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Original Article

Low-density lipoprotein receptors play an important role in the inhibition of prostate cancer cell proliferation by statins



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ABSTRACT

Background: There are some reports about the antitumor effects of statins in these days. Statins decrease the level of cholesterol in the blood by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Inhibition of this enzyme decreases intracellular cholesterol synthesis. Thus, the expression of low-density lipoprotein receptor (LDLr) is increased to import more cholesterol from the bloodstream. In this study, we assessed the effects of statins on the proliferation of prostate cancer cells, and studied the relationship between the expression of LDLr and the effects of statins.

Methods: Simvastatin was used in the experiments. We studied the effect of simvastatin on PC-3 and LNCaP cell proliferation using the MTS assay, and evaluated the expression of LDLr after administration of simvastatin by quantitative polymerase chain reaction and Western blotting. Intracellular cholesterol levels in the prostate cancer cells were measured after administration of simvastatin. Furthermore, small interfering RNA (siRNA) was used to knockdown the gene expression of LDLr.

Results: In PC-3 cells, simvastatin inhibited cell proliferation. In LNCaP cells, only a high concentration of simvastatin (100 μM) inhibited cell proliferation. In LNCaP cells, the protein level of LDLr was increased by simvastatin. In PC-3 cells, the protein levels of LDLr were unregulated. In PC-3 cells, but not in LNCaP cells, intracellular cholesterol levels were significantly decreased by simvastatin. After knocking down LDLr expression by siRNA, intracellular cholesterol levels were decreased, and cell proliferation was inhibited by simvastatin in LNCaP cells.

Conclusion: Simvastatin inhibited prostate cancer cell growth by decreasing cellular cholesterol and could be more effective in androgen-independent prostate cancer, where there is loss of regulation of LDLr expression. LDLr was shown to play an important role in the statin-induced inhibition of prostate cancer cell proliferation. These results suggest that future studies evaluating the cholesterol-lowering effects of statin may lead to new approaches to the prevention and treatment of prostate cancer.

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1. Introduction

Hyperlipidemia attracts much attention today. One of the key therapies for hyperlipidemia involves use of statins. Statins decrease the level of cholesterol in the blood by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in the mevalonate pathway.¹ Recently, some reports have demonstrated the antitumor effects of statins. Regarding prostate cancer some clinical studies, however, have presented controversial

results.^{2,3} *In vitro*, statins have biological effects that inhibit prostate carcinogenesis (e.g., cell cycle arrest, apoptosis induction, and inhibition of lipid raft signaling).^{4,5} However, the actual mechanisms underlying their antitumor effects remain unclear.

Low-density lipoprotein receptor (LDLr) plays an important role in the serum cholesterol-lowering effects of statins. Statins upregulate the expression of LDLr and increase clearance of LDL from the bloodstream. In normal cells, the expression of LDLr is dependent on intracellular cholesterol levels.⁶ However, it has also been reported that some cancer cells, including prostate cancer PC-3 cells, lack feedback regulation of LDLr, which provides an extra energy source to promote their uncontrolled growth.⁷

In this study, we evaluated the relationship between LDLr expression and the inhibition of androgen-dependent and

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androgen-independent prostate cancer cell proliferation after treatment with statins. LDLr has been shown to play an important role in the statin-induced inhibition of prostate cancer cell proliferation. These results suggest that statins could be more effective against androgen-independent prostate cancer, in which regulation of LDLr expression is lost.

2. Materials and methods

2.1. Cells and chemicals

The human prostate cancer cell lines PC-3 and LNCaP were purchased from Dainippon Pharmaceutical (Tokyo, Japan) and cultured in Roswell Park Memorial Institute-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Moregate, Bulimba, Australia). Antibodies (rabbit anti-LDLr polyclonal antibody and rabbit antihuman β -actin monoclonal antibody) were purchased from Cell Signaling (Beverly, MA, USA) and Epitomics (Burlingame, CA, USA), respectively. Simvastatin was purchased from Sigma-Aldrich.

