Statin-mediated reduction of osteopontin expression induces apoptosis and cell growth arrest in ovarian clear cell carcinoma

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Abstract. Poor prognosis in ovarian clear cell carcinoma is associated with the expression of a defined set of proteins including osteopontin (OPN) and integrin. Statins, a family of 3-hydroxy-3-methylglutaryl CoA reductase inhibitors, are currently being investigated for the treatment and prevention of cancer. In this study, we investigated the effects of simvastatin on ovarian clear cell carcinoma (OCCC) cells *in vitro* and *in vivo* and elucidated the mechanism of drug action. Changes in OPN gene expression were determined by real-time RT-PCR, and an MTT assay was performed to determine effects on cell proliferation. Finally, a xenograft tumor model was constructed to evaluate the effects of simvastatin on cell proliferation and apoptosis *in vivo*. According to our experimental results, OPN is an important protein in OCCC. Simvastatin inhibited OCCC cell pro-liferation, and the inhibition rate was approximately 40% to 50% after treatment with 10 μM simvastatin for 48 h. In the xenograft studies, simvastatin treatment resulted in a significant growth inhibition. Furthermore, the mice treated with simvastatin survived significantly longer compared to the control groups. In conclusion, simvastatin has anticancer effects *in vitro* and *in vivo*. Further confirmation of the anticancer effects of statins in future studies will increase the scope for OCCC treatment.

Introduction

Osteopontin (OPN), a secreted calcium-binding glycophos-phoprotein, originally was viewed as a mediator of bone remodeling and tissue debridement (1). However, previously it has been implicated in numerous physiological and patho-logical events including immunity, inflammation, tumor progression and cell survival (2,3). Recent studies have revealed that OPN expression correlates with the progression and metastasis of various cancers (4-6), and clinical studies have clearly shown that OPN expression is increased in ovarian cancer (7-9). In addition, OPN expression is significantly higher in the plasma of patients with epithelial ovarian cancer (9). Microarray analysis of gene expression identified genes that are highly overexpressed in ovarian cancers (10). Of these genes, OPN was highly elevated in OCCC (11). OPN immuno-localization confirmed that OCCC expression is increased in comparison to healthy ovarian epithelium (8).

We previously reported that OPN expression is increased in OCCC, and down-regulation of OPN by treatment with simvastatin, or transfection with OPN-specific siRNA *in vitro*, reduced cell invasiveness (12).

Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that prevent the conversion of HMG-CoA to mevalonate and reduce cholesterol synthesis. Statins are typically used to lower low-density lipoprotein (LDL) cholesterol for the treatment of hypercholesterolemia (13). In addition to reducing cholesterol synthesis, statins inhibit the synthesis of the cholesterol isoprenoid intermediates farnesylpyrophosphate (FPP) and geranylgeranyl pyro-phosphate (GGPP) (14). These isoprenoids attach to several cellular proteins such as oncoproteins (ras), nuclear proteins (laminins) and small guanosine triphosphate (GTP)-binding proteins (rho, rac, rab). Isoprenylation or post-translational modification is essential to signal transduction that promotes cell proliferation, differentiation and cell death (15). Various studies have described the association of statins with a decrease in the incidence of various cancers (16,17).

In a previous study, statin use lowered plasma OPN con-centrations (18). Other research has demonstrated that statins reduced both protein and mRNA OPN expression (19). In the present study, we evaluated the *in vitro* and *in vivo* effects of statin (simvastatin) treatment on cell proliferation and apoptosis in OCCC cells (RMG-1 and TOV-21G).

Materials and methods

Materials. Simvastatin was obtained from Sigma-Aldrich (St. Louis, USA) and dissolved in DMSO (Sigma-Aldrich).

Cell lines and cultures. The following ovarian cancer cell lines were used for the *in vitro* and *in vivo* studies: clear cell

