Chemopreventive effect of a vitamin D_3 analog, alfacalcidol, on colorectal carcinogenesis in mice with ulcerative colitis

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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. Repeated oral doses of 3% dextran sulfate sodium subsequent to a single intraperitoneal injection of azoxymethane induced a chronic UC resulting in an increased incidence of high-grade dysplasia and submucosal-invasive adenocarcinomas in the mouse colorectum. The active form of vitamin D_3 is a calcium-regulating hormone that increases serum calcium levels and intestinal calcium absorption. It has been reported that there is an inverse correlation between serum levels of the active metabolite of vitamin D and colorectal carcinoma stage. The features of the colitis induced in this animal model are very similar to the UC in patients in terms of both clinical and histological characteristics. Treatment with a vitamin D_3 analog, alfacalcidol, in mice prevented colitis and carcinogenesis; this is shown by inhibition of the decrease in colorectal length and inhibition of the increased incidence of colorectal dysplasia, with a reduction in the mRNA expression of the DNA-synthesizing enzyme, thymidine kinase, in colorectal tissues.
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Introduction

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 reported previously[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-invasive 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].

The active form of vitamin D_3 is a calcium-regulating hormone that increases serum calcium levels and intestinal calcium absorption [4]. One of the vitamin D_3 analogs, alfacalcidol, is known to be converted into 1α,25-dihydroxy vitamin D_3 [1,25$(OH)_2D_3$] by 25-hydroxylase in the liver after intestinal absorption and to exert regulatory effects on osteoblasts by binding to specific receptors [5]. Niv *et al.* [6] demonstrated that there was an inverse correlation between serum levels of the active metabolite of vitamin D and colorectal carcinoma stage in 84 patients. It has been reported that a vitamin D_3 analog reduced the incidence of colon tumors in rats treated with 1,2-dimethylhydrazine [7] and AZM as an antiproliferative agent and as an antiangiogenic agent [8], when administered in the postinitiation phase [9].

Thymidylate synthase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21) catalyze the formation of deoxythymidine monophosphate by the methylation of deoxyuridine monophosphate with the concomitant conversion of N^5,N^{10}-methylenetetrahydrofolic acid into 7,8-dihydrofolic acid via the de-novo pathway and the phosphorylation of thymidine via the salvage pathway, respectively [10]. High TS and TK activities have been found in rapidly proliferating tissues of normal, fetal and neoplastic tissues [11–13].

In this study, the preventive effects of a vitamin D_3 analog, alfacalcidol, 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), 6 weeks of age, were used. The animals were housed in plastic cages with wood shavings under controlled temperature ($24 \pm 0.5^{\circ}C$) and lighting (12 h of light from 06.00 to

18.00 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 8 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-D_3). The animals of the two experimental groups (30 mice) were injected intraperitoneally with 8.0 mg/kg AZM Sigma Chemical, St Louis, Missouri, 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 (molecular weight 50 000; Ensuiko Sugar Refining, Yokohama, Japan) for 7 days followed by tap water for 14 days, a total of three times. Beginning at 8 weeks of age, the animals in one of the two experimental groups were fed the same commercial diet containing a synthetic vitamin D_3 analog, 9,10-secocholr-sta-5,7,10(19)-triene-1α,3β-diol (alfacalcidol: 2.5 μg in 1 kg of diet) (ALFAROL powder; a gift from Chugai Pharmaceutical, Tokyo, Japan) for 12 weeks (AZM/DSS-D_3 group). The other 30 mice in the normal-control and AZM/DSS-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 a hematological examination of peripheral blood and killed at 20 weeks of age. The numbers of leukocytes (white blood corpuscles, $10^2/mm^3$) and erythrocytes (red blood corpuscles, $10^4/mm^3$) and the concentrations of hemoglobin (Hb; g/dl) in the obtained blood were determined. Immediately after the bleeding, the liver, spleen, kidney, uterus, adrenals, ovaries and colorectum were removed and weighed. All experimental procedures conformed to the regulations described in the US National Institutes of Health *Guide to the Care and Use of Laboratory Animals*.

