Inhibition of Cell Growth by Lovastatin Is Independent of ras Function

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ABSTRACT

We have investigated the inhibition of cell growth by lovastatin (previously known as mevinolin), an antagonist of hydroxymethylglutaryl coenzyme A reductase which blocks the processing and membrane localization of ras proteins via inhibition of polyisoprenylation. A series of NIH 3T3 cells transformed by oncogenes with activities that are dependent or independent of isoprenylated ras were studied, including cells transformed by myristylated ras protein that is isoprenylation independent. Treatment with lovastatin at concentrations ranging from 5 to 15 μM for up to 96 h resulted in a time- and dose-dependent inhibition of cell growth in all lines tested. The inhibition ranged from 25 to 50% when cells were treated with 5 μMLovastatin for 48 h, to 72–90% for cells treated with 15 μMlovastatin for 96 h. Cells transformed by v-ras, v-src, v-raf, and the myristylated ras genes displayed similar sensitivities; the parental NIH 3T3 line was the most resistant of the lines tested. Metabolic labeling of control and lovastatin-treated cells with [3H]mevalonate or tritiated lipids revealed that 15 μMLovastatin blocked the processing of both endogenous ras and v-ras proteins yet had no effect on the lipidation of myristylated ras proteins. Addition of 300 μM mevalonic acid overcame the inhibition induced by 15 μMlovastatin. Thus the inhibition of cell growth in vitro by lovastatin did not show specificity for cells the transformation of which is dependent upon isoprenylated ras protein. It is therefore likely that the inhibition of other pathways affected by lovastatin, such as cholesterol biosynthesis or the processing of other cellular proteins, are responsible for the growth inhibition by lovastatin.

INTRODUCTION

The ras group of genes, which are a highly conserved subfamily of the small GTPases, encode closely related M, 21,000 protein products (p21s) (1, 2). In addition to serving an important function in the control of normal eukaryotic growth and proliferation, mutated versions of ras proteins have been found in a wide variety of human and animal tumor systems (3). Studies in which neutralizing antibodies to ras proteins have been microinjected into NIH 3T3 cells have shown that mitogenic stimulation, and a few tumor cell lines without activated ras are still inhibited to the same degree by the antibody as are tumor lines with activated ras genes (5). Thus some human tumor cells can be described as ras dependent, although their genetic lesion(s) lie(s) outside of the ras genes.

A critical requirement for ras function is the localization of ras proteins to the cell plasma membrane from their initial synthesis in the cytoplasm (6). Membrane association occurs following posttranslational modifications that take place at the COOH terminus and include the covalent addition of lipid (7). Genetic studies indicated that the last four amino acids, especially an invariant cysteine, were required for posttranslational changes and membrane localization (8). Although it was originally believed that palmitate acid represented the obligatory lipid, it has recently been shown that palmitylation is not an obligatory step for some ras proteins. Rather, the acylation of ras proteins involves the addition of a polyisoprenoid derivative of MVA,1 which is an intermediate in the sterol-biosynthetic pathway (9–11). The polyisoprenoid is probably a farnesyl side chain (11). Following translation of p21 ras, the farnesyl moiety is apparently added to the invariant cysteine residue, which is located four amino acids from the COOH-terminus; the three amino acids downstream of this cysteine are removed; and the carboxyl group of the farnesylated cysteine is methylated (12). At this point, cysteine residue(s) NH2-terminal to the farnesylated cysteine, if present, may be palmitoylated. This process of processing steps results in an increasingly hydrophobic COOH-terminal membrane anchor for the ras proteins.

The demonstration that proper processing of both normal and transforming ras proteins is dependent on the availability of mevalonic acid-derived products has led to speculation that drugs such as compactin and lovastatin, which block the production of MVA by inhibiting the rate-limiting enzyme HMG-CoA reductase (13), may prove to be useful as chemotherapeutic agents against ras-dependent tumors (9, 14). However, since HMG-CoA reductase lies far upstream of the protein farnesylation step, blocking this enzyme may also interfere with other critical cellular processes (15). Furthermore, protein isoprenylation appears to be a common modification; in addition to ras, a large number of other proteins are labeled by MVA-derived compounds (16–18). Thus the question of specificity arises as one of critical importance in assessing the potential efficacy of lovastatin in treating ras-dependent tumors.

Using a series of cell lines transformed by defined oncogenes, the current study has sought to determine if lovastatin may have greater activity against ras-dependent cell lines than against ras-independent lines. Our in vitro studies demonstrate that under the culture conditions used here, the inhibition of cell growth by lovastatin was not selective for polyisoprenylated ras-dependent cell lines, which implies that the growth effects of lovastatin in these lines are not mediated principally through its inhibition of ras protein function.

