Statins induce apoptosis and inhibit proliferation in cholangiocarcinoma cells

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Abstract. Given the poor prognosis for cholangiocarcinoma, new and effective treatments are urgently needed. HMG-CoA reductase inhibitors (statins) reportedly exert anticancer effects in a variety of diseases, but there have been no reports of these effects in cholangiocarcinoma. In this study, we investigated the utility of statins for cholangiocarcinoma treatment. Proliferation suppression by pitavastatin and atorvastatin was investigated in the human cholangiocarcinoma cell lines HuCCT1 and YSCCC while changes in the cell cycle and intracellular signals were examined by FACS and Western blotting, respectively. Additive proliferation suppression by statins and pre-existing anticancer drugs was also investigated. HuCCT1 and YSCCC cell proliferation was dramatically suppressed by incubation with statins for 72 h or longer. Cell cycle analysis revealed a reduction in the G2M fraction and an increase in the sub-G1 fraction in statin-treated cells, while Western blotting showed increased levels of cleaved caspase-3 and a reduction in p-ERK. Furthermore, statins in combination with gemcitabine, cisplatin and 5-FU showed additive proliferation suppression. In this study, treatment of human cholangiocarcinoma cells with statins induced apoptosis via suppression of the classical MAPK pathway. Together, these results suggest that statins may be a new cholangiocarcinoma treatment option that could potentially enhance the anticancer effect of pre-existing anticancer drugs.

Introduction

Among gastroenterological cancers, cholangiocarcinoma is difficult to diagnose and only a small percentage of cases can be curatively resected surgically. The prognosis for cholangiocarcinoma is markedly poor with a five-year survival rate of <5% (1,2).

According to the study by Valle et al, gemcitabine plus cisplatin treatment was recently recommended as the primary treatment for surgically unresectable advanced or recurrent cholangiocarcinoma, but the median survival time with this treatment was still only ~11.7 months (3). Therefore, improved treatment protocols for cholangiocarcinoma are urgently needed. HMG-CoA reductase inhibitors (statins) are used to treat hypercholesterolemia by inhibiting synthesis of serum lipids such as cholesterol and triglyceride (4,5), which can prevent cardiovascular events by protecting the heart and blood vessels (6-9).

A number of recent reports demonstrated that statins inhibit not only cancer growth but also neovascularization and metastasis (10-12). The in vitro effects of statins on cancer, cell cycle regulation (13-15) and apoptosis induction (16-19) have been shown and cancer cell proliferation suppression has been confirmed (10-12). Statins also inhibit growth of cancer in vivo and reduce metastasis at clinical doses (20,21).

Moreover, clinical trials in humans and epidemiological studies demonstrated that the long-term use of statins reduces the onset and progression of colorectal, prostatic, pancreatic and breast cancers (22-25). However, to date, there are no reports as to whether statins have beneficial effects for cholangiocarcinoma. In this study, we aimed to clarify statin-induced differentiation and growth suppression in cholangiocarcinoma cells, the underlying molecular and biological mechanism of this effect, and explore the possibility of statins as a new treatment option for cholangiocarcinoma.

Materials and methods

Materials. Human extrahepatic cholangiocarcinoma HuCCT1 and human intrahepatic cholangiocarcinoma YSCCC cells were purchased from the Riken Cell Bank (Japan). Pitavastatin was provided by Kowa (Japan). Atorvastatin was supplied by Astellas (Japan). Gemcitabine and cisplatin were purchased from Toronto Research Chemicals Inc. (Canada) and Calbiochem (Germany), respectively, while 5-FU was purchased from Wako (Japan). The final concentration of dimethyl sulfoxide...
ERK 1/2 rabbit polyclonal antibody (no. 9102, Cell Signaling Technology) was then reacted overnight at 4˚C with primary antibodies (5% non-fat dry milk in TBS-0.5% Tween-20). The membrane and the membrane was blocked for 1 h with blocking solution on a 12% SDS gel.

Proteins were transferred to a PVDF membrane (Bio-Rad) and the membrane was blocked for 1 h with blocking solution (5% non-fat dry milk in TBS-0.5% Tween-20). The membrane was then reacted overnight at 4˚C with primary antibodies diluted 1:1,000. The following primary antibodies were used: ERK 1/2 rabbit polyclonal antibody (no. 9102, Cell Signaling Technology), phospho-ERK1/2 (Thr202/Tyr204) rabbit polyclonal antibody (no. 9101, Cell Signaling Technology), cleaved caspase-3 (Asp175) rabbit polyclonal antibody (no. 9664, Cell Signaling Technology), and actin goat polyclonal antibody (sc-1616, Santa Cruz Biotechnology).

