments; in preliminary experiments, this concentration of cells allowed maximum proliferation during the ensuing 1–4-day incubation. DNA synthesis was measured by the incorporation of [³H]thymidine as described (32), after the addition of 5-fluorodeoxyuridine (10 μM) to block endogenous thymidine synthesis. In some experiments, cells were cultured at a lower concentration of

Received 12/27/94; accepted 2/13/95.

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1 This work was supported by Grant CN-108 from the American Cancer Society and Public Health Service Grant AI-17653 from the NIH.

2 To whom requests for reprints should be addressed, at Liver Unit, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8887.

3 The abbreviations used are: Fmev, 6-fluoromevalonate; FDC-P1 cells, factor-dependent continuous cell line; RasDC cells, oncogenic Ras-dependent FDC-P1 cells; BCS, bovine calf serum; LPP, lipoprotein-poor plasma; IL-3, interleukin-3; HMG CoA, 3-hydroxy-3-methylglutaryl-CoA; PAGE, polyacrylamide gel electrophoresis.

1732
density (0.5 × 10^6 cells per well), and cell proliferation was quantified by counting the number of cells in replicate microtiter wells after 7 days in culture, as described previously (33). For measurements of sterol synthesis, 1 × 10^6 cells/ml were cultured in 17 × 100 mm polypropylene tubes with additions as indicated in the individual experiments. Incorporation of [1,14C]acetate into digitonin-precipitable sterols was measured in triplicate incubations as detailed previously (32).

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 counted, and the cell pellet was frozen at −90°C until activity was assayed. HMG CoA reductase activity was measured by a modification of the procedures of Brown et al. (34) and Panini et al. (35). Briefly, cell pellets were resuspended in activation buffer containing 200 mM KCl, 50 mM potassium phosphate (pH 7.4), 5 mM DTT, 5 mM EDTA, 5 mM EGTA, 25 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 a 1-min centrifugation at 16,000 × g, aliquots of the supernatant were taken for measurements of HMG CoA reductase activity (50 μl) and protein. To each reaction tube, an equal volume of 2X mix (200 mM potassium phosphate (pH 7.4), 12.5 mM DTT, 5 mM EGTA), 175 μg NADPH, and 3.75 nmol [14C] HMG CoA (Dupont Co., NEN Research Projects, 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 the addition of 2 μmol [3H]Mevalonolactone (Dupont) internal recovery standard. After a 20-min centrifugation at 16,000 × g, 50 μl of supernatant was spotted onto plastic-backed silica gel G plates and chromatographed using benzene:acetone:1:1 as the solvent system. Chromatograms were sprayed with EN3HANCE (Dupont); then mevalonate was visualized by fluorography at −90°C for 2–3 days, and [14C]mevalonate was quantitated by liquid scintillation spectrometry. Rates of HMG CoA reductase activity were calculated using [14C]mevalonate for correction and are expressed as pmol/h/10^6 cells and pmol/min/mg cell protein.

Incorporation of Mevalonate into Cellular Proteins and Lipids.
FDC-P1 and RasDC cells were cultured in complete medium supplemented with 5 μM lovastatin and with or without IL-3-containing supernatant and varying concentrations of Fmev as indicated in the individual experiments. Cells were incubated with [5-3H]mevalonolactone (specific activity, 27.8 Ci/mmol; Dupont) and harvested after a 24-h incubation, as indicated in individual experiments. Following extensive washing to remove unincorporated radioactivity, cells were lysed after resuspension in water and fractionated by a modified Bligh and Dyer method as described previously (26). Cell proteins partitioned to the interphase and were precipitated with 10% v/v trichloroacetic acid after removal of the upper methanol:water phase and lower chloroform phase. The precipitates were washed sequentially with 10% trichloroacetic acid, ice-cold acetone, and ice-cold ethanol and solubilized by heating in sample buffer containing 2% (w/v) SDS, 50 mM 2-mercaptoethanol, and 25 mM NaOH. Radioactivity incorporated into protein was quantitated in an aliquot by liquid scintillation spectrometry. The results are presented as fmol [3H]mevalonate for and are expressed as pmol/h/10^6 cells and pmol/min/mg cell protein.

