Jpn. J. Cancer Res. 85, 633–638, June 1994

Cytotoxicity of Simvastatin to Pancreatic Adenocarcinoma Cells Containing Mutant *ras* Gene

Hitoshi Ura, Takeshi Obara, Noriyuki Nishino, Satoshi Tanno, Kiyoshi Okamura and Masayoshi Namiki

Third Department of Internal Medicine, Asahikawa Medical College, 4-5-3-11 Nishikagura, Asahikawa, Hokkaido 078

Simvastatin (SV), a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, inhibits the synthesis of mevalonic acid. The dose-dependent (0.1–100 μg/ml) cytotoxicity of SV towards human (MIAPaCa-2, Panc-1, HPC-1, HPC-3, HPC-4, PK-1, PK-9) and hamster (T2) pancreatic carcinoma cell lines was determined by MTT assay. At up to 20 μg/ml of SV, the effect was reversible and was restored by 60 μg/ml mevalonic acid. Point mutation of Ki-*ras* at codon 12 in each cell line was detected by means of the modified polymerase chain reaction. The concentration of SV necessary to achieve 50% cytotoxicity was about 10 μg/ml, and at this concentration of SV, DNA synthesis assayed in terms of [^{3}H]thymidine uptake, isoprenylation of p21^{ras} examined by Western blotting and cell progression from G1 to S phase of the cell cycle analyzed by flow cytometry were all inhibited. Isoprenylation inhibitors of p21^{ras}, such as SV, are expected to be useful for the treatment of pancreatic cancer.

Key words: Pancreatic adenocarcinoma — p21^{ras} — HMG CoA reductase inhibitor

More than 90% of human pancreatic adenocarcinomas are believed to be associated with an activating mutation of the Ki-*ras* oncogene.[1] Activating mutations of Ki, Ha, and N-*ras* oncogenes are believed to contribute to carcinogenesis by distorting the normal signal transduction pathway that controls cell proliferation.[2] The *ras* oncogene product, p21^{ras} protein, is bound to the cell membrane with polyisoprenoid, an intermediate product of cholesterol biosynthesis.[3] The polyisoprenoid of the C-terminal amino acid of p21^{ras} is a farnesyl residue.[4] An inhibitor of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase blocks the post-translational farnesylation of p21^{ras}.[5] In this study, we report the cytotoxic effect of simvastatin (SV), an HMG-CoA reductase inhibitor, on human pancreatic adenocarcinoma cells that have the mutant Ki-*ras* oncogene at codon 12. The relationship between the cytotoxic effect and inhibition of farnesylation of p21^{ras} was also studied.

MATERIALS AND METHODS

Cell culture Human pancreatic carcinoma cell lines MIAPaCa-2, Panc-1, HPC-1, HPC-3, HPC-4, PK-1 and PK-9, and hamster pancreatic carcinoma cell line T2 were maintained and grown as monolayer cultures in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in air. W31 cells are the EJ-ras oncogene-transformants of the parental WKA rat fetus fibroblasts. HPC-1, HPC-3, HPC-4, and W31 were generous gifts from Dr. N. Sato.[6,7] MIAPaCa-2 was supplied by the Japanese Cancer Research Resources Bank.

Measurement of DNA synthesis Pancreatic carcinoma cells of each cell line were maintained for 24 h in complete medium with or without 10 μg/ml SV and/or 60 μg/ml mevalonic acid (Sigma, St. Louis, MO). Ten μCi of [^{3}H]thymidine (NEN, Boston, MA) was added, and the culture was continued for an additional 1 h. The cells were washed with 2 ml of phosphate-buffered saline (PBS), treated with 2 ml of 5% trichloroacetic acid (TCA), and kept overnight. Cells were washed with 5% TCA and solubilized with 0.8 ml of 1% sodium dodecyl sulfate. The radioactivity was counted in a liquid scintillation counter, LS1801 (Beckman).