2.2. Cell proliferation assay of human prostate cancer cells

Cells were seeded in a 96-well microtiter plate in 100 μ L of medium, with 10% FBS, for 48 hours. Then, the medium was aspirated and the cells were incubated with a medium containing various concentrations of simvastatin. After incubation at 37°C in 5% CO₂ for 48 hours, the number of living cells was measured, using the MTS assay (CellTiter 96 AQueous One Solution cell proliferation assay; Promega, Madison, WI, USA). The optical density of the cell lysate was expressed as fold change.

2.3. Quantification of messenger RNA levels

Messenger RNA (mRNA) levels were quantified using the CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Total RNA extraction and complementary DNA (cDNA) synthesis were then performed⁸; polymerase chain reaction (PCR) amplification was done using 2 μ L of cDNA, LDLr primer (No. Hs01092525_m1, Applied Biosystems, Waltham, MA, USA). Next, PCR was performed for one cycle of 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. For the internal control, β -actin (No. 4326315E, Applied Biosystems) transcript levels were used. Quantitation of mRNA fold changes was made using the comparative CT ($2^{-\Delta\Delta Ct}$) cycle (ΔCt) method.⁹

2.4. Western blotting assays

Cell lysates were prepared in radioimmunoprecipitation assay buffer (Pierce, Rockford, IL, USA), containing protease inhibitors (complete, without EDTA; Roche Diagnostics, Penzberg, Germany). Equal amounts of protein (30–40 μ g/lane) were electrophoresed on 4–12% sodium dodecyl sulfate–polyacrylamide gel and transferred onto nitrocellulose membranes. Each membrane was incubated with the aforementioned primary antibodies. Blots were developed with a 1:1,000 dilution of the horseradish peroxidase-conjugated secondary antibody (Cell Signaling). Proteins were visualized using the Immobilon Western HRP Reagent (Millipore, Billerica, MA, USA). A representative experiment of three independent experiments is shown in each figure.

2.5. Measurement of total cholesterol levels in vitro

Cells were cultured in a 6-well plate and incubated overnight in medium containing 10% FBS. Cells were then incubated in a culture

medium containing various concentrations of simvastatin. After 72 hours, the medium was aspirated, and cells were washed with PBS. Cholesterol was extracted with hexane:isopropanol (3:2, v/v), and the solution was transferred to glass tubes for drying by evaporation. Once the tube was dried, 200 μ L of 50mM Tris containing 0.1% Triton X-100 and 10mM sodium cholate was added to the tube, and cholesterol concentrations were measured enzymatically (Wako, Osaka, Japan). In addition, a solution of 0.1% sodium dodecyl sulfate plus 0.1N NaOH was applied to the wells and the protein concentration was measured using a DC protein assay (Bio-Rad). The total cholesterol level was calculated by dividing the result by the total protein concentration.

2.6. Small interfering RNA

Cells were transfected with ON-TARGETplus Nontargeting Pool (No. D-001810-10-05, Dharmacon, Waltham, MA, USA) and ON-TARGETplus LDLr small interfering RNA (siRNA; No. L-011073-00-0005, Dharmacon) using DharmaFect (Dharmacon). After transfection, the cells were incubated for 48 hours at 37°C in a 5% CO₂ atmosphere.

2.7. Statistical analysis

All data are expressed as means \pm standard deviation unless otherwise indicated. Differences between values were evaluated by one-way analysis of variance with Tukey *post hoc* test. In all analyses, $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effect of simvastatin on prostate cancer cell proliferation

First, we examined the effects of simvastatin on prostate cancer cell proliferation using MTS assay. The viable cell numbers of PC-3 cells decreased significantly after incubation with simvastatin in a dose-dependent manner (Fig. 1A). By contrast, simvastatin did not inhibit the proliferation of LNCaP cells except at the high concentration (100 μ M; Fig. 1B).