A peroxidase-labeled secondary antibody was then added to the membrane reacted with a primary antibody. After rinsing, the membrane was treated with chemiluminescence solution for 5 min. Specific bands were detected on X-ray film following appropriate exposure to light in the dark room or with VersaDoc 5000MP (Bio-Rad) for image processing. We evaluated the resulting bands by densitometric measurement with Image J (NIH, USA). Actin and ERK were used as internal controls for cleaved caspase-3 and p-ERK, respectively.

**Cell culture.** Human cholangiocarcinoma HuCCT1 and YSCCC cells were used in all experiments and were cultured in RPMI-1640 (Gibco BRL®) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 95% air and 5% CO₂ at 37°C. Microscopic cell images were recorded by phase-contrast imaging with a BIOREVO BZ-9000 fluorescence microscope (Keyence).

Cells were rinsed twice with ice-cold PBS and treated with 100 µl sample buffer (SDS β-ME) on ice for 30 min. The whole cell lysate was centrifuged at 12,500 rpm for 15 min at 4°C. Protein lysates (20 µl) were electrophoresed on a 12% SDS gel.

Proteins were transferred to a PVDF membrane (Bio-Rad) and the membrane was blocked for 1 h with blocking solution (5% non-fat dry milk in TBS-0.5% Tween-20). The membrane was then reacted overnight at 4°C with primary antibodies diluted 1:1,000. The following primary antibodies were used: ERK 1/2 rabbit polyclonal antibody (no. 9102, Cell Signaling Technology), phospho-ERK1/2 (Thr202/Tyr204) rabbit polyclonal antibody (no. 9101, Cell Signaling Technology), cleaved caspase-3 (Asp175) rabbit polyclonal antibody (no. 9664, Cell Signaling Technology), and actin goat polyclonal antibody (sc-1616, Santa Cruz Biotechnology).

To determine the additive growth inhibitory effect of anticancer drugs and statins, 1×10⁴ exponential growth cells were seeded in a 96-well micro-titer plate and cultured for 24 h. After confirmation of cell adherence, atorvastatin and pitavastatin were added to each well at final concentrations of 0-20 µM and the cells cultured for an additional 24-96 h. Cell proliferation was measured with the Cell Counting Kit-8 (Dojindo, Japan) graphically as the average ± SD. Student’s t-test was used for statistical analysis, and p<0.05 was considered statistically significant. Statistica software (StatSoft, USA) was used for analysis.

**Results**

**Growth suppression in cholangiocarcinoma cells by statins.** We first examined growth suppression of HuCCT1 and YSCCC cells by pitavastatin and atorvastatin using a cell proliferation assay. The proliferative activity of viable cells depending on the drug concentration and exposure time was evaluated by absorbance measurements. Compared with the control, cell proliferative activity was significantly suppressed by 20.5% (p=0.00005) and 41.0% (p=0.00001) in YSCCC cells and 18.4% (p=0.00044) and 46.5% (p=0.00014) in HuCCT1 cells following a 96-h exposure to pitavastatin at 10 and 20 µM, respectively (Fig. 1). Upon a 96-h exposure to atorvastatin at 10 and 20 µM, respectively, cell proliferative activity was significantly suppressed relative to control cells by 14.2% (p=0.00369) and 28.6% (p=0.00008) in YSCCC cells and 11.3% (p=0.00256) and 26.4% (p=0.00015) in HuCCT1 cells (Fig. 1).

We examined and recorded time-course morphological changes in cholangiocarcinoma cells induced by pitavastatin and atorvastatin using microscopy with phase-contrast imaging. For YSCCC cells, statin exposure shortened the cell diameter, reduced pseudopod formation, and decreased the cell number per view. These changes were more marked for 20 vs. 10 µM statins and for 96 vs. 48-h exposure (Fig. 2). HuCCT1 cells underwent similar morphological changes (data not shown).