MTT assay The MTT assay was performed to determine the remaining fractions according to the method described.[8] Cells were plated at 2×10^3 cells per well in a 96-well plate. Various concentrations of SV and its β-hydroxy acid form (SVA) were added to complete medium (SVA is an active form of SV). At 72 h after inoculation, the cytotoxicity and/or growth inhibition by SV was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method. Ten μl of 5 mg/ml MTT was added to the culture medium followed by incubation for 3 h at 37°C. Then MTT-formazan produced was solubilized with dimethyl sulfoxide and measured in a Multiscan Bichromatic (Labo System) at 570 nm. SV and SVA were generous gifts from Banyu Pharmaceutical Co., Tokyo.

Cell cycle analyses MIAPaCa-2 cells were plated at a density of 1×10^6 cells/75 cm^2 flask. The medium containing 1.5% FBS with SV at a concentration of 10 μg/ml was changed after 72 h serum starvation to one containing 0.5% FBS. After 12 h incubation, the cells were removed for flow cytometric analysis or transferred to the medium without SV for an additional 12 h incubation. The cells were removed from culture plates by brief trypsinization and were spun down at 1000 rpm for 5 min. Cells were fixed by re-suspension in 50% (v/v) ethanol in PBS at -20°C followed by treatment with 1% RNase solution. Cells pellets were washed with PBS, resuspended in PBS with 50 μg/ml propidium iodide and incubated at 4°C for 2 h. The DNA content was analyzed by using a flow cytometer, EPICS Elite (Coulter, Miami, FL).

Detection of point mutation in the Ki-*ras* oncogene Point mutation at codon 12 in the Ki-*ras* oncogene of pancreatic carcinoma cell lines was detected by the modified polymerase chain reaction (PCR) technique described by Haliassos *et al.*[9] Briefly, DNA from cells was extracted according to the phenol-chloroform method. Modified primers were designed to introduce a base substitution adjacent to the codon 12 of Ki-*ras* in order to create an artificial restriction site of *Msp*I on the wild-type allelic form. The sequences of the modified primers were as follows: 5′TAAACTTGTGGTAGTTGGAGCC3′(K12Nm) and 5′TCTATTGTTGGATCATATTC3′(KB12). PCR was performed at 30 cycles of amplification. Each cycle included denaturation of DNA at 94°C for 30 s, annealing of the primers at 52°C for 1 min, and enzymatic extraction at 72°C for 1 min. The amplified fragment (99 bp) was digested by *Msp*I enzyme, which gave two fragments of 78 and 21 bp in the case of a wild-type sequence or left the product undigested in the case of a mutated sequence. The fragments were analyzed by electrophoresis on 1.5% agarose gels.

Western blotting of ras proteins SV treatment (10 μg/ml) was for 24 h prior to cell lysis. Cells were treated with lysis buffer (pH 7.4 50 m*M* HEPES, 250 m*M* NaCl, 1 m*M* EDTA, 0.5% NP-40, 1 m*M* MPMSF), and proteins were separated by 13% polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically from gels to nitrocellulose membranes, vacant sites were blocked with 5% skim milk, and then the washed membranes were reacted with mouse monoclonal anti-pan-ras antibody, F111-85 (Oncogene Science, Uniondale, NY). The washed membranes were reacted with labeled sheep anti-mouse IgG antibody, and autoradiographed with X-ray film using an ECL-Western blotting kit (Amersham, Buckinghamshire, England)

RESULTS

Ki-*ras* mutation at codon 12 in pancreatic adenocarcinoma cell lines Fig. 1 shows representative electrophoretic analysis data of PCR products after *Msp* I digestion. The wild-type sequence at codon 12 of Ki-*ras* was detected from PCR products of DNA from human placenta, and visualized as a 78 bp fragment. DNA from all pancreatic adenocarcinoma cell lines examined resulted

