Differences in the effects of HMG-CoA reductase inhibitors on proliferation and viability of smooth muscle cells in culture

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Received 7 December 1998; received in revised form 16 August 1999; accepted 8 September 1999

Abstract

We investigated the influence of lovastatin, simvastatin and pravastatin on proliferation and viability of vascular smooth muscle cells (SMC) in vitro and studied the effects ofLovastatin on a mouse SMC line transgenic for a temperature-sensitive mutant of SV40 large T antigen (TAg), known to inhibit the function of p53 and pRb family members. We found that lovastatin and simvastatin inhibited cell proliferation by provoking G0:G1 phase arrest with concomitant depression of the proliferation antigen Ki-67:MIB-1. Lovastatin at high concentrations of 20 μmol/l caused cell death in the presence of serum but not under serum starved conditions, which was verified on the basis of increased DNA strand breaks, decreased DNA content and morphological alterations seen by electron microscopy. Cell death was also found for simvastatin, whereas pravastatin did not exhibit antiproliferative or cytotoxic effects. Mouse SMC transgenic for TAg did not show any impaired sensitivity to the antiproliferative and cell death inducing effect ofLovastatin, but both effects could be antagonized by the supplementation of mevalonate. The data indicate that antiproliferative and cytotoxic effects ofLovastatin are caused by the using up of products of mevalonate metabolism and do not require the presence of p53 or pRb. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cell death; p53; Proliferation; Smooth muscle cells; SV40 large TAg

1. Introduction

Coronary restenosis following percutaneous transluminal coronary angioplasty is still a major limiter of long-term clinical outcome of patients with coronary artery disease [1]. Restenotic lesions arise from neointima formation due to migration and proliferation of smooth muscle cells (SMC) and deposition of extracellular matrix, but vascular remodeling is also supposed to play an important role [2,3]. Various growth factors have been identified as stimulators of SMC proliferation and are therefore considered to be causative factors for the initiation of restenosis [1,4]. Nevertheless the mechanisms of vascular renarrowing are still not fully understood, nor is there an effectual concept for the prophylaxis of restenosis.

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors have been discussed as potential compounds for preventing restenosis. Several clinical studies have shown various benefits of treatment with these substances, such as reduction of all-cause mortality [5–9], but contradictory findings have been obtained concerning the prevention of restenosis by HMG-CoA reductase inhibitors applied systemically [10,11]. The compounds are known to significantly inhibit progression of cells through the cell cycle by inhibiting the G1/S transition [12]. This step of the cell cycle is controlled by the complex interaction of cyclin-dependent kinases (Cdks) and cyclins, all of which have to be systematically expressed and activated for cell cycle progression. The Cdk predominantly involved at the
G1/S transition is Cdk2, the activity of which is physiologically controlled by Cdk inhibitors, among which p21 [13,14] and p27 [15] have been shown to be specifically upregulated by HMG-CoA reductase inhibitors such as lovastatin. However, periodic alterations of expression levels of Cdk inhibitors are also found during progression through the cell cycle. Cdk inhibitors can be induced by various factors. In response to DNA damage, p21 has been shown to be induced by p53, a sequence-specific transcription factor which can activate genes possessing p53 consensus sites. Induction of p53 can eventually lead to G1 phase arrest as well as to apoptosis; this has led to the model wherein activation of p53 triggers transcription from the WAF1/CIP1 gene, whose product p21 accumulates in the nucleus, titrates out the activity of critical Cdk/cyclin complexes, and eventually blocks cell cycle progression [16]. However, high levels of p21 also appear to be required to maintain another group of cell cycle negative regulatory factors, the family of retinoblastoma proteins (pRb), in a constitutively active state. The complexity of cell cycle control and programmed cell death may be mirrored by the fact that the two effects are not necessarily coupled and that apoptosis can also be induced by p53-independent pathways. The following study shows the effects of lovastatin, simvastatin and pravastatin on SMC proliferation and viability. To evaluate the involvement of cell cycle negative regulatory factors such as p53 and the pRb family proteins in the effects of the statins, we used an SMC line transgenic for SV40 large T antigen (TAg), a viral oncoprotein which binds and inactivates negative regulatory factors of the cell cycle, thereby allowing us to study the functional role of p53 and pRb family members in HMG-CoA reductase inhibitor signaling on a molecular level.

