Cell cycle-specific effects of lovastatin

(mevalonic acid/p21^{ras} farnesylation/G₁ arrest/Ki-67 antibody/proliferation-associated proteins)

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ABSTRACT Lovastatin (LOV), the drug recently introduced to treat hypercholesterolemia, inhibits the synthesis of mevalonic acid. The effects of LOV on the cell cycle progression of the human bladder carcinoma T24 cell line expressing activated p21^{ras} were investigated. At a concentration of 2–10 μ M, LOV arrested cells in G₁ and also prolonged—or arrested a minor fraction of cells in—the G₂ phase of the cell cycle; at a concentration of 50 μ M, LOV was cytotoxic. The cytostatic effects were reversed by addition of exogenous mevalonate. Cells arrested in the cycle by LOV were viable for up to 72 hr and did not show any changes in RNA or protein content or chromatin condensation, which would be typical of either unbalanced growth or deep quiescence. The expression of the proliferation-associated nuclear proteins Ki-67 and p105 in these cells was reduced by up to 72% and 74%, respectively, compared with exponentially growing control cells. After removal of LOV, the cells resumed progression through the cycle; they entered S phase asynchronously after a lag of $\approx$ 6 hr. Because mevalonate is essential for the posttranslational modification (isoprenylation) of p21^{ras}, which in turn allows this protein to become attached to the cell membrane, the data suggest that the LOV-induced G₁ arrest may be a consequence of the loss of the signal transduction capacity of p21^{ras}. Indeed, while exposure of cells to LOV had no effect on the cellular content of p21^{ras} (detected immunocytochemically), it altered the intracellular location of this protein, causing its dissociation from the cell membrane and translocation toward the cytoplasm and nucleus. However, it is also possible that inhibition of isoprenylation of proteins other than p21^{ras} (e.g., nuclear lamins) by LOV may be responsible for the observed suppression of growth of T24 cells.

The drug lovastatin (LOV) was introduced to the clinic nearly 3 years ago to treat hypercholesterolemia. It suppresses synthesis of mevalonic acid by blocking activity of 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.88). However, because mevalonic acid is also in the pathway of posttranslational modification of proteins that involve attachment of thioether-linked prenyl groups (isoprenylations) such as farnesylation (1), LOV, in addition to its anticholesterol activity, is also an inhibitor of these modifications.

Recent evidence indicates that isoprenylation of the *ras* oncogene-encoded protein (p21^{ras}) is an essential step enabling this protein to anchor to the cell membrane (2, 3). Because the signal transduction activity of p21^{ras}, which is associated with its regulatory role in the cell cycle, requires its membrane attachment, suppression of the isoprenylation is expected to interfere with the cellular function of this protein. We have presently studied the effects of LOV on the cell cycle of the human bladder carcinoma T24 cell line. These cells express the activated form of p21^{ras} resulting from a single point mutation of this oncogene (4, 5). Since the cultures were maintained in the presence of fetal calf serum,

which is an exogenous source of cholesterol for the cells (6, 7), the observed effects of LOV could be attributed to its activity in suppressing isoprenylation of proteins rather than interruption of cholesterol synthesis.

MATERIALS AND METHODS

T24 cells, obtained from the American Type Culture Collection were maintained as monolayer cultures in RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μ g/ml), and 10% fetal bovine serum (GIBCO). To maintain exponential growth, cultures were trypsinized and split at a ratio of 1:10 twice a week prior to each experiment.

LOV, in the inactive lactone form, was kindly provided by Alfred W. Alberts (Merck, Sharp & Dohme Research Laboratories, Rahway, NJ). It was converted to the active form by dissolving 40 mg of the lactone in 1 ml of 100% ethanol, adding 1.5 ml of 0.1 M NaOH, heating at 50°C for 2 hr, neutralizing the solution with 0.1 M HCl to pH 7.2, and adjusting with distilled water to a volume of 20 ml. Aliquots of this stock solution (5 mM) were stored frozen.

