3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase Inhibitor, Fluvastatin, as a Novel Agent for Prophylaxis of Renal Cancer Metastasis

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ABSTRACT

Purpose: **Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, also called statins, are currently used widely as a safe, effective therapeutic in the treatment of hypercholesterolemia. Recently, statins have been recognized for their activity against cancer. In the present study, we examined the effect of a synthetic statin, fluvastatin, on the development of renal cancer.**

Experimental Design: **The effects of fluvastatin on cell viability, cell cycle, *in vitro* angiogenesis, and invasive properties were examined in murine renal cancer cell Renca. The changes in cell cycle-associated proteins, $p21^{Waf1/Cip1}$ and p53, and rac1 phosphorylation were analyzed by Western blotting. The prophylactic efficacy of fluvastatin to murine pulmonary metastasis of Renca was examined.**

Results: **Fluvastatin inhibited *in vitro* growth of Renca cells in a time- and dose-dependent manner, with up to 70% inhibition at a concentration of 10 μmol/L. This inhibitory effect was due to cell cycle arrest at the G_1 phase and induction of apoptosis accompanied by up-regulation of $p21^{Waf1/Cip1}$ and p53. The invasive properties of Renca cells through Matrigel were inhibited by fluvastatin, with decreased phosphorylation of rac1. *In vitro* angiogenesis was also inhibited by fluvastatin. Furthermore, oral administration at doses of 1 to 10 mg/kg/d, for 12 days after inoculation of Renca cells via the tail vein, significantly decreased the amount of pulmonary metastasis.**

Conclusions: **Because our results suggest that fluvastatin may effectively inhibit *in vitro* tumor growth, invasion, angiogenesis, and metastasis of Renca cells, oral administration of fluvastatin could be a novel, safe, and effective agent for preventing metastasis of renal cancer.**

Received 8/5/04; revised 9/4/04; accepted 9/15/04.

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INTRODUCTION

Numerous studies have demonstrated that elevated levels of serum cholesterol contribute to atherosclerosis and coronary artery disease, whereas reduced levels of cholesterol can significantly reduce the risk of those diseases (1–4). In recent years, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also called statins, have become well known for inhibiting the rate-limiting step in cholesterol synthesis. Statins are now widely used to treat patients with hypercholesterolemia and are recognized as safe, effective agents for this purpose (1–4). Each member of the statin family differs in terms of its specificity for HMG-CoA reductase, purity, prodrug or active administrative form, and metabolism (4). Among the statins, fluvastatin is the first entirely synthetic HMG-CoA reductase inhibitor, and it differs from the original structure in its lipophilic moiety (2–4). Among the properties of fluvastatin are low systemic exposure, a short elimination half-life, high protein binding, and a relatively low price (2). In particular, the rate of fatal rhabdomyolysis in association with fluvastatin has been reported to be lower than other statins (5) From these standpoints, fluvastatin seems safe and feasible compared with other statins.

In addition to their original role in reducing cholesterol levels, statins have also been recognized for their activity against various types of cancers, via multiple mechanisms (6–14). They have been shown to inhibit *in vitro* cancer cell proliferation by causing cell cycle arrest at the G_1-S phase and inducing apoptosis (12). Recent studies have demonstrated that fluvastatin markedly attenuates EGF-induced invasion of pancreatic cancer cells by inhibiting translocation of RhoA from the cytosol to the membrane and reducing RhoA activation (8–10). In several experimental animal models, treatment with statins at clinically relevant doses has been shown to inhibit *in vivo* tumor growth and metastasis (10, 11). Moreover, it has also been reported that high-dose cerivastatin decreased tumor growth and tumor vascularization in a murine lung cancer model (15). Recently, clinical trials investigating the possible value of statins as anticancer agents have been conducted, and the safety and effectiveness of statins have been demonstrated (16–18).

Renal cell carcinoma represents 2 to 3% of all adult cancers (19). At the time of diagnosis, approximately 30% of patients have metastatic disease, and an additional 30 to 40% develop metastases within a period of months or years after nephrectomy (19). Surgical resection remains the mainstay therapy for localized renal cell carcinoma, and metastatic renal cell carcinoma is highly refractory to conventional therapies, including radiation and chemotherapy (19). As a result, the management of advanced renal cell carcinoma remains a significant challenge to clinicians. On the basis of the previous studies investigating the safety and effectiveness of statins for cancer therapy, treatment with fluvastatin could be a novel approach in the therapy of

renal cell carcinoma. In the present study, we investigated the antitumor effects of fluvastatin on *in vitro* murine renal cancer cell proliferation, invasion, and angiogenesis. Subsequently, we examined whether fluvastatin treatment at clinically relevant doses could prevent pulmonary metastasis of murine renal cancer cells.

