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# Chemoprevention by Pravastatin, a 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Inhibitor, of *N*-Methyl-*N*-nitrosourea-induced Colon Carcinogenesis in F344 Rats

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**A potential chemopreventive action of pravastatin (Pr), a 3-hydroxy-3-methylglutaryl-coenzyme A redutase inhibitor, on colon carcinogenesis was evaluated in F344 rats. All rats at 7 weeks of age received an intrarectal dose of 2 mg of *N*-methyl-*N*-nitrosourea 3 times weekly for 2 weeks in experiment I (2 groups of 16 rats each), and for 3 weeks in experiment II (4 groups of 30 rats each). They were given drinking water containing 0 ppm (control) or 200 ppm Pr during weeks 1 to 40 in experiment I, and containing 0 ppm (control), 25 ppm, 5 ppm and 1 ppm Pr during weeks 4 to 40 in experiment II. The body weight gains, and food and water intakes were similar in all the groups. The incidence of colon carcinomas at termination of the experiment at week 40 was not different in the 200 ppm Pr and control groups in experiment I (63% vs. 69%), while it was significantly lower in the 25 ppm and 5 ppm groups, but not in the 1 ppm Pr group, compared with the control group in experiment II (50%, 48%, and 77% vs. 80%). This inhibitory effect of Pr against colon carcinogenesis was not related to the cholesterol-lowering effect of this agent. We postulate that Pr inhibits the promotion stage of colon carcinogenesis, perhaps through modulation of cholesterol synthesis *in situ* in the colonic mucosa, thereby suppressing farnesyl isoprenylation of growth-regulating proteins such as p21 ras.**

Key words: Colon cancer — Cancer chemoprevention — Pravastatin — HMG-CoA reductase

Some proteins are covalently attached post-translationally to non-steroidal isoprenoid compounds derived from intermediates in the cholesterol synthesis pathway, and some of them, such as ras protein and nuclear lamin B, are biologically active when they are anchored at the inner surface of the cell membrane after farnesyl isoprenylation.[1–4] This process is essential for intracellular signal transduction and cell growth. Much attention has been focused on growth-regulating proteins such as p21 ras, because of their involvement in the neoplastic transformation of various types of tumors including colon cancer. Therefore, the inhibition of farnesyl isoprenylation is a possible mechanism for the suppression of tumor growth. Several studies suggest that the membrane association and the biological activity of p21 ras are abolished by blocking farnesyl isoprenylation. The blocking can be achieved by using inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in the synthesis of mevalonic acid, which is a precursor of farnesyl pyrophosphate in the cholesterol synthesis pathway. *In vitro* and *in vivo* studies have demonstrated that enzyme inhibitors such as lovastatin and pravastatin (Pr), cholesterol-lowering agents, closely affect DNA synthesis and proliferation of several cell lines: human and rodent neoplastic cells,[5–14] *ras* oncogene-transfected rodent cells,[2,15,16] and human and rodent immortal cells.[1,17–20] The inhibitory effects on cell proliferation are reversed by the addition of mevalonate or farnesol, but not cholesterol.

Subsequently, we demonstrated that HMG-CoA reductase inhibitors, Pr and simvastatin, given in drinking water or diet, suppressed 1,2-dimethylhydrazine-induced colon tumorigenesis in ICR mice.[21] The present study with 2 sets of experiments was carried out to assess the preventive effect of Pr against colon carcinogenesis induced with *N*-methyl-*N*-nitrosourea (MNU), a direct-acting carcinogen, in F344 rats. Serum cholesterol, and fecal acid and neutral sterols were also determined. The results showed that Pr may effectively prevent colon carcinogenesis in rats at levels which do not exhibit a serum cholesterol-lowering effect.

## MATERIALS AND METHODS

**Animals and chemicals** Female F344/Nslc rats (Shizuoka Laboratory Animal Center, Hamamatsu), 7 weeks of age at the start of the experiment, were used. The rats were housed in plastic cages with sterilized wood chip bedding in a specific-pathogen-free animal room under constant environmental conditions with a 12 h light and dark cycle, a temperature of 22±1°C and a relative humidity of 50±10%. They had free access to

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standard laboratory pelletted chow, CE-2 (CLEA Co., Tokyo), and drinking water. The body weight and food intake were measured once weekly, and the volume of drinking water consumed was measured every other day. Aqueous solutions of HMG-CoA reductase inhibitor Pr (Sankyo Co., Tokyo) at the indicated concentrations were given as drinking water. Pr in the drinking water was confirmed to be stable at ambient temperature for one week. MNU (Nacalai Tesque, Kyoto) was dissolved in distilled water to make a 0.4% aqueous solution immediately before use for intrarectal instillation to induce colon cancer.

