Chemopreventive effect of 24R,25-dihydroxyvitamin D₃ in N,N’-dimethylhydrazine-induced rat colon carcinogenesis

Tetsuhide Taniyama, Hideki Wanibuchi, Elsayed LSalam, Yoshhis Yano¹, Shuzo Otani¹, Yoshih Nishizawa², Hiroto Mori³ and Shoji Fukushima³

First Department of Pathology, ¹Second Department of Biochemistry and Molecular Biology, Faculty of Medicine, Osaka University School of Medicine, 1-1-1 Mihara, Mondobe 565-0871, Japan and ²Institute of Immunology, Osaka University Medical School, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

In this study we investigated the effects of 24R,25-dihydroxyvitamin D₃ [24R,25(OH)₂D₃] on N,N’-dimethylhydrazine (DMH)-induced rat colon carcinogenesis. For experiments 1 and 2, 50 F344 male, 6-week-old rats were divided into five groups in each experiment. Animals were given s.c. injections of DMH once a week for 4 weeks. Those in groups 1–5 were given 24R,25(OH)₂D₃ in the diet (10, 5, 2.5, 1.25 or 0 p.p.m., respectively) during the post-initiation stage in experiment 1 and during the initiation stage in experiment 2. At termination, the numbers of aberrant crypt foci (ACF) in the rat colonic mucosa were decreased dose-dependently in rats treated with 24R,25(OH)₂D₃ during the post-initiation stage, but not in the initiation stage. For experiment 3, 15 male, 9-week-old rats were divided into three groups and given 24R,25(OH)₂D₃ in the diet (10, 5 or 0 p.p.m.). Animals were injected with 5-bromo-2’-deoxyuridine (BrdU) i.p. 1 h before death to examine DNA synthesis in the colon mucosa. BrdU labeling indices were decreased dose-dependently in colonic crypts of rats treated with 24R,25(OH)₂D₃. In experiment 4, using the murine carcinogenic protocol we could analyze our data with respect to not only one separate organ, but at the organism level. Sixty-eight male, 6-week-old rats were treated with DMH, N-methyl-N’-nitrosourea, 2,2’-dihydroxy-di-ortho-propynitrosamine, diethylaminoethylethyl nitrosamine and N-buty1-N-(4-hydroxybutyl)nitrosamine in weeks 1–4 and were then given 24R,25(OH)₂D₃ in the diet (5, 1 or 0 p.p.m.) throughout weeks 5–30. Examination of the development of tumors and preneoplastic lesions in various organs revealed that 24R,25(OH)₂D₃ inhibited colonic tumor development significantly but exerted no effects on tumor induction in other organs. In conclusion, these results strongly indicate that 24R,25(OH)₂D₃ inhibits colon carcinogenesis specifically, without any enhancement of carcinogenesis in other organs, when administered in the post-initiation phase.

Abbreviations: ACF, aberrant crypt foci; BBN, N-buty1-N-(4-hydroxybutyl)nitrosamine; BrdU, 5-bromo-2’-deoxyuridine; COX, cyclooxygenase; DEN, diethylnitrosamine; DHPN, 2,2’-dihydroxy-di-n-propynitrosamine; DMBDD treatment, combined carcinogen treatment; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 24R,25(OH)₂D₃, 24R,25-dihydroxyvitamin D₃; DMH, N,N’-dimethylhydrazine; GST-P, glutathione S-transferase placental form; MNU, N-methyl-N’-nitrosourea; ODC, ornithine decarboxylase; SAT, spermidine/spermine N⁷-acetyltransferase.

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths worldwide (1), with clear evidence of environmental determinants, especially diet. Several studies have suggested an inverse association between dietary vitamin D or calcium and colorectal cancer risk (2,3). Despite epidemiological evidence which showed beneficial effects of vitamin D₃ analogs on colon cancer, there have been relatively few animal models which have clearly demonstrated an inhibitory effect of vitamin D₃ on chemically induced colon cancer.

1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] is a major active metabolite of vitamin D₃ that has strong effects on calcium metabolism. The resultant hypercalcemia from high doses of 1,25(OH)₂D₃ is associated with major side-effects which could limit its potential usefulness as a chemopreventive agent for colon cancer. We previously reported chemopreventive effects of 22-oxacalcitriol, a new synthetic analog of 1,25(OH)₂D₃, on rat colon carcinogenesis (4). Other 1,25(OH)₂D₃ analogs (5–7) have also been reported to inhibit colon, skin, prostate and mammary carcinogenesis in rats. Another active form of vitamin D, 24R,25-dihydroxyvitamin D₃ [24R,25(OH)₂D₃] has been reported to cause mild hypercalcemia (8,9). Ikezaki et al. (10) previously reported chemopreventive effects of 24R,25(OH)₂D₃ on glandular stomach carcinogenesis in rats during the post-initiation phase. We also reported an effect of 24R,25(OH)₂D₃ on rat colon carcinogenesis when administered before, during or after carcinogen administration (11).

