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European Journal of Pharmacology 462 (2003) 15–23
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Atorvastatin inhibits expression of minichromosome maintenance proteins in vascular smooth muscle cells

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Received 22 August 2002; received in revised form 24 December 2002; accepted 7 January 2003

Abstract

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have been reported to inhibit vascular smooth muscle cell growth, a key event in the pathogenesis of proliferative vascular diseases. The mechanism by which HMG-CoA reductase inhibitors exert their antiproliferative activity is not fully understood, especially their effect on DNA replication. We therefore investigated the effects of atorvastatin on minichromosome maintenance (MCM) protein 6 and 7 expression in vascular smooth muscle cells, two proteins essential for initiation of DNA replication. Stimulation of quiescent rat aortic vascular smooth muscle cells with fetal bovine serum induced MCM6 and MCM7 protein and mRNA expression, which was potently attenuated by atorvastatin in a dose-dependent fashion. Mevalonate completely abrogated the inhibitory effect on serum-induced MCM6 and MCM7 expression, demonstrating that biosynthesis of isoprenoids was likely the specific pathway blocked by atorvastatin. Transient transfection experiments revealed that atorvastatin inhibited MCM6 and MCM7 promoter activity, implicating a transcriptional mechanism. The MCM6 and MCM7 promoters contain several E2F sites critical for their transcriptional activation. Activity of a luciferase reporter plasmid containing four E2F elements was also strongly inhibited by atorvastatin. The inhibitory effect of atorvastatin on MCM6 and MCM7 was reversed by adenoviral-mediated overexpression of E2F, indicating that their downregulation by atorvastatin involves an E2F-dependent mechanism. These findings demonstrate that MCM proteins play an essential role during the proliferation of vascular smooth muscle cells and may provide a novel therapeutic target for proliferative vascular diseases. Inhibition of MCM6 and MCM7 expression by blocking E2F function may contribute importantly to the inhibition of vascular smooth muscle cell DNA synthesis by atorvastatin.

Keywords: Smooth muscle cell, vascular; HMG-CoA reductase; Minichromosome maintenance protein; E2F

1. Introduction

Recent large clinical trials have demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors reduce cardiovascular-related morbidity and mortality in patients with atherosclerotic diseases (Packard, 1998; Sacks et al., 1996; Scandinavian Simvastatin Survival Study Group, 1994; Shepherd et al., 1995). Evidence is accumulating from clinical and experimental studies that the beneficial effects of HMG-CoA reductase inhibitors extend beyond the reduction of plasma cholesterol levels and that these agents exhibit direct vascular effects (reviewed by Takemoto and Liao, 2001). The molecular basis for the pathogenesis of proliferative vascular diseases, such as postangioplasty restenosis, transplant arteriosclerosis, and vein graft occlusion involves excessive proliferation of vascular smooth muscle cells (Braun-Dullaeus et al., 1998; Ross, 1995). HMG-CoA reductase inhibitors have been shown to prevent neointima formation and vascular smooth muscle cell growth and migration (Indolfi et al., 2000; Laufs et al., 1999; Raiteri et al., 1997; Soma et al., 1993; Yang et al., 2000). Despite recent advances in the understanding of cell cycle regulation, contradictory

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doi:10.1016/S0014-2999(03)01317-7

findings have been obtained concerning the underlying mechanisms for the antiproliferative effect of HMG-CoA reductase inhibitors on vascular smooth muscle cells. Several lines of evidence suggest that these agents attenuate the activity of key cell cycle regulators, which control progression through the G1 phase (Jakobisiak et al., 1991; Laufs et al., 1999). However, to date, no data exists investigating whether genes active in the S phase of the cell cycle, and directly involved in initiating DNA replication, are affected by HMG-CoA reductase inhibitors.

DNA replication is a highly regulated multistep process that requires the participation of a number of proteins and is largely conserved from yeast to humans (Stillman, 1996). The hexameric minichromosome maintenance (MCM) complex is the signature component for the initiation of DNA replication (reviewed by Kearsey and Labib, 1998; Tye, 1999). The MCM family of proteins constitutes a group of six proteins, including MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7. MCM proteins play a direct role in the initiation of DNA synthesis at replication origins and ensure that DNA replicates only once per mitotic cycle (Takisawa et al., 2000). The recruitment of the heterohexameric MCM complex onto replication origins during the G1 phase of the cell cycle is essential for a cascade of protein assembly that finally results in the formation of a pre-replicative complex (pre-RC). Assembly of the pre-RC begins with the binding of a six-subunit origin recognition complex (ORC) to specific origin sites in the genome determining where replication will occur (Bell and Stillman, 1992; Diffley et al., 1994). In early G1, the ORC recruits Cdc6 protein, which mediates the loading of the MCM complex to chromatin, resulting in chromatin being 'licensed' for replication in the subsequent S phase (Blow and Laskey, 1988; Coleman et al., 1996; Kubota et al., 1995). Phosphorylation of the MCM complex by the Cdc7-Dbf4 kinase (DDK) during S phase and activation of S phase-promoting cyclin-dependent kinases (CDKs) induce a conformational change in the MCM complex and promote the association of Cdc45 with the MCM at origins (Jiang et al., 1999; Mimura and Takisawa, 1998; Zou and Stillman, 1998). Upon formation of the Cdc45–MCM complex, the duplex DNA unwinding is initiated and various replication proteins, including DNA polymerases, are recruited onto unwound DNA for the initiation of DNA synthesis (Aparicio et al., 1999; Labib et al., 2000; Walter and Newport, 2000; You et al., 1999).