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carcinoma, RMG-1 (JCRB Cell Bank, Osaka, Japan) and TOV-21G (ATCC, VA, USA). Cells were cultured in RPMI-1640 (Invitrogen, CA, USA) containing 10% fetal bovine serum (Invitrogen) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Quantitative real-time RT-PCR analysis. Total RNA was isolated from cell cultures using commercial spin columns (RNeasy; Qiagen, Tokyo, Japan) in accordance with the manufacturer's instructions. cDNA synthesis was performed using the ThermoScript™ RT-PCR system (Invitrogen). Real-time quantitative RT-PCR (20) was used to measure OPN mRNA expression in the cell lines. Reactions (50 μl) contained the following reagents: 0.5 μl cDNA, 25 μl 2X iQ SYBR Green Supermix (Bio-Rad, CA, USA), 23.5 μl sterile water, and 0.5 μl (0.1 μM) OPN forward and reverse primers from a 10-μl solution (Sigma-Aldrich). The housekeeping gene glyceraldehyde-3-phosphatedehydregenase (GAPDH) was also measured. Amplification of OPN cDNA was performed using the following cycling profile: initial denaturation at 95°C for 10 min and 40 cycles of 15 sec at 95°C, and 1 min at 60°C in a Chromo4™ system (Bio-Rad). To determine the relative expression of OPN in each sample, the comparative C_T method was used as described previously (20). The GAPDH C_T value was subtracted from the OPN C_T value for each sample to calculate a ΔC_T value. The difference ($\Delta\Delta C_T$) between the ΔC_T value of each sample and the highest ΔC_T value (calibration value) was then calculated. The relative level of OPN gene expression was expressed as $2^{-\Delta\Delta C_T}$.

MTT assay. The MTT assay was performed to evaluate the effects of simvastatin on cell survival. RMG-1 and TOV-21G cells (5x10^4 cells) were seeded in 24-well plates and incubated overnight. The following day, simvastatin (0-10 μM) was added to each plate, and the plates were cultured for an additional 48 h. After this incubation, 100 μl of an MTT stock solution: 5 mg MTT/1 ml phosphate-buffered saline (PBS), was added to each well, and the plates were incubated for 4 h at 37°C. Finally, the media were replaced with 100 μl of DMSO. One hundred microliters was then transferred to 96-well plates. Absorbance was read at 550 nm on a microplate reader (Bio-Rad), and the control wells were set to 100% cell viability. Three individual experiments were performed in triplicate.

Treatment of mice with simvastatin. RMG-1 and TOV-21G cells (5x10^6 cells) in 0.1 ml of culture medium were injected subcutaneously in the left flank region of 6-week-old female NOD/SCID mice (Hokudo, Sapporo, Japan). The mice were randomly assigned to two groups: group 1, saline treatment (n=3); group 2, 15 mg/kg/day simvastatin treatment (n=3). Saline and simvastatin were injected intraperitoneally every day for 10 days starting 1 day post-implantation. Tumor volumes were measured every 5 days and calculated as a rotational ellipsoid (a x b^2 x 0.5; 'a' is the long axis, and 'b' is the transverse axis). Survival time was used as the primary endpoint.

A separate *in vivo* study was performed to measure OPN mRNA and protein expression. The mice were randomly
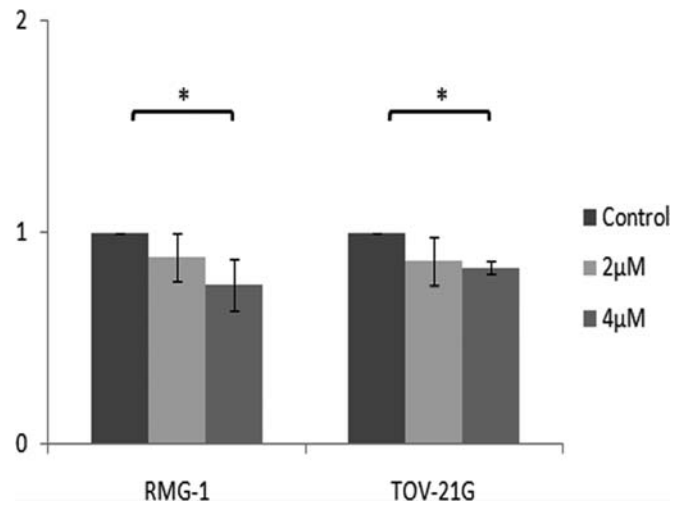

Figure 1. Simvastatin influences the levels of OPN. Two OCCC cell lines were treated with 0-4 μM simvastatin for 48 h. The levels of OPN mRNA expression were significantly decreased in this analysis (*P<0.05).