Fmev Prevents Mevalonate Incorporation into Lipids and Proteins in FDC-P1 and RasDC Cells. The effect of blocking mevalonate metabolism with Fmev on the distribution of radiolabeled mevalonate in FDC-P1 and RasDC cells was initially examined. Fmev inhibited mevalonate incorporation into lipids and proteins in a concentration-dependent manner in both cells (Figs. 1, a and b). In three other experiments, the addition of 500 μM Fmev to RasDC cells suppressed [3H]mevalonate incorporation into total lipids by 98.7 ± 0.2% (mean ± SEM; n = 3) and into protein by 97.4 ± 0.5% (n = 3). Analysis of individual lipid species by TLC demonstrated that, in blocked FDC-P1 and RasDC cells, radiolabeled mevalonate was predominantly incorporated into sterol precursors and 3β-OH sterols (squalene, lanosterol, cholesterol, and cholesteryl ester; FDC-P1 cells, 70%; RasDC cells, 74 ± 4%; mean ± SEM, n = 6). Fmev inhibited mevalonate incorporation into every fraction in a highly effective manner (data not shown), similar to the inhibition observed previously in Fmev-blocked peripheral blood mononuclear cells (26). Fluorography of [3H] mevalonate-labeled proteins, after separation by SDS-PAGE, was used to examine prenylation of specific proteins. In the unblocked (control) RasDC cells, there was an increase in [3H]mevalonate labeling of a protein with Mr ~21,000, in high levels of enrichment in the early prenylation process (Fig. 1b). Fmev completely suppressed prenylation of this and all other proteins (Fig. 1b).

Radiolabeled mevalonate accumulated in the methanol:water (aqueous) phase in Fmev-blocked cells (Fig. 1c). TLC separation of the aqueous fraction demonstrated the accumulation of mevalonate, mevalonate phosphate, and mevalonate diphosphate in Fmev-blocked cells (Fig. 1c). It should be noted that Fmev had no significant effect on the uptake of [3H]mevalonolactone by the cells (data not shown).
Thus, the decrease in mevalonate incorporation into protein was the result of inhibition of mevalonate metabolism and not merely prevention of the uptake of the radiolabeled substrate.

**Fmev Suppresses Proliferation of FDC-P1 and RasDC Cells.** To examine the effect of Fmev on proliferation, FDC-P1 and RasDC cells were cultured in medium supplemented with lipoprotein-poor plasma or with lipoprotein-containing serum to provide cholesterol for membrane synthesis, and DNA synthesis was measured after 4 days incubation. Fmev inhibited cellular proliferation in a concentration-dependent manner (Fig. 2). The presence of exogenous cholesterol, in lipoproteins (BCS), or added as molecular cholesterol (Fig. 2b) only modestly reduced the inhibitory effects of the lowest concentrations of Fmev (IC50 in LPP: RasDC, 2.9 μM; FDC-P1, 5.7 μM; IC50 in BCS: RasDC, 12.2 μM; FDC-P1, 8.3 μM). There was no further effect from adding molecular cholesterol to cells cultured in lipoprotein-containing medium (Fig. 2b). FDC-P1 and RasDC cells thus resembled transformed human cells (27) rather than normal human T cells (26) in that Fmev-mediated inhibition of proliferation was not prevented by exogenous cholesterol.