2. Materials and methods

2.1. Antibodies and cell death detection reagents

Anti-Cdk2 monoclonal mouse IgG2a antibody, anti-p53 monoclonal antibody, anti-Ki-67/MIB-1 antibody, fluorescein isothiocyanate (FITC) conjugated mouse IgG2a kappa immunoglobulin isotype control monoclonal antibody, and fluorescein conjugated AffiniPure F(ab)2 fragment of donkey anti-mouse IgG (H + L) were purchased from Dianova (Hamburg, Germany). Anti-Cdk2 rabbit polyclonal antibody was from Santa Cruz (CA). Horseradish peroxidase conjugated goat anti-rabbit IgG was obtained from BioRad (Hercules, CA). Cell death detection kit for the determination of terminal deoxynucleotidyl transferase mediated fluorescein deoxyuridine triphosphate nick end labeling of DNA strand breaks (TUNEL) was purchased from Boehringer (Mannheim, Germany).

2.2. Chemicals

Hank’s balanced solution, 0.05% trypsin, 17 mmol/l ethylenediaminetetraacetic acid and phosphate-buffered saline (PBS) were purchased from Seromed (Berlin, Germany). Lovastatin and simvastatin (Merck, Sharp and Dohme Research Laboratories, Woodbridge, NJ) and pravastatin (Bristol-Myers Squibb, Regensburg, Germany) were prepared as described elsewhere [12]. Platelet derived growth factor A/B (PDGF) was purchased from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade or the best grade available and were purchased from Boehringer (Mannheim, Germany), Serva (Heidelberg, Germany) and Merck (Darmstadt, Germany).

2.3. Cell culture

Experiments were performed on rat aortic and bovine aortic and coronary SMC in the exponential phase of growth. The cells were obtained from vascular sections by the explant technique. Identity was confirmed by immunostaining with anti-α-actin antibody. Human coronary SMC were obtained from Clonetics (Walkersville, MD, USA). SMC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% (human SMC) or 10% (rat and bovine SMC) (v/v) fetal calf serum (FCS; Boehringer Mannheim Biochemicals, Germany), 2 mmol/l-glutamine (Seromed, Berlin, Germany) and standard antibiotic conditions at 37°C in a humidified atmosphere of 5% CO₂. SMC were seeded at a density of 5–10 × 10⁵ cells/cm² cell culture flask (Nunc, Denmark) in 12 ml culture medium and grown to subconfluency for 24–48 h until lovastatin, simvastatin or pravastatin were added in concentrations of 1–20 μmol/l for another 24–72 h. Lactate dehydrogenase (LDH) levels in culture supernatants were determined by standard methods.

Mouse aortic SMC transgenic for a temperature-sensitive SV40 large TAg [17] ts A-58 mutation were maintained at the permissive temperature of 33°C. For experiments, SMC were seeded at a concentration of 2.5 × 10⁴ in 2 ml of culture medium into 35 mm dishes (Nunc, Denmark) in parallel for studies at 33°C and 39.5°C. For cells to be grown at the restrictive temperature, the dishes were shifted to 39.5°C at 12 h after seeding and maintained for 72 h until incubated with lovastatin (as the dishes at 33°C) for 48 h. The presence of TAg was confirmed by immunohistochemistry.

Cell synchronization was performed by seeding cells as indicated above, changing the media after 24 h to serum starved conditions of 0.4% FCS for 72 h before releasing them into cycling at media conditions of 10% FCS.
2.4. DNA synthesis

SMC were seeded at a concentration of 2.5 × 10^4 in 2 ml of culture medium into 35 mm dishes (Nunc, Denmark). HMG-CoA reductase inhibitors were added after 48 h at concentrations of 1–20 μmol/l as described above. Cells were cultured for another 12–168 h, after which [3H]thymidine (1.10 Tbq/mmol) (Amersham, UK) was added for 12 h. Cell preparation was performed by standard methods. Radioactivity was measured in liquid scintillation cocktails.