assigned to two groups (see above), and after tumors became visible, the mice were injected intraperitoneally every day for 10 days. Mice were then sacrificed, and the tumors were excised 24 h after the final simvastatin treatment. RNA was isolated from tissues using TRIZOL Reagent (Invitrogen) following the manufacturer's instructions. In addition, resected tumors were fixed with 10% buffered formalin and 70% ethanol. OPN protein expression was then determined by immunofluorescent staining as follows. Fixed tumor tissue was incubated with a mouse anti-human monoclonal antibody (clone 1B20, IBL, Gunma, Japan) (21) followed by an incubation with secondary antibodies tagged with Alexa Fluor® 488 and goat anti-mouse IgG (Molecular Probes, OR, USA). Immunofluorescence was visualized as described previously (22) with some modifications. Briefly, chamber slides were rinsed in PBS and incubated overnight at 4°C with the primary antibody. After 3 rinses in PBS for 10 min each, the slides were incubated with secondary antibodies for 30 sec at room temperature followed by 3 washes with PBS for 15 min each. The sections were then mounted using Fluorescent Mounting Medium (Dako Cytomation, CA, USA), overlaid with a coverslip, and examined under a fluorescent microscope (Olympus, Japan). All animal experiments were approved by the institutional animal care and use committee.

TUNEL assay for detection of apoptotic cells in vivo. To examine apoptosis in resected tumors, DNA fragmentation was measured using the TUNEL assay. This was performed using the ApopTag Fluorescein In Situ Apoptosis Detection kit (Chemicon, MA, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using Statview® 5.0 (Hulinks, Tokyo, Japan) software. The statistical differences between 2 groups were analyzed using the Mann-Whitney U-test (nonparametric data), and differences among multiple groups were analyzed using the Kruskal-Wallis test.
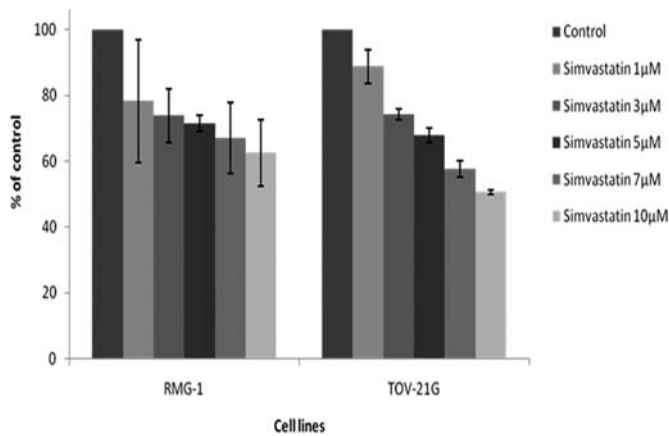
Figure 2. Inhibition of OCCC proliferation after exposure to simvastatin. Cells were treated with simvastatin (1-10 μM) for 48 h, and the MTT assay demonstrated dose-dependently reduced cell proliferation.
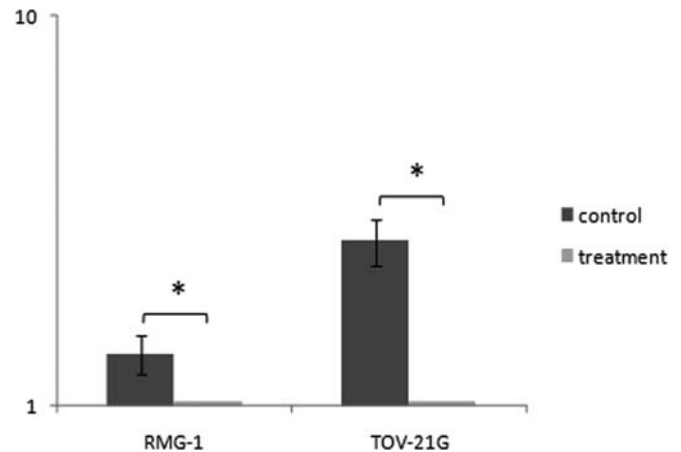

Figure 3. Simvastatin reduced OPN expression in the *in vivo* models. OPN expression was determined by real-time RT-PCR. The levels of OPN mRNA expression were significantly decreased in the simvastatin-treated groups (*P<0.05).

P<0.05 were considered significant. Survival rates were analyzed using the Kaplan-Meier method (23). The log-rank test was used to determine the difference between two groups.

