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出版情報：福岡醫學雜誌. 89 (1), pp.11-19, 1998-01-25. 福岡医学会
バージョン：
権利関係：

原　　著

Modulation of Cholesterol Metabolism Affects Tumor Growth in Hamsters

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Abstract To investigate the effects of cholesterol and cholesterol-lowering agents on cholesterol metabolism and tumor growth, 0.5% cholesterol, 2% cholestyramine, or 0.01% simvastatin was fed to hamsters with transplanted *Simian virus*$_{40}$ transformed tumor cells. Tumor weight, tissue cholesterol and DNA concentrations, and HMG-CoA reductase activities were determined. Cholesterol or cholestyramine feeding did not affect the tumor growth, however, the tumor weight and DNA concentration were decreased and tumor HMG-CoA reductase activity was increased in the simvastatin group. In conclusion, simvastatin may inhibit the DNA synthesis and growth of the *Simian virus* $_{40}$ transformed tumor cells possibly through the inhibition of cholesterol and isoprenoids synthesis in the hamster.

Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [EC 1.1.1.34] is the rate limiting enzyme of cholesterol biosynthesis and catalyzes HMG-CoA to mevalonic acid[10]. A major part of mevalonic acid is metabolized to cholesterol which is required for maintaining the mammalian cell membranes and for synthesis of bile acids and steroid hormones[10]. Mevalonic acid is also an important precursor of other isoprenoid molecules including farnesylated proteins such as *ras* proteins and lamin B, isopentenyl adenine, dolichol, haemA, and ubiquinone, which are all essential for cell proliferation, growth, and maintenance[10].

The activity of HMG-CoA reductase is regulated by negative feedback mechanism[10]. Cholesterol feeding results in an increased serum and liver cholesterol levels and a decreased enzyme activity[10]. Cholestyramine, an anion exchange resin, binds bile acids in the intestine, decreases serum cholesterol concentration, and increases hepatic HMG-CoA reductase activity[5)12)]. Recently, blockers of the enzyme have become clinically used[32)]. These agents bind to the enzyme and competitively inhibit the enzyme activity and decrease serum cholesterol level[11)]. Increased low-density lipoprotein receptors on the hepatocytes and decreased triglyceride level are also reported[14)]. The enzyme activity *in vitro* is significantly increased, indicating a compensatory increase of the enzyme mass[3)]. Of these agents, simvastatin is known to inhibit the enzyme activity both in the liver and in other tissues, whereas pravastatin preferentially inhibits the hepatic HMG-CoA reductase activity[35)].

Tumor cells usually have more rapid cell turnover rate and may require more choles-

terol and isoprenoids than normal cells[1]. It is thus considered that manipulation of cholesterol and isoprenoids metabolism may influence the tumor growth[17)20)]. Administration of cholesterol and cholestyramine may have a tumor trophic effect[22)] and HMG-CoA reductase blockers may have an inhibitory effect on the tumor growth both *in vivo*[25)] and *in vitro*[9)25)], although there are limited data yet. The mechanism is not fully understood, however, tumor cells related to *ras* oncogene were reported to be suppressed by HMG-CoA reductase inhibitors due to inactivation of *ras* proteins[24)]. We hypothesized that modulation of cholesterol metabolism by HMG-CoA reductase inhibitors may also affect the growth of tumor cells, which are not related to *ras* oncogenes, such as *Simian Virus*$_{40}$ transformed tumor cells[30)].

The aim of the present study was to investigate if simvastatin affects the tumor growth, HMG-CoA reductase activity, and the DNA content of the transplanted *Simian virus*$_{40}$ transformed tumor cells in the hamster and the effects were compared with those of cholesterol and cholestyramine.

Materials and Methods

Materials. RS- [5-^{3}H (N)] Mevalonolactone (35.0 Ci/mmol) and DL 3-[glutaryl-3-^{14}C] hydroxy-3-methylglutaryl coenzyme A (59.9 mCi/mmol) were purchased from NEN Research Products, Wilmington, DE. DL-Mevalonic acid lactone, DL-3-hydroxy-3-methylglutaryl coenzyme A, and 5 α-cholestane were from Sigma Chemical Co., St. Louis, MO. Trimethylchlorosilane was from GL Science Inc., Tokyo, Japan. Simvastatin (Lipovas) was from Banyu Co., Ltd., Tokyo. Cholestyramine (Questran) was kindly provided by Bristol-Myers Squibb Co., Ltd., Tokyo. All chemicals used in the study were of either reagent or analytical grade.