The longitudinal length of each colorectum was measured and each specimen was longitudinally sectioned into two parts; one was stored to evaluate the expression levels of TS and TK mRNA at −80°C, and the other was immediately fixed in a 10% formaldehyde buffer solution (pH 7.2), embedded in paraffin. Then 5-μm serial sections were prepared, and stained with Mayer's hematoxylin and eosin for histological examination.

Analysis of gene expression of thymidylate synthase and thymidine kinase in the colorectum

Reverse transcriptase (RT)-PCR was performed for a quantitative analysis of TS and TK mRNA levels in the colorectum. Total RNA was extracted from each colorectal sample with a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech, Little Chalfont, UK). Reverse transcription was performed using oligo-(dT) primers [0.5 ml of oligo-$(dT)_{12–18}$ primers (1.0 μg/ml) (Gibco BRL, Gaithersburg, Maryland, USA)] with a Superscript Preamplification System (Gibco BRL) according to the supplier's instructions. Once the cDNA copy had been created using the mRNA template, the PCR was conducted immediately, as outlined below. Alternatively, the cDNA was stored at −20°C until use. The PCR was performed with recombinant Taq DNA polymerase (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The RNA (1.0 μg) was subjected to RT-PCR using the primers for TS and TK cDNA for 34 cycles (each cycle consisted of denaturing at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 40 s) in a Gene Amp PCR System 2400 (Perkin Elmer, Branchburg, New Jersey, USA). RT-PCR was carried out with three sets of primers (TS: 5′-TGAATGGGGAGCTATCTTGCCA-3′ and 5′-TCGTT GGATGTGGATTATACCC-3′; TK: 5′-TAGCACAGG CGGCACACGGAGT-3′ and 5′-TGCTCCGCGATGT GACCCAGGA-3′; and β-actin: 5′-AGGCCCAGAGCAA GAGAGGCAT-3′ and 5′-CATGGCTGGGGTGTTG AAGGTC-3′). The levels of TS mRNA and TK mRNA were determined by densitometry from photographs taken with an image analyzer (AE6920-MF Densitograph; Atto, Tokyo, Japan), and are expressed as a ratio of the mRNA level of β-actin as an internal standard.

Calculations and statistics

All parameters were expressed as the mean ± SEM. Statistical analyses were performed using the unpaired *t*-test and Fisher's exact probability test. A P value less than 0.05 was considered statistically significant.

Results and discussion

AZM/DSS treatment lowered the final body weight to 88.9% of that of the normal-control group ($P < 0.05$) (Table 1). The additive treatment with the vitamin D_3 analog did not significantly improve body growth despite AZM–DSS treatment.

AZM/DSS treatment markedly altered organ weights, i.e. the weights of 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 uterus and ovaries were reduced (Table 1). The additive treatment with the vitamin D_3 analog, however, lowered the weights of the spleen ($P < 0.01$), and elevated the weights of the ovaries ($P < 0.01$), uterus ($P < 0.05$) and adrenals ($P < 0.01$).

Although the number of white blood corpuscles was not affected by AZM/DSS treatment (Table 2), the number of red blood corpuscles and the concentration of Hb were reduced to 68.5 ($P < 0.01$) and 90.9% ($P < 0.05$),

Table 1 Body growth and organ weights

	Group (*n*)		
	AZM/DSS-control (15)	AZM/DSS-D_3 (15)	Normal-control (15)
Body weight			
Initial (g)	21.1 ± 0.2	21.3 ± 0.2	21.4 ± 0.2
Final (g)	23.2 ± 1.1	24.3 ± 0.4	26.1 ± 0.6*
Growth (%)	109.9 ± 4.4	114.0 ± 1.0	122.1 ± 2.0*
Organ weight (mg/g body weight)			
Liver	61.0 ± 2.0	57.7 ± 0.5	55.8 ± 1.1*
Spleen	6.37 ± 0.49	3.67 ± 0.11**	3.10 ± 0.16**
Kidneys	14.3 ± 0.4	13.6 ± 0.2	12.8 ± 0.4*
Uterus	4.89 ± 0.24	7.22 ± 0.77*	6.32 ± 0.50*
Adrenals (× 100)	22.8 ± 1.0	37.2 ± 2.4**	17.4 ± 1.3**
Ovaries (× 100)	33.1 ± 1.8	53.0 ± 2.8**	44.6 ± 3.7*