Results

Simvastatin reduces OPN mRNA expression in vitro. To determine the influence of simvastatin on OPN expression *in vitro*, two OCCC cell lines were treated with 0-4 μM simvastatin for 48 h in 6-well plates (Fig. 1). Total RNA was isolated, and OPN mRNA expression was analyzed by real-time RT-PCR. The expression level in the 0 μM group was assigned a value of 1. Data are shown as the mean ± SD. OPN mRNA expression significantly decreased in two cell lines in 4 μM (RMG-1: 1.33 times, and TOV-21G: 1.20 times; P<0.05). This experiment was repeated 3 times with triplicate samples.

Simvastatin causes a dose-dependent decrease in ovarian clear cell carcinoma cell proliferation. To evaluate the effect of simvastatin on OCCC cell growth, two cell lines were treated with various concentrations of simvastatin for 48 h in 24-well plates. As depicted in Fig. 2, simvastatin reduced cell proliferation dose-dependently, and survival was significantly reduced to 62.5% (RMG-1) and 50.7% (TOV-21G) with 10 μM simvastatin (P<0.05). Experiments were repeated 3 times with triplicate samples.

Simvastatin reduces OPN mRNA expression in the tissues of NOD/SCID mice. To determine the effects of simvastatin on OPN expression *in vivo*, OCCC cell proliferation was measured in mice. Tumors became visible about 15 days after implantation of the two cell lines. Simvastatin was injected intraperitoneally every day for 10 days into mice. Total RNA was then isolated from freshly isolated tissue samples, and OPN mRNA expression of the subcutaneous tumor was determined by real-time RT-PCR (Fig. 3). The mRNA expression in the treatment group was assigned a value of 1.

Values are shown as the mean ± SD. Similar to the *in vitro* study results, OPN mRNA expression was significantly decreased in the treatment groups (RMG-1: 1.36 times, and TOV-21G: 2.65 times; P<0.05).

Simvastatin decreases OPN protein expression in model mice. To determine the effects of simvastatin treatment on OPN protein expression *in vivo*, immunofluorescent staining was performed to the subcutaneous tumor of the mice. As shown in Fig. 4, OPN staining was observed at the cell membrane and in the cytoplasm, and OPN staining was highly increased in the control group (Fig. 4A) in comparison to the treatment group (Fig. 4B). These data suggest that simvastatin treatment reduces OPN protein expression. Fig. 4 is presented for the tumors generated by RMG-1 cells.

Simvastatin-induced apoptosis. The TUNEL assay detects early-stage apoptosis in systems with chromatin condensation and few strand breaks. Propidium iodide (PI) was used with fluorescein (FITC). DNA in the nucleus was stained with PI (red) and FITC (green) and visualized by fluorescence microscopy. Fig. 5 is presented for the tumors generated by RMG-1 cells. Apoptosis was increased in simvastatin-treated mice (Fig. 5B) in comparison to control mice (Fig. 5A).