Epidemiological and laboratory studies indicate an inverse relationship between the risk of colon cancer development and intake of non-steroidal anti-inflammatory agents, including aspirin. One of the mechanisms by which non-steroidal anti-inflammatory agents inhibit colon carcinogenesis is related to their inhibition of prostaglandin production by induction of cyclooxygenase isozymes (COX-1 and COX-2). Overexpression of COX-2 has been observed in colon tumors (12).

Quantification of formation and growth of aberrant crypt foci (ACF) in the rodent colon has been used as a short-term bioassay to evaluate the role of nutritional components at a very early stage of colon carcinogenesis (13). Several observations have confirmed the putative association between ACF and colon cancer in both rodents (14) and humans (15). Moreover, many of the genetic alterations characteristic of colon cancer are observed in ACF (16,17).

It is important to detect chemicals which show potential to modify not only carcinogenic effects in a single organ, but throughout the whole body. If an agent prevents cancer in one organ but shows promoting effects in other organs, it should not be used for the purpose of cancer chemoprevention. The multiorgan carcinogenesis protocol consists of five carcinogens: diethylnitrosamine (DEN), N-methyl-N’-nitrosourea (MNU), N-buty1-N-(4-hydroxybutyl)nitrosamine (BBN), N,N’-dimethylhydrazine hydrochloride (DMH) and 2,2’-dihydroxy-di-n-propynitrosamine (DHPN). This protocol has clear benefits for examination of modifying effects of chemicals in
multiple organs in a single animal within a relatively short experimental period (18–20) and is based on the strong correlation between the multigorgan carcinogenesis model and long-term experimental results (21). Although 24R,25(OH)2D3 has been shown to prevent colon carcinogenesis (11), its effects on carcinogenesis of other organs has not been examined.

In this study we attempted to confirm the modifying effect of 24R,25(OH)2D3 during the post-initiation and initiation phases on DMH-induced aberrant crypt foci in rat colon carcinogenesis. In addition, the modifying effects of 24R,25(OH)2D3 on carcinogenesis in other organs were evaluated using the multigorgan carcinogenesis bioassay.

Materials and methods

Animals

Male F344 rats were obtained at 5 weeks of age (Charles River Japan Inc., Hino, Japan) and housed in plastic cages with wood chips under controlled conditions of a 12 h light/12 h dark cycle, 44 ± 5% humidity and 22 ± 2°C temperature. Diet and water were available ad libitum and body weights, food and water intake were measured weekly during the experiments.

Chemicals and diet

DMH, DEN and BBN were purchased from Tokyo Kasei Co. (Tokyo, Japan), MNU was obtained from Iwai Kagaku Yukuhin Co. (Tokyo, Japan) and DHPN from Sakai Chemical Co. (Fukui, Japan). 24R,25(OH)2D3 and the CE-2 diet (Clea Japan Inc., Tokyo) were obtained from Kureha Chemical Industries Co. (Tokyo, Japan).

Treatment

Experiment 1. Fifty, 6-week-old rats were randomly divided into five groups (10 rats/group). Animals were given s.c. injections of DMH dissolved in normal saline solution (20 mg/kg body wt) once a week for 4 weeks. After DMH treatment, the animals in groups 1–4 were given 24R,25(OH)2D3-containing diet (10, 5, 2.5 or 1.25 p.p.m. in powdered basal diet) during weeks 5–12. The animals in group 5 were given the powdered basal diet without 24R,25(OH)2D3. The total period of experimental observation was 12 weeks, after which the animals were killed under ether anesthesia and completely autopsied at the beginning of week 31. The blood and cerebrospinal fluid were used for serum chemical determinations. The livers and kidneys were removed and weighed. The blood was used for serum chemical examinations.

