Inhibition of Epidermal Growth Factor–induced RhoA Translocation and Invasion of Human Pancreatic Cancer Cells by 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Inhibitors

Toshiyuki Kusama, Mutsuko Mukai, Teruo Iwasaki, Masaharu Tatsuta, Yoshirou Matsumoto, Hitoshi Akedo, and Hiroyuki Nakamura

Departments of Tumor Biochemistry [T. K., M. M., H. A., H. N.] and Gastrointestinal Oncology [M. T.], Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511, Japan; First Department of Surgery, Yamahashi Medical University, Yamahashi 409-3898, Japan [T. K., Y. M.]; and First Department of Pathology, Hyogo College of Medicine, Nishinomiya 662-8501, Japan [T. I., H. A.]

ABSTRACT

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors prevent the conversion of HMG-CoA to mevalonate and thereby inhibit the synthesis of other products derived from this metabolite. This includes a number of small prenylated GTPases involved in cell growth, motility, and invasion. We studied the effect of HMG-CoA reductase inhibitors (fluvastatin and lovastatin) on in vitro invasion of human pancreatic cancer PANC-1 cells. Epidermal growth factor (EGF) induced a dose-dependent increase of PANC-1 cell invasion in a modified Boyden chamber assay. Stimulation of cancer cells with EGF induced translocation of RhoA from the cytosol to the membrane fraction and actin stress fiber assembly. Furthermore, Clostridium botulinum C3 transferase, a specific inhibitor of Rho, inhibited the ability of EGF to promote invasion, indicating that EGF-induced cancer cell invasion is regulated by Rho signaling. Treatment of PANC-1 cells with fluvastatin markedly attenuated EGF-induced translocation of RhoA from the cytosol to the membrane fraction and actin stress fiber assembly, whereas it did not inhibit the tyrosine phosphorylation of EGF receptor and c-erbB-2. The induction of cancer cell invasion by EGF was inhibited by the addition of fluvastatin or lovastatin in a dose-dependent manner. The effects of fluvastatin or lovastatin on cell morphology and invasion were reversed by the addition of all-trans-geranylgeraniol but not by the addition of all-trans-farnesol. These results suggest that HMG-CoA reductase inhibitors affect RhoA activation by preventing geranylgeranylation, which results in inhibition of EGF-induced invasiveness of human pancreatic cancer cells.

INTRODUCTION

Pancreatic cancer is a devastating disease with an overall 5-year survival rate of less than 1% and a median survival of approximately 5–6 months (1, 2). One reason for this poor prognosis is the propensity of pancreatic cancer cells to invade adjacent tissues and to metastasize. Pancreatic cancer is often associated with the overexpression of a variety of mitogenic growth factors and their receptors, including EGF3 and related receptors, and this overexpression has been consistently found to play a leading role in the progression of pancreatic cancer (3).

EGF has been reported to induce rapid alteration in the organization of the actin microfilament system, resulting in extensive membrane ruffling, lamellipodia formation, and cortical actin polymerization (4–6). Actin cytoskeletal reorganization is regulated by the Rho family of small GTPases (7–9). Rho, Rac, and Cdc42 regulate signal transduction from receptors in the membrane and a variety of cellular events related to cell adhesion to the extracellular matrix, cell morphology, motility, and invasion, acting as molecular switches in the cell (10, 11). Previously, we reported several findings that support the involvement of Rho in cancer cell invasion (12, 13). Furthermore, the translocation of Rho protein from the cytosol to the plasma membrane has been implicated in cancer cell invasion (14, 15).

HMG-CoA reductase inhibition causes a block of the biochemical pathway, leading to the biosynthesis of farnesyl and geranylgeranyl PPs (16, 17). These isoprenoids are covalently linked to cysteine residues located at discrete sites near the COOH-terminal end of target proteins by a reaction catalyzed by specific transferases. Protein targets for isoprenylation include small G proteins, which require this posttranslational modification to undergo a series of changes that lead to their attachment to the plasma membranes and their full function (18). Although HMG-CoA reductase inhibitors have been extensively studied as possible chemotherapeutic agents for the treatment of cancer cell growth (19), invasion (20, 21), and metastasis (20–23), no detailed data have been provided as yet on the exact mechanism(s) of their inhibitory effect on cancer cell invasion. In the present study, we show that HMG-CoA reductase inhibitors markedly attenuate EGF-induced translocation of RhoA from the cytosol to the membrane fraction, actin stress fiber assembly, and the in vitro invasive capacity of human pancreatic cancer cells.