Antitumor effects of simvastatin in ovarian clear cell adeno-carcinoma in immunodeficient mice. We determined the effects of simvastatin on tumor growth and mouse survival (Fig. 6). In the control group, tumors became visible 15 days after implantation of the two cell lines. In comparison, tumors became visible 25 days after implantation in the simvastatin-treated group. We observed significant growth inhibition compared with the control group when both cell lines were tested (P<0.05). Furthermore, we examined the effect of simvastatin treatment on the survival of immunodeficient mice. Whereas every mouse died within 80 days (RMG-1) and 65 days (TOV-21G), the mice treated with simvastatin survived significantly longer (P<0.05). The 90-day survival rate was 66.7% using the two cell lines. In this study, 15-mg/kg/day
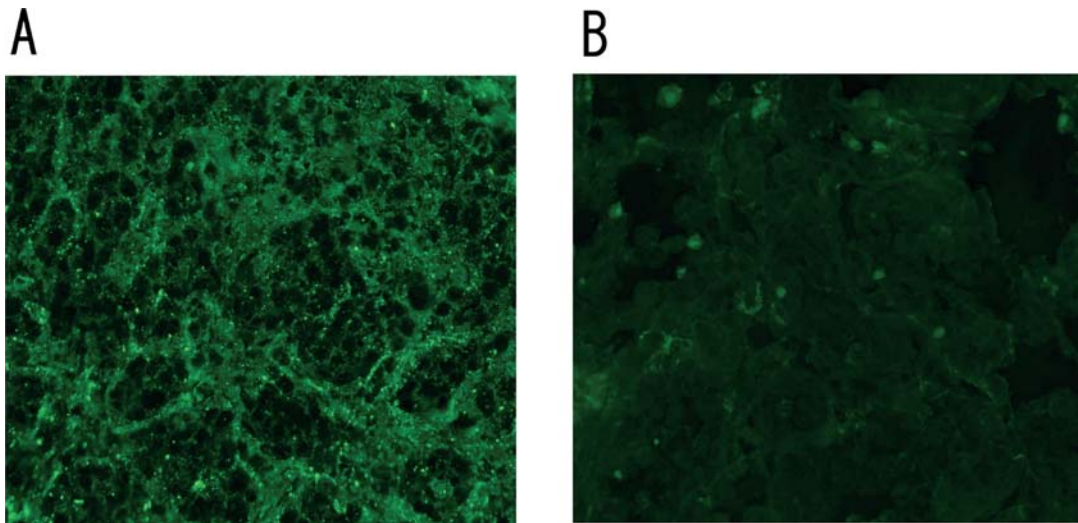
Figure 4. The effect of simvastatin on OPN protein expression in immunodeficient mice was determined by immunofluorescence. OPN expression was decreased in the simvastatin-treatment groups (B) in comparison to the control group (A).
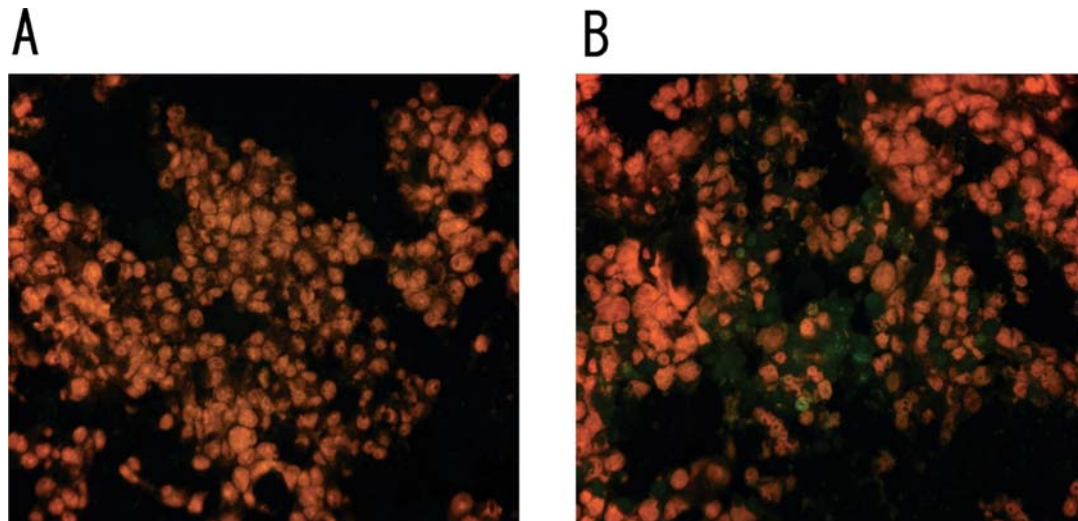

Figure 5. The detection of apoptosis by TUNEL assay. The green cells represent the apoptotic cells. Apoptosis was not detected in the control groups (A). However, apoptotic cells were detected in the simvastatin-treatment groups by the TUNEL assay (B).

simvastatin did not cause any side effects. None of the mice showed significant weight loss or any other serious side effects. Thus, 15-mg/kg/day simvastatin was considered to be safe.

Discussion

OPN is involved in cell invasion, adhesion, proliferation and apoptosis (4,5,12,21,24,25). Our previous study demonstrated that OPN is highly expressed in ovarian cancer, especially in OCCC (12). We also reported that OCCC cells transfected with OPN-specific siRNA showed the reduction of cell invasiveness significantly. These data revealed that inhibition of OPN reduced cell invasiveness *in vitro* (12).

Human OPN promoters have a number of diverse homologous cis-acting consensus sequences (26). This suggests that OPN transcription may be sensitive to statins. Indeed, statins have been found to reduce OPN expression, although the precise underlying mechanism is unclear (19).