**Animal treatments** In experiment I, all animals in 2 groups (16 rats in each group) received an intrarectal dose of 0.5 ml of MNU solution 3 times a week for 2 weeks by the procedure described previously.[22] Briefly, a metal feeding tube 8 cm long was inserted two-thirds of the way into the colon lumen through the anal orifice, and the solution was infused. The rats received 0 ppm Pr (control group) or 200 ppm Pr (Pr(H) group) in drinking water throughout the experimental period of 40 weeks. In experiment II, all animals in 4 groups (30 rats in each group) received an intrarectal dose of 0.5 ml of MNU solution 3 times a week for 3 weeks, and had 0 ppm Pr (control group), 25 ppm Pr (Pr(h) group), 5 ppm Pr (Pr(m) group) or 1 ppm Pr (Pr(l) group) in drinking water for 37 weeks starting at week 4. The dosage chosen in the Pr(H) group of experiment I was the maximum tolerated dose with no toxic effects in a chronic toxicity test in F344 rats (Sankyo Co.). After it was found that this dose had no significant effect against colon tumorigenesis, de-escalated doses of this agent were tested in experiment II. The Pr treatment was started at a different week in experiments I and II, though there was no specific reason for this. The feces were collected at week 20 for analysis of bile acids and neutral sterols. The mean dry weight of the feces/day/rat was 2.0 g in the Control group, 2.2 g in the Pr(h) and Pr(m) groups and 2.5 g in the Pr(l) group.

**Colon tumor examination** Both experiments were terminated at week 40, when all the rats were killed by exsanguination from the abdominal aorta after laparotomy under intraperitoneal nembutal anesthesia (40 mg/kg body weight). The blood was centrifuged, and the serum was stored at $-80°C$ until assay of cholesterol. Autopsy was carefully performed. The colon was excised, cut open along its length, rinsed with cold 0.9% NaCl solution to remove fecal debris, and inspected grossly. The location, size and shape of colon tumors were recorded. All of the tumors and grossly abnormal tissues or organs were histologically examined after standard processing, sectioning, and hematoxylin and eosin staining.

**Assays of serum cholesterol, and fecal bile acids and neutral sterols** Serum total cholesterol was measured by an enzymatic method using the assay kit, Determiner L-TC (Kyowa Medics, Tokyo), and a Hitachi 736-60E autoanalyzer (Hitachi, Tokyo). For bile acid analysis,[23] an aliquot of 25 mg of lyophilized feces was extracted with 5 ml of ethanol at 70°C for 2 h after addition of 0.15 $\mu$mol of 5$\beta$-pregnan-3$\alpha$, 17$\alpha$, 20$\alpha$-triol as an internal standard. The extract was dissolved in 0.5 ml of methanol and filtered, and 10 $\mu$l portions were used for bile acid analysis by high-performance liquid chromatography. The HPLC system (JASCO, Tokyo) for the analysis of bile acids relies on fluorescence detection, and involves introduction of 3$\alpha$-dehydroxysteroid dehydrogenase and $\beta$-$NAD^+$ solution into the mobile phase. For neutral sterol analysis,[24] 0.1 $\mu$mol of 5$\alpha$-cholestane as an internal standard and 1 ml of 90% ethanolic solution containing 1 *N* NaOH were added to 25 mg samples of lyophilized feces. The mixture was heated at 80°C for 1 h, then 0.5 ml of water was added and neutral sterols were extracted twice with 2.5 ml of petroleum ether. After evaporation, the neutral steroids were trifluoracetylated and analyzed on a 30 m×0.32 mm id capillary glass column coated with SPB-1 FS (Supelco, Bellefonte, PA) using a Hewlett Packard 5890 gas chromatograph (Yokogawa-Hewlett-Packard, Tokyo) with an FID detector. Operating conditions were as follows: column oven temperature, initial 220°C for 6 min and then raised at 5°C/min to 260°C; injector port temperature 230°C; detector temperature 280°C.

**Statistical methods** The data were analyzed statistically by means of the $\chi^2$ test and Student's $t$ test where appropriate. The differences among groups were considered to be significant when the $P$ value was less than 0.05.

