

Downregulation of survivin expression and elevation of caspase-3 activity involved in pitavastatin-induced HepG 2 cell apoptosis

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Abstract. The aim of the present study was to research the apoptosis of human hepatocellular carcinoma cell line HepG 2 induced by pitavastatin. HepG 2 cells were treated with increasing doses of pitavastatin or with mevalonic acid for 48 h. The proliferation of cells was detected with WST-8. The morphology of the nucleus was observed under a microscope by Hoechst 33258 staining. The apoptosis peaks were examined by flow cytometry. The expression of survivin mRNA was examined with RT-PCR. The caspase-3 activity was detected with caspase-3 colorimetric protease assay. We found that growth inhibitory effects were observed for treatment with pitavastatin at 10-50 μ M. Pitavastatin at 10 μ M induced granular apoptotic bodies of HepG 2 cells. Furthermore, pitavastatin at 10 μ M increased the appearance of sub-G1 population of HepG 2 cells. Finally, pitavastatin at 10 μ M downregulated the expression of survivin mRNA and upregulated the caspase-3 activity, which was clearly related to the HMG-CoA reductase activity. These results suggest that pitavastatin at 10 μ M induces apoptosis of HepG 2 cells, which is associated with the decreased expression of survivin mRNA and increased caspase-3 activity of HepG 2 cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer-related causes of death worldwide. In light of the very poor 5-year survival, new therapeutic approaches are mandatory (1). The 3-Hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, statins, are widely used with cholesterol-lowering drugs. The liver is the primary target organ for these drugs, and they inhibit the rate-limiting step

in cholesterol synthesis (2). Interestingly, recent studies have emphasized that some of the non-lipid-related effects of statins present potential benefits, such as anti-cancer effects *in vitro*. It has also been suggested that they increase survival time for patients with advanced hepatocellular carcinoma in combination with 5-fluorouracil (3).

Survivin is one of the apoptosis inhibitors and plays a key role in the mechanism of anti-apoptosis of tumors (4). If the activity of it is suppressed, tumor cells can undergo apoptosis and stop growing (5). In addition, survivin inhibits various modulation elements of cell apoptosis and exerts their action through the caspase enzyme system. Among them, caspase-3 is probably the one that so far best correlates with apoptosis (6). Furthermore, caspase-3 expression was detected in several human malignancies such as non-small cell lung carcinoma and gastric cancer (7,8). Statins have been proposed as promising adjunctive anti-cancer agents to treat HCC, but their mode of action is still poorly characterized (9). Pitavastatin is a novel highly potent inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (10). The mechanisms of the anti-cancer effect by pitavastatin have been poorly investigated in hepatocellular carcinoma cells. Therefore, the purpose of the present study was to examine the effect of pitavastatin treatment on the apoptosis of hepatocellular carcinoma cells (HepG 2). Here, we showed that pitavastatin induces apoptosis in HepG 2 cells, which is associated with the decreased expression of survivin mRNA and increased caspase-3 activity of HepG 2 cells.

Materials and methods

Reagents. Pitavastatin (Livalo®, NK-104) was kindly provided by Kowa Co., Ltd. (Nagoya, Japan) and Nissan Chemical Industries, Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Mevalonic acid (MEV) and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA).

Cell culture. The hepatocellular carcinoma cell line HepG 2 was originally obtained from the American Type Culture Collection (ATCC) and maintained in DMEM medium (Sigma) containing 10% fetal bovine serum (FBS). The cells

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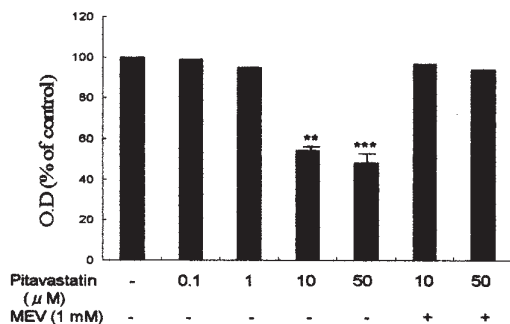


Figure 1. Growth inhibition of pitavastatin in HepG 2 cells. Cells were treated with different concentrations of pitavastatin and/or MEV (1 mM) for 48 h. The cell growth was determined using a WST-8 colorimetric assay. The results are expressed as percentages of cell growth relative to untreated control cells. The data represent the means \pm SD (n=3); **p<0.01, ***p<0.001 vs. control.

were maintained at 37°C in 5% CO₂. For experiments, the cells were seeded in 6-well culture plates and grown in complete medium to 90% confluence. Then, the cells were washed with phosphate-buffered saline (PBS) and incubated for 48 h at 37°C in 2 ml of serum-free medium containing pitavastatin or vehicle, in the presence or absence of MEV.