Accordingly, the objective of this study was to examine the effect of atorvastatin on MCM expression during the vascular smooth muscle cell cycle. We report here that the mitogen-induced expression of MCM6 and MCM7, two important regulators of DNA replication, is attenuated at a transcriptional level by the HMG-CoA reductase inhibitor atorvastatin. The mechanism by which atorvastatin inhibits MCM transcription likely involves a blockade in the release of retinoblastoma protein (Rb)-sequestered E2F during the G1 → S transition. These data provide further support for the therapeutic potential of HMG-CoA reductase inhibitors for proliferative vascular diseases, through an activity independent of lowering cholesterol levels.

2. Material and methods

2.1. Materials

Materials were obtained from the following suppliers: Dulbecco's modified Eagle's medium (DMEM), L-glutamine and OPTI-MEM from Gibco BRL (Gaithersburg, MD). Antibiotics, PerfectHyb Plus hybridization buffer and L-mevalonate were from Sigma (St. Louis, MO). Fetal bovine serum was purchased from Irvine Scientific (Santa Ana, CA). Hybond enhanced chemiluminescence nitrocellulose and nylon membranes, Rediprime II random prime labeling system, horseradish peroxidase-linked anti-mouse antibody and enhanced chemiluminescence Western blotting detection reagents were from Amersham Pharmacia Biotech (Piscataway, NJ). The goat polyclonal antibody against MCM6 (sc-9843), the mouse monoclonal antibody against MCM7 (sc-9966) and the mouse-anti goat immunoglobulin G (IgG) antibody (sc-2354) were from Santa Cruz Biotechnology (Santa Cruz, CA). The E2F-1 IgG (05-379) was from Upstate (Lake Placid, NY) and the phospho-Rb Ser 807/811 antibody (No. 9308) from Cell Signaling Technology (Beverly, MA). TRIzol reagent and LipofectAMINE 2000 were purchased from Life Technologies (Rockville, MN). [α-^{32}P] dCTP was commercially obtained from ICN (Irvine). Atorvastatin was a kindly provided by Goedecke (Freiburg, Germany).

The Dual Luciferase Reporter Assay System and pRL-CMV were purchased from Promega (Madison, MA). cDNA for MCM6 and the human MCM6 promoter pHSMCM6-Luc(−754) driven by a luciferase reporter plasmid were kindly provided by Dr. Hiroshi Nojima (Department of Molecular Genetics, Osaka University, Japan) (Ohtani et al., 1999). The human MCM7 promoter pHsMCM7-Luc(−558) luciferase reporter plasmid and MCM7 cDNA were previously described (Suzuki et al., 1998). The pE2F-Luc luciferase reporter plasmid containing four E2F enhancer elements was from Clontech (Palo Alto, CA). Adenovirus encoding human E2F-1, driven by the cytomegalovirus (CMV) immediate-early promoter (Adx-E2F), was kindly provided by Dr. Robb MacLellan (Department of Cardiology, University of California, Los Angeles, CA) (Agah et al., 1997).

2.2. Cell culture

Rat aortic vascular smooth muscle cells were prepared from the thoracic aorta of 2-to 3-month-old Sprague–Dawley rats by using the explant technique. Cells at passage three to eight were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 200 mM L-glutamine. Cells were grown to 60–70%

confluence and made quiescent by serum starvation (0.4% fetal bovine serum) for at least 24 h. Atorvastatin alone or in combination with L-mevalonate (200 μM) or the vehicle dimethyl sulfoxide (DMSO) was added 30 min prior to the addition of 10% fetal bovine serum. For all data shown, each individual experiment was performed using an independent preparation of rat aortic vascular smooth muscle cells.

2.3. Western immunoblotting

Cells were harvested at the indicated time after the addition of growth factors and sonicated in solubilization buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA, 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM sodium vanadate; 10 μg/ml each aprotinin and leupeptin; 1 mM phenylmethylsulfonyl fluoride). Cell lysates were cleared by centrifugation and protein concentrations were determined by Lowry assay. Cell lysates containing equal amount of protein were resolve by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Protein was transferred electrophoretically to a nitrocellulose membrane. After blocking in 20 mM Tris–HCl (pH 7.6) containing 150 mM NaCl, 0.1% Tween 20 and 5% (w/v) non-fat dry milk, blots were incubated with specific antibodies for MCM6, MCM7 or E2F-1. Immunoreactive bands were visualized by incubation with peroxidase-conjugated anti-mouse IgG antibody or anti-goat IgG antibody. The antigen–antibody complex was detected using an enhanced chemiluminescence detection system. Quantification of the Western blots was performed by densitometry.

2.4. RNA isolation and Northern blotting

Total RNA was isolated using TRIzol reagent as described by the manufacturer. Fifteen micrograms of total RNA was denatured in formamide and formaldehyde and electrophoresed through 1% formaldehyde-containing agarose gels. After electrophoresis, the RNA was transferred to nylon membrane by the capillary blotting and then fixed by UV cross-linking. Hybridization was performed using PerfectHyb Plus hybridization buffer as directed. Probes for MCM6 and MCM7 used in the hybridization were radiolabelled with [α-^{32}P] dCTP using Rediprime II random prime labeling system. Blots were also probed with a probe for Chinese hamster ovary gene B (CHOB), a constitutively expressed, housekeeping gene encoding a ribosomal protein, to assess equal loading of samples.