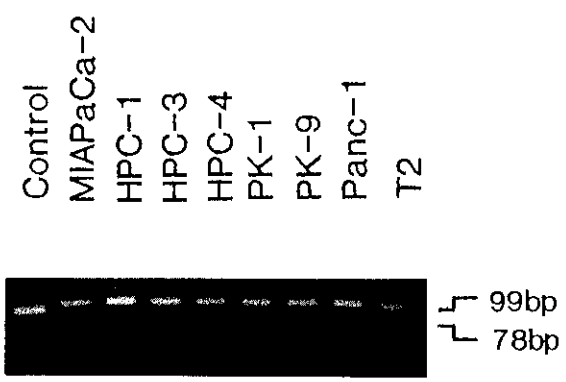

Fig. 1. PCR products were digested with *Msp*I and electrophoresed on 1.5% agarose gel. The control lane represents the result for human placenta DNA with the non mutated wild-type sequence at codon 12 of Ki-*ras* oncogene. Other lanes show the results for DNA from pancreatic carcinoma cells, and reveal mutated Ki-*ras* oncogene at codon 12.

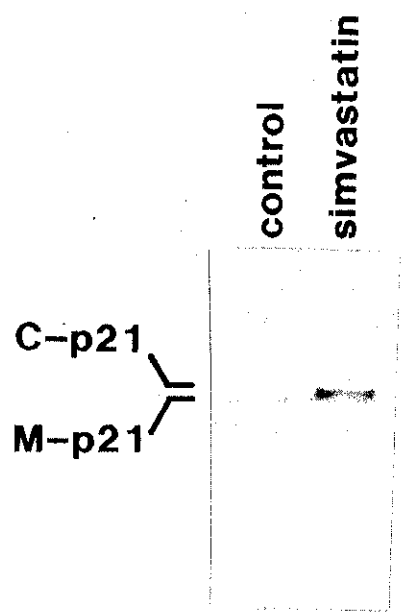

Fig. 2. Effect of simvastatin of $p21^{ras}$ analyzed by Western blotting. W31 cells were treated with 10 μg/ml of simvastatin for 24 h. After Western blotting with pan-ras antibody to p21 ras, cytosolic (C-p21) and membrane-associated, isoprenylated $p21^{ras}$ (M-p21) were separated by SDS-PAGE.

in 99 and 78 bp fragments. These results indicate the existence of point mutation in either of the guanine nucleotides at codon 12 of Ki-*ras* oncogene.

SV blocks processing of ras protein To examine whether simvastatin inhibits the posttranslational processing of ras proteins, Western blotting with anti-pan-ras monoclonal antibody was performed (Fig. 2). The *ras* oncogene-transfected cell line (W31) was selected for the experiment because the cell line overexpresses $p21^{ras}$.[7] In the absence of SV, the ras protein detected was fully processed membrane-associated $p21^{ras}$ (M-p21). Treatment with 10 μg/ml SV resulted in a decrease in M-p21 concomitant with an increase in unprocessed cytoplasmic $p21^{ras}$ (C-p21). M-p21 migrates approximately 2–3 kDa faster than the unprocessed form. Using this Western blotting method, $p21^{ras}$ was not detected in any cell line. The reasons why $p21^{ras}$ could not be detected in pancreatic carcinoma cells are discussed later.

Inhibition of DNA synthesis by SV The results in Fig. 3 show the inhibition of DNA synthesis in MIAPaCa-2 cells by simvastatin as determined by measuring [^{3}H]-thymidine uptake. DNA synthesis was suppressed to about 16% of the control level with 10 μg/ml SV. This inhibition is not due to lack of cholesterol since cholesterol is supplied in the medium with 10% FBS as low density lipoprotein (LDL).[10] Supplementation with LDL or cholesterol does not restore DNA synthesis (data not shown). However, 60 μg/ml mevalonic acid restored DNA synthesis, confirming that the inhibition of DNA synthesis is related to the mevalonic acid pathway of cholesterol synthesis. The DNA synthesis was also inhibited by SV in other pancreatic cell lines.