2.5. Staining of intracellular antigens, DNA strand breaks and DNA content for flow cytometry

Cells were fixed using formaldehyde and methanol as described recently [18]. Briefly, Cdk2 was indirectly stained using a monoclonal antibody in saturating concentrations diluted in PBS containing 0.1% BSA and 15 mmol/l sodium azide for 2.5 h at room temperature. Rat SMC were chosen for this stain since they showed the best signal to noise ratio. Cells were incubated with secondary antibody for 30 min at room temperature. FITC conjugated mouse IgG2a monoclonal antibody (non-immune antibody) was used as isotype control and secondary antibody alone was used as a control for background fluorescence. Ki-67/MIB-1 was directly stained using FITC conjugated antibodies. Cells were counterstained with 30 μmol/l propidium iodide (Di-anova, Hamburg, Germany) in the presence of 200 μg/ml RNAs (Boehringer Mannheim Biochemicals, Germany) for the determination of DNA content and for cell cycle analysis. Fluorescein deoxyuridine triphosphate nick end labeling of DNA strand breaks was performed according to the instructions provided by the manufacturer (Boehringer Mannheim, Germany).

The fluorescence intensity of 10,000 cells was determined using a FACScan flow cytometer with Lysys II software (Becton Dickinson, San Jose, CA, USA). Cell debris was excluded by dot plot analysis. Statistical analyses were performed using the built-in statistical program. Values were generated as median fluorescence of the sample minus median fluorescence of the isotype control or secondary antibody alone.

2.6. Immunoblot analysis

Cell solubilization was performed with lysis buffer containing 1% sodium dodecyl sulphate (SDS), 20 mmol/l Tris–HCl, 20 mmol/l dithiothreitol, 5 mmol/l bromophenol blue and 10% glycerol. Equal amounts (30 μg) of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (7.5% acrylamide), using buffers of Porzio and Pearson [19], and blotted onto nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Dassel, Germany) by standard methods [20]. After blotting, the membranes were stained with naphthol blue–black to assess the efficiency of transfer. Following blocking of non-specific binding by incubation in PBS, 1% Tween-20 and 5% dry milk for 4 h at room temperature, incubation with primary antibodies (polyclonal anti-Cdk2, monoclonal anti-p53) was performed for 2 h at room temperature. The antigens were revealed after incubation with horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature followed by a colorimetric reaction using a chemiluminescence system ECL™ (Amersham, UK).

2.7. Electron microscopy

Cells were washed with PBS before fixation in 200 mmol/l glutaraldehyde in PBS. Fixed cells were further processed for electron microscopic examination using standard embedding and sectioning procedures.

2.8. Statistical analysis

Data were analysed using Wilcoxon’s matched pairs signed rank test. Values are given as mean ± S.D. Linear regression analysis was performed by one-way ANOVA.

3. Experimental results

3.1. General drug effects, inhibition of cell proliferation and DNA synthesis in wild type SMC

Lovastatin and simvastatin—but not pravastatin—showed antiproliferative effects on vascular SMC in a dose-dependent manner. The effects were comparable for the various species (rat, bovine, human SMC) tested. Definitively subconfluent SMC monolayers were less sensitive to treatment with lovastatin or simvastatin than less confluent monolayers. For 5 μmol/l lovastatin after an incubation time of 48 h the reduction of cell counts ranged from 15 to 50% compared with untreated controls. In most of the cell lines tested, 5 μmol/l of lovastatin showed virtually no cytotoxic effects, but higher concentrations of the compound clearly affected cell viability in a dose-dependent manner, which was characterized by the detachment of cells from the culture flask and certain intracellular features as described below. When SMC monolayers grown for 48 h were definitively subconfluent at the time of incubation with the compound, up to 65% of cells treated with 20 μmol/l lovastatin for 48 h were detached from the cell culture flask. Less confluent cultures showed a higher sensitivity for cytotoxicity. Cell detachment and the antiproliferative effect were abolished by the addition of 100 μmol/l mevalonate. In equimolar concentrations,
the effect of simvastatin was 2- to 3-fold higher than that of lovastatin. Pravastatin, in contrast, did not exert antiproliferative and cytotoxic effects on SMC in concentrations up to 20 μmol/l tested, since it did not show a reduction in cell counts nor did it show cell detachment.

