Statin Induces Apoptosis and Cell Growth Arrest in Prostate Cancer Cells

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Abstract

Statins are a class of low molecular weight drugs that inhibit the rate-limiting enzyme of the mevalonate pathway 3-hydroxy-3-methylglutaryl-CoA reductase. Statins have been approved and effectively used to control hypercholesterolemia in clinical setting. Recent study showed statin’s antitumor activity and suggested a potential role for prevention of human cancers. In this study, we did cell viability, DNA fragmentation, and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assays to evaluate the action of statins on prostate cancer cells and used Western blotting and RhoA activation assay to investigate the underlying molecular mechanism of action. Our data showed that lovastatin and simvastatin effectively decreased cell viability in three prostate cancer cell lines (PC3, DU145, and LnCap) by inducing apoptosis and cell growth arrest at G1 phase. Both lovastatin and simvastatin induced activation of caspase-8, caspase-3, and, to a lesser extent, caspase-9. Both statins suppressed expression of Rb, phosphorylated Rb, cyclin D1, cyclin D3, CDK4, and CDK6, but induced p21 and p27 expression in prostate cancer cells. Furthermore, lovastatin and simvastatin suppressed RhoA activation and c-JUN expression, but not cyclooxygenase-2 expression. Our data showed that the antitumor activity of statins is due to induction of apoptosis and cell growth arrest. The underlying molecular mechanism of statin’s action is mediated through inactivation of RhoA, which in turn induces caspase enzymatic activity and/or G1 cell cycle. Future studies should focus on examining statins and other apoptosis-inducing drugs (e.g., cyclooxygenase-2 inhibitors or curcumin) together to assess their efficacy in prevention of prostate cancer. (Cancer Epidemiol Biomarkers Prev 2008;17(1):88–94)

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in American men (1). In the United States, an estimated 218,890 men will be diagnosed with prostate cancer and 27,050 men will die of the disease during the year 2007 (1). Risk factors of prostate cancer include old age, family history, dietary factors, ethnic background, and genetic predisposition. The most important risk factor is age — 70% of men 80 years old or older are estimated to have some histologic evidence of prostate cancer (2). High consumption of dietary fats (such as fatty acid α-linoleic acid in red meat and butter) is believed to increase the risk of prostate cancer (2). Furthermore, in African-American men, prostate cancer is found at a higher grade and stage, and they have lower survival rates (2). Clinical management of prostate cancer relies mainly on surgery before or after chemotherapy and radiation therapy (2). Potential chemopreventive agents under consideration for prostate cancer are diverse that include micro-nutrients (such as vitamin E and selenium), nonsteroidal antiinflammatory drugs, antioxidants, and statins (3). Several epidemiologic studies have shown that statins, cholesterol-lowering agents, are inversely associated with overall and high-grade prostate cancer risk (4-7). Statins affect the lipid raft in plasma membrane, which is responsible for signal transduction of key events, like cell growth, survival, and migration (4). Statins can also trigger different tumor cells to undergo apoptosis in vitro and suppress tumor growth in vivo (8-13). In high concentrations, statins can inhibit capillary tube formation of endothelial cells in vitro and in vivo (14, 15). Their effects are believed to be mediated through inhibition of Ras and RhoA activity (4, 14). To better understand the underlying molecular mechanisms that are responsible for statins’ antitumor activity, we investigated the effects of lovastatin and simvastatin on prostate cancer cell lines in vitro. Our data showed that lovastatin and simvastatin induce apoptosis and cell growth arrest in prostate cancer cell lines by inactivating RhoA and decreasing c-Jun expression.

Materials and Methods

Cell Culture, Gene Transfection, and Statin Treatment. Prostate cancer cell lines PC3, DU145, and LnCap, used in our previous study (16), were plated in cell culture dishes and grown in DMEM with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO2. To evaluate the effect of lovastatin and simvastatin, the cells were plated in DMEM for 24 h. The...
medium was then replaced either with control medium containing 0.01% DMSO (Sigma-Aldrich) or with medium containing lovastatin or simvastatin (Sigma-Aldrich), dissolved in DMSO (stock solution of 100 mmol/L) and diluted in the medium before each experiment. Some experiments were also added caspase-8 and caspase-9 inhibitors (100 μmol/L) to challenge the effectiveness of statins (both of them were obtained from R&D Systems).