Several staining techniques were applied to quantify different cell constituents. To stain cellular DNA and protein, 0.2-ml aliquots of cell suspensions ($\approx$ 2–5 $\times$ 10⁵ cells) were admixed with 2 ml of a solution containing 4',6'-diamidino-2-phenylindole (DAPI; 2 μ g/ml; kindly provided by Jan Kapuscinski of the New York Medical College), sulforhodamine 101 (10 μ g/ml; Kodak), 0.1% Triton X-100 (Sigma), 10 mM Pipes (Calbiochem), 0.1 M NaCl, and 2 mM MgCl₂ (pH 6.8) on ice. Other details of the procedure are given elsewhere (8, 9).

Simultaneous staining of DNA and RNA by acridine orange (AO) has been described (10). Briefly, the staining of the permeabilized cells was done at an AO (Polysciences) concentration of 20 μ M and a dye-to-DNA-P molar ratio >2.0 in the presence of EDTA. Under these conditions, interaction of AO with DNA results in green fluorescence with maximum emission at 530 nm, whereas binding to RNA gives red luminescence with maximum emission at 640 nm. Intensities of green and red luminescence correlate with DNA and RNA content, respectively (10).

Denaturation of DNA *in situ* by acid has been described (11). Briefly, cells fixed in 80% ethanol were resuspended in Hanks' salt solution, incubated with RNase A (Worthington), then treated with 0.1 M HCl, and stained with 20 μ M AO at pH 2.6. The red and green luminescence of cells thus stained represent the stainability of denatured and double-stranded DNA, respectively (11).

The detection of the proliferation-associated nuclear antigens Ki-67 (12, 13) and p105 (14, 15) was carried out by using the monoclonal antibody provided by Accurate Chemical and Scientific Corp. (Westbury, NY) and the 780-8 antibody from

Table 1. Effect of LOV on proliferation and viability of T24 cells

LOV, μ M	Duration of treatment, hr							
	0		24		48		72	
	P	V	P	V	P	V	P	V
0	1.0	99	2.9	99	6.4	99	16.8	99
2	1.0	99	1.4	99	3.6	98	6.5	99
10	1.0	99	1.9	99	2.6	95	3.1	93
50	1.0	99	2.1	99	2.6	66	1.9	11

Exponentially growing T24 cells were treated at 0 time with 2–50 μ M LOV. The proliferation index (P) is expressed as the multiplicity of the cell number after 24–72 hr, in relation to the cell number in cultures at 0 time. The viability index (V) represents the percentage of the cells excluding trypan blue.

Techniclone International (Tustin, CA), respectively. p21^{ras} was detected by using monoclonal antibody RAS 10 provided by DuPont. The cells, fixed briefly with 0.5% paraformaldehyde and then permeabilized with 0.1% Triton X-100 (15), were incubated with the primary (Ki-67, p105, or p21^{ras}) antibody for 30 min at room temperature and, after rinsing, with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) for 30 min, also at room temperature. Control cells were incubated with the secondary antibody only. DNA was counterstained with propidium iodide (20 μ g/ml; Polysciences) after removal of RNA with RNase A (Worthington).

Fluorescence of cells stained with DAPI or AO was measured with an ICP-22A flow cytometer (Ortho Diagnostics). In the case of cells stained with DAPI and sulforhodamine 101, the UG1 excitation filter and emission bandpass filter transmitting between 450 and 520 nm were used. Luminescence of cells stained with AO was measured

by using a BG-12 excitation filter and a combination of filters and dichroic mirrors to separate the green (515–560 nm) and red (>630 nm) components.

The green fluorescence of cells stained with the fluorescein-labeled secondary antibody for detection of Ki-67, p105, or p21^{ras} and red propidium fluorescence of DNA were measured using a Cytofluorograf II (Ortho Diagnostics) with an argon-ion laser tuned to 488 nm as the source of excitation. The fluorescence emissions of these two dyes were optically separated and measured by separate photomultipliers. Approximately 20,000 cells were measured for each sample. The data were stored and analyzed with a Compaq Deskpro 386 computer and software (ACQUICYTE) developed by Phoenix Flow Systems (San Diego, CA). The proportion of cells in particular phases of the cell cycle was estimated using MULTICYCLE and MULTI-2D programs (Phoenix Flow Systems). All experiments were repeated at least twice.