MATERIALS AND METHODS

Cell Culture and Reagents. A murine renal cancer cell line, Renca, was obtained from the American Tissue Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Tanabe Pharmaceutical Company (Osaka, Japan) provided the fluvastatin. Goat polyclonal antibody against phosphorylated rac1 (p-rac1) and rabbit polyclonal antibodies against p21, p53 (wild-type specific), and rac1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against actin was obtained from Chemicon International, Inc. (Temecula, CA).

Cell Counts and 3-(4,5-Dimethylthiazolyl)-2,5-Diphenyl Tetrazolium Bromide Assay. After incubating Renca cells overnight in 25-cm^2 flasks (5×10^4 cells/flask), the medium was changed to DMEM supplemented with either 10% FCS alone or 10% FCS with 10 μmol/L fluvastatin. Next, the total cell number in each group was counted three times. The cell viability was assessed by conducting a 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously, with minor modification (10). In brief, the Renca cells were incubated overnight in 96-well (1×10^3 cells/well) plates and then treated with the indicated concentration of fluvastatin for 72 hours. Next, 5 μL of MTT were added to each well. After 4 hours, 150 μL of dimethyl sulfoxide were added, and the optimal density was determined by using a microplate reader at 570 nm.

Cell Cycle Analysis and Determination of Apoptosis. Renca cells were first incubated overnight in 25-cm^2 flasks (5×10^4 cells/flask) and then incubated for 72 hours with the indicated concentrationof fluvastatin. Propidium iodide–stained nuclear fractions were obtained according to the manufacturer's instructions. Cell cycle data were acquired by using CellQuest software (Becton Dickinson, Heidelberg, Germany) with a flow cytometer (Becton Dickinson). To observe nuclear morphologic changes with a fluorescence microscope, untreated Renca cells and those treated with 10 μmol/L fluvastatin for 72 hours were fixed with 4% paraformaldehyde for 30 minutes at room temperature and stained with 1 mmol/L Hoechst 33258 (Sigma, St. Louis, MO).

Western Blot Analysis. Renca cells were treated with the indicated concentration of fluvastatin for the indicated time and lysed in a Tris buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 g/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Equal amounts of the resulting lysates were separated by using 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with a solution containing 5.0% skim milk and then incubated overnight with primary antibody at 4°C. Subsequently, the membranes were incubated with secondary antibody coupled to horseradish peroxidase (Amersham Biosciences, Arlington Heights, IL). The reactive proteins were visualized by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer's recommendations.

Assessment of *In vitro* Angiogenesis. *In vitro* angiogenesis was assessed based on the formation of capillary-like structures of HUVE cells cocultured with human diploid fibroblasts, as described previously (20). The experimental procedure followed the instructions provided with the angiogenesis kit (Kurabo, Tokyo, Japan) that we used. In brief, the HUVE cells were incubated in the Renca cell–conditioned medium with the indicated concentration of fluvastatin at day 1. The medium was replaced at days 4, 7, and 9. At day 11, the HUVE cells were fixed and stained by using an antihuman CD31 antibody (Kurabo) according to the provided instructions. The formation of the capillary network was observed with a microscope at ×40 magnification.

Matrigel Invasion Assay. The invasive capacities of untreated and fluvastatin-treated Renca cells were assessed by examining the invasion of cells through Matrigel-coated *trans-*

