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Original Article

Inhibitory Effect of 1α-Hydroxyvitamin D_3 on *N*-nitrosobis (2-oxopropyl)Amine-induced Cholangiocarcinogenesis in Syrian Hamsters

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Sixty-three male 5-week-old Syrian hamsters received the carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) s.c. in 5 weekly injections (the first, 70 mg/kg body, and the remaining, 20 mg/kg each). The hamsters that received BOP were given intragastric administration of 0.2 ml of medium chain triglyceride (MCT) with or without 0.04 μg of 1α-hydroxyvitamin D_3 [1α(OH)D_3] through a feeding tube for 12 weeks. Thus, 3 groups were assigned: Group 1; BOP alone (n = 20), Group 2; BOP + MCT (n = 18) and Group 3; BOP + 1α(OH)D_3 (n = 25). The mean body weight of Group 3 was lower than those of Groups 1 and 2 at the end of the experiment ($p < 0.001$, Tukey-Kramer HSD test). At the end of week 12, all surviving hamsters were put to sleep. The incidences of liver tumors were 80%, 72% and 32% in Groups 1, 2 and 3, respectively. The incidence of tumors in Group 3 was significantly lower than in Group 1 and Group 2 ($p < 0.05$, χ^2-test). All tumors were cholangiocarcinoma. These results indicated that BOP-induced cholangiocarcinogenesis was suppressed by the supplemental administration of 1α(OH)D_3.

Key words: 1α-hydroxyvitamin D_3, *N*-nitrosobis(2-oxopropyl)amine, cholangiocarcinogenesis, Syrian hamsters

Cholangiocarcinoma (CCA) is an epithelial cancer originating from the bile ducts with features of cholangiocyte differentiation. Even though CCA is rare worldwide, there has been a marked increase in the incidence of and mortality due to intrahepatic cholangiocarcinoma in the USA, the UK, Japan, and Australia [1, 2]. Since early diagnosis is difficult, effective protective measures against CCA are needed. Epidemiologic studies suggest that the risk factors for CCA are primary sclerosing cholangitis, liver fluke infection, congenital fibropolycystic liver disease, bile duct adenomas, biliary papillomatosis, hepatolithiasis,

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chemical carcinogens, chronic viral hepatitis, cirrhosis, chronic non-alcoholic liver disease and obesity [3]. Recently, an association among 1α, 25-dihydroxyvitamin D_3 [1α, 25$(OH)_2D_3$], vitamin D receptor (VDR), and cancer was recognized, as epidemiological studies indicated an inverse relationship between the level of vitamin D_3 and the risk of a variety of cancers [4]. Moreover, 1 α , 25$(OH)_2D_3$ has been used in chemoprevention and therapeutics for human tumors other than CCA [5, 6]. The expression of VDR was shown to be compatible with an overall favorable prognosis for CCA, and the human CCA cell lines with a high expression of VDR responded to high concentrations of vitamin D_3 with a decrease in cell number [7]. Previously an inhibitory effect of vitamin D_3 on colon carcinogenesis was reported in animals [8, 9]. Therefore, we examined the effect of 1α-hydroxyvitamin D_3 [1α(OH)D_3] in a CCA model.

Materials and Methods

Sixty-three male 5-week-old Syrian hamsters were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) 2 weeks before the experiment. The hamsters were housed in plastic cages (3/cage) with sterilized wooden chips as bedding in an air-conditioned room at 23 ± 2℃ and 55 ± 5% humidity with 12h alternating light and dark periods. The hamsters were maintained on a commercial pellet diet (CE-2, Clea Japan, Osaka, Japan) and tap water *ad libitum*. The calorie sources of CE-2 as fat and protein were 5.1 and 25.4%, respectively. The calcium and phosphorus contents were 1.10 and 1.05% (wt/wt), and that of vitamin D_3 was 57.5μg/kg CE-2. Body weight and appearance were recorded periodically.

N-nitrosobis(2-oxopropyl)amine (BOP) was obtained from Okayama Pharmaceutical Industries, Ltd. (Okayama, Japan) and dissolved in physiological saline just before injection. 1α(OH)D_3 (Chugai Laboratories, Tokyo, Japan) was dissolved in medium chain triglyceride (MCT) (The Nisshin Oillio Group, Ltd., Tokyo, Japan) at the concentration of 0.2μg/ml.