Statins are therapeutic drugs that effectively reduce plasma cholesterol levels and prevent coronary heart disease. Recent studies suggest that statins may be used for cancer treatment. Several statin antitumor effects have been observed: inhibition of cell proliferation, promotion of apoptosis, inhibition of angiogenesis, and prevention of metastasis (27-31). We hypothesized that the reduction of OPN expression may cause simvastatin-induced antitumor effects in ovarian clear cell carcinoma cells. Therefore, we first examined whether simvastatin treatment causes the reduction of OPN expression in OCCC cells *in vitro* and *in vivo*. Our data demonstrated that simvastatin inhibited OPN mRNA expression not only *in vitro* but also in the tumors of model mice. We think that simvastatin bound to the OPN promoter and reduced OPN mRNA expression. It is also reported that various statins deplete isoprenoids (32). Isoprenoids are important lipid moieties of G proteins and G protein subunits. Depletion of isoprenoids affects the expression of
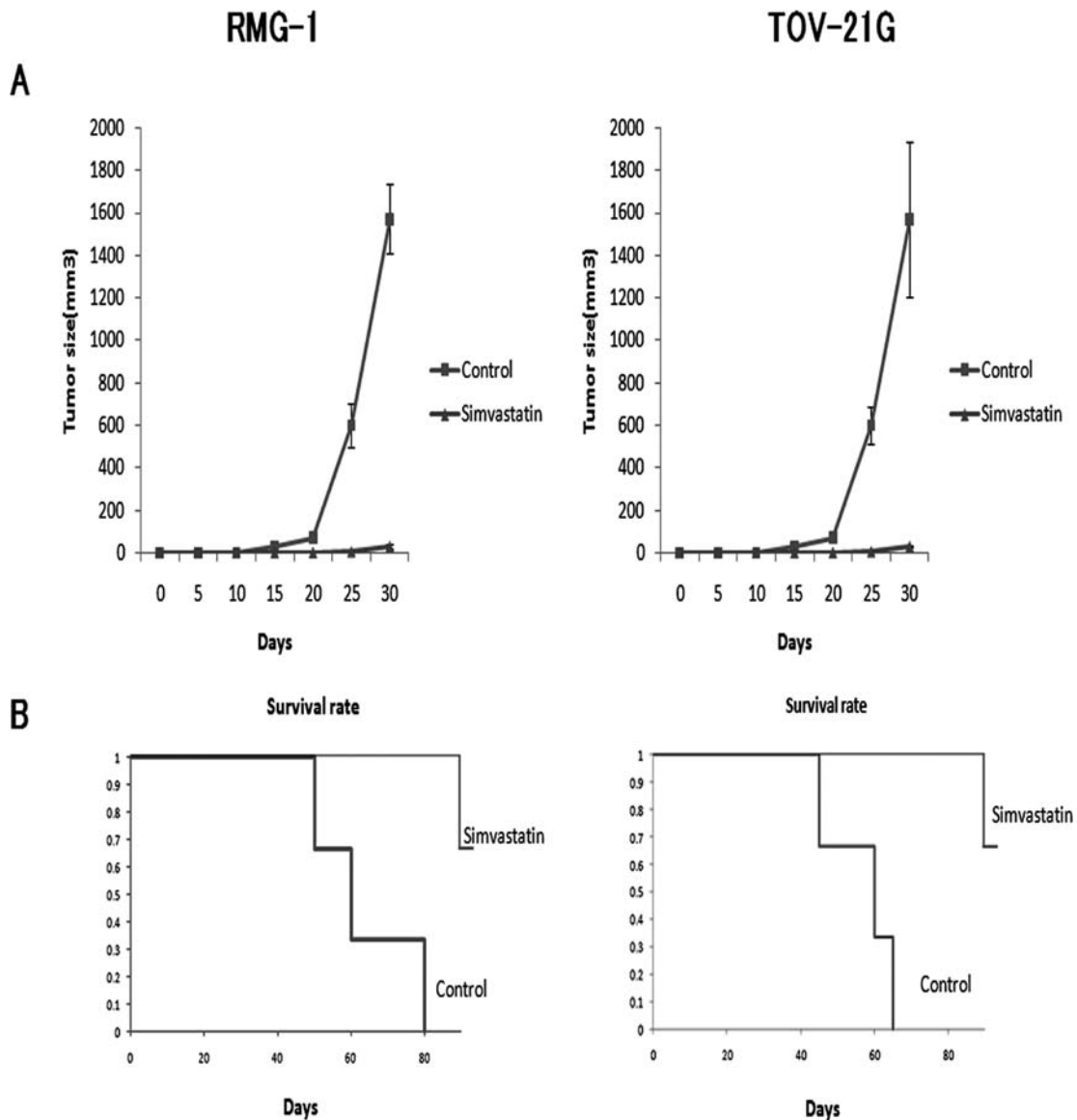
RMG-1