Experiment 2. Fifty, 6-week-old rats were randomly divided into five groups (10 rats/group). Animals were given s.c. injections of DMH dissolved in normal saline solution (20 mg/kg body wt) once a week for 4 weeks. The first injection was given at day 1 after the start of the experiment. Animals in groups 1–4 were given 24R,25(OH)2D3-containing diet (10, 5, 2.5 or 1.25 p.p.m. in powdered basal diet) during weeks 1–4. After that, animals were given basal powdered diet. Animals in group 5 were given the powdered basal diet without 24R,25(OH)2D3 throughout the experiment. The total period of experimental observation was 8 weeks. The colon was examined according to the same procedure as in experiment 1.

Experiment 3. Fifteen, 9-week-old rats were randomly divided into three groups (5 rats/group). Animals in groups 1 and 2 were given 24R,25(OH)2D3 in the diet (10 and 5 p.p.m. in powdered basal diet, respectively) for 4 weeks. The animals in group 3 were given the basal powdered diet without 24R,25(OH)2D3. The animals were treated with 5-bromo-2′-deoxyuridine (BrdU) (Sigma Japan) at a dose of 100 mg/kg body wt i.p. 1 h before death. Animals were killed under ether anesthesia at the end of week 4 for examination of the BrdU labeling index and COX-2 index in the colon.

Experiment 4. The experimental design is shown in Figure 1. Sixty-eight, 6-week-old rats were randomly divided into five groups. Animals in groups 1–3 (16 rats each in groups 1 and 2; 20 rats in group 3) were given a combined treatment (DMBDD treatment) consisting of a single i.p. administration of 100 mg/kg body wt DEN dissolved in normal saline solution at the commencement of the experiment, i.p. administrations of 20 mg/kg body wt MNU dissolved in normal saline solution on days 2, 5, 8 and 11 (a total of four times), s.c. injections of 40 mg/kg body wt DMBDD dissolved in normal saline solution on days 14, 17, 20 and 23 (a total of four times), 0.05% BBN in the drinking water for 2 weeks (during weeks 1 and 2) and 0.1% DHPN in the drinking water for 2 weeks (during weeks 3 and 4). After the DMBDD treatment, the rats were maintained without any treatment for 1 week. The rats in groups 1 and 2 then received 24R,25(OH)2D3-containing (5 p.p.m. in group 1; 1 p.p.m. in group 2) powdered basal diet and the rats in group 3 received basal diet only, as a control. In addition, eight rats in group 4 received 5 p.p.m. 24R,25(OH)2D3-containing diet from week 5 to 30 without the prior DMBDD treatment and eight rats in group 5 received basal diet during the experiment without the DMBDD treatment. The total period of experimental observation was 30 weeks. All surviving animals were exsanguinated under anesthesia and completely autopsied at the beginning of week 31. The blood was used for serum chemical determinations. The livers and kidneys were removed and weighed and all major organs were excised and fixed in 10% phosphate-buffered formalin solution. The colons were stained in 0.2%
Chemoprevention by 24,25-dihydroxyvitamin D₃

Fig. 2. Number of ACF in rats treated with 24,25(OH)₂D₃ during the post-initiation stage. □ 1 crypt; □ 2 crypts; □ 3 crypts; □ 4 crypts. *P < 0.05; **P < 0.01; ***P < 0.001 (versus control).

Fig. 3. Number of ACF in rats treated with 24R,25(OH)₂D₃ during the initiation stage. □ 1 crypt; □ 2 crypts; □ 3 crypts; □ 4 crypts.

Table II. BrdU labeling index in colon mucosa of rats treated with or without 24R,25(OH)₂D₃

<table>
<thead>
<tr>
<th>24R,25(OH)₂D₃ (p.p.m.)</th>
<th>No. of rats</th>
<th>BrdU labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
<td>7.02 ± 1.10ᵃ</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>11.67 ± 0.75</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>15.84 ± 1.72</td>
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ᵃP < 0.05 (versus control).

Fig. 4. Incidence and multiplicity of colon tumors (adenoma and/or adenocarcinoma) in rats treated with 24R,25(OH)₂D₃ following DMBDD treatment. *P < 0.05 (versus DMBDD).

Fig. 5. Area and number of GST-P-positive foci in rat livers.

was obtained from Transduction Laboratories, KY) incorporation using the avidin–biotin–peroxidase complex (ABC) method described by Hsu et al. (22). Anti-GST-P antibodies were raised as previously described (23). The avidin–biotin–peroxidase complex (ABC) method was performed as previously described.