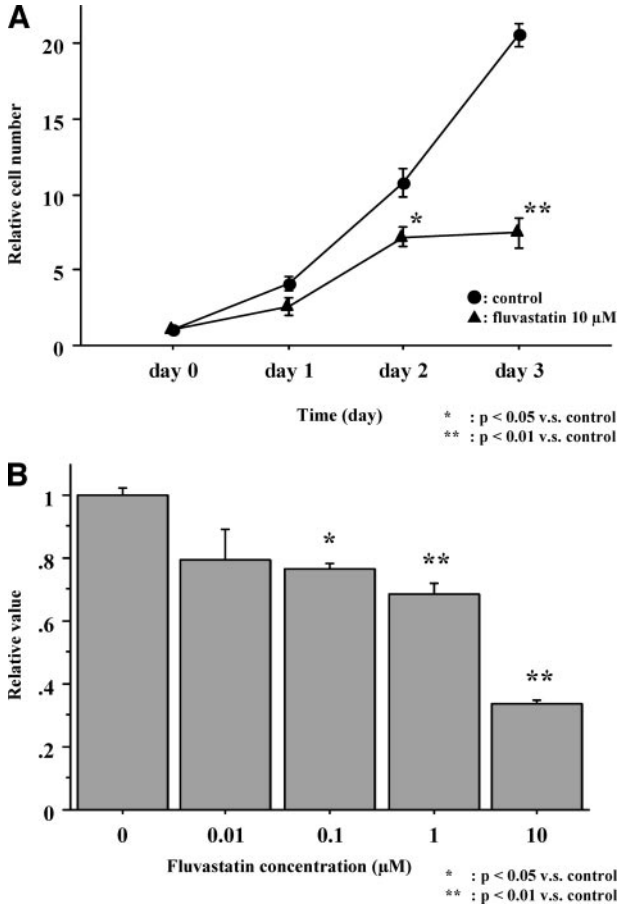

Fig. 1 Dose- and time- dependent inhibition of the proliferation of Renca cells. *A*. Renca cells were incubated for the indicated time with 10 μmol/L fluvastatin, and the total cell number was counted. *B*. Renca cells were incubated for 72 hours with the indicated concentration of fluvastatin. The cell viability was examined by applying an MTT assay. The data are presented as the means ± SE for three independent experiments.

well inserts (Becton Dickinson) with 8-μm pores in 24 wells, as described previously (8). In brief, a suspension of 2×10^5 cells in 500 mL of serum-free DMEM were added to each insert, and 750 mL of DMEM supplemented with 10% FCS were added to the bottom of each well. Thereafter, the indicated concentration of fluvastatin was added to each insert, and the plates were incubated for 24 hours at 37°C. Subsequently, the inserts were fixed in methanol, the filters were stained with 1% Toluidine blue in 1% borax, and the number of cells invading through each of the Matrigel-coated *trans*-well inserts was counted at ×40 magnification. The cell numbers were counted in at least 10 fields of each well through independent triplicate experiments.

Murine Pulmonary Metastasis Model. A total of 2×10^5 Renca cells were inoculated into male BALB/c mice via the tail vein. The mice were randomly divided into four groups of 10. One group only received PBS orally and was defined as the control group, whereas the other groups received 0.1 to 10 mg/kg/d of fluvastatin in 200 μL of PBS orally for 12 days from the day of inoculation. After that, the mice were sacrificed, their lungs were stained intratracheally with 15% India Black Ink solution, and the number of metastatic nodules in each mouse was counted. The study was conducted in accordance with the guidelines of our institute for the care and use of laboratory animals.