Cell and cell culture. *Simian virus*$_{40}$ transformed hamster (HSV) cells[30)] were cultivated with Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum. When the cells reached confluency, the cells were suspended with EDTA-trypsin and pelleted by centrifugation at 1000 g for 10 min. The cell pellet was then used for the subcutaneous transplantation.

Animal experiments. Male golden Syrian hamsters (4 weeks old, 63±3 g) were purchased from Seiwa Experimental Animals Co., Ltd., Fukuoka, Japan. After one week of acclimation period on a standard laboratory chow containing 0.075% cholesterol (Oriental Yeast Co., Ltd., Tokyo), animals were divided into 4 groups (6 animals each). They were fed either chow (control group), chow supplemented with 2% cholestyramine (cholestyramine group), 0.01 % simvastatin (simvastatin group), or 0.5% cholesterol (cholesterol group). Water was given *ad libitum* and they were kept under an ordinary light-dark cycle. After 2 weeks on the experimental diet, 8×10^{6} HSV cells were transplanted subcutaneously in the back of the hamster. All hamsters were fed the experimental diet for an additional 3 weeks and then were sacrificed by cardiac puncture under ether anesthesia between 0 AM and 2 AM. The liver and the tumor were excised and analyzed as described below. All animal experiments followed the Institution's Guidelines for Laboratory Animal in Research.

Preparation of tissue microsomes[13)]. The liver and tumor tissue were rinsed with ice cold saline and weighed. The tissue was homogenized in five volumes of cold homogenizing medium containing 0.32 M sucrose, 5 mM dithiothreitol, 30 mM EDTA, and 40 mM potassium phosphate buffer, pH 7.5, using a Potter-Elvehjem tissue grinder with a motor-driven pestle.

The homogenate was centrifuged at 13500 *g* for 20 min. The supernatant was then centrifuged twice at 105000 *g* for 1 h and the microsomal pellet obtained was then resuspended in the same buffer. An aliquot was used for protein determination according to the method of Lowry *et al*[18].

Determination of HMG-CoA reductase activity. The microsomal suspension was preincubated at 37°C for 1 h to ensure the maximal activation of HMG-CoA reductase. The reaction mixture consisted of 40 mM potassium phosphate buffer, pH 7.5, 30 mM EDTA, 5 mM dithiothreitol, 2.5 mM NADPH, 75 μM [^{14}C] HMG-CoA (10^4 dpm), and 100 to 200 μg microsomal protein in a total volume of 1 ml. The reaction was initiated by the addition of NADPH, conducted for 1 h at 37°C, and was terminated by the addition of 100 μl of 5 N HCl. [^{3}H] Mevalonic acid (4×10^5 dpm) was added to each sample as an internal standard and lactonization was carried out at 37°C for 1 h[19]. Mevalonolactone was extracted three times with 3 ml of diethyl ether[26] and the collected phase was evaporated. The residue was dissolved in 200 μl of acidic acetone, and subjected to TLC using benzene/acetone (2 : 3, v/v) as a developing solvent. The TLC plate was sprayed with anisaldehyed/H_2SO_4/acetic acid (0.3 : 0.5 : 50, v/v/v) and heated at 120°C for 1 min. After the visualization of spots, the TLC plate corresponding the spot of mevalonolactone was cut and radioactivity was counted in a liquid scintillation counter[16]. The HMG-CoA reductase activity was expressed as pmols of mevalonate formed per min per mg microsomal protein.

Determination of DNA and tissue cholesterol. DNA was assayed by the method of Burton[4] using the calf thymus DNA as the standard. Tissue cholesterol was determined as follows; The liver and tumor tissue were hydrolyzed in 10% ethanolic KOH at 70°C for 2 h. Cholesterol was extracted with n-hexane and analyzed by gasliquid chromatography as a trimethylsilyl ether derivative using 5 α-cholestane as an internal standard. The equipment and operating conditions have been described previously[34].

Serum analysis. Serum cholesterol, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) were measured by an autoanalyzer (Hitachi Autoanalyzer-736, Hitachi Koki Co., Ltd., Ibaraki, Japan). The serum high density lipoprotein and low density lipoprotein cholesterol were analyzed by electrophoresis[33].

Statistical analysis. All data were expressed as the mean ± S. D. Statistical significance was evaluated by Wilcoxon's rank-sum test. A probability value less than 0.05 was considered statistically significant.