Fmev-mediated inhibition of proliferation, unrelated to cholesterol deprivation, may have been caused by inhibitory products of mevalonate that accumulate in Fmev-blocked cells (27) or may have re-
RAS-TRANSFORMED CELLS AND FLUOROMEVALONATE

Fig. 2. Fmev suppresses proliferation regardless of exogenous cholesterol. a. FDC-P1 (Δ, A) and RasDC (O, ●) cells (3000 cells/microtiter well) were incubated in medium supplemented with 10% v/v LPP (Δ, △) or 10% v/v BCS (O, ●); varying concentrations of Fmev were added as indicated, and the cells were cultured for 4 days. WEHI-3 supernatant prepared from cells cultured in LPP or BCS was added to FDC-P1 cells. Cellular DNA synthesis was quantified by the incorporation of [3H]thymidine in replicate microtiter wells, and the percentage inhibition by Fmev was calculated. b. FDC-P1 cells (5000/microtiter well) were incubated in complete medium supplemented with BCS WEHI-3 supernatant (Δ, A) or medium supplemented with 10% v/v LPP and LPP WEHI-3 supernatant (O, •) with (●, A) or without (O, △) cholesterol (50 μg/ml) and varying concentrations of Fmev as indicated. Cellular DNA synthesis was measured after 4 days by the incorporation of [3H]thymidine. Results are mean ± SEM of triplicate determinations.

Fig. 3. Immediate suppression of proliferation of FDC-P1 and RasDC cells by Fmev. FDC-P1 (a) and RasDC (b) cells (5000 cells/microtiter well) were incubated in complete medium with (FDC-P1) or without (RasDC) WEHI-3 supernatant; varying concentrations of Fmev (no Fmev; □, 50 μM; and ■, 500 μM) were added as indicated, and the cells were cultured for 1 to 4 days. Results are mean ± SEM of triplicate determinations.

Cellular DNA synthesis was quantified by the incorporation of [3H]thymidine in replicate microtiter wells, and the percentage inhibition by Fmev was calculated. Fmev inhibited DNA synthesis by FDC-P1 cells by 57 ± 1% and by RasDC cells was blocked by Fmev and, with longer incubation periods, no capacity to escape the marked inhibition of growth was noted.

Since the onset of inhibition was rapid and there was little restoration of proliferation with addition of exogenous cholesterol to Fmev-blocked cells (Fig. 2), the possibility that some of the effects of Fmev might be related to the accumulation of mevalonate-derived inhibitors of proliferation (see Fig. 8) as described previously (27) was explored. In FDC-P1 cells, blocking mevalonate synthesis with a low concentration of lovastatin (0.5 μM) reversed the suppressive effect of Fmev (Fig. 4a, left panel), demonstrating that this Fmev-mediated inhibition was associated with the accumulation of a previously described mevalonate-derived inhibitor (27). Lovastatin at this concentration (0.5 μM) or a 10-fold higher concentration did not directly affect DNA synthesis of these cells. This result implied that mevalonate was synthesized in FDC-P1 cells at a rate that was higher than necessary to permit proliferation, since inhibiting HMG CoA reductase activity with lovastatin did not perturb cell growth. HMG CoA reductase activity in FDC-P1 cells cultured in medium supplemented with bovine calf serum (cholesterol concentration in culture medium, ≈130 μg/ml) and, therefore, with supposedly down-regulated HMG CoA reductase activity, was 162 ± 3 pmol/h/10⁶ cells (mean ± SEM, n = 3; 61 ± 1 pmol/min/mg cell protein), similar to the activity of HMG CoA reductase in fibroblasts induced by incubation in lipoprotein-deficient medium (34). Thus, increased HMG CoA reductase activity and associated enhanced mevalonate synthesis in FDC-P1 cells was associated with Fmev-mediated accumulation of mevalonate- or mevalonate phosphate-derived inhibitors and suppression of cell growth that was prevented by lovastatin.