At the end of the experiment, the cells were fixed with 10% trichloroacetic acid and stained with 0.4% sulforhodamine B (Sigma-Aldrich) in 1% acetic acid. The unit of absorbance was then read using an automated spectrophotometric plate reader at 490 nm. Cell viability was tested by exclusion of trypan blue (0.1%). The percentage of control was determined using the following equation: % control = $A_t / A_c \times 100$

where $A_t$ and $A_c$ represent the unit of absorbance in the treated and control cultures, respectively. In addition, PC3 and Du145 cells were also transiently transfected with dominant-active RhoA and vector-only constructs pUSEamp (UpState) with FuGENE6 and screened with 400 μg/mL of G418 for 3 days (FuGENE6 and G418 were both supplied by Roche Applied Science). The cells were then treated with 2 μmol/L lovastatin and simvastatin for 5 days and subjected to cell viability assay.

DNA Fragmentation Assay. Soluble DNA was extracted from both floating and attached cells after a 2-day treatment with 2 μmol/L lovastatin or simvastatin. Briefly, the cells were pelleted by centrifugation and resuspended in Tris-EDTA buffer (pH 8.0). The cell membrane was lysed on ice in a mixture of 10 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 0.5% Triton X-100 for 15 min. The lysate was centrifuged at 12,000 g for 15 min to separate soluble (fragmented) from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 μg/mL) at 37°C for 1 h, followed by treatment with protease K (100 μg/mL) in 0.5% SDS at 50°C for 2 h. The residual material was extracted with phenol/chloroform, precipitated in ethanol, electrophoresed on a 1.8% agarose gel, and stained with ethidium bromide. The gels were then photographed under UV illumination.

Figure 1. Dose-response curve. Prostate cancer cell lines were grown in a monolayer for 5 d and treated with 0.01% DMSO (control) or with lovastatin and simvastatin. The cells were then fixed with trichloroacetic acid and stained with sulforhodamine B. Absorbances were read on an automated spectrophotometric plate reader at 490 nm, and percentage of control was calculated. The experiments were triplicates and repeated twice with similar data.

Figure 2. Time-response curve. Prostate cancer cell lines were grown in a monolayer and treated with 0.01% DMSO (control) or with 2 μmol/L lovastatin (L) and simvastatin (S) for 1 to 7 d. The cells were then fixed with trichloroacetic acid and stained with sulforhodamine B. Absorbances were read on an automated spectrophotometric plate reader at 490 nm. The experiments were triplicates and repeated twice with similar data. C, control.
Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling Assay. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay was done using an APO-BRDU apoptosis kit (Phoenix Flow Systems). Briefly, the cells were treated for 2 days with 0.01% DMSO or 2 μmol/L lovastatin or simvastatin. The cells were then labeled with fluorescein dUTP and stained with propidium iodide according to the manufacturer’s protocol. Thereafter, the cells were analyzed for apoptosis using a FACScan flow cytometer (Epics Profile, Coulter Corp.).

Protein Extraction and Western Blotting. Total cellular protein from these cell lines was isolated as described previously (16, 17). Protein (50 μg) from each cell line was subjected to electrophoresis in 10% to 14% SDS-PAGE, dependent on the molecular weight of the proteins to be analyzed. The gel containing the proteins was then transferred electrophoretically to a Hybond-C nitrocellulose membrane (GE Health Sciences Corp.) at 300 mA for 2 h at 4°C and subjected to Western blotting with anti–cyclin D1 (1:2,000), cyclin D3 (1:1,000), Rb (1:2,000), phosphorylated Rb (Ser780; 1:1,000), phosphorylated Rb (Ser795; 1:1,000), phosphorylated Rb (Ser807/811; 1:1,000), p16 (1:1,000), CDK4 (1:2,000), CDK6 (1:2,000), p21 (1:2,000), p27 (1:1,000; all from Cell Signaling Technology), c-Jun (1:250; Santa Cruz Biotechnology), cyclooxygenase-2 (COX-2; 1:250; BD Biosciences), caspase-8, caspase-9, and caspase-3 inhibitors (1:250; Upstate), and anti–β-actin antibody (1:2,000; Sigma-Aldrich) by using a standard procedure (16, 17).