MATERIALS AND METHODS

Materials. C3 was kindly supplied by Dr. B. Syuto, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University (Morioka, Japan). The following materials were purchased from the sources indicated: (a) FBS, BioWhittaker (Walkersville, MD); (b) RPMI 1640, Life Technologies, Inc. (Grand Island, NY); (c) recombinant human EGF, Austral Biologicals (San Ramon, CA); (d) growth factor-reduced Matrigel, Becton Dickinson Labware (Bedford, MA); (e) Transwell chambers, Costar (Cambridge, MA); (f) PD 98059, Alexis (San Diego, CA); (g) lovastatin and genistein, Wako Pure Chemical Industries (Osaka, Japan); (h) LY 294002 and wortmannin, Biomol Research Laboratories (Plymouth Meeting, PA); (i) anti-EGFR mAb, anti-c-erbB2 polyclonal Ab, and anti-phosphotyrosine mAb 4G10, Upstate Biotechnology (Lake Placid, NY); (j) mouse anti-RhoA mAb, rabbit anti-RhoA polyclonal Ab, and protein A/G-agarose, Santa Cruz Biotechnology (Santa Cruz, CA); (k) rhodamine-phalloidin, Molecular Probes (Eugene, OR); (l) antirabbit IgG rhodamine-conjugated secondary Ab, BioSource (Camarillo, CA); and (m) all other chemicals and reagents, Sigma Chemical Co. (St. Louis, MO). Fluvastatin was isolated from the marketed product (Tanabe Seiyaku Co., Osaka, Japan). It was dissolved at 30 mM in methanol and stored at −20°C.

Cells and Cell Cultures. Human pancreatic cancer cell lines PANC-1 and ASPC-1 were obtained from the American Type Culture Collection (Manassas, VA). Human pancreatic cancer cell line MiaPaCa-2, human colon cancer cell line WiDr, and human breast cancer cell line MCF-7 were obtained from the
Japanese Cancer Research Resources Bank (Tokyo, Japan). Human pancreatic cancer cell line PSN-1 was a gift from the Genetics Division, National Cancer Research Institute (Tokyo, Japan). PANC-1 and MIAPaCa-2 cells were grown in DMEM; ASPC-1, PSN-1, and WiDr cells were grown in RPMI 1640; and MCF-7 cells were grown in MEM. All media were supplemented with 10% FBS, penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide.

**Matrigel Invasion Assay.** Cell invasion through reconstituted basement membrane Matrigel was assayed by a method reported previously (24). Briefly, polycarbonate membranes (8.0-μm pore size) of the upper compartment of Transwell culture chambers were coated with 5% Matrigel. Subconfluent cells were starved for 24 h and harvested with 0.05% trypsin containing 0.02% EDTA, washed twice with PBS, and resuspended at 1 × 106 cells/ml in serum-free medium with 0.1% fraction V BSA. The cell suspension (100 μl) was placed in the upper compartment, and the lower compartment was immediately filled with 600 μl of serum-free medium containing 0.1% BSA and the indicated ligand. After 16 h of incubation, the membranes were stained with May-Grunwald and Giemsa solution. Cells on the upper surface of the filter were removed carefully with a cotton swab, and the cells that had migrated through the membrane to the lower surface were counted in nine different fields under a light microscope at ×400 magnification. Each experiment was performed in triplicate wells and repeated three times.