In contrast to the effect noted with the parent cell line, FDC-P1, the inhibitory effect of Fmev on RasDC cells was only partially prevented by lovastatin (Fig. 4a, right panel). RasDC cell DNA synthesis in the presence of Fmev and 0.5 μM lovastatin was inhibited by 93 ± 3% (n = 3) and, with the addition of 5 μM lovastatin to Fmev-blocked cells, remained inhibited by 46 ± 7% (n = 3). The possibility that suppression of RasDC cell DNA synthesis, as measured by the incorporation of radiolabeled thymidine, did not reflect true inhibition of proliferation was examined. RasDC cells were incubated at low density to permit optimal growth for 7 days, and the number of cells in culture was quantified directly. Fmev completely prevented growth of RasDC cells assessed after 7 days of culture (Fig. 4b). Proliferation was partially restored by lovastatin such that the number of cells detected after a 7-day incubation was 48% less than that noted in control cultures. These results are consistent with those obtained from the analysis of [3H]thymidine incorporation and confirm that Fmev totally inhibited DNA synthesis and growth of RasDC cells. Unlike FDC-P1 cells, lovastatin could not overcome the inhibition by Fmev completely, implying that the activity of the transforming Ras gene product in RasDC cells had altered the functional effects of inhibition with Fmev.

One possible explanation for the failure of lovastatin to restore growth of Fmev-blocked RasDC cells was that HMG CoA reductase activity in RasDC cells was substantially higher than in FDC-P1 cells; therefore, mevalonate synthesis was not diminished sufficiently by lovastatin. HMG CoA reductase activity, measured in RasDC cells...
Lovastatin restores proliferation of Fmev-blocked FDC-P1 cells but not RasDC cells.

*a.* FDC-P1 (left panel) and RasDC (right panel) cells (5000 cells/microtiter well) were incubated in complete medium with (FDC-P1) or without (RasDC) WEHI-3 supernatant. Fmev (500 µM) and varying concentrations of lovastatin were added as indicated. After 4 days, cellular DNA synthesis was quantified by the incorporation of [3H]thymidine. Results are the means of three separate experiments; bars, SEM.

*b.* RasDC cells (500 cells/microtiter well) were incubated in complete medium with or without Fmev (500 µM) and lovastatin (5 µM) as indicated. After 7 days, cellular proliferation was quantified by counting the number of cells in each well and subtracting the background (cell-free, complete medium). Results are the means of triplicate determinations; bars, SEM.

**Fig. 5.** Exogenous IL-3 restores proliferation of Fmev-blocked RasDC cells. RasDC cells (5000 cells/microtiter well) were incubated in complete medium with or without lovastatin (to prevent accumulation of a mevalonate-derived inhibitor), Fmev (500 µM), and with or without WEHI-3 supernatant (IL-3) as indicated. Cellular DNA synthesis was quantitated after 2 days (upper panel) or 4 days (lower panel). Results are the means of two (0.5 µM lovastatin) and four (5 µM lovastatin) experiments; bars, SEM.

Exogenous IL-3 restores proliferation of RasDC cells. Next, the capacity of IL-3 to overcome the inhibitory effect of Fmev on the growth of RasDC cells was investigated. The RasDC cells were grown in lipoprotein-containing medium with or without lovastatin to prevent the accumulation of an inhibitory product of mevalonate in the Fmev-blocked cells. Control RasDC cells proliferated in the absence of exogenous growth factors. As noted above, Fmev markedly suppressed growth (Fig. 5). Supplementation of the cultures with lovastatin, either 0.5 µM or 5 µM, failed to restore proliferation. Suppression of cell growth that was not reversed by lovastatin was greater with prolonged incubation of RasDC cells (>4 days; data not shown).
Fmev but not Lovastatin Depletes Total Cellular Ras Content. The final experiments examined the effect of Fmev on membrane localization of Ras and compared it with the action of lovastatin. Since it was necessary to compare concentrations of Fmev and lovastatin that were equal in their ability to block the formation of the precursor of prenylation, farnesyl 1-diphosphate, preliminary studies were carried out to establish the equivalent inhibitory concentrations. The incorporation of the precursor acetate into the end-product cholesterol was used as an estimate of the formation of farnesyl 1-diphosphate, and the ability of Fmev and lovastatin to inhibit synthesis was quantitated. The effect of 500 μM Fmev was comparable to that of 50 μM lovastatin (control untreated RasDC cells, 135.9 ± 2.8 pmol [1-14C]acetate incorporated into digitonin-precipitable sterols/h/10⁶ cells; Fmev, 4.9 ± 0.3 pmol/h/10⁶ cells; lovastatin, 4.1 ± 0.7 pmol/h/10⁶ cells).