RhoA Activation Assay. PC3 and Du145 cells were seeded in cell culture dishes overnight in DMEM with 10% fetal bovine serum and then in DMEM without fetal bovine serum for an additional 12 h. To activate the RhoA, we cultured the cells in DMEM with 10% fetal bovine serum for 6 h and treated the culture with 2 μmol/L lovastatin or simvastatin for additional 6 h, after which the total cellular protein was extracted in an ice-cold lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, 150 mmol/L NaCl, 1 mmol/L Na2 EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPi, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, and 1 μmol/L/mL leupeptin. The activated GTP-bound Rho protein in the cell lysates was pulled down by using a recombinant glutathione S-transferase–tagged rhotein-Rho binding domain (Upstate) and analyzed with Western blotting by using an anti-RhoA antibody (Santa Cruz Biotechnologies). Levels of the activated RhoA protein were normalized with total RhoA from cell lysates that were not subjected to the pull-down assay (18).

Results

Lovastatin and Simvastatin Reduce Tumor Cell Viability. To determine statin antitumor activity in prostate cancer, we cultured three human prostate cancer cell lines in a monolayer and treated them with lovastatin or simvastatin. We first conducted dose-response studies of lovastatin and simvastatin in these cell lines and found 2 μmol/L dose to have the optimal effect on prostate cancer cells (Fig. 1). We therefore chose this dose for the time course experiment. After treating the three cell lines for 1 to 7 days with 2 μmol/L statins, we observed a trend of reduced cell viability over time, but maximum effect was achieved by 7 days of treatment (Fig. 2). We then did the DNA fragmentation and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assays to assess the underlying mechanism responsible for statin-reduced cell viability. The DNA fragmentation assay showed that all three cell lines underwent apoptosis after a 2-day treatment with 2 μmol/L of lovastatin or simvastatin (Fig. 3A), and quantitative measurement of apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay showed the percentage of cancer cells that underwent apoptosis after treatment with these drugs (Fig. 3B).

Lovastatin and Simvastatin Induce Apoptosis through Cytochrome c–Dependent and Cytochrome c–Independent Signaling Pathway. To better understand the underlying molecular pathway for lovastatin...
Similarly, in Du145 cells treated with lovastatin or simvastatin for 2 d. Both floating and adherent cells were collected for protein extraction (see Materials and Methods), and caspase activation was analyzed using Western blotting. The experiments were repeated once.

Lovastatin and Simvastatin Induce Tumor Cell Growth Arrest. To determine the effect of lovastatin and simvastatin on tumor cell growth, we did cell cycle analysis and detected expression of cell cycle–related genes. When PC3 cells were treated with 2 μmol/L of lovastatin or simvastatin for 2 days, G1 phase of the cell cycle was shifted from 45.1% to 70.5% and 67.3%, respectively, whereas S phase of the cell cycle was reduced from 29.0% to 13.1% and 16.1%, respectively. These data suggest that both lovastatin and simvastatin can reduce tumor cell proliferation. Then we evaluated expression levels of the G1 phase–related genes (e.g., cyclin D1 and Rb) at the molecular level and found both lovastatin and simvastatin inhibited expression of Rb, phosphorylated Rb, cyclin D1, cyclin D3, CDK4, and CDK6, whereas p21 and p27 expression was induced in PC3 and Du145 cells (Fig. 7), indicating that both apoptosis and cell growth arrest in G1 phase of the cell cycle are important in reducing cell viability in PC3 and Du145 cells. However, compared with untreated cells, COX-2 protein expression was found to be either unchanged or even increased after 1 or 2 μmol/L of lovastatin and simvastatin treatment (Fig. 8).