WST-8 assay. The cell proliferation was evaluated using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay. HepG 2 cells (1x10⁴ cells/well) were seeded into 96-well plates in 100 μ l of culture medium overnight, and then treated with various concentrations of pitavastatin for 48 h. Next, 10 μ l of WST-8 reagent solution (Cell Counting kit, Dojindo Laboratories, Japan) was added and incubated for 2 h. Cell viability was determined according to the manufacturer's instructions.

Hoechst 33258 staining. To observe nucleus changes occurring during apoptosis, the chromation-specific dye Hoechst 33258 was used (11). Cultures were fixed for 5 min with 4% formaldehyde in PBS at 37°C and then permeabilized by treatment with a mixture of ethanol/acetic acid (3:1) for 10 min at 25°C. After being washed with PBS, the cells were stained with 1 μ g/ml Hoechst 33258 in PBS for 10 min at room temperature and then washed again. Apoptosis was determined morphologically after staining the cells with Hoechst 33258 using fluorescence microscopy.

Flow cytometric analysis. Cells were harvested by trypsinization, washed twice with ice-cold PBS, re-suspended in ice-cold PBS and fixed with 70% ethanol. When ready to stain with propidium iodide (PI), the cells were centrifuged. After the ethanol was removed, the cells were washed once in PBS. The cell pellets were then re-suspended in 1 ml of PI/Triton X-100 staining solution (0.1% Triton X-100 in PBS, 0.2 mg/ml RNase A and 10 μ g/ml propidium iodide) and incubated for at least 30 min at room temperature. The stained cells were analyzed using a FACScan flow cytometer in combination with BD Lysis II software (Becton Dickinson).

RT-PCR for the detection of survivin mRNA expression. Survivin mRNA expression in HepG 2 cells was determined by

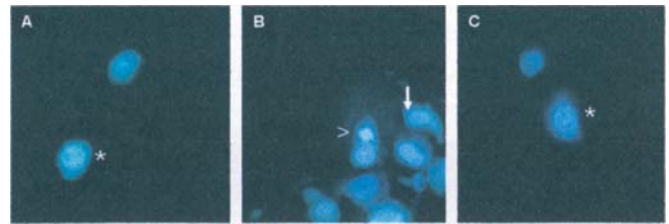


Figure 2. Fluorescence photomicrographs of HepG 2 cells with Hoechst 33258 staining. The assessment of nuclear morphology of the cell was performed after HepG 2 cells were incubated for 48 h with vehicle (A), with 10 μ M pitavastatin (B) or with 10 μ M pitavastatin and MEV (C) respectively. (*) Normal nuclear structure; (→) cytoplasmic change; (-) nuclear fragmentation (Hoechst 33258x400).

RT-PCR analysis. To prepare the samples for the analysis, we isolated the total RNA from HepG 2 cells using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The primers used were survivin sense: 5'-GGA CCACCGCATCTCTACAT-3', antisense: 5'-GCACTTTCT TCGCAGTTTCC-3' (12); and GAPDH sense: 5'-ACCACA GTCCATGCCATCAC-3', antisense: 5'-TCCACCACCCT GTTGCTG-3'. The reaction parameters of survivin were as follows: 95°C for 5 min, 94°C for 1 min, 54°C for 1 min, 72°C for 1 min for 30 cycles and 72°C extension for 10 min. The PCR products were separated in 2% agarose gels, visualized by staining with ethidium bromide and analyzed with Image 1.62.

Caspase-3 activity assay. To analyze caspase-3 activity, HepG 2 cells (1x10⁶ cells) were treated with reagents for 48 h. The caspase-3 activity was measured with a caspase-3 colorimetric protease assay kit (Medical & Biological Laboratories Co., Ltd., Japan) according to the manufacturer's instructions.

Statistical analysis. Data are presented as means \pm SD. Statistical analysis was performed with SPSS 10.0 software. P<0.05 was considered statistically significant.

Results

Growth inhibition of HepG 2 cells by pitavastatin. To elucidate the optimal administration for pitavastatin in HepG 2 cells, the effect of pitavastatin (0.1-50 μ M) on HepG 2 cell proliferation was examined by the WST-8 assay. Compared with the control, pitavastatin at 10 and 50 μ M markedly inhibited proliferation of HepG 2 cells (Fig. 1). The treatment of HepG 2 cells with up to 1 μ M pitavastatin had no significant effect on cell viability. Growth inhibition by pitavastatin was completely abolished by MEV (1 mM).