2.5. Transient transfection and luciferase assay

Rat aortic vascular smooth muscle cells were grown to 70–80% confluence in six-well plates and placed in OPTI-MEM I medium. For analysis of the MCM6 and MCM7 promoter activities, 1 μg of the pHSMCM6-Luc(− 754) or pHsMCM7-Luc(− 558) were transfected using Lipofect-AMINE 2000. Twenty-four hours after the transfection, cells were starved in DMEM medium containing 0.4% fetal bovine serum for 24 h. Cells were then pretreated with the indicated concentrations of atorvastatin 30 min prior to the addition of 10% fetal bovine serum and stimulated for 24 h. For analysis of the pE2F-Luc reporter plasmid activity, 1 μg of DNA was transfected in DMEM containing 5% fetal bovine serum for 24 h. Atorvastatin was added at a final concentration of 20 μM and cells were harvested for analysis at the indicated time points. Luciferase activity was assayed using a Dual Luciferase Reporter Assay System according to the manufacturer's instructions. Transfection efficiency was adjusted by normalizing firefly luciferase activities to the *Renilla* luciferase activities generated by co-transfection with 10 ng pRL-CMV. All experiments were repeated al least three times with different cell preparations.

2.6. Adenoviral infection

Rat aortic vascular smooth muscle cells were infected with 100 PFU/cell Adx-E2F in DMEM containing 0.4% fetal bovine serum for 24 h. After further starvation for 24 h, cells were pretreated with atorvastatin for 30 min and 10% fetal bovine serum was added for 12 h. Recombinant type 5 adenovirus expressing green fluorescent protein (GFP) gene was generated and used as a control vector (Adx-GFP) in all experiments.

2.7. Statistics

Data were expressed as mean ± standard error of the mean (S.E.M). Statistical significance was determined using the Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Atorvastatin inhibits phosphorylation of the retinoblastoma protein

G1 → S phase transition requires increased phosphorylation of the Rb protein to release the S phase transcription factor, E2F, which regulates expression of genes encoding the enzymatic machinery for DNA synthesis (Weinberg, 1996; Dyson, 1998). We therefore investigated the effect of atrovastatin on Rb phosphorylation at specific phosphorylation sites, such as Ser807/811, which mediate CDK-dependent regulation of Rb function and are important for cells to exit G1 and enter S phase (Connell-Crowley et al., 1997). Compared to quiescent rat aortic vascular smooth muscle cells, phosphorylation of Ser807/811 increased significantly after 24-h stimulation with 10% fetal bovine serum (Fig. 1). Pretreatment with atorvastatin substantially

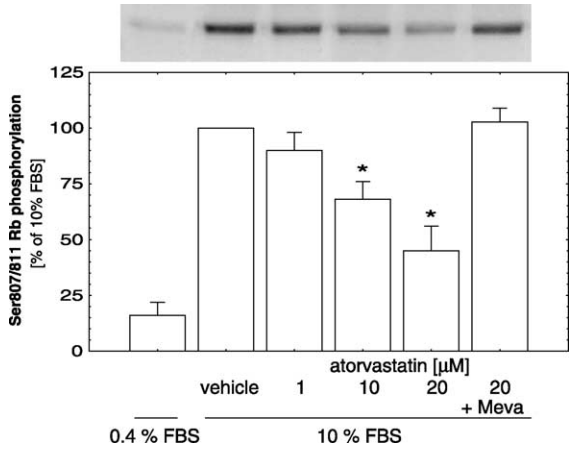

Fig. 1. Atorvastatin inhibits retinoblastoma protein phosphorylation in rat aortic vascular smooth muscle cells. Quiescent rat aortic vascular smooth muscle cells (0.4% fetal bovine serum) were stimulated with 10% fetal bovine serum for 24 h. Cells were preincubated with the indicated concentrations of atorvastatin alone (1–20 μM) or in combination with L-mevalonate (Meva, 200 μM) or vehicle (DMSO) 30 min prior to addition of the 10% fetal bovine serum. Whole cell proteins (75 μg) were assayed by Western immunoblotting using an anti-phospho-Rb Ser 807/811 antibody. Results were expressed as percent of the mitogenic induction of retinoblastoma protein phosphorylation at Ser 807/811. The autoradiogram is representative of three separate experiments and data are expressed as mean ± S.E.M, *$P<0.05$ vs. 10% fetal bovine serum.

inhibited mitogen-induced phosphorylation of Ser807/811 in a dose-dependent fashion (55.5 ± 9.7% inhibition (20 μM) vs. 10% fetal bovine serum, $n=3$, $P<0.05$). Treatment with mevalonate (200 μM) completely reversed this inhibitory effect, indicating that atorvastatin inhibited Rb phosphorylation by blocking the synthesis of isoprenoid metabolites. Mevalonate alone, however, had no effect on Rb phosphorylation at Ser807/811 (data not shown). In combination, these findings suggest that atorvastatin inhibits vascular smooth muscle cell proliferation, at least in part, by inhibition of Rb phosphorylation at specific phosphorylation sites, such as Ser807/811.