RESULTS

Proliferation of T24 cells was suppressed in cultures containing LOV (Table 1). This effect was evident at 2 μ M LOV and was concentration dependent. At a concentration of 10 μ M, in the absence of any significant cell death, cells reached a plateau in growth by 24 hr. LOV was clearly cytotoxic at 50 μ M; significant cell death was seen after 48 hr, and nearly all cells were dead after 3 days.

The cell cycle distribution in cultures exposed to a cytostatic concentration of LOV was unchanged during the initial 6 hr of treatment. However, as the time of exposure to LOV increased, the proportion of cells in S phase progressively decreased concomitant with an increase in G₁-phase cells. The percentage of cells in G₂ plus M remained rather constant throughout the treatment (Fig. 1 and Table 2). The effect of

FIG. 1. The cell cycle distribution of T24 cells growing in the absence (A) or presence of 10 μ M LOV for 24 (B) or 48 (C) hr.

Table 2. Effect of LOV alone and in combination with mevalonate on the cell cycle distribution of T24 cells

LOV, μ M	Mevalonate, μ M	Treatment, hr	% cells		
			G ₁	S	G ₂ + M
0	0	—	43 $\pm$ 3	39 $\pm$ 3	17 $\pm$ 3
0.4	0	18	57	29	14
2.0	0	18	68	14	18
10.0	0	6	41	44	15
10.0	0	24	77 $\pm$ 3	8 $\pm$ 4	15 $\pm$ 3
10.0	0	48	82 $\pm$ 4	4 $\pm$ 2	14 $\pm$ 6
10.0	8	24	77	7	16
10.0	40	24	63	21	16
10.0	200	24	53	33	14

The data are the percentage (mean values of duplicate cultures $\pm$ SD if more than two cultures per point were analyzed) of the cells in the G₁, S, and G₂ plus M phases of the cell cycle in cultures treated with LOV alone and with LOV plus mevalonate. LOV or LOV plus mevalonate were added at 0 time, and the cells were assayed after 24 hr.

LOV in reducing the proportion of S-phase cells was very pronounced. For example, in cultures exposed to 10 μ M LOV for 24 hr, the number of cells in S phase decreased from 39% to 8% and after 48 hr to 4%. Addition of exogenous mevalonate prevented the cytostatic effects of LOV in a dose-dependent manner (Table 2).

The cells arrested in G₁ were able to enter S phase after removal of LOV from the cultures (Fig. 2). The first cells entering S phase were seen 6 hr after drug removal. The entrance to S phase was rather asynchronous, and the number of cells in S progressively increased between 6 and 20 hr. The highest rate of entrance to S was observed between 12 and 18 hr. The number of cells in S peaked at the 20th hour after drug removal (58%) and then started to decrease, concomitant with an increase in the number of cells in G₂ plus M. After 24 hr of recovery time, relatively few cells remained in G₁ (18%).

Cells arrested in G₁ by LOV for up to 48 hr were characterized by an RNA and protein content similar to that of G₁ cells from exponentially growing cultures (Table 3). Also, the sensitivity of DNA *in situ* to denaturation in nuclei of cells arrested by LOV in G₁ was not changed compared with G₁ cells from the control cultures.

The LOV-induced cell arrest in G₁ was accompanied by a decrease in expression of the proliferation-associated nuclear proteins Ki-67 (Fig. 3) and p105 (Table 3). Both the proportion of cells expressing these proteins above the background

FIG. 2. Reversibility of arrest of T24 cells in G₁ by LOV. Exponentially growing cells were treated with 10 μ M LOV for 48 hr. The cells were then washed and cultured in absence of the drug (48-0). The cell cycle distribution was assayed 6-24 hr after removal of LOV.

Table 3. Changes in RNA and protein content, sensitivity of DNA *in situ* to denaturation (α_t), and changes in content of Ki-67, p105, and p21^{ras} proteins in T24 cells treated with 10 μ M LOV for 24 and 48 hr