MATERIALS AND METHODS

Construction of Gene Encoding a Myristylated ras Protein. A double-stranded oligonucleotide encoding the first 15 amino acids of the v-src protein and the first four amino acids of ras was synthesized (sense strand: 5'-GGCCACCATTTGAGTGACAGGAAGCAGCTATTGGAGCCCAACTGCGGATGCAGAGATACGACCACTATGACG-3'). For the annealed double-stranded oligonucleotide, the 5' end encodes a SacII site overhang, and the 3' end a HindIII site overhang; the HindIII occurs naturally at amino acid 4 of v-ras1. The main portion of the ras gene was derived from a HindIII/XhoI fragment from the plasmid pBW1225. This fragment encodes amino acids 4–189 of the v-ras1 protein including a cysteine-to-serine mutation at amino acid 186 which blocks the COOH-terminal posttranslational processing of ras protein. These two fragments were inserted into a derivative of the expression

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1 The abbreviations used are: MVA, mevalonic acid; HMG-CoA, hydroxymethylglutaryl coenzyme A; D-MEM, Dulbecco’s modified Eagle’s medium.
vector pGV16 (19), pBW1160 (20), by ligation to vector linearized by double digestion with SacI and XhoI, which each cleave pBW1160 once. The resulting plasmid is designated pJDCl06.

Derivation and Culture of Cell Lines. Morphological transformation was induced by calcium phosphate transfection of the NIH 3T3 (clone 7) line with the following plasmids; pBW1423 for v-ra\(^2\); pBW1631 for c-ra\(^3\) (21); pJDCl2 (22) for v-src; pJDCl106 for the myristylated ras; and MSV3611 proviral DNA for v-raf (23). With the exception of the v-ra plasmid, each of the above plasmids contains neo as a linked selectable marker. All cell lines except the v-ra line were generated by transfection of colonies with cloning cylinders, and expansion into cell lines. The v-ra\(^-\) line was generated by cloning an individual focus of transformed cells and expansion into a cell line. Cells were cultured in D-MEM supplemented with 10% fetal bovine serum (GIBCO) and 0.6 μM G418 where required.

Growth Inhibition Assays. On day 0, cells were trypsinized and plated at 10\(^4\)/well in 8 triplicate sets in 24-well plates (Costar). On day 3, cells were fed with fresh medium containing either 0, 5, 10, or 15 μM lovastatin. After 48 h (day 5), one triplicate set of each concentration was trypsinized and counted in a Coulter Counter. The other triplicate sets were fed fresh medium with or without lovastatin at this time. Two days later (96 h total treatment) the other triplicate sets were trypsinized and counted. For experiments in which MVA was included, 300 μM MVA was added together with 15 μMLovastatin at both day 3 and day 5.

Preparation of Lovastatin. Lovastatin was generously provided by Alfred Alberts of Merck, Sharp & Dohme Co. To convert the inactive lactone form of lovastatin to the active form, the drug was dissolved in ethanol, heated at 50°C in 0.1 N NaOH, neutralized with HCl, and stored unfiltered as a 4 mg/ml stock at -20°C.

Metabolic Labeling and Immunoprecipitation of ras Proteins. Cells were plated at a density of 2 x 10\(^4\)/75-cm\(^2\) flask (Costar). Labeling with \(^{35}\)S-methionine was carried out in 2 ml of methionine-free D-MEM (Flow Laboratories) supplemented with 10% regular D-MEM, 2% fetal bovine serum, and 200 μCi/ml \(^{35}\)S-Trans-label (ICN). Labeling with \(^{3}H\)myristate or \(^{3}H\)palmitate (NEN-Dupont) was carried out in regular medium with 10% fetal bovine serum for 6 h with 0.5 and 2.0 mCi/ml, respectively. For the labeling experiments, lovastatin treatment (15 μM) was for 24 h prior to cell lysis, including the labeling period.

Cells were lysed as described (24). For analysis of \(^3\)S-labeled cells, lysates containing equal numbers of acid-precipitable counts were analyzed. The entire lysate from one flask was analyzed for the \(^3\)H-labeled cells. Lysates were immunoprecipitated with the ras-specific monoclonal antibody Y13–259 (25). Following a 2-h antibody incubation, 60 μl of a 10% suspension of protein A-Sepharose coated with rabbit-anti rat IgG were added for 45 min. The immunoprecipitates were washed 4 times with wash buffer as described (24), boiled in sodium dodecyl sulfate sample buffer, and loaded on 15% polyacrylamide gels.