RasDC cells cultured with 500 μM Fmev or 50 μM lovastatin were separated into soluble (cytosolic) and particulate (membrane) fractions, and Ras was detected by immunoblotting. After a 24-h incubation, there was little effect of either agent on the distribution of immunodetectable Ras (data not shown). By 2 days, however, both Fmev and lovastatin caused a substantial diminution in membrane-associated Ras in RasDC cells (Fig. 7a). This decrease in Ras was not affected by the presence of IL-3-containing WEHI-3 supernatants (data not shown). In lovastatin-blocked RasDC cells, but not Fmev-blocked cells, there was accumulation of unprenylated Ras, identified by its location and slower migration characteristics, in the cytoplasm (Fig. 7a, lower panel). Of note, since Fmev caused a depletion of membrane Ras without accumulation of cytosolic Ras, there was a resultant striking diminution in total cellular Ras in Fmev-blocked cells, corresponding with the marked inhibition of proliferation observed after 2 days (Figs. 3 and 5). Total cytosolic and membrane proteins were largely unaffected by either lovastatin or Fmev (Fig. 7a, upper panel). Similar results were obtained with FDC-P1 cells (Fig. 7c). These results indicate that Fmev not only effectively prevents the posttranslational modification of proteins by prenylation and blocks the localization of Ras to the membrane but also decreases total cellular Ras.

Since lovastatin partially restored proliferation of Fmev-blocked RasDC cells, the effect of lovastatin on Fmev-mediated Ras depletion was examined. Concentrations of lovastatin that allowed proliferation of Fmev-blocked RasDC cells (5 μM) had no effect on the content or distribution of Ras (determined after 2 days) and did not alter the depletion of membrane-associated Ras by Fmev (500 μM; Fig. 7b). Similar results were obtained when a source of exogenous IL-3 was added (data not shown).

DISCUSSION

These results provide evidence for three different mechanisms whereby cellular proliferation may be blocked by Fmev (Fig. 8): (a) if cells are dependent on endogenous sources of cholesterol for new membrane production, Fmev-mediated inhibition of sterol synthesis effectively prevents growth. This is a characteristic of all cells (26, 27); (b) accumulation of mevalonate-derived inhibitory compounds in Fmev-blocked cells rapidly stops cell growth. This may be a characteristic of transformed cells, regardless of the exact basis of transformation, since a number of leukemia and lymphoma cell lines behave similarly (27); and (c) as demonstrated herein, when cell proliferation requires oncogenic Ras function, inhibition of prenylation blocks expression of the Ras-transformed phenotype. Exploitation of these diverse inhibitory effects of Fmev may permit targeted therapy against growth of malignant cells.