Apoptosis in HepG 2 cells induced by pitavastatin. To investigate whether HepG 2 cells underwent apoptosis when they were treated with pitavastatin, the cells were stained with propidium iodide, followed by an examination of the appearance of sub-G1 population using a flow cytometry. After treatment with 10 μ M of pitavastatin for 48 h, cell death became apparent. As evidenced by the appearance of sub-G1 population, the apoptotic index in HepG 2 cells was 24.3%. Regarding the specificity of the effect of pitavastatin, MEV (1 mM) abolished the induction of apoptosis by pitavastatin

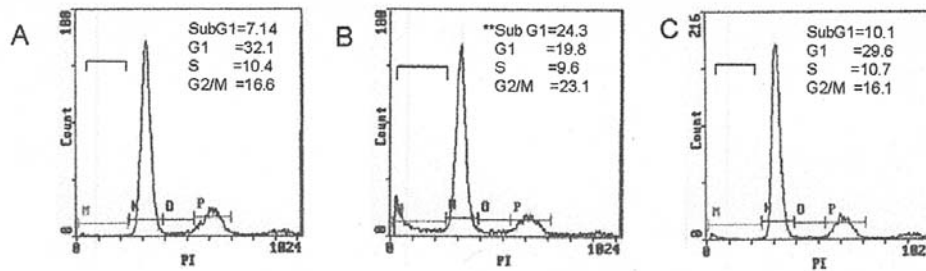


Figure 3. Cell cycle analysis of HepG 2 cells treated with pitavastatin by flow cytometry. Subconfluent monolayers of HepG 2 cells were treated with pitavastatin (10 μ M) alone, or pitavastatin (10 μ M) and MEV (1 mM) for 48 h. The cells were fixed with ice-cold 70% ethanol, treated with RNase A and stained with propidium iodide in the dark. The percentage of non-apoptotic and apoptotic cells within each cell cycle was observed by flow cytometry. The data represent the means \pm SD (n=3); **p<0.01 vs. control.

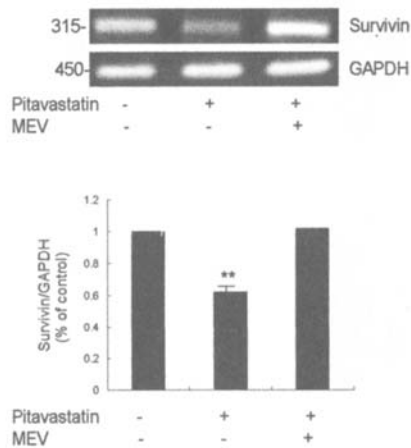


Figure 4. Survivin mRNA expression following treatment with pitavastatin. Subconfluent monolayers of HepG 2 cells were treated with pitavastatin (10 μ M) alone, or pitavastatin (10 μ M) and MEV (1 mM) for 48 h. After the treatment, RNA was extracted. Levels of survivin and GAPDH mRNA were examined by RT-PCR. The two top panels show the expression levels of survivin and GAPDH mRNA; the bottom bar graphs shows the expression levels of survivin relative to those of GAPDH. The data represent means \pm SD (n=3); **p<0.01 vs. control.

(Fig. 3). Furthermore, we also observed the morphologic changes of the nucleus by Hoechst 33258 staining. In the control group, the nuclei of the HepG 2 cells were round and homogeneously stained (Fig. 2A). However, pitavastatin treated HepG 2 cells showed granular apoptotic bodies (Fig. 2B), which were clearly related to the HMG-CoA reductase activity due to the addition of Mevalonic acid (MEV) which could recover the morphologic changes of the nucleus (Fig. 2C). These results demonstrate that pitavastatin induces apoptosis of HepG 2 cells.

Downregulation of survivin mRNA expression by pitavastatin in HepG 2 cells. Survivin, a member of the IAP family, is a bifunctional protein that suppresses apoptosis and regulates cell division (4). Forty-eight hours after the treatment with reagents, the expression of survivin mRNA was detected by RT-PCR. The statistical analysis showed that the expression of survivin mRNA in HepG 2 cells was downregulated significantly by pitavastatin at 10 μ M compared with the control (p<0.01) (Fig. 4) and MEV abolished the pitavastatin-reduced expression of survivin mRNA.

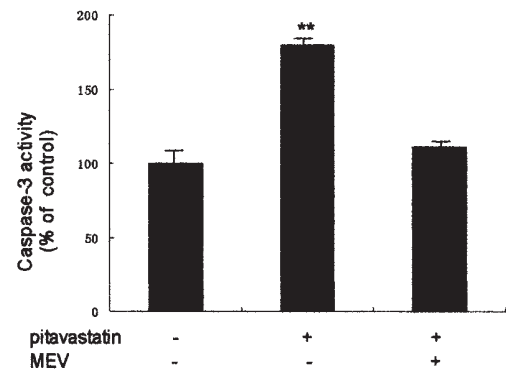


Figure 5. Pitavastatin-induced activity of caspase-3. Subconfluent monolayers of HepG 2 cells were exposed to pitavastatin (10 μ M) alone, or pitavastatin (10 μ M) and MEV (1 mM) for 48 h. The caspase-3 activity was measured with a colorimetric protease assay. The results are expressed as percentages of caspase-3 activity relative to untreated control cells. The data represent means \pm SD (n=3); **p<0.01 vs. control.