3.2. MCM6 and MCM7 mRNA and protein expression in rat aortic vascular smooth muscle cells is attenuated by atorvastatin

Initiation and completion of S phase in the cell cycle requires the function of MCM gene products (Kearsey and Labib, 1998; Tye, 1999). To investigate the effect of HMG-CoA reductase inhibitor treatment on MCM expression, quiescent rat aortic vascular smooth muscle cells were pretreated with atorvastatin for 30 min and stimulated with 10% fetal bovine serum to re-enter the cell cycle. Northern blot analysis revealed that stimulation with fetal bovine serum resulted in a marked increase of MCM6 and MCM7 mRNA expression after 12 h (4.2 ± 0.7- and 5.3 ± 0.9-fold induction vs. quiescent cells). This induction of MCM6 and MCM7 mRNA was substantially downregulated by treatment with atorvastatin (54.8 ± 6.2%, 64.5 ± 5.1% inhibition vs. 10% fetal bovine serum after 12 h treatment with 20 μM atorvastatin, $n=3$, $P<0.05$). The inhibition of MCM6 and MCM7 mRNA expression observed after administration of atorvastatin was dose dependent (Fig. 2). At the concentrations used, no cytotoxic effects were observed, as evidenced by the lack of cell detachment and failure to take up the vital dye trypan blue.

Western analysis revealed that the inhibition of the mitogenic induction of MCM6 and MCM7 mRNA by atorvastatin correlated with a significant dose-dependent attenuation of MCM6 and MCM7 protein expression 24 h after growth stimulation (49.8 ± 6.8%, 59.9 ± 7.1% inhibition vs. 10% fetal bovine serum after 24 h treatment with 20 μM atorvastatin, $n=3$, $P<0.05$, Fig. 3). Co-treatment with mevalonate (200 μM) completely reversed the inhibitory effect of atorvastatin on MCM6 and MCM7 mRNA and protein expression. Mevalonate alone, however, had no effect on MCM6 and MCM7 expression (data not shown). These data demonstrate that atorvastatin regulation of MCM expression was unrelated to its blockade of cholesterol biosynthesis. Under the same experimental conditions, we also analyzed the inhibitory effect of atorvastatin on the mitogenic induction of MCM6 and MCM7 expression by platelet-derived growth factor (PDGF) BB (20 ng/ml) and insulin (1 μM). Similar to the findings on fetal bovine serum stimulated rat aortic vascular smooth muscle cells, atorvastatin exhibited a comparable inhibition on PDGF + insulin induced MCM6 and MCM7 mRNA and protein expression (data not shown). Taken together, these findings indicate that the inhibition of MCM6 and MCM7 protein expression by atorvastatin results from an inhibition of MCM6 and MCM7 mRNA expression. Reduced MCM6 and MCM7 mRNA levels by atorvastatin could reflect either decreased transcription and/or mRNA stability.

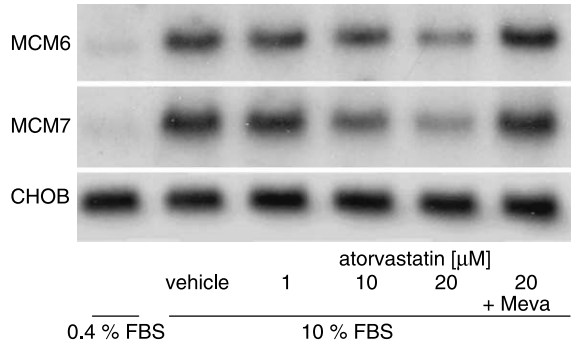

Fig. 2. Atorvastatin inhibits growth-induced MCM6 and MCM7 mRNA expression in rat aortic vascular smooth muscle cells. Quiescent rat aortic vascular smooth muscle cells (0.4% fetal bovine serum) were preincubated with atorvastatin alone (1–20 μM) or in combination with L-mevalonate (Meva, 200 μM) or vehicle (DMSO) 30 min prior to growth stimulation with 10% fetal bovine serum. Twelve hours after stimulation, cells were harvested and total RNA was analyzed for MCM6 and MCM7 mRNA expression by Northern blotting. Co-hybridization for CHOB, a constitutively expressed housekeeping gene encoding a ribosomal protein, was employed to assess equal loading of samples. Each autoradiogram is representative of three separate experiments.