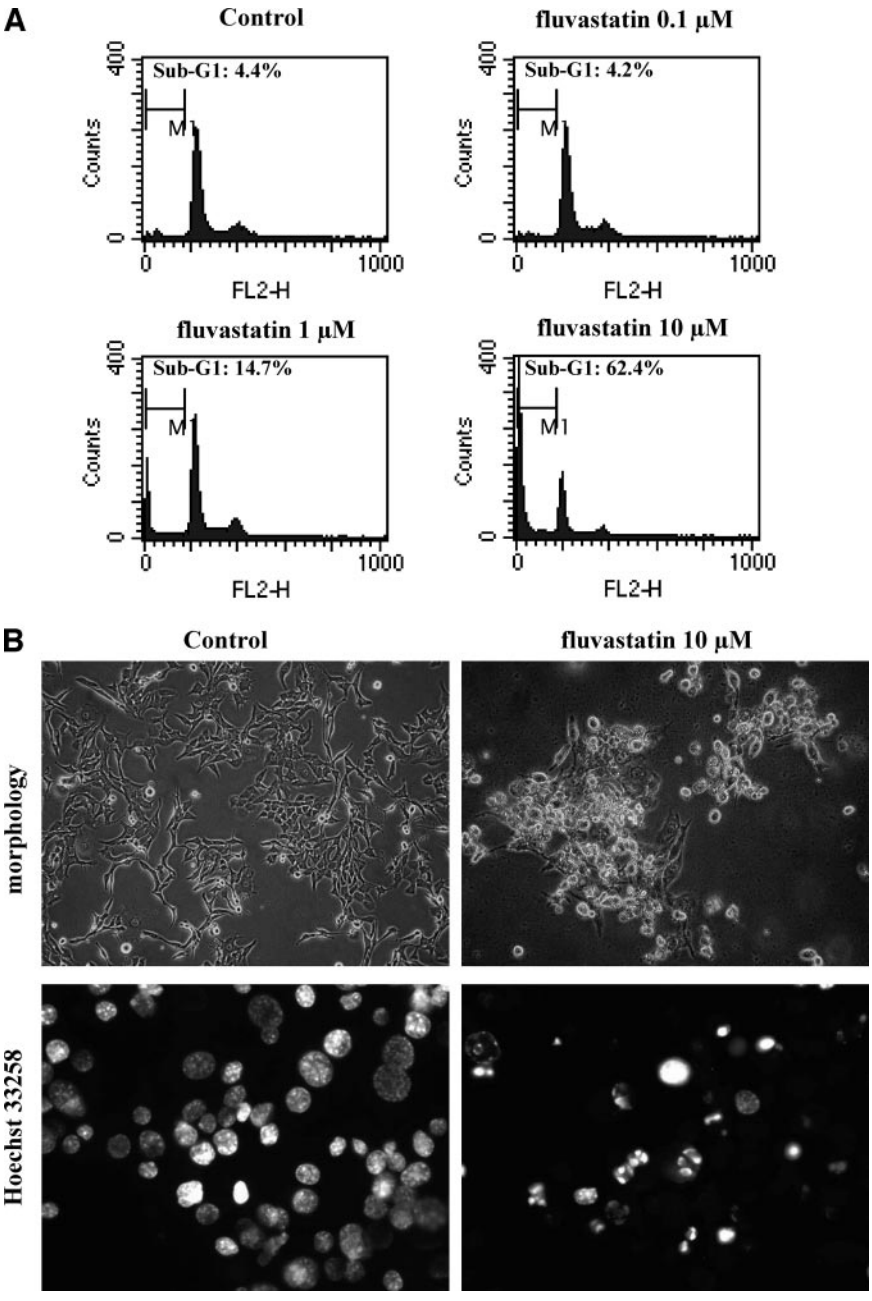

Fig. 2 Inhibition of cell cycle progression and induction of apoptosis by treatment with fluvastatin. *A*. Renca cells were incubated for 72 hours with the indicated concentration of fluvastatin, and the cell cycle distributions were analyzed by using a flow cytometer. The sub-G_1 population of each group is shown. *B*. Renca cells were incubated for 72 hours with 10 μmol/L fluvastatin, and their morphologic changes were observed either by phase-contrast microscopy (*top panels*) or under a fluorescence microscope after staining with Hoechst 33258 (*bottom panels*).

RESULTS

Fluvastatin Inhibited the Proliferation and Viability of Renca Cells. We first examined the effects of fluvastatin on Renca cell proliferation and viability. Renca cells were either left untreated or incubated with 10 μmol/L fluvastatin; and triplicate observations of the total cell number were taken after 0, 24, 48, and 72 hours. Although no significant difference was found between the numbers of untreated and treated cells at 24 hours, the total number of fluvastatin-treated cells was significantly decreased at 48 and 72 hours compared with the number of untreated cells (Fig. 1*A*). The effect of fluvastatin on Renca cell viability was examined based on the results of the MTT assay, in which Renca cells were either left untreated or incubated for 72 hours with 0.01 to 10 μmol/L fluvastatin. Significantly decreased cell viability was observed at 0.1 μmol/L, with up to 70% reduction at 10 μmol/L (Fig. 1*B*).

Fluvastatin Led to Cell Cycle Arrest at G_1 Phase and Induced Apoptosis. We next examined the effect of fluvastatin on the cell cycle. Renca cells were either left untreated or incubated for 72 hours with 0.1 to 10 μmol/L fluvastatin, and the cell cycle was analyzed with a flow cytometer. The sub-G_1 population of untreated cells was 4.4%, whereas those of cells incubated with 0.1, 1, and 10 μmol/L fluvastatin were 4.2%, 14.7%, and 62.4%, respectively (Fig. 2*A*). In addition, the effect of fluvastatin on cell and nuclear morphology was examined. Cells incubated for 72 hours with 10 μmol/L fluvastatin exhibited a decidedly round, swollen morphology as compared with untreated cells (Fig. 2*B*, *top panels*). In addition, the nuclei of treated cells appeared lobated and severely fragmented and contained highly condensed chromatin, which are findings typical of apoptosis (Fig. 2*B*, *bottom panels*).

Fluvastatin Inhibited *In vitro* Angiogenesis and Renca Cell Invasion. HUVE cells were incubated for 11 days with Renca-conditioned medium in the presence or absence of 0.1 to 10 μmol/L fluvastatin. The formation of vascular-like structures by the HUVE cells was impaired by treatment with fluvastatin in a dose-dependent manner (Fig. 3). Next, we examined the effect of fluvastatin on Renca cell invasion by using the Matrigel invasion chamber. The number of Renca cells invading through the chamber was significantly decreased by treatment with 1 or 10 μmol/L fluvastatin ($P = 0.0031$ or $P < 0.0001$, respectively; Fig. 4).