Lovastatin and Simvastatin Suppress RhoA Activation and c-Jun Expression. Mechanistically, statins block farnesyl PPi and geranylgeranyl PPi synthesis, which is required for activation of certain proteins (e.g., Ras and Rho; ref. 4). Farnesylated Ras and geranylgeranylated Rho translocate onto cell membrane to execute their biological activities (4). To determine RhoA activity in statin-treated prostate cancer cells, we used the RhoA activation assay, which showed that lovastatin and simvastatin suppressed RhoA activation in the treated cells compared with the untreated cells (Fig. 9A). We then assessed RhoA downstream gene expression (e.g., c-Jun; ref. 19), showing that lovastatin and simvastatin indeed inhibited expression of c-Jun, which in turn reduced expression of cyclin D1 and other cell cycle–related genes (Fig. 7).

Furthermore, to test whether the effects of statin indeed is through suppression of RhoA activity, we transiently transfected the dominant-active RhoA or vector-only constructs into PC3 and Du145 cells and then treated these cells with lovastatin and simvastatin. Our data showed that the dominant-active RhoA induced cell growth compared with the vector-only–transfected cells (Fig. 9B). However, lovastatin and simvastatin inhibited cell viability at similar level for both dominant-active RhoA–transfected and the vector-only–transfected cells (Fig. 9B). In addition, lovastatin and simvastatin suppressed cyclin D1 expression at similar level for both the dominant-active RhoA–transfected and the vector-only–transfected cells (Fig. 9C).
indicating that lovastatin and simvastatin indeed inhibit RhoA activity for their antitumor effects.

Discussion

In this study, we determined the effects of statins in prostate cancer cell lines in vitro and investigated the underlying molecular mechanisms responsible for their antitumor activity. Our data showed that lovastatin and simvastatin reduced tumor cell viability by inducing apoptosis and cell growth arrest. Additionally, lovastatin and simvastatin suppressed activation of a small GTPase RhoA, then the downstream gene expression of, for example, c-Jun and cell cycle–related genes, but not that of COX-2. The results of this study indicated the usefulness of statins in efficient control of prostate cancer, but future studies should investigate the effects of statins in combination with other apoptosis-inducing drugs (such as COX-2 inhibitors or curcumin) in prostate cancer in vitro and in vivo.

Among cancer cell lines that respond to statin treatment (8-15), prostate cancer seems to be particularly sensitive to statins, which induced these cancer cell lines to undergo apoptosis (4, 20). There are two main apoptosis pathways, i.e., the pathways of mitochondrial cytochrome c–induced caspase-9 and p53 and Fas–induced caspase-8 activation (21-23). During the activation of cytochrome c–dependent apoptosis, induction of mitochondrial permeability transition and cytochrome c release are the most important consequential events (21-23). Our previous study confirmed that cytosolic cytochrome c was increased as early as 6 h after treatment of esophageal and colon cancer cell lines with...
a COX-2 inhibitor NS398; thereafter, caspase-9, caspase-3, and poly(ADP-ribose) polymerase were further activated, leading to the degradation of DNA into nucleosomal fragments. However, p53, Fas, tumor necrosis factor-α, and death receptor-5 use a pathway distinct from that of cytochrome c. In that pathway, when caspase-8 proenzyme is activated, it activates caspase-3 to trigger apoptosis. However, there is some crosstalk between the pathways, for example, the activated caspase-8 can also trigger activation of caspase-9 (23). In our current study, statins induced both caspase-8 and caspase-9 activation in prostate cancer cell lines as early as 18-h treatment, although caspase-8 seems to be more activated in 1-day treatment with statins than caspase-9, both are activated in mediating statin-induced apoptosis as a whole. In addition, this study failed to show dose-dependent suppression of prostate cancer cells with lovastatin and simvastatin, which may be due to their induction of COX-2 expression at higher doses. Based on our current knowledge, one may hypothesize that induction of COX-2 is one of the potential side effects of statins. Therefore, our data suggest that the combination of statins with other apoptosis-inducing drugs (e.g., COX-2 inhibitors or curcumin) should have synergistic or additive effects.

