Abstract. An increased incidence of colorectal carcinoma is known to occur in patients with ulcerative colitis (UC), which displays a cycle of recurrence-remission in the colorectal mucosa. Fluvastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, is a hypocholesterolemic agent effective in animals and humans. Repeated administration of 3% dextran sulfate sodium subsequent to a single intraperitoneal injection of azoxymethane induces chronic UC resulting in an increased incidence of high-grade dysplasia and submucosal-invasive adenocarcinomas in the mouse colorectum. The effects of fluvastatin as an antioxidant on colorectal carcinogenesis in mice with UC were investigated. Treatment with fluvastatin in mice with UC abolished the anemia caused by colorectal carcinogenesis, and markedly lowered plasma lipid levels resulting in a reduction of colitis and carcinogenesis, shown by inhibition of the decrease in colorectal length, the increased number of foci of gland loss with inflammatory cell infiltration indicating the severity of UC and incidence of colorectal dysplasia, respectively, with a reduction in anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody (a biological marker of in vivo oxidative DNA damage)-positive cells of the colorectal mucosa and the activity of the DNA-synthesizing enzyme thymidine kinase in colorectal tissues.

An increased incidence of colorectal carcinoma is known to occur in patients with ulcerative colitis (UC) (1), which displays a cycle of recurrence-remission, i.e., periods of ulceration and regeneration of the colorectal mucosa. As previously reported (2), three administrations of 3% dextran sulfate sodium (DSS) subsequent to a single intraperitoneal injection of azoxymethane (AZM) induced a chronic UC resulting in an increase of high-grade dysplasia and submucosal-invading adenocarcinomas in the mouse colorectum. The features of the colitis induced in this animal model are very similar to those in patients, in terms of both clinical and histopathological characteristics, i.e., diarrhea, occult blood, melena, mucosal inflammatory cell infiltration, crypt abscess formation and mucosal erosion (3). Oxidative stress and cellular damage are hallmarks of UC, i.e., the activities of phagocytic leukocytes are greatly increased in the colons of UC patients, resulting in an enhanced generation of pro-oxidant molecules. UC also manifests as deficiencies in antioxidant defenses, presumably due to excessive inflammation (4). Serum nitrite levels and nitric oxide synthase activity are increased in patients with active UC and in UC biopsies (5). It has been suggested that hypercholesterolemia may potentiate the carcinogenicity of a chemical carcinogen, 1,2-dimethylhydrazine, in rats via an increase of lipid peroxidation and decrease in the activity of peroxidase in the colorectum (6). Myoinositol hexaphosphate, phytic acid may act as an antioxidant to inhibit the generation of reactive oxygen species from H2O2 by chelating metals, resulting in chemoprevention of cancer (7).

Fluvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, is a hypocholesterolemic agent effective in animals and humans (8, 9). Statins have been known to inhibit the production of mevalonate, a precursor for cholesterol and for geranylgeranyl diphosphate, which is essential for the prenylation of small proteins involved in signal transduction (10). Statins increase the synthesis of cellular low density lipoprotein (LDL) receptors, causing a decrease in plasma levels of LDL (11), and have been known to slow the progression of coronary heart disease (12). Lipophilic fluvastatin can easily cross the cell membrane (13), and can reduce the susceptibility of LDL to oxidation (9). Administration of fluvastatin to nude mice reduced both...
metastatic tumor formation in the liver and the growth of established liver metastases (14).

In this study, the effects of a fluvastatin on colorectal carcinogenesis in mice treated with AZM and DSS were investigated.

**Materials and Methods**

*Animals and chemicals.* Forty-five specific pathogen-free female CBA/J mice (Charles River Japan, Tokyo, Japan), six weeks of age, were used. The animals were housed in plastic cages with steel shavings under controlled temperature (24±0.5°C) and lighting (12 h of light from 0600 to 1800 h), and were permitted free access to a commercial diet (CE-2, CLEA Japan, Tokyo, Japan) and tap water at the animal research center of Tokyo Medical and Dental University (Tokyo, Japan).

At eight weeks of age, the animals were divided into three groups of 15 mice each; one control group (Normal-Control) and two experimental groups (AZM/DSS-Control and AZM/DSS-Fluvastatin). The animals of the two experimental groups (30 mice) were injected intraperitoneally with 8.0 mg/kg AZM (Sigma Chemical, St. Louis, MO, USA). In the control group, 15 mice received 0.1 ml of a 0.9% NaCl solution by the same procedure. Two weeks after the intraperitoneal pretreatment with AZM, the animals in the two experimental groups were given distilled water containing 3% (w/v) synthetic DSS (mol wt 50,000; Ensuiko Sugar Refining, Yokohama, Japan) for seven days followed by tap water for 14 days, a total of three times. Beginning at eight weeks of age, the animals in one of the two experimental groups were fed the same commercial diet containing synthetic fluvastatin-Na (100 mg in 1 kg of diet) (LOCHOL™; a gift from Tanabe Seiyaku Co., Ltd., Osaka, Japan. Lot 9680028) for 12 weeks. The other 30 mice in the Normal-Control and AZM-Control groups received the same commercial diet alone for 12 weeks.