TOV-21G

Figure 6. Antitumor effects of simvastatin in immunodeficient mice. (A) Tumor growth in the treatment groups was significantly inhibited in comparison with the control groups using both cell lines (P<0.05). (B) Mice treated with simvastatin survived significantly longer (P<0.05). At 90 days, the survival rate was 66.7% using both cell lines.

glycoproteins such as OPN. These data demonstrate the relationship between OPN expression and simvastatin treatment.

In general, various cancers are sensitive to statin treatment (33-35). Similarly, ovarian cancer cells may be sensitive to simvastatin treatment (12). Our data demonstrate that 10 μM simvastatin significantly reduced the survival of the two cell lines to 62.5% (RMG-1) and 50.7% (TOV-21G). The mechanism of OPN-mediated cell proliferation has not been clearly elucidated (4,36). Elgavish et al (37) reported that OPN treatment increased epithelial cell proliferation. Our previous study also suggested that OPN induced cell invasiveness in vitro by binding αvβ1, αvβ3, and α5β1 integrins in OCCC (12). These results revealed that OPN promoted the proliferation of ovarian epithelial cancers including OCCC.

Statins inhibit cancer cell proliferation in vitro and in vivo by the inhibition of HMG-CoA reductase and the depletion of isoprenoids (32). The HMG-CoA reductase inhibitor-dependent decrease in cell proliferation is due to the prevention of G1 to S phase transition in the cell cycle, down-regulation of cyclin-dependent kinases that facilitate cell cycle progression or up-regulation of cell cycle inhibitors (33). The highest concentration of simvastatin achieved in the plasma of patients receiving a 40-mg/day treatment was approximately 7.2 μM (38). In addition, Spek et al (39) reported that the maximum tolerated dose of simvastatin was 15 mg/kg/day. Thus, much higher concentrations of simvastatin could be used. Our results demonstrated that one of the mechanisms of the anti-proliferative activity of simvastatin in OCCC cells was the down-regulation of OPN expression, in addition to HMG-CoA reductase inhibition and isoprenoid depletion.
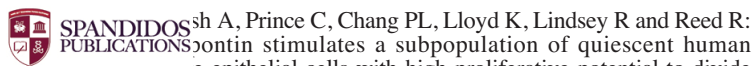
Studies have demonstrated an association between OPN expression and the resistance to apoptosis, and that OPN inhibits apoptosis in a variety of cells (4). In addition, statins induce apoptosis through both the extrinsic and intrinsic

pathways. Shibata *et al* (34) reported that lovastatin induced p53-independent mitochondrial-mediated apoptosis in a mouse mammary carcinoma. The sensitivity of different cell types to statin-induced apoptosis varies; acute myeloid leukemic and neuroblastoma cells are more sensitive than acute lymphoblastic leukemic cells (40). Apoptosis becomes evident following treatment with high concentrations of statins, and statin-induced cell inhibition is observed at lower concentrations. In this study, simvastatin exerted an apoptotic effect on OCCC cells, accompanied by growth inhibition. The simvastatin-mediated induction of apoptosis occurred concomitantly with a decrease in OPN expression and growth suppression. In accordance with previous studies, we treated with simvastatin at a dose of 15 mg/kg/day in the *in vivo* studies (39). Our *in vivo* study demonstrated that simvastatin inhibited the formation and growth of OCCC cells in immuno-deficient mice. In addition, we determined that a high dose of simvastatin did not significantly induce weight loss or other serious side effects.

In this study, we examined the *in vitro* and *in vivo* effects of simvastatin on OCCC cell lines. Our data demonstrate that simvastatin reduced OPN expression and induced cell growth arrest and apoptosis. There seems to be considerable overlap of the various mechanisms by which statins exert their beneficial effects. Future studies are needed to reveal the detailed relationship between statins and OPN expression. In addition to the above-mentioned mechanisms, the anti-inflammatory, immunomodulatory, and radiosensitizing properties of statins may contribute to cancer inhibition. Further confirmation of the anticancer effects of statins will increase the therapeutic scope for OCCC.

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