Results

To decide appropriate doses of the agents and the experimental period, preliminary studies were carried out. When 0.1%, 0.5%, or 1% cholesterol was fed, the liver of hamsters fed 1% cholesterol became marked fatty liver macroscopically. When 1%, 2%, or 5% cholestyramine, or 0.01%, 0.1%, or 0.2% simvastatin was fed, the animal weight did not increase in 5% cholestyramine, 0.1% and 0.2% simvastatin groups, and some animals of these groups died. Thus, 0.5% cholesterol, 2% cholestyramine, and 0.01% simvastatin were employed in this study.

The HSV tumors larger than 3 cm in diameter had central necrosis, probably due to insufficient blood supply to the center of the tumor. Thus, the animals were sacrificed at 3 weeks after transplantation of HSV cells, when tu-

Table 1 Effects of Cholesterol-lowering Agents and Cholesterol on Body Weight, Liver Weight, Tumor Weight, and Serum Transaminases in Hamsters

	Body weight	Tissue weight		Serum transaminase	
	(g)	Liver (g)	Tumor (g)	GOT (IU/l)	GPT (IU/l)
Cont	135±12	6.9±0.9	1.9±0.1	128±29	114±32
CS	131±9	4.6±0.3[a]	2.0±0.1	143±37	136±37
SIMV	136±11	4.9±0.5[a]	1.5±0.2[a]	147±35	136±22
CH	128±5	5.6±0.5[a]	2.0±0.2	118±32	116±49

Note. The hamsters were fed chow (Cont), 2% cholestyramine (CS), 0.01% simvastatin (SIMV), and 0.5% cholesterol (CH) diet for 5 weeks. The data are expressed as the means ±SD of 6 animals in each group.
[a]: $P<0.05$ versus controls.

mor size was equal to or less than 2 cm in diameter and there was no necrotic part in the tumors.

All animals were active and survived throughout the experimental period. The tumor generally became palpable within 6 days after the transplantation of HSV cells in all hamsters. The body, liver, and tumor weight and serum transaminases at the end of the experiment are shown in Table 1. The body weight and serum GOT and GPT were not significantly different among the groups, indicating no harmful side effects of these treatments on liver function and nutrition. The liver weight was significantly decreased by each treatment. The tumor weight was significantly lower only in the simvastatin-treated group.

The serum cholesterol was significantly higher in the cholesterol group and lower in the cholestyramine and simvastatin groups than in the controls (Fig. 1). The proportion of hight density lipoprotein cholesterol was not significantly different among the groups (control group: 67.8±6.9%, cholesterol group: 63.5±5.0%, cholestyramine group: 68.3±3.9%, simvastatin group: 62.3±2.6%). Treatments with cholestyramine and simvastatin significantly decreased the liver cholesterol, however, tumor

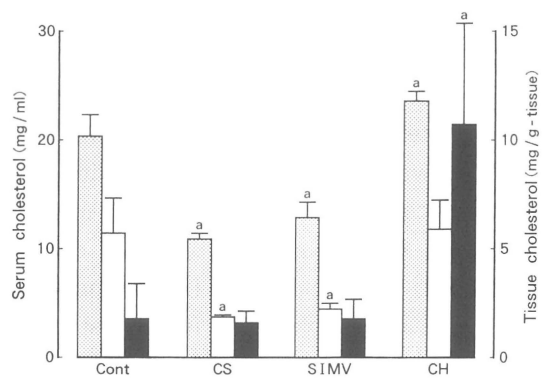

Fig 1. Effects of cholesterol-lowering agents and cholesterol on serum, liver, and tumor cholesterol levels in hamsters. The hamsters were fed chow (Cont), 2% cholestyramine (CS), 0.01% simvastatin (SIMV), and 0.5% cholesterol (CH) diet for 5 weeks. The shaded, open, and closed columns represent the mean of the serum, liver, and tumor cholesterol, respectively, and the bar represents the SD of 6 hamsters in each group. a: $P<0.05$ versus controls.

cholesterol content was not significantly changed. Cholesterol feeding significantly increased tumor cholesterol content, though it had no apparent effect on liver cholesterol.

Both cholestyramine and simvastatin feeding increased the hepatic microsomal HMG-CoA reductase activity. In contrast, cholesterol feeding decreased the enzyme activity (Fig. 2).

The hepatic HMG-CoA reductase activity was not statistically different between the cholestyramine and simvastatin groups. In the tumor tissue, treatment with simvastatin significantly increased the microsomal HMG-CoA reductase activity whereas cholestyramine and cholesterol feeding had no significant effect on the enzyme activity as compared with the control group (Fig. 2).