3.2. Levels of LDLr in prostate cancer cells after treatment with simvastatin

We then evaluated the effect of simvastatin on LDLr levels in prostate cancer cells. As shown in Fig. 2A, simvastatin significantly decreased LDLr mRNA expression in PC-3 cells. However, LDLr mRNA levels significantly increased after simvastatin treatment in LNCaP cells. In the same manner as mRNA, LDLr protein levels also

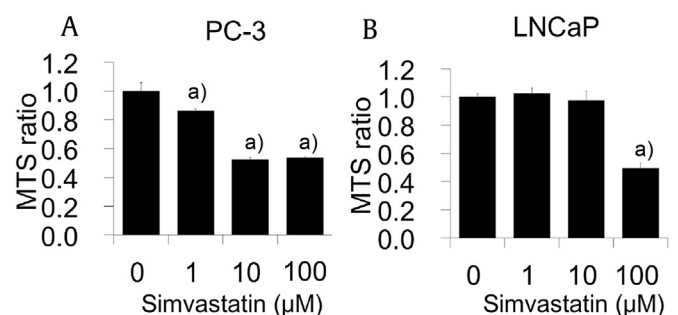


Fig. 1. Effects of simvastatin on prostate cancer cell proliferations. (A, B) Cells were incubated with simvastatin-containing media. The inhibitor effects of simvastatin on cell proliferation were evaluated at 48 hours with MTS assay. Data are expressed as mean \pm SD ($n = 4$). ^{a)} $P < 0.01$ versus simvastatin 0 μ M.

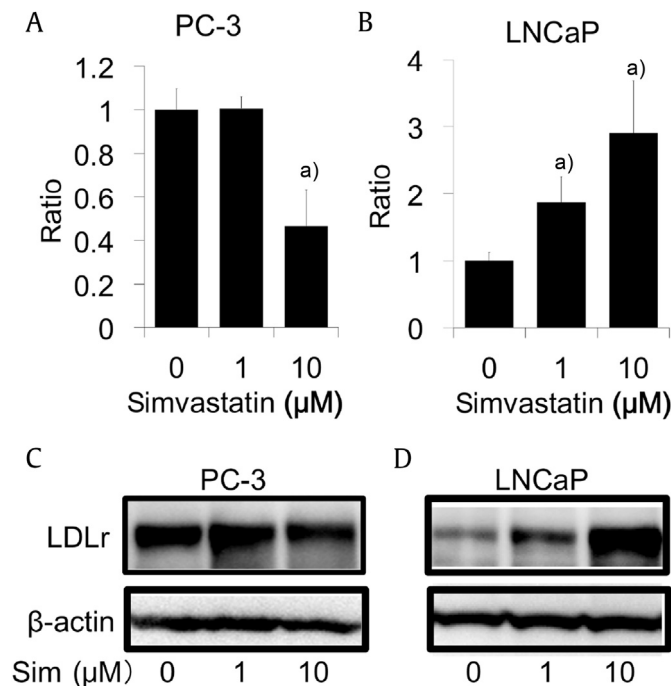


Fig. 2. Low-density lipoprotein receptor (LDLr) expression levels in prostate cancer cells after treatment with simvastatin. (A, B) Cells were incubated with simvastatin-containing media. RNA samples were extracted and assayed by quantitative real-time polymerase chain reaction. Data are expressed as means \pm SD ($n = 5$). ^{a)} $P < 0.01$ versus 0μM. (C, D) Cells were incubated in simvastatin simvastatin-containing media for 48 hours and analyzed for LDLr protein levels by Western blotting. Beta-actin protein levels were used as the internal control. Sim, simvastatin.

increased in LNCaP cells, whereas in PC-3 cells, LDLr protein levels remained unchanged after simvastatin treatment (Fig. 2C).

3.3. Intracellular cholesterol levels in prostate cancer cells after simvastatin treatment

Cholesterol levels are known to affect prostate cancer cell proliferation.¹⁰ In PC-3 cells, simvastatin decreased intracellular cholesterol levels in a dose-dependent manner (Fig. 3A). However, LNCaP cells appeared to be resistant to any change in intracellular cholesterol levels with simvastatin (Fig. 3B).

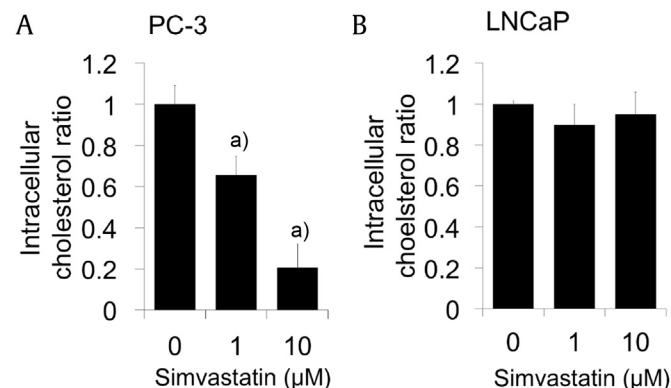


Fig. 3. Total cholesterol mass after 72-hour treatment with various concentrations of simvastatin. (A, B) Data are expressed as means \pm SD ($n = 3$). ^{a)} $P < 0.01$, versus 0μM.