## RESULTS

**Body weight, food intake and water consumption** The body weight gains of rats were similar in all groups in each experiment. The mean body weights in the control and Pr(H) groups of experiment I were 130 g and 131 g at week 1, and 221 g and 223 g at week 40, respectively. In experiment II, the body weights averaged about 113 g in all groups at week 1, and 228–231 g at week 40. The food intake was similar among the groups throughout the experiments; the mean amount in each group was 9–10 g/day/rat in experiment I, and 10–11 g/day/rat in experiment II. The amount of water consumed was also similar among the groups throughout the experiments; the mean volume in each group was 18–20 ml/day/rat in experiment I and 17–21 ml/day/rat in experiment II. Based on water intake, the amount of ingested Pr/kg body weight/day was computed. The mean amount ranged from 21 to 16 mg in the Pr(H) group, from 3.1 to 1.8 mg in the Pr(h) group, from 0.61 to 0.39 mg in the Pr(m) group and from 0.12 to 0.07 mg in the Pr(l) group. The average

Table I. MNU-induced Colon Tumors in F344 Rats Given De-escalated Doses of Pr in Drinking Water

| Treatment[a] groups | No. of rats examined | No. of rats with tumors | No. of tumors per rat | No. of tumors per tumor-bearing rat |
|---|---|---|---|---|
| Exper. I : | | | | |
| Control | 16 | 11 (69%) | 1.3±1.2[b] | 1.8±1.1[b] |
| Pr(H) | 16 | 10 (63%) | 0.7±0.6 | 1.1±0.3 |
| Exper. II : | | | | |
| Control | 30 | 24 (80%) | 1.5±1.3 | 1.8±1.2 |
| Pr(h) | 30 | 15 (50%)[c, d] | 0.8±1.0[c] | 1.7±0.7 |
| Pr(m) | 29 | 14 (48%)[c, d] | 0.7±1.0[c, d] | 1.5±0.9 |
| Pr(l) | 30 | 23 (77%) | 1.2±0.9 | 1.6±0.7 |

*a*) All rats received an intrarectal dose of 2 mg of MNU 3 times a week for weeks 1–2 in experiment I and for weeks 1–3 in experiment II, and had drinking water containing Pr at 0 ppm (control group) or 200 ppm (Pr(H) group) during weeks 1–40 in experiment I and at 0 ppm (control group), 25 ppm (Pr(h) group), 5 ppm (Pr(m) group) or 1 ppm (Pr(l) group) during weeks 4–40 in experiment II. Experiments were terminated at week 40.
*b*) Mean±SD.
*c, d*) Significantly different from control group (c) and Pr(l) group (d) : $P<0.05$.

Table II. Number of Colon Tumors, and Size and Depth of Invasion

| Treatment[a] groups | Size (diameter) | | | Depth of invasion | |
|---|---|---|---|---|---|
| | 1–4 mm | 5–8 mm | 9–12 mm | Mucosa | Submucosa -serosa |
| Exper. I : | | | | | |
| Control | 14 (70%) | 4 (20%) | 2 (10%) | 17 (85%) | 3 (15%) |
| Pr(H) | 9 (82%) | 2 (18%) | 0 | 4 (36%) | 7 (64%) |
| Exper. II : | | | | | |
| Control | 27 (61%) | 15 (34%) | 2 (5%) | 26 (59%) | 18 (41%) |
| Pr(h) | 20 (80%) | 4 (16%) | 1 (4%) | 17 (68%) | 8 (32%) |
| Pr(m) | 12 (57%) | 6 (29%) | 3 (14%) | 15 (71%) | 6 (29%) |
| Pr(l) | 22 (59%) | 14 (38%) | 1 (3%) | 24 (65%) | 13 (35%) |

*a*) See text or Table I.

values were 18 mg, 2.4 mg, 0.46 mg and 0.09 mg, respectively. No visible sign of toxicity due to Pr was apparent.

**Colon tumor development** The incidence and multiplicity of colon tumors at week 40 are summarized in Table I. In experiment I, administration of Pr (Pr(H) group) had no significant effect on colon tumor incidence, although there was a tendency for decreased tumor multiplicity. In experiment II, however, administration of high (Pr(h) group) and moderate (Pr(m) group) levels of Pr significantly inhibited the incidence and multiplicity (number of tumors per animal) of colon tumors compared to the 0 (control group) and low (Pr(l) group) levels of Pr. There was, however, no difference between the control and Pr(l) groups or between the Pr(h) and Pr(m) groups.