Parameter	Cells	Cell treatment		
		LOV		
		None	24 hr	48 hr
RNA	All	42.4	38.3 (-10)	41.2 (-3)
	G ₁	36.0	33.6 (-7)	37.7 (+5)
Protein	All	81.0	75.2 (-9)	67.2 (-17)
	G ₁	64.6	57.3 (-11)	60.0 (-7)
α_t	All	0.37	ND	0.38 (+2)
	G ₁	0.39	ND	0.39
Ki-67	All	29.6	20.0 (-32)	8.1 (-72)
	G ₁	21.6	15.5 (-37)	6.6 (-69)
p105	All	24.1	14.0 (-42)	7.4 (-69)
	G ₁	23.0	12.5 (-46)	6.0 (-74)
p21 ^{ras}	All	34.8	ND	31.3 (-11)
	G ₁	27.1	ND	28.6 (+6)

The data represent mean fluorescence intensities of either all cells (G₁ + S + G₂ + M) or only cells in G₁ phase. The nonspecific component of cell fluorescence of cells stained with Ki-67, p105, or p21^{ras} antibodies (after treatment with secondary antibody only) has been subtracted. The numbers in parentheses indicate the percent change of the measured parameters in the LOV-treated cultures, compared with the respective controls. ND, not determined.

level and the mean fluorescence intensity of either all or only G₁ cells were reduced in cultures treated with LOV for 24-48 hr. In contrast, expression of p21^{ras}, detected immunocytochemically, was not changed in cells growing in the presence of LOV for up to 48 hr (Table 3).

Intracellular localization of p21^{ras}, however, was altered in cells treated with LOV, as shown in Fig. 4. Namely, a dissociation of the antigen detected by the p21^{ras} antibody, from the cell periphery toward inner portions of cytoplasm and nucleus, was evident in cells exposed to LOV.

DISCUSSION

The data show that LOV arrested T24 cells in the G₁ phase of the cell cycle. The arrest was pronounced after 18 hr but could not be seen during the initial 6 hr of exposure to the drug. Because the T24 cell doubling time is $\approx$ 18 hr and the duration of G₁ is 7 hr (estimated based on the growth curves and proportion of cells in the respective phases of the cell cycle), this would indicate that the cells that were already in G₁ at the time of addition of LOV completed progression through this phase. However, the cells that divided and entered G₁ in the presence of the drug became delayed in progression and were effectively prevented from initiating DNA replication.

LOV had little effect on the proportion of cells in G₂ plus M. Because few cells traversed S phase in the drug's presence, the rate of entrance to G₂ was decreased, and thus fewer cells were dividing (M cells; see the growth curves). The presence of a relatively large number of G₂ plus M cells therefore indicates that cell progression through G₂ was also delayed in the presence of LOV.

The cells arrested by LOV in G₁, even 48 hr after addition of the drug, had an RNA and protein content similar to that of the G₁ cells from exponentially growing cultures. There was no evidence, therefore, of unbalanced growth, which otherwise characterizes cells arrested in the cycle by most antitumor drugs (16). Also, there was no evidence of decreased RNA content, which would be typical of cell entrance to a deep quiescent state (10, 17). Likewise, the sensitivity of DNA *in situ* to denaturation, which correlates

FIG. 3. DNA content and expression of the proliferation-associated nuclear protein detected by the Ki-67 antibody in exponentially growing T24 cells (A) and in cells treated with 10 μ M LOV for 24 (B) and 48 (C) hr. The cells treated with secondary antibody have only minimal fluorescence (D). (Left) Bivariate cell distributions in the form of contour maps. (Right) Univariate distributions of Ki-67 of all cells.

with the degree of chromatin condensation and is generally increased when cells enter the G_0 state (11), was also unchanged. Considering these metabolic features, the cells arrested in G_1 by LOV were indistinguishable from cells normally traversing the early portion of G_1 in exponentially growing cultures. According to the subdivision of the cell cycle based on metabolic characteristics of the cells (18), the cells arrested by LOV can be classified as G_{1A} cells.

Expression of the nuclear antigen detected by the Ki-67 antibody was decreased in cells arrested by LOV almost to the background level. This antigen, which is generally expressed in cycling cells, but is minimal in noncycling cells (12, 13), is widely used as marker of the tumor growth fraction (e.g., ref. 19). The cellular function of the protein reacting with the Ki-67 antibody has not yet been revealed. The antigen has nucleolar localization, appears to be associated with the nuclear matrix (20), and possibly interacts with DNA polymerase. Interestingly, and in contrast to LOV, cell arrest

in the G_1 phase in subconfluent cultures or at the G_1/S interphase as a result of hydroxyurea treatment (21) does not lead to a decrease in Ki-67 expression.