**Apoptosis induction in cholangiocarcinoma cells by statins.** We examined cell morphological changes by staining and FACS. Cells were stained with propidium iodide and subjected to cell cycle analysis. Compared with the control, statin-treated cells showed a reduction in side scatter (SSC) and forward scatter (FSC) and a tendency toward reduced cell diameter and density (Fig. 3). Compared with DMSO-treated controls, 96-h exposure to 20 µM pitavastatin and atorvastatin reduced the G2M fraction from 19.01 to 13.72 and 14.21%, respectively, and increased the subG1 fraction from 2.47 to 14.4 and 11.36%, respectively, which indicated that apoptosis was induced (Fig. 3 and Table 1). These changes were more marked at 96 than at
No changes were observed for control cells exposed to DMSO at the same concentration (Fig. 3). Similar phenomena were observed for HuCCT1 cells. (Data for 10 µM in HuCCT1 cells are not shown).

Table I. Changes in the cell cycle by exposure to statin in cholangiocarcinoma YSCCC cells.

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>48 h Control</th>
<th>Pitavastatin (20 µM)</th>
<th>Atorvastatin (20 µM)</th>
<th>96 h Control</th>
<th>Pitavastatin (20 µM)</th>
<th>Atorvastatin (20 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (%)</td>
<td>64.76</td>
<td>72.55</td>
<td>70.96</td>
<td>66.67</td>
<td>48.59</td>
<td>49.13</td>
</tr>
<tr>
<td>S (%)</td>
<td>14.9</td>
<td>10.29</td>
<td>8.22</td>
<td>12.04</td>
<td>14.65</td>
<td>24.92</td>
</tr>
<tr>
<td>G2 M(%)</td>
<td>19.79</td>
<td>14.26</td>
<td>23.46</td>
<td>19.01</td>
<td>13.72</td>
<td>14.21</td>
</tr>
<tr>
<td>Sub G1 (%)</td>
<td>2.24</td>
<td>5.07</td>
<td>1.24</td>
<td>2.47</td>
<td>14.4</td>
<td>11.36</td>
</tr>
</tbody>
</table>

48 h and at 20 than 10 µM statin. No changes were observed for control cells exposed to DMSO at the same concentration (Fig. 3). Similar phenomena were observed for HuCCT1 cells. (Data for 10 µM in HuCCT1 cells are not shown).

Changes in proteins involved in survival signals (caspase-3 and ERK) in cholangiocarcinoma cells. To investigate the effect of statins on caspase-3 and ERK, we collected cells after exposure to statins for different periods and examined the changes in intracellular signaling proteins by Western blotting.

In YSCCC cells, exposure to 20 µM pitavastatin time-dependently elevated cleaved caspase-3 levels (cleaved caspase-3/actin) to 120.0 and 281.2% 48 and 96 h after addition, respectively. Exposure to atorvastatin temporarily decreased
Enhanced effect of anticancer drugs by statins in cholangiocarcinoma cells. We used a cell proliferation assay to compare the proliferation suppressive effect in cholangiocarcinoma cells of anticancer drugs used either alone or in combination with statins. Proliferation suppression was more marked in cells pretreated with statins for 48 h prior to anticancer drug treatment compared to cells exposed to an anticancer drug alone. After exposure to pitavastatin (20 µM) for 48 h, gemcitabine, cisplatin and 5-FU were added and the YSCCC cells were cultured for another 48 h. Compared with control cells exposed to these anticancer drugs (40 µM) alone, cell proliferative activity was significantly attenuated to 72.7% (p=0.00051), 36.6% (p=0.00002) and 62.2% (p=0.00235), respectively (Fig. 5A).

Similarly, after exposure to pitavastatin (20 µM) for 48 h, gemcitabine, cisplatin and 5-FU were added to HuCCT1 cells and cultured for another 48 h. Compared with control cells exposed to these anticancer drugs (40 µM) alone, cell proliferative activity was significantly attenuated to 81.1% (p=0.00047), 85.9% (p=0.00314) and 81.6% (p=0.00432), respectively (Fig. 5B) (data not shown for atorvastatin).

Discussion

Chronic bile stasis and ensuing chronic inflammation are reported to cause genome injury by releasing a variety of cytokines and growth factors in situ, such as IL-6, AKT, EGF and MAPK, which results in persistent cell differentiation...
and reduced amounts of apoptosis (26,27). We previously proposed treating cholangiocarcinoma with the HER family tyrosine kinase inhibitor CI-1033 in combination with radiation to target the Fas-FasL pathway (28) and therapy with the