**Immunofluorescence.** Cells were seeded onto 35-mm culture dishes and incubated for 1 h. Subsequently, the cells were incubated for 24 h in serum-free medium containing 0.1% BSA with the indicated additions and challenged with 1 nm EGF for the indicated time periods at 37°C. Cells were then fixed with 5% paraformaldehyde in PBS for 10 min, washed with PBS, permeabilized in 1% Triton X-100 in PBS for 15 min, washed, and blocked with 1% BSA in TTBS. For visualization of filamentous actin, the cells were exposed to rhodamine-phalloidin for 30 min at 37°C and washed with TTBS. After final washes, coverslips were mounted on the dishes using a 50% solution of glycerol in PBS. The cells were examined under a fluorescence microscope (Olympus BX50, Olympus, Tokyo, Japan).

**For RhoA staining, fixed and permeabilized cells were incubated for 2 h with rabbit anti-RhoA Ab diluted in 1% BSA-TTBS (1:200) for 2 h at 37°C, rinsed three times with TTBS, and then incubated for 30 min with a 1:80 dilution of antirabbit IgG rhodamine-conjugated secondary Ab.**

**Immunoblotting and Immunoprecipitation.** Serum-starved subconfluent cells were washed with cold PBS and solubilized in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.1% SDS, 1% sodium deoxycholate, 2 mM sodium vanadate, 50 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C. The lysis was homogenized and centrifuged at 15,000 rpm for 30 min at 4°C to remove debris, and the protein concentration was determined by the method of Lowry et al. (25). The proteins were separated by electrophoresis on SDS-PAGE gels and transferred onto nitrocellulose membranes. The blot membranes were scanned as described above.

**Separation of Particulate and Cytosolic Fractions.** Separation of cytosolic and membrane fractions was performed as described previously (14). Serum-starved subconfluent cells were incubated with EGF for the indicated time periods at 37°C. The cells were then washed with cold PBS and lysed by freeze-thawing in ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM EDTA, 1 mM MgCl, 10 mM sodium fluoride, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000 × g for 30 min at 4°C, and the supernatant was collected as the cytosolic fraction. Pellets were homogenized in the above-mentioned lysis buffer containing 2% Triton X-114 and centrifuged at 800 × g for 10 min at 4°C. The supernatant was collected and is referred to here as the membrane fraction. The protein concentrations in the cytosolic fraction and the membrane fraction were measured using a DC protein assay kit and adjusted to the same concentration with the lysis buffer, and then these fractions were subjected to immunoblotting. The blot membranes were scanned as described above.

**Statistical Analysis.** The statistical significance of the differences between groups was calculated by applying Student’s two-tailed t test, and P < 0.05 was considered significant.

**RESULTS**

**Effect of EGF on the in Vitro Invasiveness of Human Pancreatic Cancer Cells.** The possibility that EGF stimulates the invasiveness of human pancreatic cancer cells was studied in a Matrigel invasion assay in Transwell culture chambers. Cells were incubated for 16 h in the absence or presence of 0.01–1 nm EGF in the lower compartment of the culture chambers, and then the cells that passed through the Matrigel-coated membranes to the lower surface of the filters were counted. As shown in Fig. 1A, the invasive capacity of PANC-1 cells was stimulated by EGF in a dose-dependent manner. During this incubation period, the number of PANC-1 cells increased approximately 10% in the presence of 1 nm EGF (data not shown).

As a prelude to identifying the signaling mechanism(s) by which EGF stimulates invasion, we initially assessed the effects of inhibitors of MAPK (PD 98059) and PI3K (LY 294002 or wortmannin) on PANC-1 cell invasion stimulated by EGF. When cells were incubated for 16 h with 10 μM PD 98059 in the Transwell chamber, EGF-induced cancer cell invasion was not altered (Fig. 1B). In contrast, 2 μM LY 294002 or 0.5 μM wortmannin inhibited EGF-induced cancer cell invasion significantly (Fig. 1B). Genistein, an inhibitor of tyrosine phosphorylation, also inhibited the cancer cell invasion in response to EGF (Fig. 1B). None of the inhibitors altered the capacity of the cells to exclude trypan blue, indicating that under the experimental conditions used in the present study, the inhibitors did not injure the cells.