Another proliferation-associated antigen, p105, is localized in interchromatin granules of the cell nucleus, and its expression is generally decreased in noncycling cells (14, 15). The level of p105 increases just prior to entrance to S phase and then rises again during S phase and the G_2 -to-M transition (14). Expression of this protein, similar to that of Ki-67 antigen, was markedly depressed in T24 cells arrested in the cycle by LOV (Table 3).

Addition of exogenous mevalonate prevented the LOV-induced G_1 arrest. In all probability, therefore, the observed effects of this drug on the cell cycle of T24 cells were mediated via suppression of mevalonate synthesis. The present data are thus in agreement with earlier observations showing that inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase suppress cell proliferation and arrest cells in G_1 (22–24). The early data on the role of mevalonate in the cell cycle have been discussed in the context of the suppression of cholesterol synthesis. Most cells, however, utilize exogenous cholesterol from the serum (6, 7). In more recent studies, therefore, the role of protein isoprenylation, in general, has also been considered (24). In light of the latest findings that farnesylation of p21^{ras} is essential for its attachment to the cell membrane (2, 3, 25) and the evidence that this protein plays a regulatory role in the cell cycle, primarily in G_1 (reviewed in ref. 26), these data call for a new interpretation. Namely, it is possible that the observed cell cycle effects (G_1 and G_2 arrest) induced by inhibitors of mevalonate synthesis are a direct consequence of an impairment of isoprenylation of p21^{ras}. However, in addition to p21^{ras}, nuclear lamins A and B (27, 28) as well as several other proteins (29) undergo isoprenylation in the cell. It is not known whether these proteins have any role in regulation of the cell cycle. If they have such a role, inhibition of their isoprenylation by LOV may also be the causative factor involved in the suppression of cell growth. This possibility should be considered especially in light of the most recent evidence (published while this paper was under review) that inhibition of cell growth by LOV was not selective for isoprenylated *ras*-dependent lines of NIH 3T3 cells (30).

Because it was possible to detect p21^{ras} immunocytochemically in the LOV-treated cells, the farnesyl moiety is not a part of the epitope detected by the antibody used. The content of p21^{ras} was unchanged in cells arrested by LOV, compared with the untreated control cells. Impaired isoprenylation of p21^{ras} therefore had no apparent effect on the synthesis or turnover of this protein. Localization of the antigen detected by p21^{ras} antibody, however, was altered by LOV, compatible with the possibility discussed above that impairment of farnesylation of this protein results in its inability to attach to the cell membrane and participate in signal transduction.

The effects of LOV were presently studied on human bladder carcinoma T24 cells, which are known to have activated *ras* (4, 5). Their malignant transformation is a consequence of a single point mutation in c-Ha-*ras*; the product (p21^{ras}) differs by three amino acid substitutions compared with the p21^{ras} of nontransformed cells (4, 5). None of the substitutions is at Cys-186, which is the site of farnesylation of p21^{ras}. The presumed effects of inhibition of isoprenylation of p21^{ras} therefore were evaluated on the transformed cells whose cell cycle control malfunctions at the point regulated by p21^{ras}. As is evident, the effects of LOV were dramatic, and nearly all cells were prevented from entering S phase. Thus, inhibition of mevalonate synthesis, regardless of whether induced in apparently normal, non-transformed muscle (31), 3T3 cells or fibroblasts (32), immortal but not malignant Chinese hamster ovary cells (23,

FIG. 4. Localization of the antigen detected by monoclonal antibody RAS 10 in exponentially growing control T24 cells (A) and in T24 cells growing for 24 hr in the presence of 10 μ M LOV (B). The cells were stained with the antibody in suspension (as described for detection of p21^{ras} by flow cytometry). Peripheral localization of the antigen in these spherical cells (A) is compatible with its presence on the cell membrane. Translocation of the antigen toward the center of the cell is seen in the LOV-treated cells (B).

24), malignant cells (32, 33), or as presently shown, transformed cells that have activated *c-ras*, invariably leads to cessation of cell proliferation and arrest in G₁. Differences in the sensitivity to inhibition of the mevalonate synthesis between particular cell types, however, may exist (30, 33).

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