DNA synthesis was assessed by monitoring [3H]thymidine incorporation. Lovastatin, simvastatin and pravastatin were used at concentrations of 1–20 μmol/l for 12–168 h. Inhibition of DNA synthesis in adherent cells was observed to be dose-dependent for lovastatin and simvastatin. No inhibitory effect was found for pravastatin. Fig. 1 shows representative data of rat aortic SMC incubated with lovastatin. Values are found for pravastatin. After 48 h of incubation with the HMG-CoA reductase inhibitor, [3H]thymidine incorporation was virtually blocked by 20 μmol/l lovastatin, whereas 5 μmol/l of the compound caused a decrease of incorporation by 23%, and concentrations of up to 2.5 μmol/l lovastatin failed to show a clear inhibitory effect. After 168 h, DNA synthesis of SMC treated with 5 μmol/l lovastatin was slightly increased compared with controls, probably due to the fact that untreated cultures had reached full confluence. Similar results were obtained for human and bovine SMC.

Flow cytometric cell cycle analysis on unsynchronized SMC revealed that lovastatin and simvastatin caused a G0/G1 phase arrest. Rat aortic SMC treated with 5 μmol/l lovastatin for 24 h displayed a significantly (P < 0.05) increased portion of G0/G1 phase cells and diminished portion of S phase cells compared with untreated controls, whereas the portion of G2/M phase cells was comparable in both populations (Table 1).

The expression of the nuclear proliferation-associated antigen Ki-67/MIB-1 as a parameter of cell proliferation was evaluated by flow cytometry, allowing the identification of the subpopulation expressing the antigen on a single cell level. The subpopulation of bovine coronary SMC expressing Ki-67 made up about 20% of all cells and was suppressed by more than one third under the influence of 5 μmol/l lovastatin after an incubation time of 48 h (Fig. 2). The antiproliferative effect was found when the medium was supplemented with 10% FCS as well as under serum deficient conditions when cells were stimulated by 10 ng/ml PDGF. The effect of 2.5 μmol/l simvastatin was comparable to suppression by 5 μmol/l lovastatin.

### 3.2. Induction of cell death in wild-type SMC

To evaluate whether lovastatin is capable of inducing cell death in vascular SMC, we quantified DNA content, detected DNA strand breaks by the TUNEL reaction and performed electron microscopy on adherent and detached SMC exposed to the compound at concentrations up to 20 μmol/l. The cells were stained with propidium iodide for comparison of their DNA content and run on a FACScan using a doublet discrimination module. Fig. 3A shows typically the influence of 20 μmol/l lovastatin on DNA content in rat aortic SMC after 48 h of incubation with the compound. Untreated controls were used as reference. Detached cells consisted of subpopulations with normal DNA content (30% of cells) and those with decreased DNA content (70% of cells). Adherent cells under the influence of 20 μmol/l lovastatin and SMC exposed to 5 μmol/l lovastatin showed normal DNA content. Comparable data were found for human and bovine SMC.

Flow cytometric determination of DNA strand breaks by the TUNEL reaction revealed a significant increase in DNA fragmentation in detached SMC exposed...
posed to 20 μmol/l lovastatin for 24–72 h. For rat aortic SMC the mean fluorescence intensity was 6.96 ± 2.34 arbitrary units vs 3.33 ± 0.41 for untreated controls (n = 7, P < 0.05). Human SMC showed mean values of 9.28 ± 4.66 vs 5.81 ± 2.59, respectively (n = 5, P < 0.05). Adherent cells showed no increase in DNA strand breaks. Fig. 3B shows representative data obtained from rat aortic SMC exposed to 20 μmol/l lovastatin for 24 h and 48 h. At least 45% of detached cells yielded increased values of DNA strand breaks compared with controls.

We also examined treated SMC electron microscopically for signs of cell death. Many of the detached cells from cultures exposed to high concentrations of lovastatin for 48 h exhibited various degrees of margination and compaction of nuclear chromatin, the hallmark of apoptosis (Fig. 4). The cells contained large numbers of structured, electron dense and lucent cytoplasmic granules. Their boundaries appeared entire and the presence of microtubules vouched for the intactness of their cytoskeletons. On the other hand, necrotic cells with discontinuous plasma membranes and obvious loss of cytoplasmic structural integrity were also found among the detached cells. Adherent cells revealed no signs of cell death in the range of concentrations of the compound tested and these cells regained the ability to proliferate after withdrawal of the drug.