Because EGF is known to stimulate the small GTPase Rho (7), the role of Rho in EGF-induced cancer cell invasion was tested by suppressing Rho function with C3 (a specific inhibitor of Rho). When PANC-1 cells were pretreated with 5 μg/ml C3 for 24 h in the presence of 10% FBS, EGF-induced cell invasion was suppressed to approximately 13% in comparison with cells not treated with C3 (Fig. 1C). These results indicate that Rho and PI3K are essential for EGF-induced cancer cell invasion.

**Effects of HMG-CoA Reductase Inhibitors on Cell Morphology and Cytoskeletal Organization.** Because HMG-CoA reductase inhibitors cause cell rounding by prevention of isoprenylation proteins important for cytoskeletal organization (25–27), we first studied the effect of fluvastatin or lovastatin on the morphology of human pancreatic cancer cells. Typical shapes of PANC-1 cells are shown in Fig. 2. Untreated PANC-1 cells were flat and well spread, but exposure of the cells to 15 μM fluvastatin resulted in cell retraction from the substratum, rounding up, and loss of contacts between neighboring cells. The same morphological changes were observed when lovastatin was used instead of fluvastatin. After a 24-h incubation of PANC-1 cells with 15 μM fluvastatin or lovastatin, cell growth was not altered significantly, and these morphological changes were reversed 24 h after the removal of fluvastatin (data not shown).

The role of geranylgeranylated or farnesylated proteins was studied by treating cells with GGOL or FOL in the presence of fluvastatin. The cells treated with fluvastatin in the presence of GGOL (10 μM) were well spread, like the control cells (Fig. 2C), but the cells treated...
with fluvastatin in the presence of 10 μM FOL remained spindle-shaped (Fig. 2D). These results indicate that geranylgeranylation of Rho is required for the well-spread morphology of PANC-1 cells.

The exposure of cells to growth factors has been shown to cause cytoskeletal reorganization and altered cell morphology, which have accordingly been implicated in the enhancement of cell migration and invasion (6, 7, 9). Therefore, we investigated whether EGF stimulation results in actin cytoskeletal rearrangements in PANC-1 cells. As shown in Fig. 3, serum-starved cells displayed low levels of F-actin as judged by phalloidin staining (Fig. 3A). The treatment of cancer cells with 1 nM EGF resulted in a dramatic increase in polymerized cortical actin (Fig. 3B). In contrast, cells preincubated with 15 μM fluvastatin for 24 h were round, and EGF-induced cortical actin polymerization was greatly reduced (Fig. 3C). The effect of fluvastatin on EGF-induced actin stress fiber assembly was reversed by the addition of GGOL (data not shown).

**Inhibitory Effects of HMG-CoA Reductase Inhibitors on EGF-induced Cell Invasion.** The effect of HMG-CoA inhibitors on the *in vitro* invasiveness of EGF-stimulated cells was assessed using the Matrigel invasion assay. The migration of PANC-1 cells through the Matrigel-coated polycarbonate membrane was inhibited by the addition of fluvastatin or lovastatin in a dose-dependent manner (Fig. 4A). Half-maximal inhibition was estimated to occur at about 3 μM fluvastatin or lovastatin, and approximately 70% inhibition was observed with 15 μM fluvastatin or lovastatin (Fig. 4A). The inhibitory effect of fluvastatin or lovastatin on EGF-induced PANC-1 cell invasion was completely reversed 24 h after removal of the inhibitors. The presence of 15 μM fluvastatin or lovastatin did not reduce PANC-1 cell attachment to Matrigel-coated dishes 1 h after seeding and did not affect the cell growth during the 16-h incubation period (data not shown). These results suggest that fluvastatin and lovastatin inhibit the migration of the cells toward the chemoattractant EGF located in the lower chamber.

The role of geranylgeranylated or farnesylated proteins was studied by treating cells with GGOL or FOL. Whereas neither GGOL nor FOL induced control cell invasion without EGF stimulation, the inhibitory effect of fluvastatin on EGF-induced PANC-1 cell invasion was reversed by the addition of GGOL, but not by the addition of FOL (Fig. 4B).