Induction of caspase-3 activity by pitavastatin in HepG 2 cells. Caspases, the cytoplasmic aspartate-specific cysteine proteases, have been shown to play a central role in the apoptotic signaling pathway. Caspase-3, a member of the caspase family, was shown to play an essential role in apoptosis induced by a variety of stimuli (13-15). Finally, we examined whether caspase-3 activity was increased during pitavastatin-induced apoptosis in HepG 2 cells. Fig. 5 shows the appearance of the caspase-3 activity following exposure to 10 μ M pitavastatin for 48 h, thus indicating that pitavastatin efficiently activated caspase-3 in HepG 2 cells. As expected, MEV also suppressed the pitavastatin-induced caspase-3 activity.

Discussion

It has recently been suggested that extra hepatic effects of statins might play a potentially beneficial role in cancer therapy. The presumption is based on experimental data obtained both *in vitro* and *in vivo* (16,17). Simvastatin (1-30 μ M) was demonstrated to have a cancer preventing ability (18). Fluvastatin inhibited the proliferation of Huh 7 cells by inducing apoptosis and G1/S cell cycle arrest. A maximal decrease in cell number by 90% was observed at 50 μ M and the IC₅₀ of fluvastatin was 10 \pm 3 μ M in Huh 7 cells (9). Gel shift

assay revealed that cerivastatin at 25 ng/ml displayed a potent anti-invasive effect on human breast cancer cells (MDA-MB-231 cells), which could be related to NF- κ B inhibition (19). Statin *in vitro* models have been shown to interact with and increase the efficiency of chemotherapeutic agents, such as 5-fluorouracil, cisplatin or doxorubicin (20).

Due to metabolic and morphological similarities, monocytic HepG 2 cells have been accepted as a good model of hepatocellular carcinoma. Pitavastatin, also known as NK-104, is a novel highly potent inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (10). In the present study, pitavastatin significantly suppressed the proliferation of HepG 2 cells. Moreover, the nuclear morphology and apoptosis rate analyzed quantitatively by flow cytometry suggested that an apoptotic cell death mechanism was also potentially involved in this direct cytotoxicity (Figs. 2 and 3).

Tumor development and progression as well as resistance to most oncologic therapies result mainly from a lacking response to apoptotic stimuli (21). Survivin, a member of the IAP family, is a bifunctional protein that suppresses apoptosis and regulates cell division. Previous studies showed that there is a close relationship between survivin and malignant tumors. The expression of survivin is highly cancer-specific and is one of the top four transcripts uniformly upregulated in human cancers, but not in normal tissues (22). The overexpression of survivin appears to correlate with aggressive tumor behavior and poor prognosis in non-small cell lung cancers (23), neuroblastomas (24) and hepatocellular carcinoma (25). Additionally, survivin overexpression is also correlated with insensitivity to chemotherapy and radiotherapy in cancers (26). Inhibiting the expression of survivin can induce tumor cell apoptosis, sensitize tumor cells to chemotherapy and radiotherapy and inhibit tumor-angiogenesis (27,28). Survivin has become an ideal target for the diagnosis and treatment of cancer. To determine whether the apoptosis of HepG 2 cells by pitavastatin was mediated by a decrease in survivin mRNA expression, HepG 2 cells were treated with pitavastatin (10 μ M) for 48 h, as shown in Fig. 4. RT-PCR analysis revealed that pitavastatin suppressed survivin mRNA expression levels compared with the control. This finding demonstrates that the apoptosis of HepG 2 cells by pitavastatin is associated with the downregulation of survivin mRNA expression.

In addition, the mechanisms by which survivin inhibits cell apoptosis and cell division are still highly controversial (29). Survivin may regulate apoptosis by directly inhibiting the activity of caspases and by mainly suppressing the activity of caspase-3 and caspase-7 (30). Disruption of survivin-microtubule interactions could result in the loss of survivin's anti-apoptosis function and increase caspase-3 activity, a mechanism involved in cell death during mitosis (31). Caspase-3 is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis (32). In the present study, we found that the pitavastatin-induced apoptosis of HepG 2 was also related to the increase in caspase-3 activity (Fig. 5).

In summary, we have shown that pitavastatin, which is a well-characterized and well-established pharmaceutical, induces apoptosis of HepG 2 cells through nuclear degradation, suppressing the survivin mRNA and increasing

caspase-3 activity. The significance of these findings for the anti-cancer effects of pitavastatin should be characterized in more detail.

Acknowledgements

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