3.4. Effects of LDLr knockdown on LNCaP cells treated with simvastatin

To further determine whether LDLr plays an important role in the statin-induced inhibition of prostate cancer cell proliferation, LDLr expression was reduced by transfection with a siRNA against LDLr. The amount of LDLr protein was reduced markedly by the siRNA (Fig. 4A). The reduced level of LDLr in LNCaP cells following siRNA transfection was associated with decreased intracellular cholesterol levels after treatment with simvastatin (Fig. 4B). Simvastatin also inhibited LNCaP cell proliferation after transfection of the siRNA (Fig. 4C), consistent with the known role of intracellular cholesterol in cell proliferation.

4. Discussion

In this study, we showed that the mechanism of the effect of simvastatin on prostate cancer cells depends on the expression of LDLr. It has been reported that statins inhibit the development of prostate cancer cells, but the mechanism has not been clear. To the best of our knowledge, this is the first report showing that the LDLr plays an important role in the statin-induced inhibition of prostate cancer cell proliferation.

Statins are taken by many hyperlipidemic patients worldwide. In addition, statins have been investigated for carcinoma prevention or as cures for cancer. There are many clinical reports on the relationship between statins and prostate cancer outcomes. Recently, large population-based retrospective cohort studies showed that the use of statins after diagnosis was associated with a decreased risk of prostate cancer mortality¹¹; furthermore, statin use was associated with a reduced risk for prostate cancer.² Concerning the outcomes after radical therapy, statin use was associated with reductions in the risk of biochemical recurrence after both radical prostatectomy¹² and radiation therapy.¹³ However, it is reported that the use of statins did not reduce the risk of prostate cancer overall, but was associated with a reduced risk of advanced (especially metastatic or fatal) prostate cancer.¹⁴ We showed that simvastatin had more antitumor effects on androgen-independent than on androgen-dependent prostate cancer cells. These findings indicate that statins may reduce the development of more aggressive prostate cancers.

The expression of LDLr is regulated by intracellular cholesterol levels in normal cells. Lipoprotein treatment decreased LDLr expression to prevent too much cholesterol from entering normal cells.⁶ However, LDLr expression is increased by statins, which inhibit HMG-CoA reductase and decrease intracellular cholesterol levels. As a result, statins reduce blood cholesterol levels by increasing LDL uptake.¹⁵ In this study, LDLr expression in LNCaP cells was increased by simvastatin, as in normal cells, but not in PC-3 cells. Regarding prostate cancer, PC-3 and DU145 cells are reported to lack feedback regulation of sterol regulatory element-binding protein-2 (SREBP2), which controls LDLr expression in accordance with intracellular cholesterol levels; the lack of SREBP2 feedback is at least in part explained by the lack of LDLr feedback in the cells.⁷ It was also reported that androgens regulate lipogenesis through activation of the SREBP pathway in prostate cancer,¹⁶ and dysregulation of SREBPs by androgens occurred during progression to androgen independence in LNCaP cells.¹⁷ Chen and Hughes-Fulford⁷ and Sekine et al¹⁸ have shown that LDL and remnant lipoproteins downregulated LDLr expression in LNCaP cells, but not in PC-3 cells. In this study, androgen-dependent LNCaP cells were able to regulate LDLr expression in response to simvastatin, whereas androgen-independent PC-3 cells could not. Thus, we suggest that androgen dependency has an important role in the regulation of LDLr expression in prostate cancer cells.