To examine the cell type specificity of inhibition, the effect of fluvastatin on the invasiveness of a variety of cancer cells was studied. Fluvastatin inhibited the EGF-induced invasiveness of ASPC-1 and MIAPaCa-2 human pancreatic cancer cells and WiDr human colon cancer cells (Table 1). Furthermore, fluvastatin inhibited the serum-induced invasiveness of PSN-1 human pancreatic cancer cells and
In vitro experiments in triplicate wells. PANC-1 cells were incubated for 24 h in medium containing 0.1% BSA in the absence or presence of fluvastatin (15 μM). After further incubation with EGF (1 nM) for 15 min, cells were stained with rhodamine-phalloidin to visualize actin stress fibers. A, untreated cells; B, cells treated with EGF; C, cells treated with fluvastatin and EGF.

MCF-7 human breast cancer cells, indicating that the inhibitory effect of fluvastatin was not specific to PANC-1 cells.

**Inhibitory Effect of Fluvastatin on EGF-induced Translocation of RhoA from the Cytosol to the Membrane Fraction.** Because EGFR tyrosine kinase activity has been shown to be involved in cell motility (28), we sought to determine whether fluvastatin would inhibit tyrosine phosphorylation of EGFR and c-erbB-2. PANC-1 cells were preincubated for 24 h with or without fluvastatin and stimulated with EGF, and cell lysates were immunoprecipitated with anti-EGFR Ab or anti-c-erbB-2 Ab, followed by immunoblotting with antiphosphotyrosine Ab. As shown in Fig. 5, pretreatment with fluvastatin did not attenuate the EGF-stimulated phosphorylation of either receptor.

Because RhoA must be targeted to the plasma membrane for its activation (14, 15), we first examined the effect of fluvastatin on the translocation of RhoA protein from the cytosol to the membrane fraction in PANC-1 cells. In serum-starved PANC-1 cells, approximately 26% of the RhoA protein was present in the membrane fraction without any stimulation. The incubation of these cells with 15 μM fluvastatin caused RhoA to decrease in the membrane fraction and to accumulate in the cytosolic fraction in a time-dependent manner (Fig. 6A). Translocation of RhoA from the membrane to the cytosol became evident after 60 min of fluvastatin treatment. To determine whether the inhibition of cell invasion by fluvastatin is mediated by suppression of RhoA activation, we examined the effect of fluvastatin on the translocation of RhoA after stimulation with EGF. Western blot analysis showed that stimulation of PANC-1 cells with 1 nM EGF for 30 min resulted in a 1.7-fold increase in the amount of RhoA translocated from the cytosol to the membrane fraction, as compared with control (Fig. 6, B and C). To confirm RhoA localization in PANC-1 cells under culture conditions, cells were immunostained with a RhoA-specific Ab. In control cells, RhoA immunostaining was confined largely to the cytosol (Fig. 7A). In contrast, the stimulation of PANC-1 cells with EGF resulted in the translocation of RhoA to the membrane at the edges of lamellae (Fig. 7B). The translocation of RhoA from the cytosol to the membrane fraction in response to EGF was markedly blocked by pretreatment with fluvastatin in a dose-dependent manner (Fig. 6, B and C, and Fig. 7D). The effect of fluvastatin on EGF-induced translocation of RhoA was blocked by the addition of GGOL, but not by the addition of FOL (Fig. 6, B and C, and Fig. 7, E and F).

### DISCUSSION

Cancer cells require more cholesterol than normal cells (29, 30). This requirement seems to be satisfied by a higher HMG-CoA reductase activity or a higher activity of low density lipoprotein receptor. HMG-CoA reductase activity was shown to be higher in colorectal cancer not expressing low density lipoprotein receptor, and these features are associated with decreased patient survival (31, 32). These data suggest the involvement of high HMG-CoA reductase activity
high levels of mevalonate-derived metabolites, such as isoprenoid compounds, in the biological aggressiveness of cancer.