The tumor or DNA content was significantly increased in the cholestyramine group and decreased in the cholesterol and simvastatin groups compared to the control group (Fig. 3).

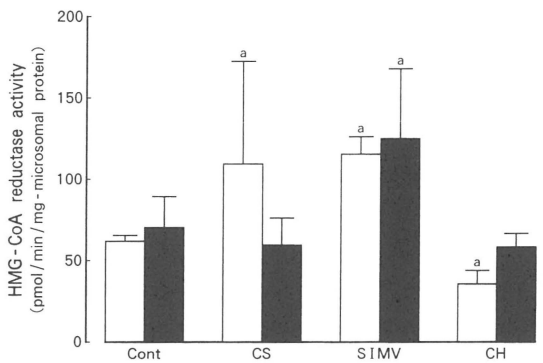

Fig 2. Effects of cholesterol-lowering agents and cholesterol on 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity of the liver and the transplanted tumor cells in hamsters. The hamsters were fed chow (Cont), 2% cholestyramine (CS), 0.01% simvastatin (SIMV), and 0.5% cholesterol (CH) diet for 5 weeks and 8×10^6 *Simian virus*$_{40}$ transformed cells were transplanted in their back in the second week. The HMG-CoA reductase activity was expressed as pmols mevalonic acid formed per min per mg microsomal protein. The open and closed columns represent the mean of the HMG-CoA reductase activity of the liver and the tumor tissue, respectively, and the bar represents the SD of 6 hamsters in each group. a: $P<0.05$ versus controls.

Discussion

The present study has shown that cholesterol and two cholesterol-lowering agents influenced differently the cholesterol metabolism in the liver and in the tumor tissue. Cholesterol is mainly synthesized in the liver[8] and its synthetic rate is regulated by serum and liver cholesterol concentrations[3)10)]. In the present study, cholesterol feeding resulted in a marked increase of serum cholesterol level and an insignificant change of liver cholesterol content. Hepatic HMG-CoA reductase activity was decreased as expected[10]. These effects are ascribed to the well-known negative feedback regulation of cholesterol synthesis to maintain homeostasis in cholesterol metabolism[10]. Hamsters are known to be moderately susceptible

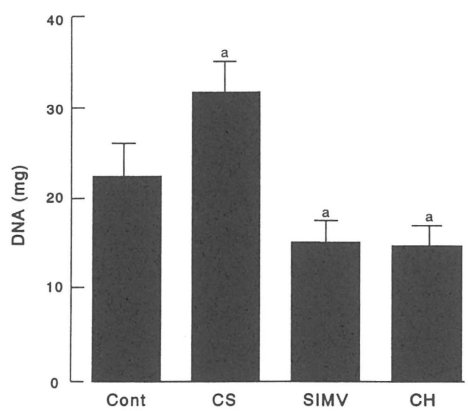

Fig 3. Effects of cholesterol-lowering agents and cholesterol on the tumor DNA content in hamsters. The hamsters were fed chow (Cont), 2% cholestyramine (CS), 0.01% simvastatin (SIMV), and 0.5% cholesterol (CH) diet for 5 weeks and 8×10^6 *Simian virus*$_{40}$ transformed cells were transplanted in their back in the second week. The closed columns represent the mean of the total DNA content of the tumor and the bar represents the SD of 6 hamsters in each group. a: $P<0.05$ versus controls.

to cholesterol feeding and higher concentration of cholesterol in diet was reported to increase liver cholesterol content significantly[22].

Cholestyramine, an insoluble quaternary ammonium anion exchange resin, inhibits the absorption of bile acids from the gastrointestinal tract[12]. The loss of ionized bile acids in the intestine results in a decreased solubility of cholesterol and triglycerides as mixed micelle and in a decreased intestinal absorption[12]. The increased loss of bile acids from the enterohepatic circulation stimulates conversion of cholesterol to bile acids in the liver[5]. The decreased intestinal absorption and increased degradation of cholesterol result in a marked decrease in serum and tissue cholesterol levels, which increases HMG-CoA reductase activity and cholesterol synthesis in the liver[6][21][22]. The results of the present study were consistent with the previous reports as long as serum and liver cholesterol metabolism was concerned.