For cancer chemotherapy to be practical, malignant cells must be particularly sensitive to the effects of the agent, and/or normal cells...
Fig. 7. Fmev depletes membrane-associated and total cellular Ras. a. RasDC cells, incubated for 2 days in complete medium with or without 500 μM Fmev or 50 μM lovastatin as indicated, were separated into soluble (cytosolic) and particulate (membrane) fractions, and Ras proteins were identified by immunoblotting. Parallel samples were stained for protein with Coomassie blue after SDS-PAGE. Upper panel, Coomassie blue stain of SDS-PAGE gel (3 × 10^6 cells/lane). Lower panel, fluorogram demonstrating localization of Ras. b. Immunodetectable Ras in RasDC cells, incubated for 2 days in complete medium with or without 5 μM lovastatin, and 500 μM Fmev as indicated before cell fractionation and immunoblotting of Ras (3 × 10^6 cells/lane). c. Immunodetectable Ras in FDC-P1 cells incubated for 2 days in complete medium supplemented with WEHI-3 supernatant and with or without 500 μM Fmev or 50 μM lovastatin as indicated before cell fractionation and immunoblotting of Ras (3 × 10^6 cells/lane).

must be specifically protected from inhibition of growth. In normal human T cells and fibroblasts, Fmev only inhibits proliferation when cells are cultured in the absence of exogenous cholesterol (26). When these cells are cultured in medium supplemented with lipoprotein cholesterol, Fmev has no effect on proliferation. Thus, in normal cells, lipoprotein cholesterol not only provides a source of sterol necessary for membrane synthesis but also prevents accumulation of mevalonate-derived inhibitory compounds. In some colon cancer cells, the capacity of exogenous lipoproteins to provide cholesterol for membrane synthesis is apparently lost (40). Lovastatin-mediated inhibition of cholesterol synthesis in these cell lines suppresses growth and proliferation and is not restored by exogenous lipoproteins. Growth of cancer cells with similar defects in the use of exogenous lipoprotein cholesterol for membrane synthesis would also be inhibited by Fmev, whereas normal cells would be protected.

The failure of exogenous cholesterol to restore growth of Fmev-blocked FDC-P1 cells was not the result of abnormalities in receptor-mediated uptake of lipoprotein cholesterol, however. Thus, molecular cholesterol also failed to restore proliferation to these cells, demonstrating that defective recognition of lipoproteins was not the basis of the inability of exogenous cholesterol to overcome Fmev-mediated inhibition. Furthermore, proliferation of Fmev-blocked FDC-P1 cells was restored once mevalonate-derived inhibitors were prevented from accumulating, establishing that these cells could use exogenous lipoproteins to provide cholesterol essential for membrane synthesis. Taken together, the current results are consistent with previous findings with other transformed cells (27) that the lack of growth restoration to the parental FDC-P1 cell line by serum lipoprotein cholesterol is a manifestation of the failure of normal down-regulation of mevalonate synthesis by lipoprotein cholesterol, since decreasing mevalonate synthesis with lovastatin restored growth.

In normal cells, mevalonate synthesis is tightly controlled by regulation of HMG CoA reductase activity (41), whereas poorly regulated or unregulated activity is a hallmark of some transformed cells (42–46). Lipoprotein cholesterol decreases mevalonate synthesis by decreasing transcription of the gene encoding HMG CoA reductase and increasing degradation of the enzyme (41). In contrast, the activity of HMG CoA reductase is poorly regulated by lipoprotein cholesterol in various transformed human leukemic and lymphoma cell lines (27). With Fmev, mevalonate-derived inhibitors accumulate in these transformed cells and prevent proliferation (27). One possible explanation for unregulated or poorly regulated HMG CoA reductase activity in FDC-P1 cells is that the cells may have undergone subtle change(s) during selection for an immortal phenotype (30). Although insufficient to support growth factor-independent proliferation, these changes may have resulted in the loss of normal regulation of HMG CoA reductase activity, a common alteration in transformed hematopoietic cells (47). This conclusion is supported by measurements of cellular HMG CoA reductase activity. Both FDC-P1 cells and RasDC cells demonstrated rates of HMG CoA reductase activity 5–10-fold higher than those in fibroblasts similarly incubated in lipoprotein-containing serum, and activity was equivalent to that observed in fibroblasts maximally induced by incubation in lipoprotein-depleted medium (34). As a result of markedly higher rates of mevalonate synthesis, these cells may exhibit greater sensitivity to Fmev-mediated inhibition from increased accumulation of mevalonate-derived inhibitory products.