For evaluation of the amount of necrotic cells, unfixed detached SMC exposed to 20 μmol/l lovastatin were stained with propidium iodide to test for the intactness of the cell membranes. Up to 25% of detached cells after 24 h and more than 50% after 48 h treatment with lovastatin stained positively and thereby exhibited a typical sign of necrosis. The time course of cell death was also measured by determining LDH levels in culture supernatants. LDH levels started to increase distinctly after 48 h incubation with 20 μmol/l lovastatin. LDH levels did not rise when 100 μmol/l mevalonate was added to the media (data not shown). Cell death was also found for simvastatin but not for pravastatin.

We also tested cell viability under the influence of serum deprivation by means of DNA content measurements. Depriving bovine SMC of serum for 48 h resulted in cell cycle arrest, but not in cell detachment or reduction of DNA content. Interestingly, when 20 μmol/l lovastatin was added to growth arrested cultures for an incubation time of 48 h, no cell detachment was observed. Flow cytometric analysis revealed that the cells simply remained in their cell cycle arrested state but did not reveal a reduction of DNA content.
Fig. 3. (A) Flow cytometric quantification of DNA content. Rat aortic SMC were cultured as described in the legend to Fig. 1. Then 5 or 20 μmol/l lovastatin (lov.) was added for 48 h. SMC were permeabilized with methanol and stained with 30 μmol/l propidium iodide. (B) Flow cytometric quantification of DNA strand breaks as determined by the TUNEL reaction. Cells were prepared as described for (A) and treated with the compound for 24 or 48 h. The analysis was performed using a FACScan cytometer.

Fig. 4. (A) Electron micrograph of a bovine coronary SMC grown for 48 h under serum conditions as described in the legend to Fig. 1. (B) Representative electron micrograph of a detached bovine coronary SMC after 48 h of exposure to 20 μmol/l lovastatin, showing chromatin condensation, thickening of the nuclear membrane and cytoplasmic granules typical of apoptosis (× 10,000).
3.3. Cell cycle regulatory effects of HMG-CoA reductase inhibitors on p53 and Cdk2 expression

Since lovastatin is known to affect G1/S transition at least by the upregulation of Cdk inhibitor activity [13–15], we were interested in studying the quantitative regulation of p53 and the downstream kinase Cdk2. Flow cytometric analysis was used for the determination of Cdk2 expression in rat aortic SMC. After incubating unsynchronized cells for 24–48 h with 5 μmol/l lovastatin, we found a moderate but significant downregulation of Cdk2 levels compared with untreated controls. Treated cells revealed a mean fluorescence intensity of 6.01 ± 2.51 arbitrary units vs 7.92 ± 2.17 for untreated controls (n = 5, P < 0.05). Moderate downregulation of Cdk2 after 48 h of incubation with 5 μmol/l lovastatin was also found by immunoblotting performed on total cell lysates of bovine SMC. To evaluate the influence of the cell cycle distribution on Cdk2 expression, bovine SMC were synchronized and tested for Cdk2 levels and run for cell cycle analysis. Cells were seeded in the presence of 10% FCS before arresting them under serum starved conditions (0.4% FCS) for 72 h. The cells were then released into cycling by supplementing 10% FCS. Fig. 5 shows that resting cells expressed distinctively lower Cdk2 levels than cycling cells, and that SMC passing the cell cycle for the second time (36–48 h) expressed higher Cdk2 levels than those in first cell cycle passage (18–24 h). However, when correlating the Cdk2 levels with the cell cycle distribution, no change in Cdk2 levels could be detected between 18 h, when the majority of cells was in the G1 phase, and 24 h, when most cells were in the S phase. With regard to the influence of lovastatin on Cdk2 levels, a moderate downregulation could be detected after 48 h when compared with the untreated control as well as with the culture supplemented with lovastatin and its inhibitor mevalonate. Downregulation of Cdk2 under lovastatin could not be detected after 24 h, although at this time point the cell cycle arrest was already obvious.

We also studied the expression of p53 under the influence of lovastatin in the same cell cultures as depicted in Fig. 5. No increase in p53 levels could be demonstrated due to the treatment with 5 μmol/l lovastatin for all time points tested. To further evaluate this phenomenon, unsynchronized bovine SMC were incubated with 5 and 20 μmol/l lovastatin and harvested after 5, 8, 12 and 24 h incubation time. Again, no increase in p53 levels could be demonstrated (data not shown). Since the exact timing of the peak elevation of p53 levels might be a critical step, a transgenic cell line with functionally depleted p53 and pRb function was employed to study the lovastatin effects on cell proliferation and on cell death under these conditions.