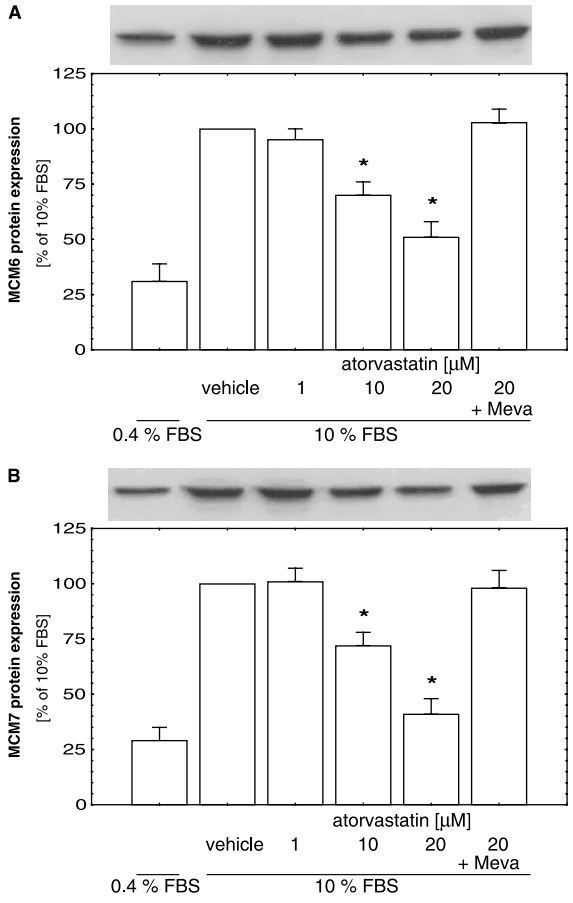

Fig. 3. Atorvastatin inhibits growth-induced MCM6 and MCM7 protein expression in rat aortic vascular smooth muscle cells. Rat aortic vascular smooth muscle cells were growth-arrested in G_0/G_1 by incubation in 0.4% fetal bovine serum for at least 24 h (0.4% fetal bovine serum). Cells were then preincubated with atorvastatin alone (1–20 μM) or in combination with L-mevalonate (Meva, 200 μM) or vehicle (DMSO) 30 min prior to growth stimulation with 10% fetal bovine serum. Twenty-four hours after stimulation, whole cell proteins (40 μg) was subjected to immunoblotting using specific MCM6 (panel A) and MCM7 (panel B) antibodies. Data are expressed as mean ± S.E.M and each autoradiogram is representative of three separate experiments, $*P<0.05$ vs. 10% fetal bovine serum.

3.3. Atorvastatin inhibits MCM6 and MCM7 promoter activity

To examine the effect of atorvastatin on MCM6 and MCM7 transcription, we transiently transfected rat aortic vascular smooth muscle cells with human MCM6 and MCM7 promoter fragments driving expression of the luciferase reporter gene. The MCM6 promoter fragment employed contains a 789-bp DNA segment (−754 to +35) and the MCM7 promoter fragment consists of a 505-bp DNA segment (−558 to −54). Growth induction with 10% fetal bovine serum significantly upregulated MCM6 (Fig. 4A) and MCM7 (Fig. 4A) promoter activity (2.8 ± 0.2- and 5.6 ± 0.9-fold induction vs. quiescent cells, $n=3$, $P<0.05$). This growth-related induction of MCM6 and MCM7 transcriptional activity was significantly downregulated by atorvastatin in a dose-dependent fashion (61.1 ± 5.1%, 63.2 ± 4.4% inhibition vs. 10% fetal bovine serum after 24 h (20 μM), $n=3$, $P<0.05$). Co-treatment with mevalonate (200 μM) preserved mitogen-dependent MCM6 and MCM7 transcription. These data demonstrate that atorvastatin inhibits MCM6 and MCM7 expression, at least in part, at the transcriptional level.

3.4. Atorvastatin inhibits E2F promoter activity

The promoter regions of MCM6 contains five E2F sites, while three E2F sites have been identified in the MCM7 promoter. This transcription factor has been shown to be primarily responsible for the coordinated increase in MCM

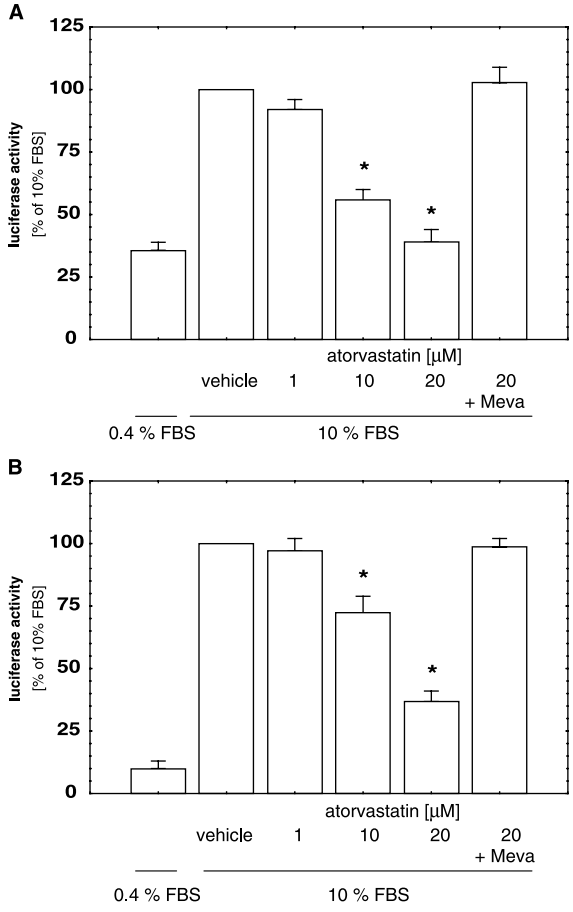

Fig. 4. Atorvastatin attenuates MCM6 and MCM7 transcriptional activation. Rat aortic vascular smooth muscle cells were transiently transfected with 1 μg pHsMCM6-Luc (−754) (panel A) and pHsMCM7-Luc (−558) (panel B) promoter-fragments driving expression of luciferase reporter genes. Transfected cells were serum-starved for 24 h (0.4% fetal bovine serum). Thirty minutes before growth stimulation with 10% fetal bovine serum, cells were pretreated with the indicated concentrations of atorvastatin alone (1–20 μM) or in combination with L-mevalonate (Meva, 200 μM) or vehicle (DMSO). Twenty-four hours after stimulation, luciferase activity was assayed. Transfection efficiency was adjusted by normalizing firefly luciferase activities to *Renilla* luciferase activities generated by co-transfection with pRL-CMV. All experiments were repeated al least three times with different cell preparations. Data are expressed as mean ± S.E.M., $*P<0.05$ vs. 10% fetal bovine serum.