In RasDC cells, Fmev-mediated suppression of proliferation was also associated with the accumulation of mevalonate-derived inhibitors of growth since decreasing mevalonate synthesis, by the addition
of low concentrations of lovastatin, permitted some proliferation in the absence of supplemental growth factors and nearly normal proliferation in response to IL-3. One implication of these results is that Fmev blocked the Ras-transformed phenotype and returned the RasDC cells to IL-3 dependence. This is inferred from the observation that Fmev-mediated suppression of the IL-3-independent growth of RasDC cells was not fully restored by blocking the accumulation of mevalonate-derived inhibitors with lovastatin, whereas restored IL-3-dependent growth was observed. The difference in cell growth in these two circumstances should reflect the requirement for oncogenic Ras for IL-3-independent growth. During the first 2 days of culture, when proliferation was already markedly suppressed, the amount of total cellular and membrane-associated Ras in Fmev-blocked cultures remained significantly decreased independent of the presence of IL-3. These results are consistent with the conclusion that Fmev (in the presence of lovastatin) blocked the growth-promoting effects of transforming Ras by depleting cellular and/or membrane-associated Ras but permitted IL-3-dependent growth of these same cells. Thus, Fmev inhibited the growth of RasDC cells, not only by causing accumulation of a mevalonate-derived inhibitor, but also by blocking the capacity of transforming Ras to promote cytokine-independent growth.

Although IL-3 supported the growth of RasDC cells, proliferation was somewhat decreased by the presence of both 500 μM Fmev and 5 μM lovastatin during the 4-day incubation. One possible explanation for this observation is that mevalonate-derived inhibitors accumulated, despite decreasing mevalonate synthesis with 5 μM lovastatin. Alternatively, the decrease in IL-3-supported proliferation may reflect some requirement for membrane-association of oncogenic Ras in Ras-transformed RasDC cells or a threshold amount of cellular Ras that is required for growth of these cells. In contrast, membrane association of normal Ras in untransformed FDC-P1 cells was apparently less important since complete restoration of growth was accomplished with the addition of lovastatin to Fmev-blocked cells, thereby further decreasing any synthesis of prenylated proteins. This latter result suggests that oncogenic and normal Ras have quite different requirements for prenylation in order to function and is in agreement with the observation that proliferation of untransformed Rat1 cells was not suppressed by directly preventing farnesylation of normal Ras, whereas proliferation of oncogenic Ras-transformed cells was inhibited by blocking farnesylation (21).

The role of cellular Ras in IL-3-dependent proliferation of cells has been suggested (4, 48, 49) but remains controversial. On the one hand, IL-3 activates Ras (48) and antiseis mesenchymal cell transformation directed against c-ras, and its downstream effector c-raf inhibit IL-3-dependent growth (4, 49). On the other hand, when the common β subunit of the IL-3 receptor that transduces growth signals is mutated so that Ras cannot be activated, growth factor-dependent proliferation is unaltered (50). This latter observation suggests that IL-3-dependent growth does not require a signaling pathway using activated Ras. The results reported herein support the conclusion that normal, membrane-associated Ras is not required for IL-3-dependent proliferation. In contrast, when IL-3 is replaced by oncogenic Ras, membrane association of the transforming Ras product appears to be necessary for its function(s). The apparent lack of a requirement for membrane-associated Ras for cytokine-dependent growth is consistent with previous reports of the effect of directly preventing Ras farnesylation (20, 21) as well as of previous studies of the effects of Fmev (26, 27). Thus, mitogen-stimulated peripheral blood T lymphocytes proliferated, although prenylation of Ras was completely blocked by Fmev (26). Likewise, Fmev-blocked myeloid and lymphoid cell lines proliferated if mevalonate-derived inhibitors did not accumulate (27). Taken together with the observation in the current studies that IL-3 can sustain the proliferation of Fmev-blocked RasDC cells and FDC-P1 cells, it appears that normal Ras may not be essential or may function in intact cells without membrane localization and at far lower total cellular concentrations than required for transformation. Alternatively, only very small amounts of membrane-associated or total cellular Ras may be sufficient. It is clear from the present experiments, however, that ongoing prenylation of Ras is not required for IL-3-dependent cell growth.