3.4. Inhibition of cell proliferation and induction of cell death in mouse SMC transgenic for a temperature-sensitive mutant of SV40 large TAg

To evaluate the causative role of cell cycle negative regulatory parameters such as p53 and pRb family proteins in the lovastatin-induced inhibition of cell proliferation and the induction of cell death, murine aortic SMC expressing a temperature-sensitive SV40 large TAg were incubated with the compound as de-
scribed above. The cells were cultured at the permissive temperature of 33°C, which allows the expression of an active TAg, and in separate cultures at the restrictive temperature of 39.5°C, with reduced levels of TAg to complex with proteins such as p53 and pRb. In addition, wild-type murine aortic SMC were used as controls at a temperature of 37°C.

To evaluate the antiproliferative effect of lovastatin in the absence of cell death, SMC cultures were treated with low doses of lovastatin (0.5–1.5 µmol/l) for 48 h. None of the cultures, transgenic at 33°C and 39.5°C as well as wild-type at 37°C, showed cell detachment above the background levels of untreated controls. Cell viability was assessed by trypan blue testing. Fig. 6 shows a representative cell counting experiment performed in duplicate. Values are given as mean relative cell counts after 48 h exposure to lovastatin, with the highest cell count of each cell line set as 100%. At both the permissive and restrictive temperatures, transgenic cells were equally inhibited by lovastatin (linear regression analysis, \( P = 0.64 \)). Furthermore, no diminished sensitivity of the TAg expressing cells could be detected when compared with wild-type SMC. The addition of 50 µmol/l mevalonate promptly abolished the antiproliferative effect in both the transgenic and wild-type cell lines (data not shown). Compared with SMC of other species, the murine SMC tested showed a higher sensitivity to the treatment with lovastatin, based on the fact that rat, bovine and human SMC required more than 1.5 µmol/l of the compound to exhibit a clear antiproliferative effect.

To study the mechanism of lovastatin-induced cell death in murine SMC expressing TAg, higher concentrations of lovastatin up to 20 µmol/l were used for an incubation period of 48 h applied to transgenic SMC at the permissive and restrictive temperature as well as on wild-type mouse SMC at 37°C. Fig. 7 shows the phenomenon of cell detachment under the influence of lovastatin. In the transgenic cell line, at a concentration of 20 µmol/l lovastatin, the vast majority of cells were detached from the culture flask and detached cells showed patterns of reduced DNA content comparable to those depicted for other cell lines above. In the presence of TAg, SMC thereby failed to show an inhibition of cell detachment and DNA reduction, whereas the addition of 100 µmol/l mevalonate to the culture media completely abolished these effects. The phenomenon of cell detachment was observed at both the restrictive and the permissive temperatures at comparable levels, whereas wild-type mouse SMC were even less susceptible to undergoing this effect.

4. Discussion

We studied the influence of lovastatin, simvastatin and pravastatin on arterial SMC by investigating their effects on cell proliferation and cell viability. Cell proliferation was studied by cell counting, expression of Ki-67/MIB-1, [3H]thymidine incorporation or cell cycle analysis. Lovastatin and simvastatin showed clear antiproliferative and cytotoxic effects on SMC, with simvastatin being 2–3-fold stronger than lovastatin, whereas pravastatin showed no inhibition of cell growth or cytotoxic effects in vitro, due to its hydrophilicity causing poor penetration into non-hepatic cells [21].

Various studies have reported the cell death inducing effect of some HMG-CoA reductase inhibitors on a variety of cell lines [22–26]. To evaluate the mechanisms of cytotoxic effects, we studied DNA content, DNA strand breaks and SMC morphology under the influence of lovastatin. Our data indicate that the cytotoxic effect displayed by some HMG-CoA reductase inhibitors is caused by the withdrawal of metabolites of the mevalonate pathway. The data indicate, furthermore, that this cytotoxic effect on SMC is characterized by apoptosis-like features such as the increase in the number of DNA strand breaks, reduction of DNA content, and exhibition of typical morphological signs such as the compaction of chromatin and intracellular granules. On the other hand, signs of necrotic cell death were also found, but the time course of the propidium iodide incorporation into unfixed nuclei and the release of LDH indicate that necrotic events occur as a secondary phenomenon. However, a minority of SMC, characterized by persistent adherence to the cell culture
Fig. 7. Cell detachment of transgenic and wild-type mouse aortic SMC. Cell cultures were seeded as indicated in the legend to Fig. 1. Experiments on cells transgenic for temperature-sensitive TAg were performed at the permissive temperature of 33°C, wild-type SMC were kept at 37°C. The cultures were incubated with 5 and 20 μmol/l lovastatin for 48 h. Mevalonate at a concentration of 100 μmol/l was used as an inhibitor of lovastatin (× 400).