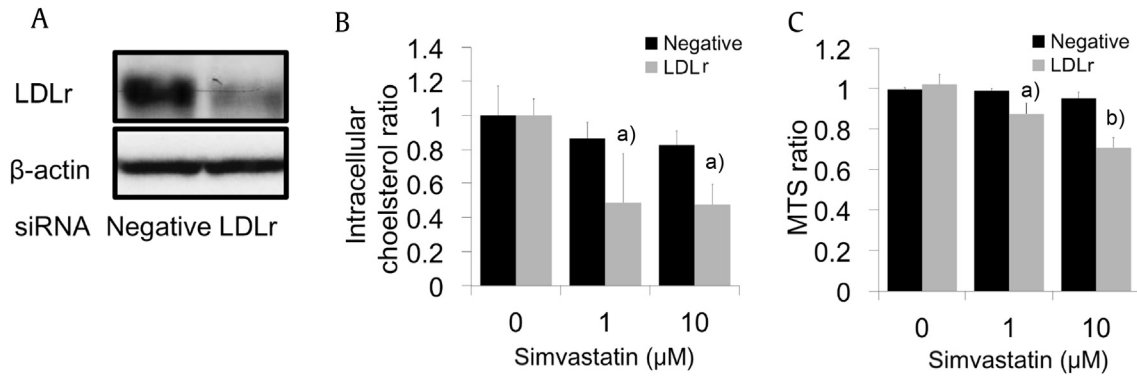


Fig. 4. Effect of low-density lipoprotein receptor (LDLr) knockdown on LNCaP cells treated with simvastatin. (A) Transfection of LNCaP cells with LDLr small interfering RNA (siRNA) or negative siRNA was performed as described in the "Materials and Methods" section. After transfection, the cells were incubated for 48 hours before harvesting for Western blotting. Negative siRNA-transfected cells were used as controls. (B) After transfection, the cells were incubated with simvastatin-containing media. The total cholesterol mass was evaluated after 72 hours of treatment with various concentrations of simvastatin. Values are expressed as the means \pm SD ($n = 3$). ^a $P < 0.01$, vs. 0 μ M. (C) After transfection, the cells were incubated with simvastatin-containing media. The number of viable cells was evaluated using MTS assay after 48 hours of treatment with various concentrations of simvastatin. Values are expressed as means \pm SD ($n = 3$). ^a $P < 0.05$ versus 0 μ M. ^b $P < 0.01$ versus 0 μ M.

Cholesterol is vital for cell membrane integrity, cellular metabolism, and cell signaling in cellular proliferation. Clinically, it has been shown that high cholesterol levels are associated with an increased risk of aggressive prostate cancer.^{19,20} In addition, cholesterol promotes prostate cancer cell line growth *in vitro* and in xenograft models by inducing Akt activation²¹ and *de novo* steroidogenesis.²² We showed that simvastatin inhibited cell proliferation and decreased cellular cholesterol levels in PC-3 cells, which could not increase LDLr expression sufficiently to cover the deficiency in cellular cholesterol after treatment with simvastatin. In addition, the reduced level of LDLr in LNCaP cells following siRNA transfection was associated with decreased cellular cholesterol levels and basal cell proliferation. Murtola et al²³ reported that simvastatin inhibited LNCaP cell proliferation in culture medium without cholesterol, and the effect was prevented by LDL. These findings indicate that a decrease in cholesterol levels is one of the mechanisms underlying statin-induced inhibition of prostate cancer cells.

Our study had several limitations. First, the clinical plasma concentrations of statins are in the range of 10–100nM²⁴ but the concentration in prostate tissue is unknown. Thus, there is a possibility that our experimental concentration of simvastatin was unrealistically high. Furthermore, we evaluated the effect of statins only *in vitro*. Despite these limitations, the results are interesting because there has been no previous report of any relationship between the antitumor effects of statins and LDLr expression in prostate cancer.

In summary, statins inhibited prostate cancer cell growth by decreasing cellular cholesterol, and they could be more effective against androgen-independent prostate cancer, in which there is loss of regulation of LDLr expression. Besides, androgen-deprivation therapy for prostate cancer is well-known to significantly increase the levels of total cholesterol and triglycerides.²⁵ These results suggest that statins have the clinical potentials of not only improving hyperlipidemia, but also inhibiting the progression of castration refractory prostate cancer. Future studies on the cholesterol-lowering effects of statins may lead to new approaches for the prevention and treatment of prostate cancer.

Conflicts of interest

All authors have no disclosures to declare.

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