The implication of the current results is that Fmev might be an effective and selective inhibitor of the proliferation of malignant cells. Therapy of Ras-transformed and other malignant cells by Fmev-mediated inhibition of Ras prenylation and accumulation of mevalonate-derived inhibitors may be possible since normal cells are spared from comparable inhibitory effects. Lovastatin, like Fmev, blocks proliferation of cells by a mechanism separate from its ability to inhibit synthesis of cholesterol (26, 41, 51). However, unlike Fmev, these additional effects of lovastatin, resulting from inhibition of mevalonate synthesis and prevention of the synthesis of a necessary non-sterol product, limit its potential ability to inhibit the growth of Ras-transformed cells selectively since all cells are similarly affected. Although the non-sterol product has not been identified, available evidence indicates that the product is not prenylated Ras (52) but rather is derived directly from mevalonate or the mevalonate phosphates (26). Thus, lovastatin inhibited the proliferation of NIH 3T3 cells transformed with either prenylated Ras or myristoylated Ras, demonstrating that, although Ras was associated with the plasma membrane by myristoylation, lovastatin remained capable of suppressing growth (52). High concentrations ofLovastatin (50 μM) inhibited the proliferation of FDC-P1 cells, whereas concentrations of Fmev that were equally effective at blocking the synthesis of cholesterol from the precursor acetate did not suppress FDC-P1 cell growth if accumulation of mevalonate-derived inhibitors was prevented (see above). This observation further supports the contention that the essential, non-sterol mevalonate product is synthesized from mevalonate or mevalonate phosphate(s).

In summary, selective inhibition of the proliferation of Ras-transformed cells may be achieved by suppressing mevalonate metabolism with Fmev. In addition to preventing membrane association of oncogenic Ras by blocking protein prenylation, Fmev depletes total cellular Ras and thus interferes with cell growth by two Ras-dependent mechanisms. In contrast, farnesyltransferase inhibitors prevent prenylation of Ras but do not deplete cellular Ras. Fmev also inhibits the proliferation of cells with poorly regulated HMG CoA reductase activity and consequent enhanced mevalonate synthesis by causing the accumulation of inhibitory products derived from mevalonate or mevalonate phosphate(s). Since HMG CoA reductase activity is tightly regulated in normal cells, this effect of Fmev is also selective for transformed cells. Farnesyltransferase inhibitors have no such effect, since only farnesylation of proteins is blocked by these compounds. Finally, Fmev blocks the synthesis of cholesterol. In malignant cells with defects in the use of exogenously supplied cholesterol in plasma lipoproteins (40), Fmev but not farnesyltransferase inhibitors would interfere with membrane synthesis and cell growth. Once again, normal cells are protected from the effects of Fmev by the presence of lipoprotein receptors that permit uptake of plasma lipoproteins and release of cholesterol needed for cell proliferation. Fmev may, therefore, be useful in therapy and offer advantages compared to farnesyltransferase inhibitors in preventing the uncontrolled growth of malignant cells.

ACKNOWLEDGMENTS

We thank Dr. H. Scott Boswell for kindly providing FDC-P1 and RasDC cells, Lan Nguyen and Angela Diem for excellent technical assistance, and Dr. Maureen Howard for generously furnishing recombinant murine IL-3.
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Suppression of the Proliferation of Ras-transformed Cells by Fluoromevalonate, an Inhibitor of Mevalonate Metabolism

Jennifer A. Cuthbert and Peter E. Lipsky


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