Fluvastatin Attenuated rac1 Phosphorylation and Up-Regulated p21 and p53. Rac1, a member of the family of Rho-like GTPase, has been reported to mediate the distinct actin cytoskeleton changes required for cell invasion and sustained activation of rac1 induces invasion and metastasis (21). As described above, treatment with fluvastatin inhibited the invasive capacity of Renca cells. Thus, we examined whether treatment with fluvastatin affects the rac1 phosphorylation level. Renca cells were either left untreated or incubated for 48 hours with 0.1 to 10 μmol/L fluvastatin, and the entire cell lysates were subjected to Western blotting. Although the total rac1 expression levels did not change, rac1 phosphorylation was inhibited by treatment with fluvastatin at 10 μmol/L (Fig. 5*A*). In addition, Renca cells were either left untreated or incubated for 0 to 48 hours with 10 μmol/L fluvastatin, and changes of rac1 phosphorylation were analyzed. The results indicated attenuation of rac1 phosphorylation at 24 hours (Fig. 5*B*).

As described above, treatment with fluvastatin led to cell cycle arrest, which has been shown to result from increased $p21^{WAF/CIP1}$ expression (12). Thus, we examined the effect of fluvastatin on the expression levels of $p21^{WAF/CIP1}$ and p53, which is an upstream regulator of $p21^{WAF/CIP1}$. The treatment with fluvastatin resulted in up-regulation of both proteins (Fig. 5).

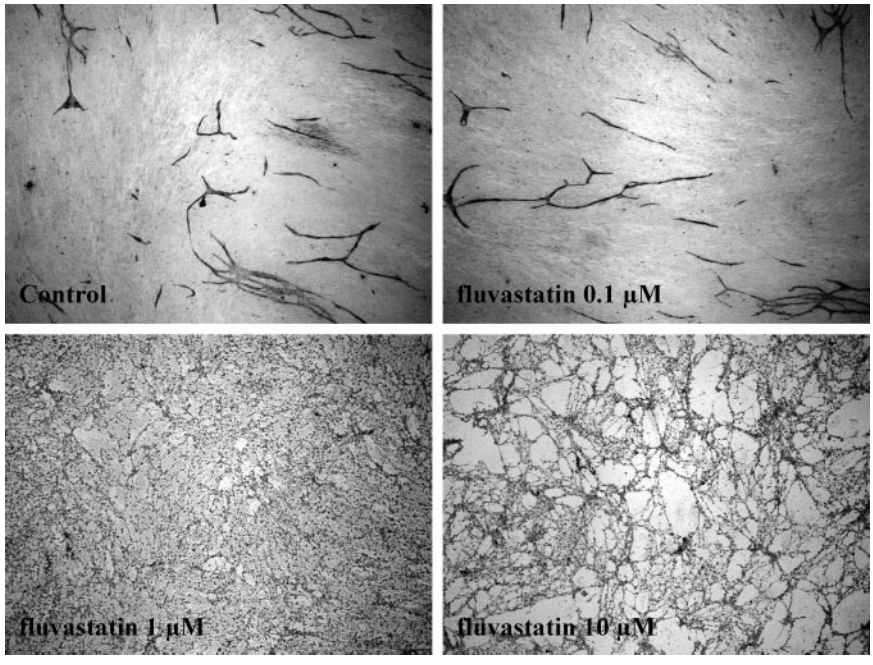

Fig. 3 Dose-dependent inhibition of *in vitro* angiogenesis by treatment with fluvastatin. HUVE cells were incubated in a Renca cell–conditioned medium, with various concentrations of fluvastatin. After incubation for 11 days, the HUVE cells were fixed and stained with an antihuman CD31 antibody. The formation of the capillary network was observed with a microscope at ×40 magnification.