All HMG-CoA reductase inhibitors in clinical use (lovastatin, simvastatin, pravastatin, and fluvastatin) are reversible inhibitors that interfere with catalysis of the rate-limiting step in cholesterol biosynthesis. HMG-CoA reductase inhibitors, including simvastatin and lovastatin, prevent cell proliferation by inducing a block of the cell cycle in G1 phase (33–35). Pravastatin was demonstrated to inhibit the cell cycle in G1 of cancer cell proliferation and actin stress fiber assembly (Fig. 3C). Pretreatment of cells with fluvastatin reversed the addition of GGOL, but not by the addition of FOL (Figs. 4B, 6B, and 7, E and F).

Pharmacokinetic studies have shown that HMG-CoA reductase inhibitors are targeted to the liver and have elimination half-lives of 1.2–3 h (37). Despite their short systemic exposure times, HMG-CoA reductase inhibitors have been demonstrated to inhibit lung metastasis (20, 22) and lymph node metastasis (21). In the present study, the translocation of RhoA from the cytosol to the membrane fraction in PANC-1 cells became evident after 1 h of fluvastatin treatment (Fig. 6A), and EGF-induced translocation of RhoA from the cytosol to the membrane fraction was blocked by the pretreatment of cells for 2 h with fluvastatin (Fig. 6B). Koch et al. (26) demonstrated that the translocation of ADP-ribosylated Rho protein from the membrane to the cytosol fraction in NIH 3T3 cells was observed after 6 h of treatment with lovastatin. These data suggest that membrane binding of Rho protein is inhibited within several hours when geranylgeranylation of Rho protein is prevented by treatment with HMG-CoA reductase inhibitors.

On the other hand, farnesylated Ras proteins are associated with...
findings suggest, at least in our assay system, both activation of tyrosine kinase receptors and geranylgeranylation of RhoA are necessary for cancer cell invasion. Growth factors mediate their biological effects by binding to and activating receptors with tyrosine kinase activity, leading to a series of molecular events that are ultimately manifested as biological responses (43, 44). Multiple receptor targets, including PI3K (45, 46), Src (47), and phospholipase Cγ (45, 48), contribute to efficient signaling for growth factor-induced cell migration. We demonstrated that induction of cell invasion by EGF was inhibited by the PI3K inhibitors wortmannin and LY 294002 (Fig. 1B). Consistent with these results, Asslan et al. (42) demonstrated that EGF enhances HMG-CoA reductase activity through a PI3K pathway independent of the extracellular signal-regulated kinase/MAPK or c-Jun-NH₂-terminal kinase/stress-activated protein kinase pathways. Furthermore, PI3K activity, which is localized in the membrane fraction, is decreased by HMG-CoA reductase inhibitors (49). Together, these data suggest the involvement of PI3K activity in the tyrosine kinase receptor-RhoA invasion cascade via HMG-CoA reductase activity.

Our results highlight the importance of HMG-CoA reductase metabolism in cancer cell invasion in response to EGF and suggest that HMG-CoA reductase inhibitors affect EGF-induced translocation of RhoA and cancer cell invasion by preventing geranylgeranylation of RhoA. A significant inhibitory effect of fluvastatin or lovastatin on EGF-induced PANC-1 cell invasion was observed at a concentration of 1.5 μM, and half-maximal inhibition was estimated to occur at about 3 μM (Fig. 4A). Although the inhibitory fluvastatin concentration was higher than the peak plasma concentration of fluvastatin (0.1–1.5 μM) detected in clinical trials (34), the inhibitory concentration of lovastatin was comparable to the plasma concentration of lovastatin (0.1–3.9 μM) measured in a Phase I study in patients with cancer (50). HMG-CoA reductase inhibitors are well absorbed, actively targeted to the liver, and safe for use in long-term treatment (37). These data strongly suggest that these inhibitors have the potential to prevent pancreatic cancer cell invasion and liver metastasis at concentrations that might be relatively nontoxic to normal cells in comparison to most presently used anticancer agents.

REFERENCES


Inhibition of Epidermal Growth Factor-induced RhoA Translocation and Invasion of Human Pancreatic Cancer Cells by 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Inhibitors

Toshiyuki Kusama, Mutsuko Mukai, Teruo Iwasaki, et al.