Data are means ± SEM.
AZM, azoxymethane; DSS, dextran sulfate sodium; D_3, vitamin D_3.
Significantly different from AZM/DSS-control: $^{**}P<0.01$ and $^{*}P<0.05$, respectively.

Table 2 Blood features

	Group (*n*)		
	AZM/DSS-control (15)	AZM/DSS-D_3 (15)	Normal-control (15)
WBC (10^2/mm^3)	39.0 ± 8.9	30.5 ± 4.9	39.0 ± 8.3
RBC (10^4/mm^3)	451 ± 39	366 ± 25*	658 ± 22**
Hb (g/dl)	11.0 ± 0.4	10.8 ± 0.3	12.1 ± 0.3*

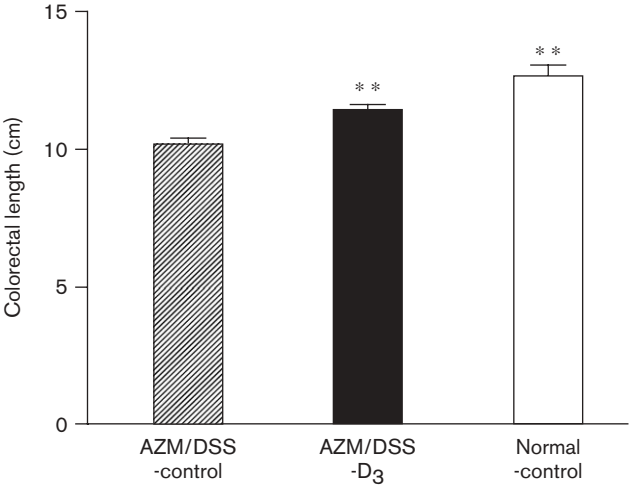
Data are means ± SEM.
AZM, azoxymethane; DSS, dextran sulfate sodium; D_3, vitamin D_3; Hb, hemoglobin; RBC, red blood corpuscles; WBC, white blood corpuscles.
Significantly different from AZM/DSS-control; $^{**}P<0.01$ and $^{*}P<0.05$, respectively.

respectively. The additive treatment with the vitamin D_3 analog did not alter the hemorrhagic anemia.

The colorectal length in AZM/DSS-treated mice (AZM/DSS-control group) was markedly reduced to 80.2% of that in the normal-control group ($P < 0.01$) (Fig. 1). The additive treatment with the vitamin D_3 analog partially prevented the shrinking of the colorectum ($P < 0.01$). No open ulcer was found, as mice in the experimental groups were killed 3 weeks after the last administration of DSS. The number of foci of gland loss with inflammatory cell infiltration, which indicates the severity of UC, in mice treated with the vitamin D_3 analog (AZM/DSS-D_3 group) was, however, reduced compared with that in the AZM/DSS-treated mice. AZM/DSS treatment induced high-grade dysplasia (Fig. 2b): 27.9 sites/mouse in number and 184.1 mm^2/mouse in cumulative area, although high-grade dysplasia was not found in the normal-control group (Fig. 2a). The additive treatment with the vitamin D_3 analog, however, markedly reduced the high-grade dysplasia in both number and region to approximately 5.0% of those in the AZM/DSS-control group ($P < 0.01$) (Fig. 3). In this study, no submucosal-invasive adenocarcinomas were found in any colorectal samples.

Expression levels of TS and TK mRNAs in the entire colorectum in the AZM/DSS-control group were markedly elevated to 1.7 and 9.9 times the levels in the normal-control group, respectively ($P < 0.01$) (Fig. 4). The additive treatment with the vitamin D_3 analog, however, significantly lowered the gene expression level of TK, but not TS, to less than 70% of that in the AZM/DSS-control group ($P < 0.05$).