*Experimental procedures and measurements.* Changes in body weight were recorded every week throughout the experiment. All animals were anesthetized with ether, bled by cardiac puncture for hematological examination of peripheral blood, and sacrificed at 20 weeks of age. The numbers of leukocytes (WBC; 102/mm3) and erythrocytes (RBC; 108/mm3), and the concentrations of hemoglobin (Hb; g/dl) in the obtained blood were immediately determined. Plasma levels of lipids, HDL cholesterol and LDL cholesterol were determined by the methods of the animal research center of Tokyo Medical and Dental University (4223-4228 (2006)).

*Body growth and organ weights.* AZM-DSS treatment lowered the final body weight to 88.9% of that of the Normal-Control group (p<0.05) (Table I). The additive fluvastatin treatment improved body growth, despite the AZM-DSS treatment, but not significantly.

The AZM-DSS treatment markedly altered organ weights, i.e., the weights of the liver (p<0.05), spleen (p<0.01), kidney (p<0.05) and adrenals (p<0.01) were augmented compared with those of the Normal-Control group, though the weights of the uterus and ovaries were reduced (Table I). However, the additive fluvastatin treatment lowered the weights of the spleen (p<0.01) and kidney (p<0.01), and elevated the weights of the ovaries (p<0.01) and uterus (p<0.05).

*Features of blood.* The number of WBC was not affected by AZM-DSS treatment (Table I). The number of RBC and the concentration of Hb were reduced to 68.5% (p<0.05) and 90.9% (not significantly) of those values in the AZM-Control group. The number of RBC and Hb were increased to 130.8% (p<0.05) and 107.3% (not significantly) of those values in the AZM-Control group (Table I).

The additive treatment with fluvastatin markedly reduced the plasma levels of total cholesterol (p<0.01), free cholesterol (p<0.01), LDL cholesterol (p<0.01) and phospholipids (p<0.05) compared to those in AZM-DSS-treated mice (Table II).

*Colorectal length, the severity of UC and the incidence of colorectal dysplasia.* The colorectal length in AZM-DSS-treated mice (AZM-Control group) was markedly reduced to
80.2% of that in the Normal-Control group (p<0.01) (Table III). The additive treatment with fluvastatin partially prevented the shrinking of the colorectum (p<0.01). No open ulcer was found since mice in the experimental groups were sacrificed three weeks after the last administration of DSS. However, the number of foci of gland loss with inflammatory cell infiltration, i.e., indicating the severity of UC, in fluvastatin-treated mice (AZM/DSS-Fluvastatin group) was reduced to less than 50% of that in the AZM-DSS-treated mice (p<0.01). AZM-DSS treatment induced high-grade dysplasia (Figure 1B); 27.9 sites/mouse in number and 184.1 mm²/mouse in cumulative area (Table III), though high-grade dysplasia was not found in the Normal-Control group (Figure 1A). However, the additive treatment with fluvastatin markedly reduced the high-grade dysplasia in both number and region to less than 3.0% of that in the AZM-Control group (p<0.01). In the present study, no submucosal-invasive adenocarcinomas were found in any of the colorectal samples.

**Immunohistochemical study using an anti-8-hydroxy-2'-deoxyguanosine antibody as a biological marker of in vivo oxidative DNA damage.** The 8-OHdG-positive cells (yellow-colored cells) were distributed throughout the mucosal tissue in normal-control (Figure 2A) and AZM-DSS-treated mice (Figure 2B). However, additive treatment with fluvastatin reduced 8-OHdG-positive cells in number and density (Figure 2C).