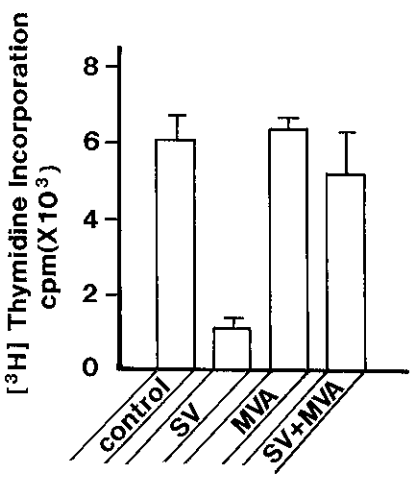

Fig. 3. Inhibition of [^{3}H]thymidine incorporation by simvastatin. MIAPaCa-2 cells were treated for 24 h with DMEM containing 5% FBS with the following additions: control= none, SV=10 μg/ml simvastatin, MVA=60 μg/ml mevalonic acid. The mean of triplicate assays is indicated by columns and standard error by bars.

Cytotoxic effect of SV and SVA in pancreatic adenocarcinoma cells As shown in Fig. 4, SV caused dose-dependent cytotoxicity in MIAPaCa-2 cells as measured by the MTT method. SVA was more cytotoxic. The concentrations of SV and SVA necessary to achieve 50% cytotoxicity (IC50) were 2.6 μg/ml and 0.52 μg/ml, respectively. The values of IC50 of SV and SVA for each cell line are presented in Table I.

Cell cycle-specific effects of SV The cell cycle distribution of MIAPaCa-2 cells growing in the absence or presence of 10 μg/ml SV for 12 h is shown in Fig. 5. Cells in the S phase increased during the first 12 h after medium change in the absence of SV (Fig. 5b). However,

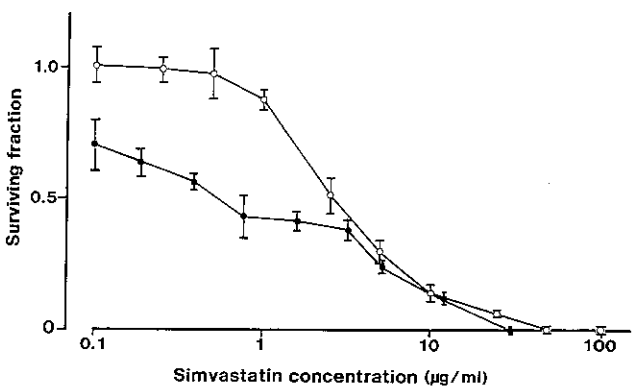

Fig. 4. Cytotoxic effect of simvastatin and its dihydroxy acid form (active form of SV). The cell density of viable MIAPaCa-2 cells was determined by MTT assay and expressed as the relative ratio to the untreated control cultures. (○) simvastatin; (●) β-hydroxy acid simvastatin.

Table I. Growth-inhibitory Concentration (IC50) of Simvastatin and Its Active Form in Human and Hamster Pancreatic Carcinoma Cell Lines

Cell line	IC50 (μg/ml)	
	SV	SVA
MIAPaCa-2	2.6	0.52
Panc-1	6.2	1.2
PK-1	12.0	0.68
PK-9	23.0	2.1
HPC-1	1.2	0.96
HPC-3	2.7	0.78
HPC-4	21.0	2.3
T2	0.8	0.46

MIAPaCa-2, Panc-1, PK-1, PK-9, HPC-1, HPC-3, HPC-4: Human pancreatic carcinoma cell lines. T2: Hamster pancreatic carcinoma cell line. SV: Simvastatin. SVA: β-Hydroxy acid of simvastatin.