Simvastatin inhibits the cholesterol synthesis in the liver and the other organs, whereas pravastatin mainly inhibits liver cholesterol synthesis, by a competitive inhibition of HMG-CoA reductase[35]. In the present study, the serum and liver cholesterol levels were decreased and the activity of hepatic HMG-CoA reductase was increased in the simvastatin group. The results were consistent with those of previous reports, showing that HMG-CoA reductase inhibitors induced HMG-CoA reductase protein mass as an adaptation against the suppressed synthesis of cholesterol[29]. As a result, the enzyme activity, when being measured *in vitro*, was increased, because the inhibitors had been lost during the preparation of the microsomal fraction[2][23]. Because of the limited methodologies[19][26], it is difficult to assess how far *in vivo* enzyme activity is suppressed. However, it is very probable that an increased enzyme activity *in vitro* indicates that HMG-CoA reductase is blocked by the agents *in vivo*[3].

It is reported that tumor growth is self-limiting and that tumor HMG-CoA reductase activity is not regulated by the serum cholesterol concentration[1][28]. In this study, the HMG-CoA reductase activity of the tumor was not inhibited despite of the significantly high concentrations of serum and tumor cholesterol levels in the cholesterol-fed animals and the enzyme activity and cholesterol content of the HSVtumors were not changed by administration of cholestyramine which did not have direct effect on tumor cells, even when serum cholesterol was significantly decreased. The results suggested the lack of the feedback regulation of cholesterol synthesis in the tumor tissue[1]. In contrast, simvastatin increased the enzyme activity of the tumor tissue. This supports the hypothesis that simvastatin could inhibit HMG-CoA reductase of the HSV tumor cells as well as the hepatocyte[29].

Cholesterol feeding decreased the total tissue DNA and increased the cholesterol content of the tumor. The tumor weight itself, however, was similar to that of the controls. Since the total tissue DNA is considered to correlate with the number of cells, if DNA ploidity pattern is unchanged, the results may indicate that the cell multiplication was decreased and that each cell was enlarged by the accumulation of intracellular cholesterol. The effect of cholesterol feeding on carcinogenesis still remains controversial. Some case control studies and experimental models have shown increased incidences of cancers of various organs and others have failed[15][31], suggesting multifactorial effects of cholesterol on tumor carcinogenesis. Cholestyramine was reported to induce tumor growth and to increase the tis-

sue DNA content[6)21)22)]. The significant increase in the tissue total DNA of the cholestyramine-treated animals in the present study has confirmed the reported effect. Unchanged tumor weight and tissue cholesterol content suggest that each cell might have a decreased intracellular cholesterol and smaller cytoplasm. In contrast, simvastatin decreased both the tumor weight and tissue total DNA. The mechanism of inhibition of the tumor growth could not be clarified in the present study, however, we consider that it might be due to the reduction in the metabolites of mevalonic acid, which are essential for cell proliferation[7)9)27)]. The present study suggests a possibility that HMG-CoA reductase inhibitors, especially simvastatin, might be effective as an adjuvant therapy in patients with malignant tumors.

In conclusion, simvastatin had an inhibitory effect on the HMG-CoA reductase activity, tissue total DNA, and growth of the transplanted HSV tumors in the hamsters, however, cholesterol and cholestyramine had no apparent inhibitory effects. Further studies to clarify the inhibitory mechanism and to evaluate the clinical efficacy are highly warranted.

Acknowledgments

The authors acknowledge Drs. H. Yamashita, K. Date, T. Ogawa and M. Ueki for their helpful suggestions and Dr. Brian T. Quinn for his assistance in preparing the manuscript.

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(Received for publication October 30, 1997)

(和文抄録)

ハムスターにおけるコレステロール代謝異常が腫瘍増殖におよぼす影響

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コレステロール代謝と腫瘍増殖に対するコレステロールおよびコレステロール降下剤の効果を調べるため，*Simian virus*$_{40}$ より作成した腫瘍細胞を移植したハムスターに0.5%コレステロール，2%コレスチラミン，0.01%シンバスタチンをそれぞれ経口投与した．その後，腫瘍の重量，組織内のコレステロール，DNAの濃度およびHMG-CoA還元酵素の活性を測定した．コレステロールとコレスチラミンは腫瘍増殖に対し何ら影響をおよぼさなかったが，シンバスタチンは腫瘍の重量とDNA濃度を減少させ腫瘍内のHMG-CoA還元酵素の活性を上昇させた．結果として，シンバスタチンはハムスターにおいて *Simian virus*$_{40}$ により作成した腫瘍細胞のDNA合成と腫瘍増殖を抑制する可能性があり，それにはおそらくコレステロールとイソプレノイドの合成阻害が関与していると考えられた．