Simvastatin in combination with meclofenamic acid inhibits the proliferation and migration of human prostate cancer PC-3 cells via an AKR1C3 mechanism

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Abstract. Statins have become of interest in research due to their anticancer effects. However, the exact mechanism of their anticancer properties remains unclear. The authors previously reported that statins decrease intracellular cholesterol levels in androgen-independent prostate cancer cells. In de novo androgen synthesis, cholesterol is the primary material and certain enzymes have important roles. The present study aimed to determine whether simvastatin alters the expression of androgen synthesis-associated enzymes in androgen-independent prostate cancer cells. A novel combination therapy of statins and other drugs that inhibit the overexpression of enzymes involved in androgen synthesis was explored. The cytotoxicity of simvastatin and meclofenamic acid was assessed in prostate cancer cells using MTS and migration assays. Testosterone and dihydrotestosterone concentrations in the culture medium were measured using liquid chromatography-tandem mass spectrometry. RAC-α-serine/threonine-protein kinase (Akt) phosphorylation was detected by western blot analysis. Following treatment with simvastatin, aldo-keto reductase family 1 member C3 (AKR1C3) expression increased in PC-3 (>60-fold) and LNCaP-LA cells, however not in 22Rv1 cells. Small interfering (si)RNA was used to clarify the effects of AKR1C3 expression. The reduction in AKR1C3 expression in PC-3 cells following siRNA transfection was not associated with basal cell proliferation and migration; however, treatment with simvastatin decreased cell proliferation and migration. The combination of simvastatin and meclofenamic acid, an AKR1C3 inhibitor, further enhanced the inhibition of cell proliferation and migration compared with treatment with either drug alone. Furthermore, treatment with simvastatin attenuated insulin-like growth factor 1-induced Akt activation; however, the combination of simvastatin and meclofenamic acid further inhibited Akt activation. These results suggest that the combination of simvastatin and meclofenamic acid may be an effective strategy for the treatment of castration-resistant prostate cancer.

Introduction

A statin is a drug used to treat hyperlipidemia and functions by inhibiting 3-hydroxy-3-metylglutaryl coenzyme A reductase. Statins have gained much recent attention due to their anticancer effects. Previous studies have shown that statins can prolong survival, while others have reported no benefits in cancer patients (1). Concerning prostate cancer, the anticancer effect of statins is controversial (2,3). We previously reported that intracellular cholesterol levels are decreased in androgen-independent prostate cancer cells after treatment with simvastatin (6); however, alterations in androgen synthesis-related enzymes are not clear.

In this study, we determined whether simvastatin alters the expression of enzymes involved in androgen synthesis in CRPC cells. We also explored a new combination therapy...
of statins and other drugs that inhibit the overexpression of androgen synthesis-related enzymes.

**Materials and methods**

**Cells and chemicals.** Human prostate cancer cell lines PC-3, LNCaP, and 22RVI were purchased from DS Pharma Biomedical (Osaka, Japan) and cultured in RPMI 1640 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% FBS (Moregate BioTech, Bulimba, Australia). PC-3 is an androgen receptor-negative human prostate cancer cell line (7). LNCaP-LA cells, which were generated from LNCaP cells, were cultured in medium containing 10% charcoal-stripped fetal bovine serum (FBS) for more than 3 months.

**Measurement of testosterone and dihydrotestosterone (DHT) in culture medium.** Cells were cultured on a 6-well plate and incubated overnight in medium containing 10% FBS. Cells were then incubated with or without simvastatin (5 µM). After 48 h, androstenedione (100 µM) was added to the medium. After 24 h, culture medium was collected, and testosterone and DHT concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (ASKA Pharmaceutical Medical Co., Ltd., Kawasaki, Japan). RIPA buffer was added to wells and protein concentration was measured by the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Testosterone and DHT levels were calculated by dividing the results of the protein assay by the total protein concentration.

**RT-qPCR.** Transcript levels were quantified using the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s protocol. Total RNA was extracted, cDNA was synthesized (8), and polymerase chain reaction (PCR) amplification was performed, using 2 µl cDNA and the StAR, CYP11A1, CYP17A1, aldo-keto reductase family 1 member C3 (AKR1C3), HSD3B1, HSD3B2, SRD5A1, SRD5A2, and AKR1C2 primers (9). Total RNA was extracted, cDNA was synthesized (8), and polymerase chain reaction (PCR) amplification was performed, using 2 µl cDNA and the StAR, CYP11A1, CYP17A1, aldo-keto reductase family 1 member C3 (AKR1C3), HSD3B1, HSD3B2, SRD5A1, SRD5A2, and AKR1C2 primers (9). After 48 h, androstenedione (100 µM) was added to the medium. After 24 h, culture medium was collected, and testosterone and DHT concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (ASKA Pharmaceutical Medical Co., Ltd., Kawasaki, Japan). RIPA buffer was added to wells and protein concentration was measured by the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Testosterone and DHT levels were calculated by dividing the results of the protein assay by the total protein concentration.