### Discussion

DSS is a synthetic, sulfated polysaccharide that induces colitis in rodents, which clinically and histologically resembles that in human UC. As previously reported by Okayasu et al. (3), DSS was administered via the drinking fluid for 3-7 days,
followed by water administration for 1-2 weeks to permit healing of the colonic mucosa. The animals were subjected to several DSS-cycles to simulate the course of UC observed in humans, which is characterized by periods of active inflammation separated by periods free of disease. The hyperproliferation of cells in the inflammation-associated damage-regeneration cycle has been shown to contribute to the fixation of genetic and epigenetic alterations and promoted the development of colorectal dysplasia and carcinoma (21). Seril et al. (22) have reported that the consumption of a diet containing twice (90 mg iron/kg diet) the normal level of iron (45 mg/kg diet) increased colorectal tumor development in DSS-treated mice (from 30% to more than 80%), together with the expression levels of inducible nitric oxide synthase and nitrotyrosine. Furthermore, the dietary antioxidant N-acetylcysteine decreased inflammation-driven epithelial cell proliferation (23). Oxidative stress and inflammation play important roles in the development of colorectal cancer in UC patients. Sulphasalazine, which is a prodrug and is split into sulphapyridine and 5-aminosalicylic acid by bacteria in the colon, inhibited the colorectal inflammation resulting in the reduction of tumor regions with high-grade dysplasia in AZM-DSS treated mice (24).

Fatty diets composed of much meat and little fiber have been known to increase the risk of colorectal carcinogenesis, associated with hypercholesterolemia and hydroxyl radical formation (6, 25). The products of lipid peroxidation, formed by the reaction of reactive oxygen and nitrogen species with cell membranes, form etheno- and propane-DNA adducts leading to base transition mutations. Nitric oxide-mediated nitration of 5-methylcytosine and subsequent base deamination leads to C to T transition. Nitric oxide has also been found to inhibit the function of DNA repair proteins and might thereby act to impair the removal of DNA lesions. The most commonly observed oxidized adduct in human tissues is 8-OHdG, which has been found to cause predominantly G to T transversions in vitro (26), which is often seen in oncogenes and tumor suppressor genes that have been mutated (27).

AZM is known as a procarcinogen, which becomes an alkylating agent with carcinogenic activity following metabolic activation in the host (28). DSS has been found to be negative in the Ames test for mutagens (29). However, nine cyclic administrations of DSS induced nine low- and four high-grade dysplasias and two carcinomas in 25 mice in our previous study (30). Inflammation-associated regenerative atypia is thought to be difficult to differentiate from dysplasia. Our histological diagnosis was supported by the findings of diffuse labelling of tumor cells with bromodeoxyuridine (BrdU) and activities of TS and TK throughout the colorectal mucosa, i.e., BrdU uptake and both activities of TS and TK in mucosal tumors were higher than in non-tumorous tissues (2). Thus, structural and cellular atypia pointed to a diagnosis of high-grade dysplasia. Accelerated epithelial cell turnover caused by
chronic inflammation and epithelial damage might predispose the mucosa to DNA damage. AZM-DSS reduced body growth to 90% of the control, but the additive treatment with fluvastatin slightly increased growth despite the AZM-DSS treatment. Although AZM-DSS treatment increased the weight of the organs except the ovary and uterus, fluvastatin had a tendency to normalize the altered weights. Additive treatment with fluvastatin abolished the anemia caused by colorectal carcinogenesis, and markedly lowered plasma lipid levels resulting in a reduction in colitis, as shown by a reduced pathology score of UC and an inhibition of the decrease in colorectal length. It is also reduced in the incidence of colorectal carcinogenesis, shown by a reduced incidence of colorectal dysplasia, reduced anti-8-OHdG antibody-positive cells in number and density and reduced the activity of the DNA-synthesizing enzyme TK in the colorectum.

The intake of dietary fiber has been known to be beneficial to the microbial conversion of bile acid and cholesterol in the colorectum (31), resulting in a reduction of precancerous lesions (32). Dietary antioxidants may alleviate oxidative stress and decrease the risk of cancer in UC patients. On the other hand, hormone replacement therapy in postmenopausal women has been known to lower the levels of serum cholesterol (33), bone fractures and the incidence of colorectal cancer (34). Since Mundy et al. reported that statins enhanced the expression of bone morphogenetic protein-2 in vitro and increased bone formation in mice (35), statins have been shown to increase bone mineral density and reduce the risk of fracture in postmenopausal-older women (36-38). The present results obtained suggest that colorectal carcinogenesis can be prevented by not only ingesting low fat-diets but also lowering circulating plasma levels of lipids. Anyway, the present findings indicate that the lowering of plasma lipid levels by fluvastatin may lead to less oxidative DNA damage in the mucosa, resulting in a reduction of colorectal carcinogenesis. Our results, together with other studies (33-38), suggest that the hypcholesterolemic agent, fluvastatin, may have a role in the treatment of colorectal carcinogenesis in patients with UC, post-menopausal women with hypercholesterolemia, osteoporosis and breast cancer, and possibly individuals with hereditary or familiar high risk colorectal cancer.

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