Figure 3. Statin-dependent apoptosis in cholangiocarcinoma cells. Quantitative FACS analysis is shown. (A) Morphological changes were induced by pitavastatin and atorvastatin in YSCCC cells. Forward scatter (FSC) and side scatter (SSC) were reduced after exposure to 20 µM pitavastatin (ii) or atorvastatin (iii) for 48 h compared with DMSO for 48 h (i), and 20 µM pitavastatin (v) or atorvastatin (vi) for 96 h compared with DMSO for 96 h (iv). Red and yellow dots indicate a concentration of cells. (B) Propidium iodide staining shows changes in the cell cycle. A marked reduction in the G2M fraction, as well as the appearance of the sub-G1 fraction, was observed upon exposure to 20 µM pitavastatin (v) or atorvastatin (vi) for 96 h.
EGFR inhibitor ZD-1839 and radiation to target the EGFR-RAS-PI3K-AKT pathway (29). However, these reports of combination therapy are not in practical use, prognosis from cholangiocarcinoma remains poor.
Statins have a variety of effects (30). We demonstrated that two types of hydrophobic statins, pitavastatin and atorvastatin, induced apoptosis in cholangiocarcinoma cells after exposure for as long as 72-96 h and inhibited proliferation in a dose- and time-dependent manner. In general, there are two types of statins: a) natural or fermented products: lovastatin (Mevacor), simvastatin (Zocor) and pravastatin (Pravachol); and b) synthesized products: fluvastatin (Lescol), atorvastatin (Lipitor), cerivastatin (Lipobay) and rosuvastatin (Creator). Pravastatin and cerivastatin are hydrophilic, while simvastatin and lovastatin are hydrophobic (25,31).

By inhibiting HMG-CoA reductase, statins deplete mevalonic acid, a cholesterol precursor, and inhibit cholesterol synthesis. However, multiple aspects of statins beyond their cholesterol-reducing effects have attracted attention and are thought to be dependent on their structural characteristics, such as hydrophilicity and hydrophobicity (25).

Studies on breast, gastrointestinal and prostatic cancer, as well as leukemia and lymphoma, have shown an anticancer effect of statins, but these have yet to be definitively proven. On the other hand, hydrophilic statins have no inhibitory effect on HMG-CoA reductase activity in cells with an extrahepatic origin, while statins are involved in mevalonic acid synthesis and could potentially increase carcinogenic risk (32-34).

Meanwhile, since hydrophobic statins inhibit HMG-CoA reductase activity in cultured human hepatocytes and cells with an extrahepatic origin, the use of hydrophobic statins such as simvastatin and lovastatin should not increase this risk (33,34), and this has been confirmed epidemiologically (35). Therefore, the results of this study showing that the hydrophobic statins pitavastatin and atorvastatin inhibited cholangiocarcinoma cell proliferation are consistent with these previous findings.

On the other hand, sensitivity to statins has been reported to be varied and statin-induced cell cycle arrest and susceptibility to apoptosis differ among breast and prostatic cancer, myeloma, and melanoma cells (30,36,37). Although the cause of these differences is unclear, statins likely do not influence isoprenylation of proteins but rather act on pathways that are affected by different growth characteristics or genetic mutations present in various cancer cells (37).

The reduction in p-ERK/ERK and the elevated caspase-3 levels we detected could indicate that the cells underwent apoptosis that was dependent on the MAPK pathway. Statins were previously reported to enhance the effect of anticancer drugs and radiation therapy in other cancer cells, such as prostatic and pancreatic cancer cells (38-40), and the results of this study demonstrated that statins combined with anticancer drugs such as gemcitabine, cisplatin and 5-FU enhanced their effects in cholangiocarcinoma cells (Fig. 5). Therefore, use of statins has the potential to be a new treatment option for cholangiocarcinoma.

The inclusion of statins in anticancer treatment regimens may reduce the amount of anticancer drugs needed to achieve therapeutic effects, and as such could reduce the side-effects associated with anticancer drugs. Our results could have significant clinical importance since cholangiocarcinoma has a markedly poor prognosis.

In this study, we used two types of cells and two types of statins. Future in vitro and in vivo experiments using different cholangiocarcinoma cells will be necessary to examine the relationship between statin treatment and genetic mutation in cancer cells (37), as well as selection of appropriate statin type, concentration, exposure time, and which anticancer drugs will be most useful for combination therapies. Results from these studies could eventually provide a foundation for future clinical trials.

In conclusion, long exposure to statins inhibited p-ERK and subsequently induced apoptosis in cholangiocarcinoma cells. Moreover, statins were found to have an additive impact on the anticancer effect of pre-existing anticancer drugs in cholangiocarcinoma cells. Statins could thus be a new treatment option for cholangiocarcinoma.

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