RESULTS

Morphological Properties of Transformed Cell Lines. To compare the effect of lovastatin on cell lines that were transformed by oncogenes that are ras dependent or ras independent, NIH 3T3 cells, which require normal (c-ras) function for passage through the cell cycle, were transformed by a variety of oncogenes and expanded as cell lines. For lines that would be dependent upon exogenous ras, cells were transformed by mutationally activated v-ras or by overexpression of c-ras. We also took advantage of the ability of ras protein to be transforming when its membrane association is driven by myristylation at its NH\(_2\) terminus in the absence of COOH-terminal posttranslational changes by inducing transformation with a nonfarnesylated, myristylated transforming ras protein the COOH terminus of which had been mutated to prevent posttranslational processing (26, 27). Since the transforming activity of the myristylated ras protein is independent of farnesylation, the effects of lovastatin on these transformants should distinguish those of lovastatin in specifically blocking farnesylation of ras protein from its other effects in ras-transformed cells. Cells were also transformed with v-src and v-raf, which are ras dependent and ras independent, respectively (4).

Fig. 1 represents photomicrographs of the cell lines. The parental NIH 3T3 line exhibits a flat, nonrefractile morphology, with fully contact-inhibited growth. In contrast, the v-ra transformed cells have an elongated, refractile appearance and tend to pile on top of each other, as do the v-src-transformed cells, although they have a rounded, refractile morphology. The c-ras, v-ras, and myristylated ras (myr-ras) transformed cells display a morphology somewhat in between the v-ra- and the v-src-transformed cells. Some of the cells are elongated, while others are rounded (especially for v-ras), and all the ras-transformed lines lack contact inhibition and pile up extensively. Thus all of the oncogenes produce alterations in cell morphology and a loss of monolayer-restricted growth in NIH 3T3 cells. In addition, the growth rates of the oncogene-transformed cells exceed that of the parental line (data not shown).

Growth Inhibition by Lovastatin. To assess their relative sensitivity to lovastatin, the cell lines were treated with 5, 10, and 15 μMlovastatin for up to 96 h. This concentration range has been shown previously to inhibit the growth of cultured cell lines, including NIH 3T3. As noted by others, a dramatic, dose-dependent rounding of the cells was observed after 24 h lovastatin treatment (28). This morphological effect, which was especially pronounced at 15 μMlovastatin, was similar in the untransformed parental line and in the transformed lines (not shown).

Following 48 h treatment with lovastatin, cell growth was inhibited by 25–45% for 5 μM treatment and by 40–65% for 15 μM treatment (Fig. 2A). All lines tested displayed a dose-dependent inhibition over the concentration range tested. Furthermore, the degrees of inhibition of the ras-transformed cell lines, including two independently derived myr-ras transformants, were virtually identical and within 10% of the inhibition seen for the v-src- and the v-ras-transformed lines. Only the parental NIH 3T3 line differed significantly in its response to lovastatin; the inhibition of this line was less than for any of the transformed lines (Fig. 2A). Ninety-six h of treatment of the cell lines with the same concentration range of lovastatin also resulted in a similar pattern of inhibition that was dose dependent for each line. At this time point, the inhibition of the v-src, v-ras, and myr-ras lines was almost identical (Fig. 2B), while the c-ras and v-ra lines were somewhat less inhibited, and again, the NIH 3T3 line was the least inhibited of the lines tested. Furthermore, the inhibition of every line, at each concentration, was greater at 96 h of treatment than at 48 h, although the extent of inhibition and the variation among the lines were greater at 96 h than after 48 h of treatment (Fig. 2B). At 5 μM the inhibition ranged from 40 to 80%, while at 15 μM the range was from 70 to 90%. Thus growth inhibition in these studies was both time and concentration dependent.

Lovastatin Blocks Processing of ras Proteins. To confirm that lovastatin was inhibiting the processing of the authentic (non-myristylated) endogenous and exogenous ras proteins, cells were labeled with \(^{35}\)S-methionine, \(^{3}H\)myristate, or \(^{3}H\)palmitate, in the presence or absence of lovastatin, and lysates of cell extracts were immunoprecipitated with the anti-ras monoclonal antibody Y13–259 (Fig. 3). In the absence of lovastatin, the ras proteins detected under these conditions represent fully processed forms, which migrate approximately 2–3 kDa faster than an unprocessed primary ras translation product. Normal NIH
LOVASTATIN-INHIBITED CELL GROWTH AND ras FUNCTION

Fig. 1. Phase-contrast photomicrographs of the cell lines used in this study. A, normal NIH 3T3; B, v-ras-transformed line; C, v-src-transformed line; D, c-ras-transformed line; E, v-ras-transformed line; F, myr-ras-transformed line 4-3; G, myr-ras-transformed line 4-4. Cells were plated at 8 x 10^4/60-mm dish 2 days prior to photomicroscopy. Original magnification, ×85.