Although recent epidemiologic studies showed statins’ antitumor activity (5-7), controversial data based on epidemiologic observations were also published in recent years (24). The conclusion from a metaanalysis of randomized controlled trials was that statins have a neutral effect on cancer and cancer death risk. No type of cancer was found affected by statin use, and no subtype of statin affected the risk of cancer. This report aroused concerns in the research community (25-27). Our current study, together with those of others, did show the antitumor activity of statins in prostate cancer cell lines. The logical follow-up to the controversy would be to design a large-scale randomized trial to assess the efficacy of statins in preventing and controlling prostate cancer.

Molecularly, statins inhibit the rate-limiting enzyme of the mevalonate pathway 3-hydroxy-3-methylglutaryl-CoA reductase, and in turn, they block farnesyl PPI and geranylgeranyl PPI synthesis, which is required to activate certain proteins, such as Ras and Rho (4). Our current data showed that lovastatin and simvastatin inhibited RhoA activity and c-Jun expression and then significantly increased the enzymatic activity of caspase-8 and caspase-3 and moderately that of caspase-9. Although the level of apoptosis induced by statins was not much different among these cell lines, statins-induced reduction of cell viability was significantly different, i.e., LnCap cells showed the least reduction of cell viability but the highest level of apoptosis after treatment with statins, whereas PC3 and DU145 cells showed more reduction in cell viability by statins but similar level of apoptosis. This difference may be because of statin’s effects on regulation of expression of G1-phase cell cycle–related genes by reducing Rb, phosphorylated Rb, CDK4, and CDK6 expression, but increasing that of p21 and p27, which clearly showed G1 cell cycle arrest. Gene expression in different prostate cancer cell lines is so complex; thus, lovastatin and simvastatin can trigger more apoptosis in one tumor cell line (e.g., LnCaP) but induce both apoptosis and growth arrest in others (PC3

**Figure 8.** Modulation of COX-2 expression by statins. Prostate cancer cell lines were grown and treated with 1 or 2 μmol/L lovastatin and simvastatin for 5 d. Total cellular protein was extracted and subjected to Western blotting analysis of COX-2.

**Figure 9.** Suppression of RhoA activation by statins. A. RhoA activation assay. Prostate cancer cells were first subjected to RhoA activation cultures (see Materials and Methods) and then treated with or without 2 μmol/L lovastatin and simvastatin for 6 h. The total cellular protein lysates were then pulled down with a Rho-binding domain and then subjected to Western blotting (see Materials and Methods). B. Cell viability assay. The cells were transiently transfected with dominant-active RhoA and the vector-only constructs with FUGENE6 and screened with 400 μg/mL G418 for 3 d, and the cells were then treated with 2 μmol/L lovastatin and simvastatin for 5 d and subjected to cell viability assay. C. Western blotting assay. The cells were subjected the same gene transfection and statin treatment. The total cell lysates were then subjected to Western blotting assay with anti–cyclin D1 and anti–β-actin antibodies.
and Du145). Previous studies showed that statins induced different tumor cells to undergo apoptosis by activating caspase-8 or that they induced tumor cell growth arrest at G1 phase by inducing p21 (28-30). We observed both phenomena. In addition, although others (31, 32) have shown higher concentrations (10 or 20 μm/L) of lovastatin and simvastatin induced COX-2 protein expression in various cell lines, our data showed that a low concentration (2 μm/L) of lovastatin and simvastatin also induced COX-2 expression in prostate cancer cell lines. Moreover, our data suggested that inactivation of RhoA may be the molecular mechanism by which statin mediates its antitumor activity. Our data showed that dominant-active RhoA induced cell growth, but lovastatin and simvastatin inhibited cell viability at similar level for both dominant-active RhoA–transfected and the vector-only–transfected cells (Fig. 9B). In addition, lovastatin and simvastatin suppressed cyclin D1 expression at similar level for the both dominant-active RhoA–transfected and the vector-only–transfected cells (Fig. 9C). Otherwise, the inhibition level of cell growth could be different in the dominant-active RhoA–transfected and the vector-only–transfected cells if lovastatin and simvastatin affect different molecular pathways other than RhoA. Further studies will be required to investigate how inhibition of RhoA activity leads to suppression of tumor cell growth and induction of apoptosis.

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References

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