The tumors were located diffusely in the distal half of the colon at 0–9 cm from the anus, which had been bathed with the instilled MNU solution, and were plaque-shaped or polypoid in both experiments. Histologically all the tumors were well-differentiated adenocarcinomas. No significant differences were observed in the size or invasion of the tumors in the two experiments, except that significantly more tumors invaded below the submucosal layer in the Pr(H) group, presumably due to the toxic effect of treatment with a very large dosage of Pr (Table II). Low grade dysplastic tumors (adenomas) were not found. There were no other pathologic findings in the gastro-intestinal tract or other organs in any group in either experiment. Thus, the data demonstrated that Pr administered orally inhibited the development of MNU-induced colon tumors, as observed in experiment II, but a very high dose of Pr in experiment I did not show any inhibitory properties.

**Serum cholesterol, and fecal bile acids and neutral sterols** Total cholesterol levels were measured in the serum of 8 rats in each group. The mean value in the Pr (H) group was increased slightly, but not significantly,

Table III. Fecal Bile Acids and Neutral Sterols at Week 20 in Experiment II

| Treatment[a] groups | Bile acids (μmol/g dry feces) | | | Neutral sterols (μmol/day/rat)[d] | | |
|---|---|---|---|---|---|---|
| | Primary bile acids | Secondary bile acids | Total | Cholesterol | Coprostanol | Total |
| Control | 0.9±0.1[b] | 1.9±0.2 | 2.8±0.3 | 4.3±0.5 | 7.4±1.5 | 11.7±1.8 |
| Pr(h) | 0.9±0.1 | 1.9±0.4 | 2.8±0.4 | 4.4±1.4 | 8.8±2.7 | 13.2±4.0 |
| Pr(m) | 0.9±0.02 | 2.0±0.2 | 2.9±0.2 | 5.1±1.2 | 8.6±1.4 | 13.7±2.6 |
| Pr(l) | 0.8±0.1 | 1.8±0.2 | 2.6±0.2 | 4.1±0.9 | 5.2±1.8[c] | 9.3±2.6[c] |

*a*) See text or Table I.
*b*) Mean±SD, each group consisted of 5 samples.
*c*) Significantly different from Pr(h) and Pr(m) groups: $P<0.05$.
*d*) Coprostanone was not detected.

compared with the control group in experiment I: 96±15 (SD) mg/dl and 87±13 mg/dl, respectively. In experiment II, serum cholesterol levels were also not affected by Pr: 89±4 (SD) mg/dl in the Pr(h) group, 91±11 mg/dl in the Pr(m) group, 94±6 mg/dl in the Pr(l) group and 93±7 mg/dl in the control group.

The levels of bile acids and neutral sterols analyzed in 5 fecal samples of each group of experiment II are summarized in Table III. Administration of different levels of Pr had no effect on the concentrations of primary bile acids (α-muricholic acid, β-muricholic acid, hyocholic acid and chenodeoxycholic acid), secondary bile acids (hyodeoxycholic acid, deoxycholic acid and lithocholic acid) and total bile acids. The levels of cholesterol, coprostanol and total neutral sterols were also not affected by Pr. Coprostanone was not detected in any group.

## DISCUSSION

The present study clearly demonstrated that continuous administration of Pr, an HMG-CoA reductase inhibitor, during the promotion and progression stages may prevent colon carcinogenesis induced by MNU in F344 rats. It is noteworthy that a high dose (25 ppm) and a medium dose (5 ppm) of Pr in drinking water decreased the tumor incidence and multiplicity to the same extent, but a very high dose (200 ppm) and a small dose (1 ppm) were ineffective. The results are consistent with another study by us, in which treatment with 30 ppm Pr in drinking water reduced the incidence of azoxymethane-induced colon tumors in F344 rats, but treatment with 150 ppm Pr did not (unpublished data). These results in rats and others in mice[21] indicate that the prevention of colon tumorigenesis by HMG-CoA reductase inhibitors is reproducible in animal models. Inhibition of colon tumorigenesis as observed in the present study strongly suggests that an optimum dose of Pr can reduce the risk of colon cancer development, but a large dose or a small dose can not. The effective doses of Pr were given during the post-initiation phase after carcinogen treatment for 3 weeks. The findings that the size and the invasion of developed tumors were not different among all the groups suggest that Pr treatment had no effect on tumor growth, but affected an early stage of carcinogenesis. However, we do not know from the present and previous[21] studies whether Pr affects the initiation phase. Although several studies have demonstrated that HMG-CoA reductase inhibitors including Pr inhibit the growth of established tumor cells *in vivo* and *in vitro*,[5-14] this was not the case in the present study using a colon tumor model.