Animals received the carcinogen BOP s.c. in 5 weekly injections (the first at 70mg/kg, and the others at 20mg/kg each) [10]. The animals that received BOP were given intragastric administration of 0.2ml of MCT with or without 0.04μg of 1α(OH)D_3 through a feeding tube 3 times weekly for 12 weeks. Thus, 3 groups were assigned: Group 1; BOP alone (n = 20), Group 2; BOP + MCT (n = 18) and Group 3; BOP + 1α(OH)D_3 (n = 25). These treatments were done without anesthesia. Hamsters were observed daily and weighed once every 4 weeks. At the end of week 12, all surviving hamsters were put to sleep, and pathological (macroscopic and histological) examinations were done exactly as has been previously described in detail [11]. When a macroscopic liver tumor was diagnosed as cholangiocarcinoma by histological examination, that animal was included among the hamsters with liver tumors. Serum calcium levels were measured using the o-cresolphthalein complexone (oCPC) method (Clinimate CA, Sekisui Medical Co. Ltd., Tokyo, Japan) in heart blood taken at autopsy.

The χ^2-test, Student's *t*-test and the Tukey-Kramer HSD test were used for statistical analysis where appropriate.

Results

During the experiment there were no notable changes in the general appearance of the hamsters. The mean body weight of Group 3 was lower than those of Groups 1 and 2 at the end of the experiment ($p < 0.001$, Tukey-Kramer HSD test) (Fig. 1).

Table 1 summarizes the data on the incidence of cancer. The incidences of liver tumors were 80%, 72% and 32% in Groups 1, 2 and 3, respectively. The incidence of tumors in Group 3 was significantly lower than those in Groups 1 and 2 ($p < 0.05$, the χ^2-test). There were no metastases in these animals. Macroscopically, liver tumors were whitish and single or multiple (Fig. 2) in all 3 groups. All tumors were cholangiocarcinoma. Microscopically, these have a bile duct pattern but do not contain bile. The duct-like structure is lined by cuboidal or columnar cells that are accompanied by abundant connective tissue stroma (Fig. 3). The portal tract is enlarged and contains clear spaces, mainly representing proliferated bile ducts (Figs. 3, 4). The serum calcium levels of hamsters given 1α(OH)D_3 (16.2 ± 1.9mg/dl, mean ± SD, n = 22) were significantly higher than those of the groups not given 1α(OH)D_3 (12.0 ± 1.2mg/dl, n = 22) ($p < 0.001$, Student's *t*-test).

Table 1 Incidence and numbers of hamsters with liver tumor

Group	Treatment	No. of hamster	Body weight (g) (mean ± SD)	Size (mm) Range (mean)	No. of hamster with liver tumors (%)
1	BOP	20	100 ± 30.0	0.5–10 (4.2)	16 (80)
2	BOP + MCT	18	119 ± 15.0	0.8–16 (4.5)	13 (72)
3	BOP + 1α(OH)D_3 In MCT	25	71.4 ± 19.7*	0.5– 9 (3.4)	8 (32)**

MCT: medium chain triglyceride
*Significantly different from Group 1 and 2, $p < 0.001$ by Tukey-Kramer HSD test.
**Significantly different from Group 1 and 2, $p < 0.05$ by χ^2-test.

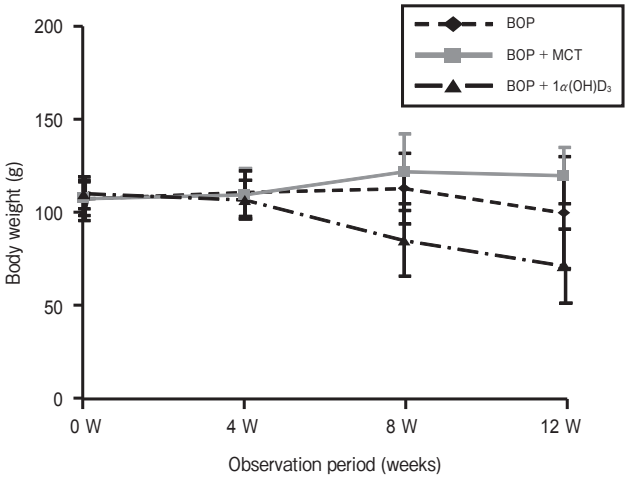

Fig. 1 Growth curve of male Syrian hamsters.