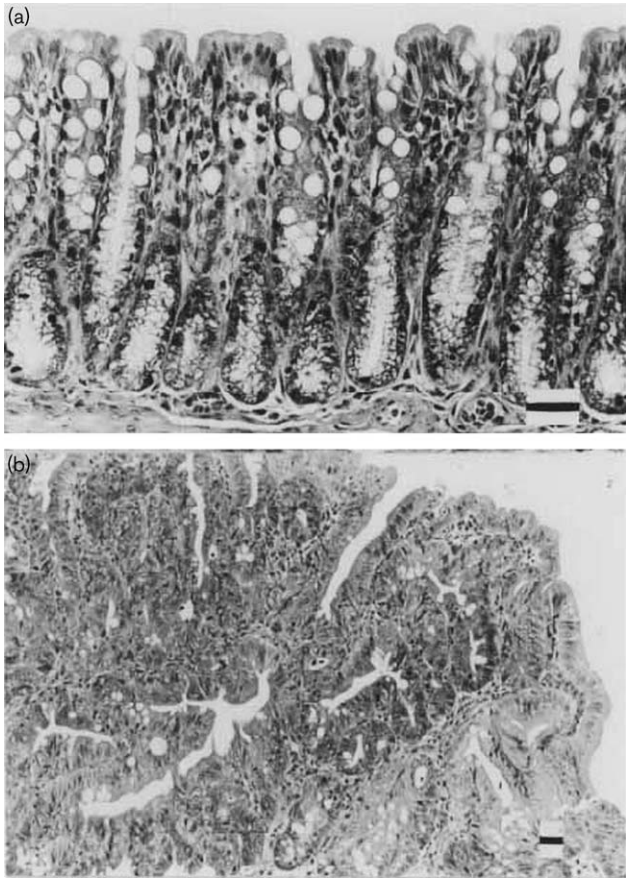
Fig. 1



Colorectal length in each group. Data are means ± SEM. **Significantly different from AZM/DSS-control; $P<0.01$. AZM, azoxymethane; DSS, dextran sulfate sodium; D_3, vitamin D_3.

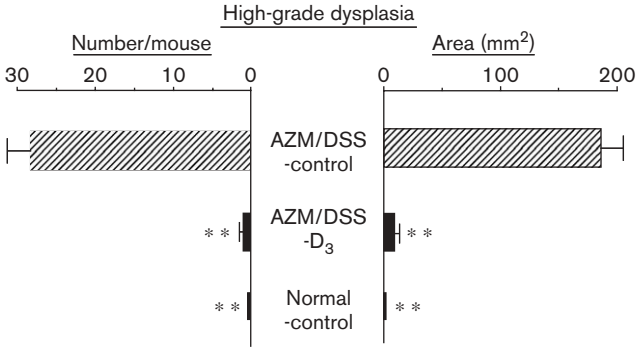
DSS is a synthetic, sulfated polysaccharide that induces a colitis in rodents, which clinically and histologically resembles human UC. The hyperproliferation of cells in the inflammation-associated damage–regeneration cycle has been shown to contribute to the fixation of genetic and epigenetic alterations, and promote the development of colorectal dysplasia and carcinoma [14]. AZM is known as a procarcinogen, which becomes an alkylating agent with carcinogenic activity following metabolic activation in the host [15]. DSS has been found to be negative in the Ames test for mutagens [16]. Nine cyclic administrations of DSS, however, induced nine low-grade dysplasias, four high-grade dysplasias and two carcinomas in 25 mice in our previous study [17]. Inflammation-associated regenerative atypia is thought to be difficult to distinguish from dysplasia. Our histological diagnosis was supported by the findings of diffuse labeling of tumor cells with bromodeoxyuridine and activities of TS and TK throughout the colorectal mucosa, i.e. bromodeoxyuridine uptake and activities of TS and TK were higher in mucosal tumors than in nontumorous 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 treatment