flask even under high concentrations of lovastatin, regained the ability to proliferate after withdrawal of the drug. Since it has been shown that cells can be adapted to grow in the presence of statins by upregulation of the HMG-CoA reductase gene [27,28], our finding probably identifies the surviving cells as good upregulators of the HMG-CoA reductase gene allowing the cells to overcome the detrimental effects of lovastatin.

Studying HMG-CoA reductase inhibitors with a view to their potential for performing G0/G1 phase arrest and inducing cell death highlights the question of the involvement of the p53 pathway [16], including its downstream kinase Cdk2 [29,30]. The role of the Cdk inhibitors p21 and p27 for the statin-induced cell cycle arrest has recently been reported [13–15], but little is known about the quantitative regulation of Cdk2 under the treatment with the compounds. We found a moderate downregulation of Cdk2 levels under the influence of lovastatin. To evaluate whether this phenomenon may be causally related to the G0/G1 arrest, we corre-
lated Cdk2 expression levels with the cell cycle distribution. It could be shown that resting SMC express less Cdk2 than cycling SMC, but no substantial difference in Cdk2 levels was observed between G1 and S phase cells. This also indicates that the smaller portion of S phase cells under lovastatin treatment does not account for the diminished Cdk2 levels. However, we did not observe a clear decrease in Cdk2 levels after short incubation with the compound, but the cell cycle arrest was already obvious at that time point. We therefore conclude that the downregulation of Cdk2 may be a secondary phenomenon to lovastatin treatment rather than a causal factor for cell cycle arrest.

Activation of p53 is capable of arresting cells in the late G1 phase and may also induce apoptosis, especially after DNA damage [16,31,32]. The p53 pathway is thereby a ubiquitous mechanism for controlling cell proliferation. Although upregulation of p53 has been found in mammary epithelial cells after lovastatin treatment [33], certain cancer cell lines have revealed lovastatin induced growth arrest and cell death in the absence of p53 [13,34]. However, little is known about the regulatory effects of the compound on the proliferation and viability of vascular SMC. Our data did not show an increase in p53 synthesis under the influence of lovastatin at various incubation times. Elevated levels may only be present for a limited time period and may therefore be difficult to detect. To better investigate a functional involvement of p53 in the regulation of cell death and the inhibition of cell proliferation, a transgenic cell line expressing a TAg was employed. TAg is known to complex and inactivate cell cycle negative regulatory proteins, among which p53 and proteins of the pRb family play a pivotal role. The cell line we used has recently been characterized and has been shown to express the TAg under in vitro conditions [17]. Our data did not indicate the inhibition of the antiproliferative and cytotoxic effects of lovastatin in the presence of TAg, whereas both effects were completely abolished by supplementation with mevalonate. We also showed that 20 μmol/l lovastatin does not exert cytotoxic effects on growth arrested SMC, whereas the cytotoxic and antiproliferative effect is seen — but can be antagonized by mevalonate — when HMG-CoA reductase is inhibited by lovastatin in proliferating SMC. Taking these results together, our data provide evidence that the antiproliferative effect of lovastatin does not require the presence of p53 or pRb, but is dependent on the supplementation of mevalonate. Furthermore, the induction of cell death in SMC after incubation with high concentrations of lovastatin may be explained by the using up of metabolites of the mevalonate pathway and can be considered a general cytotoxic effect independent from p53.

Some HMG-CoA reductase inhibitors have been shown to inhibit SMC proliferation and to induce cell death in concentrations that are distinctly higher than systemic concentrations achieved under therapy for the lowering of cholesterol levels [35]. It therefore appears doubtful whether the inhibition of SMC proliferation (i.e. in the prevention of restenosis) is an issue under systemic therapy with HMG-CoA reductase inhibitors. However, this study was not focussed on this question and further investigations are needed to evaluate the cell cycle and cell death pathways and their consequences for further clinical demands.

References