3T3 cells labeled with [35S]methionine exhibited a low level of endogenous ras proteins at M, 21,000–22,000 (Fig. 3A, Lane 1). As expected, the v-ras protein migrated as a doublet (29); the upper band represents the viral protein that is phosphorylated at Thr-59 and the lower band represents the nonphosphorylated form (Fig. 3A, Lane 2). The two myr-ras lines, since they also contain Thr-59, express a doublet of M, 26,000 and 28,000; their slower migration rate results from the additional 15 amino acids at their NH2 terminus and their lack of COOH terminal processing (Fig. 3A, Lanes 3 and 4). In the presence of 15 μM lovastatin, the endogenous ras doublet was replaced by a single band (M, 22,000) (Fig. 3A, Lanes 5 and 6), while both the phosphorylated and nonphosphorylated v-ras proteins migrated approximately 2 kDa slower, indicating a lack of farnesylation, proteolytic cleavage, and carboxymethylation. In contrast, no effect on the migration of the myr-ras protein was seen (compare Lanes 4 and 6). We conclude that lovastatin has blocked the posttranslational modifications of newly synthesized c-ras and v-ras proteins but has not altered the myr-ras proteins.

When the cells were labeled with [3H]myristate (Fig. 3B), as expected no label was incorporated into the endogenous ras or the v-ras proteins (Fig. 3B, Lanes 1 and 2). The myr-ras proteins did, however, incorporate label in the presence or absence of lovastatin, indicating that they were myristylated, and no change in the migration of myr-ras protein was seen in the lovastatin-treated cells (Fig. 3B, Lanes 3–5). The slight reduction in label in Lane 5 compared to Lane 4 probably reflects the smaller number of cells in the flasks treated with lovastatin.

Labeling of cells in the presence or absence of lovastatin showed that lovastatin blocked the processing of newly synthesized endogenous ras and v-ras proteins, but no effect was noted on the processing of the myr-ras proteins. The cycling of palmitate on and off previously processed ras proteins continued in the presence of lovastatin, as shown by metabolic labeling with [3H]palmitate (data not shown).

Mevalonic Acid Overcomes the Effects of Lovastatin. Since lovastatin blocks the enzyme HMG-CoA reductase, which converts HMG to MVA, MVA can overcome effects of lovastatin that are due to its inhibition of HMG-CoA reductase. Repre-
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Fig. 2. Inhibition of cell growth by 48- or 96-h lovastatin treatment. In A, cells were treated for 48 h with lovastatin at the concentrations indicated, and the number of cells obtained were presented as the percentage of untreated controls. In B, cells were treated for 96 h.

Representative lines were therefore treated with lovastatin in the presence of MVA to determine the extent to which the effects of lovastatin on the cell lines resulted from HMG-CoA reductase inhibition (Fig. 4). When 300 μM MVA was added together with 15 μM lovastatin, cell growth was restored to within 15% of untreated controls, at both 48 and 96 h. In addition, no cell rounding was observed for cells treated simultaneously with MVA and lovastatin (data not shown). We conclude that the major effects of lovastatin on the cell lines depend upon its inhibition of HMG-CoA reductase.

DISCUSSION

Based on the recent observation that proper processing of ras proteins depends on products derived from the sterol-biosynthetic pathway, we have here investigated the possibility that the compound lovastatin, which blocks the rate-limiting step in this pathway, may specifically inhibit the growth of cells which are dependent on ras function for their growth. Our results demonstrated, in accordance with earlier reports (9–11), that under the in vitro culture conditions used here, lovastatin blocked the processing of both endogenous ras and v-ras proteins. As expected, no effect was seen by lovastatin on the processing of a control transforming ras protein that became membrane associated via myristylation, which is a form of acylation that does not depend on the sterol pathway. Despite these biochemical differences, lovastatin induced similar, dose-dependent, biological changes (morphological changes and growth inhibition) in transformed lines, regardless of whether the transforming ras protein was prenylated. A similar degree of growth inhibition was also observed for lines transformed by v-src and v-raf, although transformation by v-raf has been shown to be independent of endogenous ras. Since it has been shown that transforming ras genes can provide the essential function of endogenous ras (30), and the myristylated ras gene should also provide this function, it is unlikely that the lack of specificity for growth inhibition seen here is due to the inhibi-
by this compound may be overly optimistic, although it remains possible that specificity might be seen under different in vitro culture conditions or in tumorigenicity studies carried out in animals.

The lack of specificity by lovastatin demonstrated here should not restrict further attempts to pharmacologically block the membrane localization of ras protein. However, it may be more appropriate to focus on targets downstream from HMG-CoA reductase which are more directly involved in the posttranslational processing of ras, such as the farnesylation step itself (35). Even such inhibitors of downstream targets may also affect macromolecules in addition to ras. As antagonists of farnesylation, carboxymethylation, or related events are identified, their degree of specificity for ras transformation may be readily evaluated by comparison with their ability to inhibit myr-ras transformed cells.

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