mRNA during the G1 → S phase transition (Ohtani et al., 1999; Suzuki et al., 1998). To determine the mechanism by which the HMG-CoA reductase inhibitors downregulate MCM6 and MCM7 expression, we investigated the effect of atorvastatin on the activity of a luciferase reporter plasmid driven by multiple E2F binding sites. Treatment with atorvastatin (20 μM) resulted in a marked inhibition of the promoter activity of this reporter plasmid (Fig. 5, 65.3 ± 5.8% inhibition after 36 h, $n = 3$, $P < 0.05$). These data show that the downregulation of MCM6 and MCM7 transcription by atorvastatin occurs, at least in part, through an inhibition of transactivation of E2F elements in the MCM6 and MCM7 promoters.

3.5. Adenoviral overexpression of E2F-1 reversed the inhibitory effect of atorvastatin on MCM6 and MCM7

In order to further investigate the role of E2F in the attenuation of MCM6 and MCM7 induction by atorvastatin, we employed an adenoviral expression vector to overexpress E2F-independent of Rb phosphorylation during the cell cycle. Adx-GFP-infected rat aortic vascular smooth muscle cells were used as control to assess potential virus-mediated effects. In Adx-GFP-infected cells, stimulation with fetal bovine serum resulted in a marked increase of MCM7 mRNA (Fig. 6A) and protein (Fig. 6B), which was potently blocked by treatment with 20 μM atorvastatin. Infection of rat aortic vascular smooth muscle cells with the recombinant Adx-E2F virus resulted in a strong overexpression of E2F-1 protein as determined by Western immunoblotting (Fig. 6B). Infection of quiescent cells with Adx-E2F increased the level

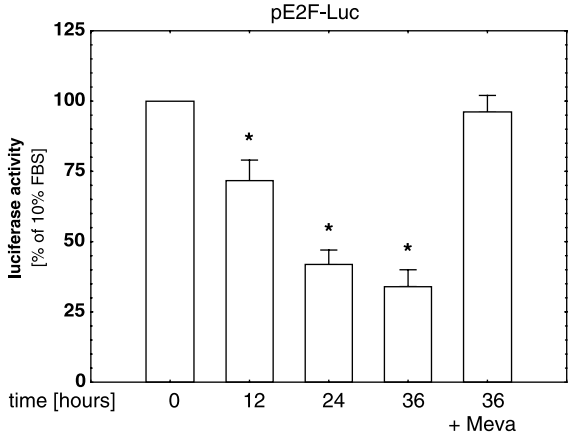

Fig. 5. Atorvastatin inhibits growth-induced E2F transcriptional activation. Rat aortic vascular smooth muscle cells were transiently transfected with 1 μg pE2F-Luc driving expression of luciferase reporter genes. Transfected cells were serum-starved for 24 h. Thirty minutes before stimulation with 10% fetal bovine serum, cells were pretreated with atorvastatin (20 μM) alone or in combination with L-mevalonate (Meva, 200 μM). Luciferase activity was assayed at the indicated time points. Transfection efficiency was adjusted by normalizing firefly luciferase activities to *Renilla* luciferase activities generated by co-transfection with pRL-CMV. All experiments were repeated al least three times with different cell preparations. Data are expressed as mean ± S.E.M., *$P < 0.05$ vs. untreated cells.