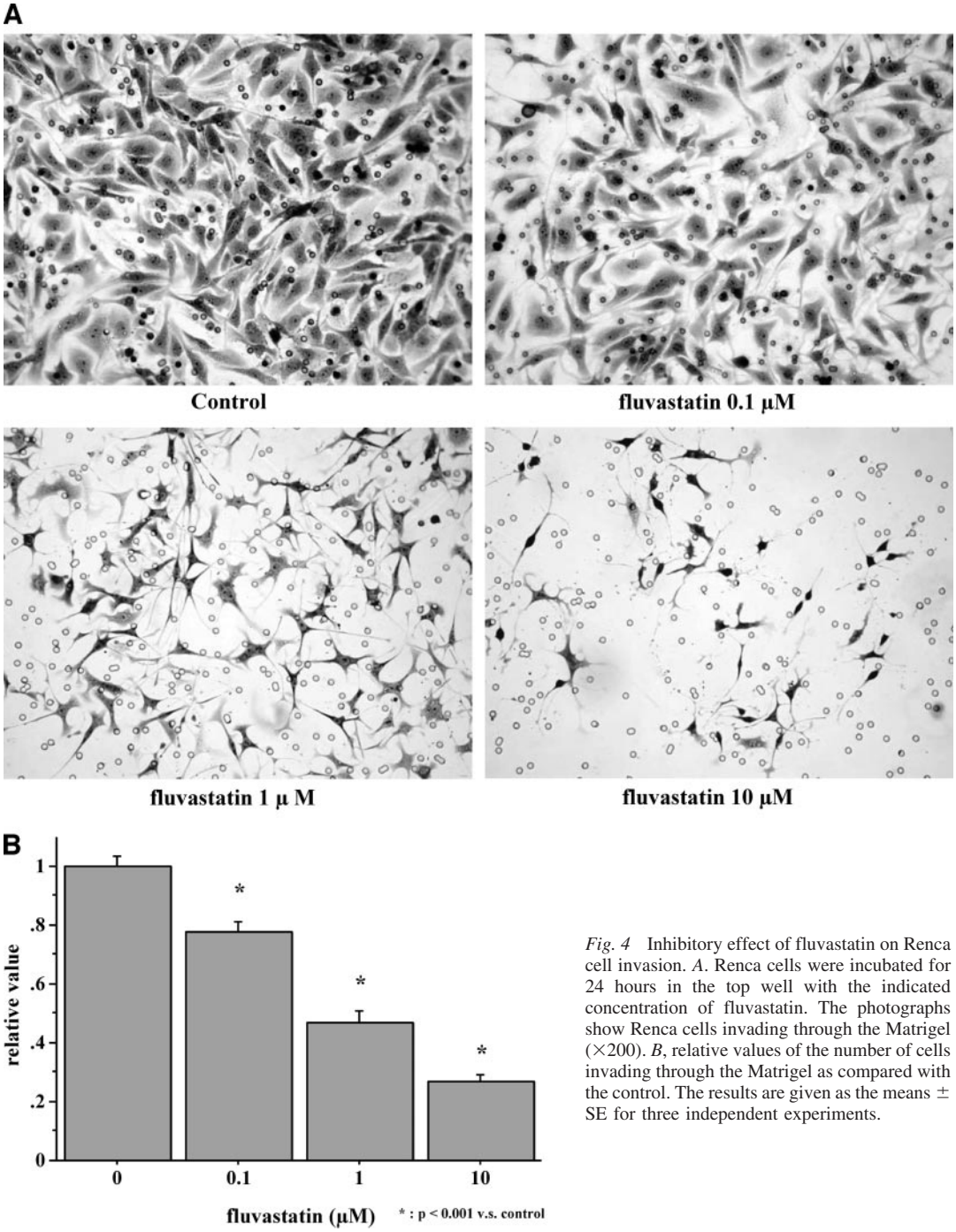

Fig. 4 Inhibitory effect of fluvastatin on Renca cell invasion. *A*. Renca cells were incubated for 24 hours in the top well with the indicated concentration of fluvastatin. The photographs show Renca cells invading through the Matrigel (×200). *B*, relative values of the number of cells invading through the Matrigel as compared with the control. The results are given as the means ± SE for three independent experiments.

Fluvastatin Prevented Pulmonary Metastases of Renca Cells in Mice. We finally examined whether oral administration of fluvastatin at clinically relevant doses could prevent experimental pulmonary metastasis of Renca cells. Renca cells were inoculated into male BALB/c mice via the tail vein, 0.1 to 10 mg/kg/d of fluvastatin was orally administered for 12 consecutive days, and the number of metastatic nodules was counted for each mouse. The fluvastatin was well tolerated in all mice. There was no significant difference between the mean number of metastatic nodules in the control group (313 ± 21 nodules, $n = 19$) and that in the group receiving 0.1 mg/kg/d (314 ± 28 nodules, $n = 10$). Significant inhibitory effects were found, however, in the groups receiving 1 mg/kg/d (138 ± 35 nodules, $n = 10$, $P < 0.0001$) and 10 mg/kg/d (46 ± 25 nodules, $n = 10$, $P < 0.0001$; Fig. 6).