Fig. 2



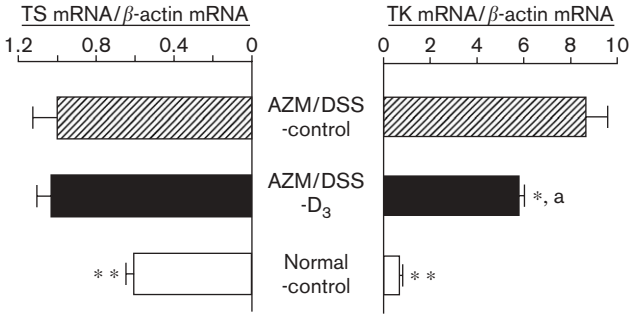
Histopathological structure of the colorectal tumorous region with high-grade dysplasia (b) (original magnification × 148) in mice treated with azoxymethane and 3% dextran sulfate sodium compared with normal mucosa (a) (× 400, hematoxylin and eosin). Scale bar in the right-bottom corner of each figure indicates 25 μm in horizontal length.

Fig. 3



High-grade dysplasia in the colorectum: number (left) and area (right) in each group. Data are means ± SEM. **Significantly different from AZM/DSS-control; $P < 0.01$. AZM, azoxymethane; DSS, dextran sulfate sodium; D_3, vitamin D_3.

Fig. 4



Expression levels of thymidylate synthase (TS) mRNA (left) and thymidine kinase (TK) mRNA (right) as a ratio of the β-actin mRNA level in the colorectum. Data are means ± SEM. Significantly different from AZM/DSS-control: $^{**}P < 0.01$ and $^{*}P < 0.05$, respectively. [a]Significantly different from normal-control; $P < 0.01$. AZM, azoxymethane; DSS, dextran sulfate sodium; D_3, vitamin D_3.

with the vitamin D_3 analog slightly increased growth despite the AZM/DSS treatment. Although AZM/DSS treatment increased the weight of all the organs except the ovary and uterus, the vitamin D_3 analog had a tendency to normalize the altered weights without adrenals. Additive treatment with the analog prevented shrinkage of the colorectum, which was affected by a cycle of recurrence–remission in colorectal mucosa, resulting in a reduced incidence of colorectal dysplasia and a reduced expression of TK mRNA in the colorectum.

An inverse correlation has been reported between serum levels of the active metabolite of vitamin D and the stage of colorectal carcinoma [6]; low levels of exposure to sunshine and vitamin D intake are suspected to increase the risk of colorectal carcinoma [18]. The intake of dietary fiber has been considered beneficial to the microbial conversion of bile acid and cholesterol in the colorectum [19], resulting in a reduction in the incidence of precancerous lesions [20]. The vitamin D receptor is known to function as a receptor for the secondary bile acid, lithocholic acid, which is both hepatotoxic and a potential enteric carcinogen. Activation of the vitamin D receptor by lithocholic acid or vitamin D was reported to induce the expression *in vivo* of CYP3A, a cytochrome P450 enzyme that detoxified lithocholic acid in the liver and intestine [21]. A synthetic vitamin D_3 analog reduced human rectal crypt cell production rates in biopsy specimens obtained from patients with UC [22]. Slattery *et al.* [18]demonstrated that the Fok1 vitamin D receptor polymorphism interacted with the androgen receptor to alter colon cancer risk. Polymorphism of the vitamin D receptor gene is associated with increased mRNA expression of the vitamin D receptor gene and increased serum levels of 1,25-dihydroxy

vitamin D. Thus, reduced levels of vitamin D receptor in mice treated with AZM/DSS can be enhanced by additive treatment with a vitamin D_3 analog. It might be possible to explain the protective effects of vitamin D and its receptor against colorectal inflammation and/or carcinogenesis. Taken together, our study and other studies might indicate that the active metabolite of vitamin D inhibits colorectal cancer, and that it should be developed as an inhibitor of colorectal epithelial cell proliferation and the neoplastic process, or as a promoter of cellular differentiation.

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