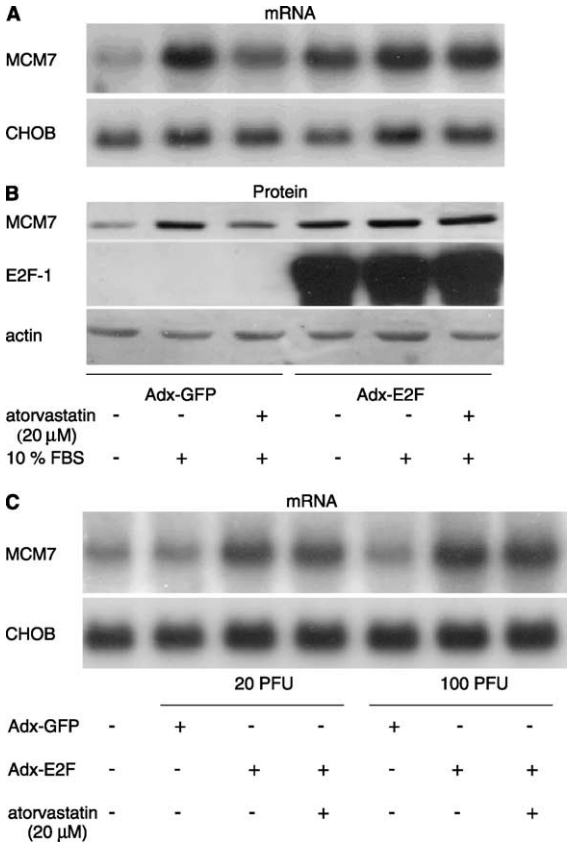

Fig. 6. Inhibition of MCM7 expression by atorvastatin is reversed by adenoviral-mediated overexpression of E2F. Quiescent rat aortic vascular smooth muscle cells were infected with 100 PFU/cell adenovirus overexpressing human E2F-1, driven by the cytomegalovirus immediate-early promoter (Adx-E2F) for 24 h. Infection of rat aortic vascular smooth muscle cells with recombinant type 5 adenovirus expressing green fluorescent protein (Adx-GFP) served as control. After infection, cells were pretreated with atorvastatin (20 μm) 30 min prior to stimulation with 10% fetal bovine serum. Twelve hours after stimulation, cells were harvested and total RNA was analyzed for MCM7 mRNA expression by Northern blotting (panel A). Twenty-four hours after stimulation, whole cell proteins (40 μg) was analyzed by immunoblotting using a specific MCM7 antibody (panel B). Overexpression of human E2F-1 was monitored by immunoblotting with a specific human E2F-1 antibody (panel B). Serum-starved rat aortic vascular smooth muscle cells were infected for 24 h with 20 or 100 PFU/cell Adx-E2F or Adx-GFP and treated with atorvastatin (20 μM) as indicated for 12 h. Total RNA was analyzed for MCM7 mRNA expression by Northern blotting. Co-hybridization for CHOB, a constitutively expressed housekeeping gene encoding a ribosomal protein, was employed to assess equal loading of samples (panel C). Each autoradiogram is representative of three separate experiments.

of MCM7 (mRNA and protein) as E2F activated S phase gene expression demonstrating that MCM7 expression in rat aortic vascular smooth muscle cells is regulated principally by E2F. In rat aortic vascular smooth muscle cells, ectopically expressing E2F, atorvastatin was ineffective in suppressing serum-induced MCM7 mRNA and protein. This finding suggests that atorvastatin exerts its effect at, or upstream of E2F, release from phosphorylated Rb. Similarly, atorvastatin had no effect on MCM7 expression in quiescent cells infected with either 20 PFU/cell or 100 PFU/cell

Adx-E2F (Fig. 6C). Comparable results were obtained for MCM6 (data not shown). In combination, these data strongly suggest that the inhibitory effect of atorvastatin on fetal bovine serum induced MCM6 and MCM7 expression is mediated by preventing E2F release from Rb, rather than by directly inhibiting E2F activity.

4. Discussion

Proliferation of vascular smooth muscle cells in the arterial wall is a key event in restenosis and atherosclerosis (Braun-Dullaeus et al., 1998; Ross, 1995). HMG-CoA reductase inhibitors have been reported to inhibit neointima formation and vascular smooth muscle cell proliferation independent of their cholesterol-lowing ability, suggesting pleiotropic vascular effects of these agents (Indolfi et al., 2000; Laufs et al., 1999; Raiteri et al., 1997; Soma et al., 1993; Yang et al., 2000). We report here the novel finding that the mitogen-induced expression of MCM6 and MCM7 in vascular smooth muscle cells, two important regulators of DNA replication, is attenuated at the transcriptional level by the HMG-CoA reductase inhibitor atorvastatin by blocking function of the transcription factor E2F.

Numerous studies have reported antiproliferative activities of HMG-CoA reductase inhibitors in a variety of vascular (Indolfi et al., 2000; Laufs et al., 1999; Raiteri et al., 1997; Soma et al., 1993; Yang et al., 2000) and non-vascular cell lines (Borner et al., 1995; Danesh et al., 2002; Jakobisiak et al., 1991). The mechanisms by which these agents abrogate vascular smooth muscle cell growth are not fully defined and may vary between malignant and normal cells. HMG-CoA inhibitors have been reported to inhibit cell proliferation by interfering with gene products regulating cell cycle progression. In particular, they have been shown to inhibit Rb phosphorylation in vascular (Laufs et al., 1999; Yang et al., 2000) and cancer (Jakobisiak et al., 1991) cells, thereby blocking the G1 → S phase transition. The cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$, an important negative regulator of cell growth, blocks cyclin/cyclin-dependent kinase activity and phosphorylation of Rb, resulting in G1 arrest (Morgan, 1995; Hengst and Reed, 1996). $p27^{kip1}$ has been shown to be specifically upregulated by HMG-CoA reductase inhibitors in vascular smooth muscle cells (Laufs et al., 1999; Weiss et al., 1999). Laufs et al. have demonstrated that the HMG-CoA reductase inhibitor simvastatin attenuates mitogen-induced vascular smooth muscle cell proliferation and Rb hyperphosphorylation, which was associated with increased levels of $p27^{kip1}$. Similarly, we observed that atorvastatin inhibited phosphorylation of Rb at serine 807/811 in rat aortic vascular smooth muscle cells, providing further evidence that Rb is an important target for the antiproliferative activity of HMG-CoA reductase inhibitors. Phosphorylation of Rb at serine 807/811 is mediated primarily by cyclinD/CDK4 (Knudson and Wang, 1996). Serine 807/811 phosphorylation plays a more important role in disrupting c-Abl binding to Rb than in promoting the release of sequestered E2F (Knudson and Wang, 1996). Rb contains multiple CDK phosphorylation sites that regulate its conformation and ability to bind to other cell cycle regulators, such as E2F. Although Rb contains 16 putative CDK phosphorylation sites, their hierarchical contribution to regulating E2F binding remains to be clearly established (Knudson and Wang, 1996). Laufs et al. (1999), however, have shown that simvastatin potently inhibited global phosphorylation of Rb, suggesting that HMG-CoA reductase inhibitors effectively target the subset of serines and threonines that control E2F release. In contrast, in studies where vascular smooth muscle cells were derived from mice expressing the SV40 large T antigen, Sindermann et al. (2000) have shown that the antiproliferative effect of the HMG-CoA reductase inhibitor, lovastatin, occurred independent of decreased Rb phosphorylation.