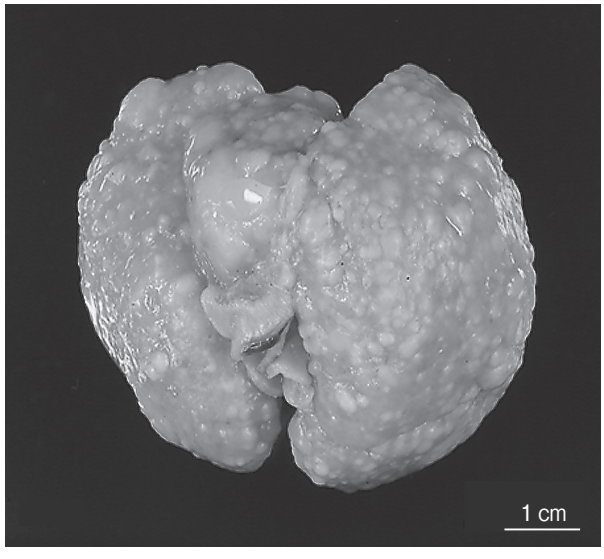

Fig. 2 Macroscopic appearance of liver tumors in a hamster in Group 1. This appearance was the same in Groups 2 and 3.

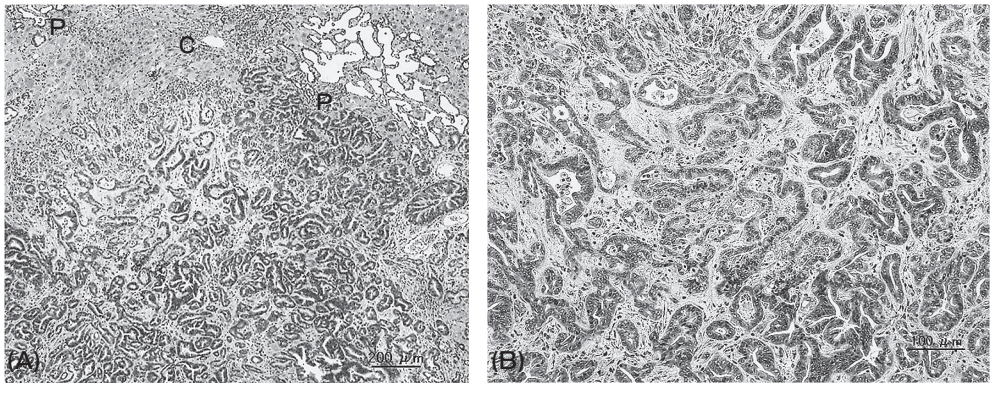

Fig. 3 **A**, Histological appearance of tubular adenocarcinoma, centrilobular area (C) and portal tract (P) in a hamster in Group 1. This appearance was the same in Groups 2 and 3 (H & E stain); **B**, Higher-power view of tubular adenocarcinoma (H & E stain). Scale bar: **A**, 200 μm; **B**, 100 μm.