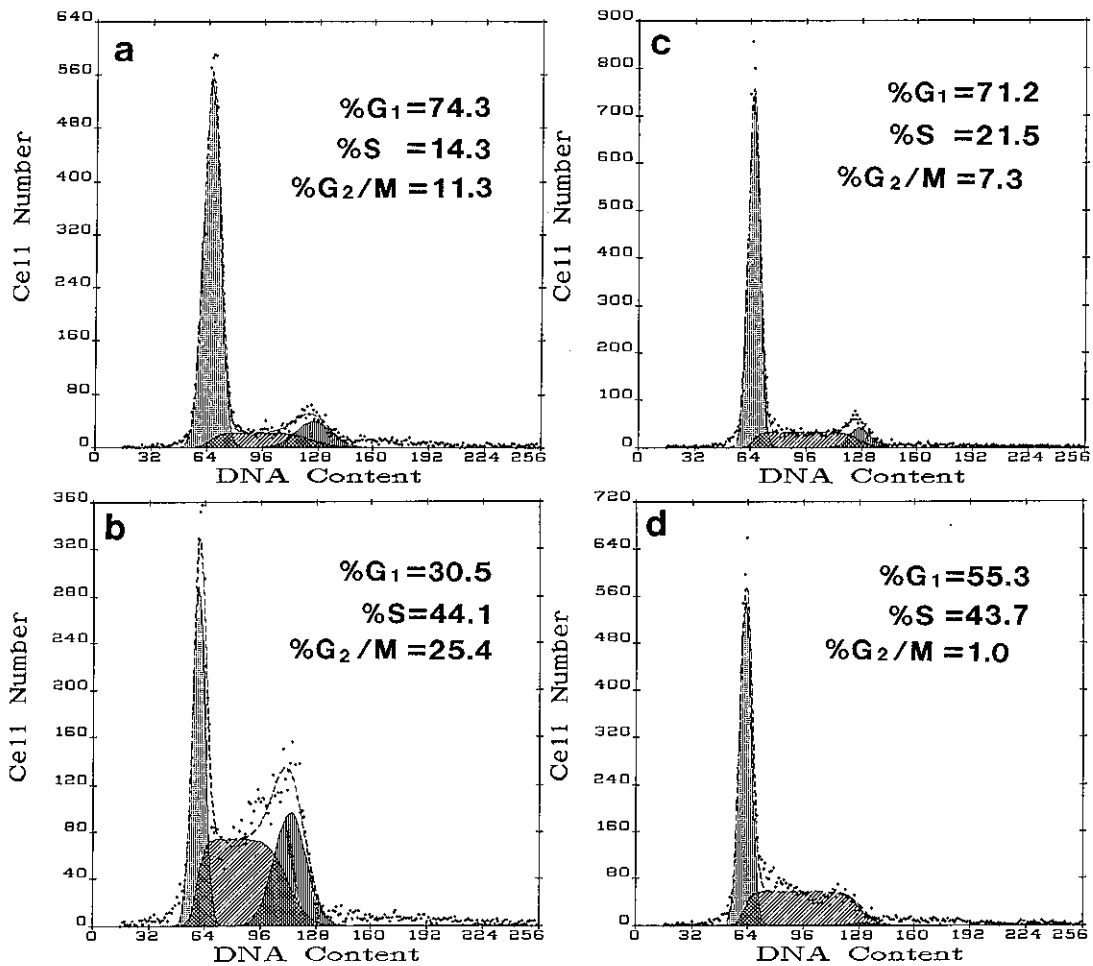

Fig. 5. DNA histogram of MIAPaCa-2 cells treated with simvastatin. Cells incubated in the medium containing 0.5% FBS for 72 h (a) were transferred to the medium with 1.5% FBS containing 0 (b) or 10 μg/ml (c) of simvastatin. After this incubation, cells treated with simvastatin were transferred to the medium without simvastatin for 12 h (d).

the proportion of cells in the S phase in the presence of SV remained constant through this period (Fig. 5c) compared with that in the steady state of the cell cycle after serum starvation (Fig. 5a). After removal of SV, cells moved from G1 to S phase over a 12 h period (Fig. 5d). After this period, all cells moved through the S, G2, and M phases as a synchronized cell population.

DISCUSSION

In this report we have described the cytotoxic effects of SV and its active form SVA on human pancreatic adenocarcinoma cells. SVA was 10 to 20 times more cytotoxic than SV. The cytotoxic effects were caused mainly by the inhibition of cellular DNA synthesis, as shown by measurements of [^{3}H]thymidine uptake. At doses of less than 20 μg/ml of SV for 24 h, DNA synthesis was restored by 60 μg/ml of mevalonic acid but doses greater than 40 μg/ml of SV for 24 h, or even 20 μg/ml for 72 h were cytotoxic and the addition of mevalonic acid did not restore DNA synthesis (unpublished data). These results suggest that the cytotoxic effect of SV is related to metabolites of mevalonic acid, including isoprenoids. Isoprenoids modify cysteine at the fourth position from the carboxyl-terminus of p21^{ras}.[11] Recent evidence indicates that isoprenylation of p21^{ras} is an essential step enabling this protein to anchor to the cell membrane.[12, 13] The signal-transducing activity of p21^{ras} requires this membrane attachment, and suppression of the isoprenylation is believed to interfere with the cellular func-