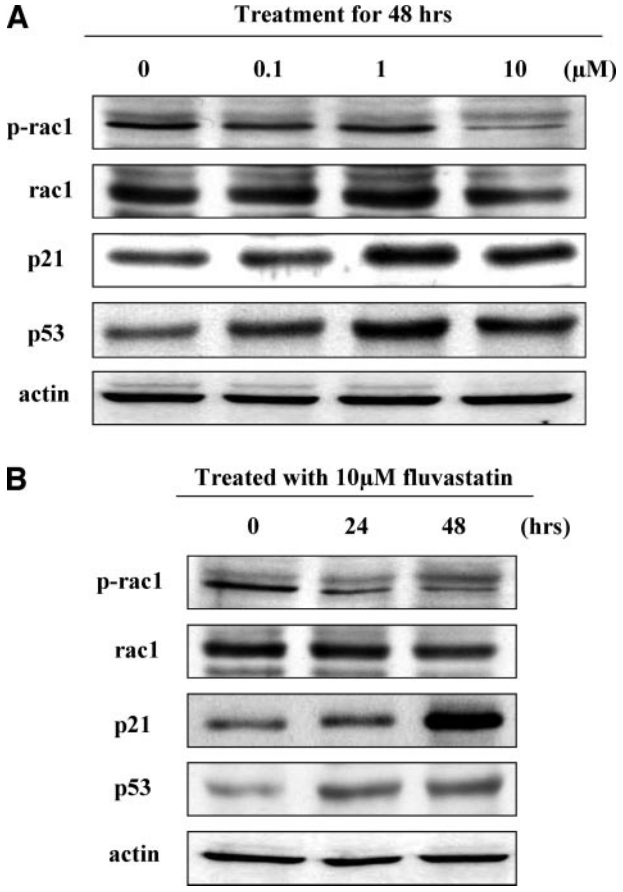

Fig. 5 Western blot analyses of the effect of fluvastatin on the expression levels of p-rac1, rac1, p21, and p53. Actin was used as an internal control. *A*. Renca cells were incubated for 48 hours with the indicated concentration of fluvastatin. The entire cell lysates were subjected to Western blotting. A decrease in rac-1 phosphorylation and increases in p21 and p53 were found. *B*. Renca cells were incubated for up to 48 hours with 10 μmol/L fluvastatin. The entire cell lysates were subjected to Western blotting. A decrease in rac-1 phosphorylation and increases in p21 and p53 were found.

DISCUSSION

Recently, investigation of the anticancer activity of statin-family drugs has focused on several types of cancer (6–14). Clinical trials investigating the possible value of statins as anticancer agents have also been conducted, and the safety of statins has been documented extensively (16–18). The therapeutic efficacy of statins against renal cell carcinoma, however, has not yet been investigated. This is the first study using an experimental model to demonstrate the therapeutic efficacy of a statin-family drug, fluvastatin, against *in vitro* renal cancer growth, invasion, angiogenesis, and pulmonary metastasis.

Within the statin family, fluvastatin is entirely synthetic and has a distinct biopharmaceutical profile, including a high rate of absorption, a short systemic exposure time, virtually no active circulating metabolites, and a high degree of protein binding, which enables fluvastatin to act as a vascular statin at a lower concentration than other statins (2) Furthermore, the average wholesale price of fluvastatin is 40 to 50% lower than that of the other available statins, and it thus may be considered the most cost-effective of these agents (2). Fluvastatin is usually administered orally at daily doses of 20 to 80 mg in patients with hypercholesterolemia, and the plasma concentration typically reaches around 1.0 μmol/L (3, 4). In the present study, we have examined whether clinically relevant doses of fluvastatin could be potentially effective as anticancer agents against murine renal cancer cells from the standpoints of proliferation, invasion, angiogenesis, and metastasis.

First, we examined the effect of fluvastatin at concentrations of 0.1 to 10 μmol/L on the proliferation and cell cycle of renal cancer cells. Even at relatively low concentrations of fluvastatin (0.1 and 1 μmol/L), we found decreased Renca cell proliferation, with up to 70% inhibition at 10 μmol/L. Because treatment with fluvastatin resulted in increased sub-G_1 population and nuclear fragmentation of Renca cells, we considered the inhibitory effect to be due to the induction of apoptosis. Statins have been shown to synchronize tumor cells by blocking the transition of G_1-S in the cell cycle, thereby causing it to exert its antiproliferative effect, which may be attributable to an increase in $p21^{WAF/CIP1}$, a cyclin-dependent kinase inhibitor (12). Although it has been suggested that statins inhibit cancer cell growth via a p53-independent mechanism (22–24), we found that fluvastatin increased the expression of $p21^{WAF/CIP1}$ accompanying increased expression of p53 in Renca cells.

Renal cell carcinoma has been reported to be morphologically characterized by a high degree of vascularization (25, 26).