**Statistical analysis.** Data are expressed as the mean ± standard deviation. Differences between values were evaluated by one-way ANOVA using Tukey’s post hoc analysis and Student’s t-test. *P* < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Simvastatin altered the expression of genes encoding steroidogenic enzymes in androgen-independent prostate cancer cells.** We examined PC-3, LNCaP-LA and 22RVI cells to determine whether simvastatin alters genes that encode steroidogenic enzymes in androgen-independent prostate cancer cells. After treatment with simvastatin, the expression of AKR1C3 was increased in PC-3 and LNCaP-LA cells (Figs. 1A and 2A) but not in 22RVI cells (data not shown). Moreover, the fold change was more than 60 times in PC-3 cells. Conversely, the expression of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1) was decreased in PC-3 and LNCaP-LA cells (Figs. 1A and 2A) but not in 22RVI cells (data not shown). Moreover, simvastatin increased steroid 5 alpha-reductase 1 (SRD5A1) expression in PC-3 (Figs. 1A) but not in LNCaP-LA or 22RVI cells (data not shown). The expression of steroidogenic acute regulatory protein (StAR), cytochrome P450 family 11 subfamily A member 1 (CYP11A1), cytochrome P450 family 17 subfamily A member 1 (CYP17A1), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (HSD3B2), steroid 5 alpha-reductase 2 (SRD5A2), and aldo-keto reductase family 1 member C2 (AKR1C2) did not change following treatment with simvastatin (data not shown).
Effects of AKR1C3 expression on testosterone and DHT levels in PC-3 cell culture medium. To determine whether increased levels of AKR1C3 affect the de novo synthesis of intracellular androgen, we measured the testosterone and DHT levels in culture medium following treatment with simvastatin by LC-MS/MS. Simvastatin significantly increased both testosterone (Fig. 2A) and DHT (Fig. 2B) levels after the addition of androstenedione. These data show that the up-regulation of AKR1C3 is functional. AKR1C3 inhibition increased the simvastatin-induced inhibition of cell proliferation and migration. PC-3 is an AR-negative human prostate cancer cell line. Therefore, there is a possibility that an increase in testosterone and DHT levels does not affect cell viability. In contrast, the overexpression of AKR1C3 promotes angiogenesis and aggressiveness in PC-3 cells (9). To further determine whether increased AKR1C3 expression affects simvastatin-induced cell viability, AKR1C3 expression was reduced by transfection with siRNA against AKR1C3. siRNA treatment inhibited the expression of AKR1C3 mRNA in PC-3 cells (Fig. 3A). The reduction in AKR1C3 expression in PC-3 cells following siRNA transfection was not associated with basal cell proliferation and migration; however, siRNA transfection with simvastatin significantly decreased both cell proliferation (Fig. 3B) and cell migration (Fig. 3C and D) compared to simvastatin alone.

Meclofenamic acid increased the simvastatin-induced inhibition of cell proliferation and migration. Some drugs are reported to inhibit AKR1C3. Meclofenamic acid is an NSAID as well as one of the best inhibitors of AKRs, especially AKR1C3 (10). Therefore, we evaluated the combinatorial effects of simvastatin and meclofenamic acid in PC-3 cells. Treatment
Statins have recenty been studied for their pleiotrophic effects, which may make them relevant for cancer prevention or treatment. Clinical reports have shown that statin use is beneficial for overall survival and cancer-specific survival both before and after prostate cancer diagnosis (1,2). In contrast, Platz et al reported that the use of statin drugs was not associated with the overall risk of prostate cancer (3). In vitro, statins exert many biological activities that inhibit prostate cancer progression (e.g., lowering raft cholesteral content, inhibiting cyclin-dependent-kinase-2 activity, decreasing IGF1 receptor expression, and increasing AKR1C3 expression) (4,5,11,12). These results indicate that statins have anticancer potential.

Combination of simvastatin and meclofenamic acid inhibited IGF1-induced Akt activation. AKR1C3 overexpression induces Akt activation in PC-3 cells (9). We previously showed that simvastatin without IGF1 decreases IGFR1 expression strongly in PC-3 cells (4). IGF1-Akt activation is a well-known pathway in prostate cancer. We hypothesized that inhibiting simvastatin-stimulated AKR1C3 expression with an AKR1C3 inhibitor would have a synergistic effect on simvastatin-blocked IGF1-induced Akt activation. Therefore, the effects of the combination of simvastatin and meclofenamic acid on IGF1-induced Akt activation were evaluated in PC-3 cells. Treatment with either simvastatin or meclofenamic acid alone attenuated IGF1-induced Akt activation, whereas the combination of simvastatin and meclofenamic acid further inhibited Akt activation (Fig. 5).