Phosphorylation of Rb results in a conformational change that releases the sequestered S phase transcription factor, E2F, enabling it to transactivate target genes encoding the enzymatic machinery for DNA synthesis (Weinberg, 1996; Dyson, 1998). The question whether HMG-CoA reductase inhibitors affect pathways downstream of Rb phosphorylation and G1 → S phase transition, such as DNA replication, in vascular cells has not been addressed. This is an important issue for understanding the mechanism of action for atorvastatin because in prostate cancer cells lovastatin blocks E2F function through mRNA destabilization and accelerated proteolysis (Park et al., 2001). To evaluate whether HMG-CoA reductase inhibitors affect E2F-dependent S phase gene expression, we examined the effect of atorvastatin on MCM6 and MCM7 expression in vascular smooth muscle cells. In this study, we demonstrated that atorvastatin potently attenuated the mitogen-induced expression of MCM6 and MCM7 in vascular smooth muscle cells. The MCM6 and MCM7 promoters contain functional E2F binding sites (Ohtani et al., 1999; Suzuki et al., 1998), suggesting that E2F is responsible for the coordinate expression of MCMs during progression through the cell cycle. Consistent with a central role for E2F in regulating MCM expression, we found that atorvastatin potently inhibited transcription of a luciferase reporter driven by multiple E2F elements. These data importantly imply that E2F is a major target for the antiproliferative effects of HMG-CoA reductase inhibitors in vascular cells.

Induction of MCM6 and MCM7 gene expression by adenoviral-mediated overexpression of E2F was refractory to inhibition by atorvastatin. This finding provides further evidence that atorvastatin attenuates MCM6 and MCM7 expression primarily by blocking E2F release from phosphorylated Rb. Regulation of E2F function by atorvastatin in vascular smooth muscle cells, therefore, is distinct from the effect of lovastatin on prostate cancer cells to accelerate the degradation of E2F mRNA and protein. Although HMG-CoA reductase inhibitors may employ different mechanisms to block E2F function in vascular cells versus cancer cells,

they accomplish the same critical effect to prevent E2F-regulated S phase gene expression, such as MCMs.

Inhibition of HMG-CoA has many ramifications, including the loss of important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate and geranylgeranylpyrophosphate. Farnesylation and geranylgeranylation are important steps in posttranslational modification and lipid attachment of a variety of proteins, including Ras and Rho GTP binding proteins, which play a key role in cell proliferation (Casey, 1995; Goldstein and Brown, 1990; Olson et al.,1995). An increased RhoA activity has been recently found to be associated with reduced expression of $p27^{kip1}$ in the vasculature of hypertensive rats (Seasholtz et al., 2001). Moreover, Rho GTPase has been shown to be important for cyclin D1 expression in the G1 phase of the cell cycle (Welsh et al., 2001). These reports suggest that HMG-CoA reductase inhibitors exert their antiproliferative effects on vascular cells mainly by blocking Rho GTPase via inhibition of the protein prenylation induced by mevalonate metabolites. Data presented in this study extend these observations and suggest that inhibition of the HMG-CoA/mevalonate pathway also impairs S phase gene expression. We have demonstrated that the inhibition of MCM expression by atorvastatin is mediated through an inhibition of mevalonate biosynthesis providing further evidence for the important role of isoprenoid intermediates in regulating cell cycle progression.

In conclusion, our data provide evidence that the antiproliferative efficacy of atorvastatin may be, at least in part, mediated by an inhibition of DNA replication and S phase gene expression. The transcriptional regulation of E2F and its target genes MCM6 and MCM7 may be critical regulatory events in atorvastatin-mediated inhibition of vascular smooth muscle cell proliferation. Pharmacologic inhibition of MCM expression, therefore, may provide a novel therapeutic approach for vascular proliferative diseases, such as atherosclerosis and postangioplasty restenosis.

Acknowledgements

This study was supported by a National Institutes of Health Grant HL 58328 to Willa A. Hsueh. Dennis Bruemmer was supported by a grant from MSD Sharp & Dohme (Acute Coronary Syndrome 2000) and by a research fellowship from the Gonda (Goldschmied) Diabetes Center, University of California, Los Angeles. The authors thank Dr. Hiroshi Nojima for providing human MCM6 cDNA and the human MCM6 reporter plasmid.

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