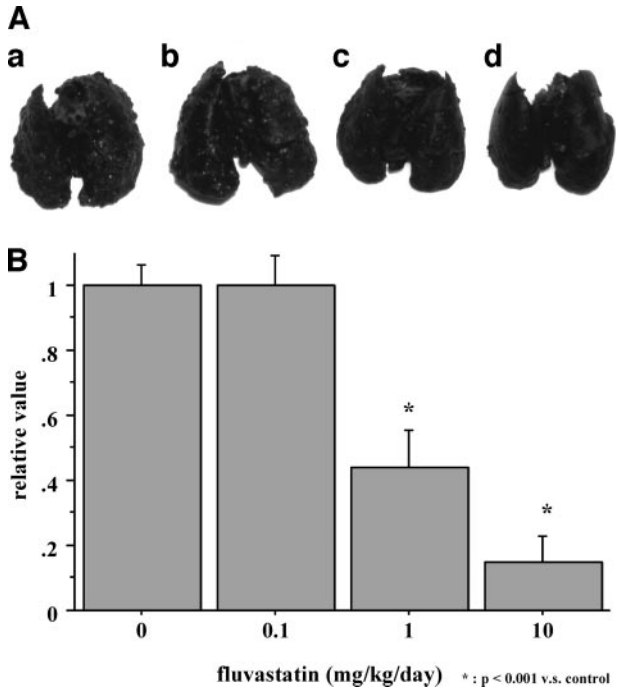

Fig. 6 Inhibition of pulmonary metastasis of Renca cells by orally administered fluvastatin. *A*. Gross appearances of pulmonary metastases were observed. Mice were treated daily with PBS only (*a*; control), 0.1 mg/kg (*b*), 1 mg/kg (*c*), or 10 mg/kg (*d*) fluvastatin for 12 days from the day of inoculation. *B*, relative values of the number of metastatic nodules compared with that of the control group. The results are given as the means ± SE ($n = 10$ in each group).

Therefore, angiogenesis could be a potential target for new therapeutic approaches against renal cell carcinoma. The effects of statins on angiogenesis are still controversial. Low doses of statins have been shown to promote Akt-dependent vascular endothelial cell survival in serum-deprived media and induce vascular structure formation (15, 27, 28), whereas high doses of statins have been shown to inhibit angiogenesis (15). In the present study, we examined the effects of fluvastatin at doses of 0.1 to 10 μmol/L on *in vitro* angiogenesis and found inhibitory effects at these concentrations. Although we did not observe any enhancement of *in vitro* angiogenesis by fluvastatin at these doses, clinicians should be aware of the possibility that statins might promote tumor angiogenesis, as described previously.

Statins have also been shown to inhibit cell signaling pathways associated with the invasive and metastatic properties of cancer, including the Rho family of small GTPase (8–10, 12). Some recent studies have demonstrated that fluvastatin inhibits *in vitro* pancreatic cancer cell invasion not only by preventing the translocation of RhoA from the cytosol to the membrane, but also by reducing RhoA activation (8–10). In addition to RhoA, rac1 is another small GTP-binding protein whose role in invasion of human renal cancer cells has been studied (21). Engers *et al.* (21) have reported that increased rac1 activation inhibited invasion of a human renal cancer cell line, clearCa-28, by up-regulation of TIMP-1 and TIMP-2. Another study, however, demonstrated that constitutively activated rac1 promoted cell invasiveness across type-I collagen, as well as collagen-dependent MMP-2 activation, in HT1080 fibrosarcoma cells (29). Thus, the role of rac1 activation in cellular invasion seems at least partly cell type and cell substrate specific. These previous studies suggest that rac1 might play a key role in the modulation of invasive capacities of renal cell carcinoma. Thus, in the present study, we examined the changes of rac1 phosphorylation levels and found that fluvastatin markedly reduced the phosphorylation of rac1 and significantly inhibited the *in vitro* invasive property of Renca cells. Therefore, we suggest that the decreased phosphorylation of rac1 due to fluvastatin might be responsible for the reduced invasive properties of the Renca cells, at least in part.

Although statins have been shown to inhibit *in vitro* cancer cell proliferation, their effects on *in vivo* tumor growth are not well defined. In particular, at present, there is only one study investigating the effect of fluvastatin on cancer metastasis, in which it was reported that orally administrated fluvastatin reduced the experimental liver metastasis of pancreatic cancer. Patients with renal cell carcinoma frequently develop distant metastasis, and metastatic renal cell carcinoma is well known to be refractory to standard therapies (19). Thus, the prevention of metastasis is an extremely important issue in treating patients with renal cell carcinoma. We investigated whether orally administered fluvastatin can prevent pulmonary metastasis of Renca cells. Oral administration of fluvastatin to mice at doses of 1 to 10 mg/kg/d for 12 days after inoculation of Renca cells significantly decreased the number of metastatic pulmonary nodules. Fluvastatin was well tolerated in all of the mice in our study. Our results strongly suggest that orally administered fluvastatin at the doses used for patients with hypercholesterolemia would have prophylactic effects against metastasis of renal cell carcinoma.

In conclusion, we have demonstrated that fluvastatin, administered at the doses recommended for the treatment of hypercholesterolemia, markedly inhibited renal cancer cell proliferation, invasion, and *in vitro* angiogenesis, resulting in reduced pulmonary metastasis. Treatment with fluvastatin has been shown to be well tolerated and cost-effective for the majority of patients with hypercholesterolemia (2). Therefore, oral administration of fluvastatin could be a safe and potentially effective treatment for preventing the invasion and pulmonary metastasis of renal cancer cells.

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