Discussion

The main finding of the present study was that simvastatin increased AKR1C3 expression in androgen-independent prostate cancer cells. Furthermore, the combination of simvastatin and meclofenamic acid, an AKR1C3 inhibitor, further suppressed PC-3 cell proliferation, migration, and Akt activation compared with simvastatin alone.

Statins have recentely been studied for their pleiotrophic effects, which may make them relevant for cancer prevention or treatment. Clinical reports have shown that statin use is beneficial for overall survival and cancer-specific survival both before and after prostate cancer diagnosis (1,2). In contrast, Platz et al reported that the use of statin drugs was not associated with the overall risk of prostate cancer (3). In vitro, statins exert many biological activities that inhibit prostate cancer progression (e.g., lowering raft cholesteral content, inhibiting cyclin-dependent-kinase-2 activity, decreasing IGF1 receptor expression, and increasing ANXA10 expression) (4,5,11,12). These results indicate that statins have anticancer potential.

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase, one of the most important players in cholesteral biosynthesis. In androgen-dependent prostate cancer cells, statins do not lower intracellular cholesterol levels by up-regulating the low density lipoprotein receptor in the same manner as in normal cells (6). Conversely, statins decrease intracellular cholesterol levels in androgen-independent prostate cancer cells, which cannot regulate low density lipoprotein receptor expression (6). In this study, we focused on the decrease in intracellular cholesterol levels following treatment with statins in CRPC. Recent reports have shown that de novo androgen synthesis is a therapeutic objective in CRPC (13). In the androgen synthesis pathway, cholesterol is the primary material. Therefore, we examined the effects of statins on androgen synthesis-related enzymes in CRPC cells.

Simvastatin increased the expression of AKR1C3 in PC-3 cells. AKR1C3 exhibits 5α-, 17β- and 20α-hydroxysteroid dehydrogenase activities (14). The expression of AKR1C3 is increased in several human cancers, including kidney (15) and breast (16). Concerning prostate cancer, localized, metastatic and recurrent prostate cancer has high levels of AKR1C3 (17-19). In addition, elevated AKR1C3 expresion promotes the aggressiveness of PC-3 cells, which lack AR (7). These data indicate that increased levels of AKR1C3 induce prostate cancer progression not only by synthesizing...
intracellular androgen but also by androgen-independent mechanisms.

Medical agents such as non-steroidal anti-inflammatory drugs (NSAIDs), steroids, flavonoids, cyclopentane derivatives, and benzodiazepines inhibit AKR1C3 (20). One example of an NSAID is meclofenamic acid, which inhibits AKR1C3, cyclooxygenase-1, and cyclooxygenase-2 more strongly than other NSAIDs (21,22). Meclofenamic acid inhibits androgen-independent prostate cancer progression both in vitro and in vivo (23). In this study, AKR1C3 siRNA did not affect PC-3 cell proliferation and migration, whereas meclofenamic acid inhibited these processes, suggesting that meclofenamic acid also has anticancer effects without the AKR1C3 mechanism. The combination of simvastatin and meclofenamic acid inhibited PC-3 cell proliferation, migration, and Akt activation to a greater extent than simvastatin or meclofenamic acid alone. Previous reports have described combination therapy using statins and NSAIDs for the treatment of prostate cancer, which works by inhibiting NF-kB (24) or IL-6 (25). These results reveal that the inhibition of AKR1C3 is an underlying mechanism of the combination therapy of simvastatin and meclofenamic acid.

The present study had several limitations. First, three androgen-independent prostate cancer cell lines responded to simvastatin with different levels of AKR1C3 expression. Prostate cancer is very heterogenic. In particular, androgen-independent prostate cancer cell lines have a different genetic background (26). Therefore, our results may not be applicable to all CRPCs. In addition, we evaluated the effects of simvastatin and meclofenamic acid only in PC-3 cells, and studied the changes of gene expressions following simvastatin treatment only in one single time rather than performing a time-course experiment. Concerning Akt activation by IGF-1, we also checked only in one single time and IGFIRe phosphorylation status was not evaluated. Moreover, in vivo models are required to show whether the combination of simvastatin and meclofenamic acid may have a curative influence on CRPC.

In summary, simvastatin increased AKR1C3 expression in androgen-independent prostate cancer cells, and the combination of simvastatin and meclofenamic acid further inhibited PC-3 cell proliferation, migration, and Akt activation compared with simvastatin alone. These results suggest that the combination of statin and NSAIDs may be an effective strategy for the treatment of prostate cancer.

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References