It has been established that ras oncogene activation is an important early event in colon carcinogenesis in humans and animal models.[25-27] This genomic alteration was also observed in rat colon tumors induced by MNU, which was used in the present study.[27] It has been detected not only in carcinomas, but also in aberrant crypt foci (putative preneoplastic lesions) of the colon.[26, 28, 29] Farnesyl isoprenylation of oncogenic ras protein p21 ras is a crucial step in the transformation of initiated and p21 ras-producing cells.[1-4] Thus, it has been postulated that the antiproliferative activity attributable to blockage of the activity of HMG-CoA reductase in various types of neoplastic and transformed cells results from reduced production of farnesyl isoprenoid. On the other hand, recent investigations showed that inhibitors of farnesyl protein transferase block the farnesylation of p21 ras, thereby inhibiting the growth of ras-mutated neoplastic and transformed cells in *in vitro* and *in vivo* studies.[30-33] In view of the association of p21 ras farnesylation and cell proliferation, it is possible that the depletion of farnesyl residue owing to treatment with Pr is responsible for the inhibition of colon tumorigenesis observed in the present experiments.

In the present study, administration of Pr inhibited colon tumor development, but had no effect on serum cholesterol or fecal bile acid output. Previous studies have also demonstrated no lowering effect of serum cholesterol by HMG-CoA reductase inhibitors in rats and

mice.[9, 34, 35] Cholesterol can be absorbed to the extent of 2–4 mg/kg body weight/day in humans and 30–50 times this amount in rodents, while cholesterol biosynthesis can vary from 120 mg/kg body weight/day in humans to 10 mg/kg body weight/day in rats.[36] Thus, in rats HMG-CoA reductase inhibitors even at a small dosage may block the cholesterol biosynthesis pathway without lowering cholesterol and bile acids in the body, as observed in the present study. It is possible that a large dose of HMG-CoA reductase inhibitors may initially inhibit the activity of HMG-CoA reductase and later stimulate cholesterol synthesis after induction of the enzyme, presumably due to a compensatory increase in enzyme protein synthesis.[37, 38] These actions of the enzyme inhibitors in rats might be responsible for the potential inhibition of colon carcinogenesis in the Pr(h) and Pr(m) groups, but not in the Pr(H) group. Bile acids, particularly secondary bile acids, are known to be tumor promoters in colon carcinogenesis.[39, 40] The previous study in mice with Pr treatment showed a decrease of 1,2-dimethylhydrazine-induced colon tumor yields along with decreased concentration of fecal bile acids, especially secondary bile acids.[21] However, it can be concluded that the tumor development was not associated with bile acids in the present study, because the concentrations of secondary bile acids as well as total bile acids in the feces did not change in the Pr(h) and Pr(m) groups. Thus, we consider that the inhibitory effect of Pr against colon carcinogenesis is attributable essentially to the inhibition of isoprenoid production by this agent, but not to altered bile acid metabolism, in these animal models. It is possible that the anti-carcinogenic effect of Pr is dissociable from the serum-cholesterol-lowering activity of this agent.

Two-fold higher HMG-CoA reductase activity coupled with greater isoprenoid production of human lung adenocarcinoma cell line than normal human fibroblasts, which may be related to increased requirement for up-regulated cell growth, was reported.[5] Human malignant glioma cell line underwent apoptosis in the presence of a very low concentration of lovastatin (100 n*M*), while normal glial cells in the presence of a thousand-fold higher concentration of the agent underwent only reversible inhibition of DNA synthesis and proliferation.[8] Moreover, DMBA-initiated and TPA-promoted skin papillomas in mice regressed upon continuous administration of lovastatin, which induced a block of p21 ras membrane localization and a decrease of PKC isozyme activation in the tumors.[41] It has been demonstrated that hepatomas of rats, mice and humans completely lack the negative feedback control mechanism of HMG-CoA reductase by cholesterol in cholesterol biosynthesis.[42, 43] Thus, it might be postulated that the continuous administration of an appropriate dose of HMG-CoA reductase inhibitors, as observed in the Pr(h) and Pr(m) groups in the present study, can suppress tumor promotion of initiated colonic cells by reducing the extent of protein farnesylation, in conjunction with reduced isoprenoid synthesis. However, to clarify fully the mechanism underlying the anticarcinogenic activity of those agents, further studies in relation to cholesterol synthesis *in situ* in the colonic mucosal cells are needed.

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