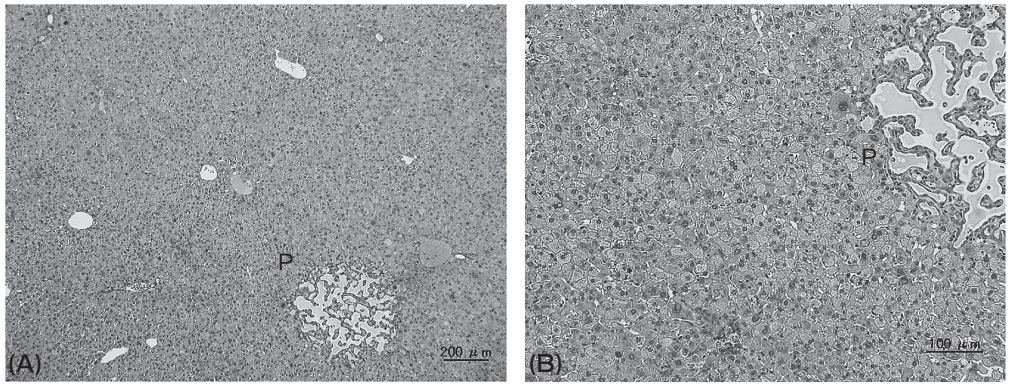

Fig. 4 **A**, Histological appearance of hepatic lobules and portal tract (P) in a hamster in Group 3 (H & E stain); **B**, High-power view of portal tract (P) (H & E stain). Scale bar: **A**, 200 μm; **B**, 100 μm.

Discussion

In the present study, we first showed that administration of $1\alpha(OH)D_3$ inhibited BOP-induced cholangiocarcinogenesis in Syrian hamsters. The administered $1\alpha(OH)D_3$ is metabolized in the liver to $1\alpha, 25(OH)_2D_3$, which acts hormonally through its specific VDR in the body [12] including cancerous cells [13, 14]. A recent study demonstrated that treatment with $1\alpha, 25(OH)_2D_3$ in CCA cell lines with a high expression of VDR significantly reduced cell proliferation in a dose-dependent manner [7]. The present result was consistent with this and provided experimental evidence of the chemopreventive action of $1\alpha(OH)D_3$ or $1\alpha, 25(OH)_2D_3$ in cholangiocarcinogenesis.

We did not use a vitamin D_3-deprived diet as a control diet, since the supplementation of vitamin D_3 to the basal diet might be a practical experiment in future clinical work. There is considerable evidence that tumor growth is an angiogenesis-dependent event [15]. Mantell demonstrated that $1\alpha, 25(OH)_2D_3$ significantly inhibited vascular endothelial growth factor (VEGF)-induced endothelial cell sprouting and elongation *in vitro* in a dose-dependent manner and had a small, but significant, inhibitory effect on VEGF-induced endothelial cell proliferation through specific stages of the angiogenic process [16]. Recently, Kisker isolated vitamin D binding protein-macrophage activating factor (DBP-maf) generated from systemically available DBP by a human pancreatic cancer cell line [17]. Also, Kalkunte indicated that DBP-maf inhibited human endothelial cell proliferation by inhibiting DNA synthesis, and that DBP-maf inhibited VEGF signaling by decreasing VEGF-mediated phosphorylation of VEGF-2 and extracellular signal-regulated kinase (ERK)1/2, a downstream target of the VEGF signaling cascade [18]. Although the relationship between this antiangiogenetic activity of vitamin D_3 and the effects of DBP-maf was unclear, the present study suggests that the chemopreventive effects of $1\alpha(OH)D_3$ in cholangiocarcinogenesis may be exerted through the inhibition of angiogenesis by $1\alpha, 25(OH)_2D_3$ or DBP-maf. Also, it was indicated that macrophages activated with DBP-maf were highly tumoricidal against a variety of malignancies [19], which might have been part of the mechanism of anti-carcinogenic action of vitamin D_3.

With regard to the hypercalcemia observed in our study, it has been suggested that the anti-carcinogenic effect of $1\alpha(OH)D_3$ is mediated by hypercalcemia, as $1\alpha, 25(OH)_2D_3$ was found to decrease the number of cell lines derived from human colon cancer *in vitro* [20]. Furthermore, the mean body weight in Group 3 was lower at the end of the experiment through hypercalcemia in this study. It was reported that the clinical manifestation of hypercalcemia induced appetite loss [21]. According to Fair [22], the restriction of calories by 10 to 40% decreased cell proliferation and increased apoptosis through anti-angiogenic processes, resulting in the potent anticancer effect of caloric restriction. Thus, the weight loss that occurred presumably through elevated extracellular Ca^{2+} may be a feasible factor in the cancer prevention observed in this study.

Although further investigation is necessary to determine the precise mechanism of our results in

view of the many physiological actions of vitamin D_3 [23], our study may lend support to the finding that vitamin D_3 plays a preventive role in cholangiocarcinogenesis, as indicated by experimental studies *in vitro* [7].

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