tion of this protein. From the results of Western blotting in W31, it appears that SV inhibits this post-translational modification of $p21^{ras}$ at the same concentration as the growth-inhibitory dose. We expected that isoprenylation of $p21^{ras}$ in pancreatic carcinoma cells would be inhibited by SV, though we could not detect $p21^{ras}$ in pancreatic carcinoma cells. Presumably only small amounts of $p21^{ras}$ protein are expressed in pancreatic carcinoma cells, or the antibody used in Western blotting was inadequate. There were differences in the IC50 values of SV in the pancreatic cell lines (0.8–23 μg/ml), though only small differences were observed in the IC50 values of SVA (0.46–2.3 μg/ml). SV is a lactone prodrug that undergoes hydrolysis to form active SVA in cell microsomes.[14] The effects of SVA are independent of hydrolysis and this could explain the relatively uniform IC50 values of SVA. The β-hydroxy acid form, 6′-hydroxy form, 6′-hydroxymethyl form and 6′-exomethylene form of SV are metabolites of SV.[14] The difference of survival curve shapes between SV and SVA may thus arise from the cytotoxic activity of these metabolites.

The cell cycle distribution in cultures exposed to 10 μg/ml of SV was unchanged during the initial 6 h of treatment. However, as the time of exposure to 10 μg/ml of SV increased, the proportion of cells in S phase progressively decreased concomitantly with an increase in G1 phase cells, compared with the control cells. Without SV, S phase cells increased up to 12 h. Lovastatin (HMG-CoA reductase inhibitor) also induced G1 arrest of the cell cycle progression on the human bladder carcinoma T24 cell line expressing activated $p21^{ras}$.[15] It is possible that G1 arrest induced by the inhibitor of mevalonate synthesis is a direct consequence of impairment of isoprenylation of $p21^{ras}$. An anti-ras antibody introduced by microinjection into c-Ha-*ras* proto-oncogene-expressing cells specifically blocked the progression from late G1 to S phase produced by the insulin-like growth factor I.[16] Our result and these results[16] suggest the involvement of $p21^{ras}$ in the cell cycle, particularly between the G1 and S phases. However, in addition to $p21^{ras}$, nuclear lamins A and B and several other proteins undergo isoprenylation in the cell.[17, 18] It is known that 26 kDa isoprenylated proteins containing isopentenyl adenosine regulate the cell cycle at the G1 phase.[19] If they have such a role, the inhibition of isoprenylation by SV may also be a causative factor involved in the suppression of cell progression. The present results suggest SV is a potential therapeutic agent for pancreatic carcinoma, although there would be difficulty in clinical trials of this compound, because the growth-inhibitory effect of HMG-CoA reductase inhibitor is also observed in non-malignant cells, including non-transformed muscle,[20] 3T3 cell fibroblasts,[21] and Chinese hamster ovary cells.[22] In our experiment, the cytotoxic dose of SV was about 10 μg/ml (24 μM) in pancreatic carcinoma cells, and it has been reported that the same concentration of HMG-CoA reductase inhibitor inhibits proliferation of non-malignant cells.[20–22] Additionally, the concentration of HMG-CoA reductase inhibitor necessary to inhibit mevalonate biosynthesis is 100 times less than that needed to inhibit isoprenylation of $p21^{ras}$.[23] The mechanisms of growth inhibition by HMG-CoA reductase inhibitor in non-malignant cells may be related to isoprenylated proteins, including non-mutated $p21^{ras}$. Experiments using more specific inhibitors of farnesylation of $p21^{ras}$[24] of pancreatic carcinoma cells are now being conducted.

(Received December 20, 1993/Accepted March 4, 1994)

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