Serum and urine examination
Experiments 1 and 4. Urine samples were collected from the animals in the morning on the day of death. Blood samples were collected at autopsy and centrifuged at 3000 r.p.m. for 15 min. Urine and serum samples were analyzed for calcium and phosphate levels (urine, Hitachi-710 electrolyte analyzer; serum, Hitachi-7150; Hitachi, Tokyo, Japan). The organs were embedded in paraffin and used for sectioning with routine hematoxylin and eosin staining for histopathological examination. Three slices from the liver were used for immunohistochemical staining of glutathione S-transferase placental form (GST-P)-positive foci.

Table II. BrdU labeling index in colon mucosa of rats treated with or without 24R,25(OH)₂D₃

Experiment 5. ODC and SAT activities were measured as previously described by Otani et al. (24) and Matsui et al. (25). Frozen rat colonic epithelium samples were suspended in 0.5 ml of 50 mM Tris containing 0.25 M sucrose and disrupted with a homogenizer for 30 s. The homogenized suspensions were centrifuged at 100 000 g for 30 min and the supernatant was assayed for ODC and SAT activity by measurement of the amount of radioactive putrescine produced from [5-⁴C]ornithine and the amount of moiety transferred from [1-⁴C]acetylcoenzyme A to spermidine, respectively.

Immunohistochemical staining
Experiment 5. At week 4, all animals were killed under ether anesthesia. The colons were removed, flushed with phosphate-buffered saline, cut open along the longitudinal median axis and then divided in half. The attached colonic mucosa was scraped off with glass slides. The colon mucosa specimens were used for ornithine decarboxylase (ODC) and spermidine/spermine N₁-acetyltransferase (SAT) assays.

ACF count assay
Experiments 1, 2 and 4. After fixation colons were stained in 0.2% methylene blue for 1 min and examined for ACF by light microscopy. All the organs were embedded in paraffin and used for sectioning with routine hematoxylin and eosin staining for histopathological examination. Three slices from the liver were used for immunohistochemical staining of glutathione S-transferase placental form (GST-P)-positive foci. Animals in group 2 received basal diet only during the experiment. The colons were removed, fixed in methanol/Carnoy’s fixative and serial sections (3 µm thick) were made. Sections were used for immunohistochemical detection of BrdU and COX-2 (the COX-2 antibody was not dose-dependent. No deposition of calcium was observed from [1-⁴C]acetylcoenzyme A to spermidine, respectively.

Statistical analysis
Data were analyzed using Student’s t-test (Stat View SE+ Graphics; Abacus Concepts Inc., USA).

Results
Experiment 1
All rats survived until the final termination. Final body and relative liver and kidney weights did not significantly differ among the groups. Water consumption was increased in groups treated with 24R,25(OH)₂D₃, however, the differences were not dose-dependent. No deposition of calcium was observed
in kidneys of rats treated with 24R,25(OH)2D3. Urine calcium levels were dose-dependently increased in rats treated with 24R,25(OH)2D3 (Table I). Serum calcium levels were also dose-dependently increased in treated animals but the increases in serum calcium levels were slight. Serum phosphorus levels were comparable among the groups.

The number of ACF in the colon dose-dependently decreased in rats treated with 24R,25(OH)2D3, even at the lowest dose (1.25 p.p.m.) administered (Figure 2). Although ACF larger than four crypts per foci were correlated with the treatment doses, statistical significance was not observed. In addition, the size and number of ACF were also significantly correlated with the treatment doses.

**Experiment 2**

All rats survived until the final termination. Final body and relative liver and kidney weights did not significantly differ among groups. Water consumption was increased in groups treated with 24R,25(OH)2D3. No deposition of calcium was observed in kidneys of rats treated with 24R,25(OH)2D3. The kidney weight in group 1 was significantly different from number of ACF did not differ significantly among the groups group 3 because there were two rats with large tumors in the (Figure 3). The number of ACF appears to be higher in the distal colon in all of the carcinogen-treated groups.

**Experiment 3**

All rats survived until the final termination. Final average body weights of the animals did not differ significantly among groups 1–3. Table II summarizes the data for the BrdU labeling index of the distal colon. The BrdU labeling indices of the colonic epithelium in 24R,25(OH)2D3-treated groups were lower than in the control group in a dose-dependent manner, in particular, group 1 was significantly decreased compared with the controls, group 3. COX-2 immunoreactivity was localized in the perinuclear cytoplasm of colonic crypt cells, as well as in a few mesenchymal cells detected in between the crypts. COX-2 indices in rat colon mucosa were not significantly different in each group (0.8% in group 1, 0.76% in group 2 and 1.21% in group 3).

**Experiment 4**

Two rats in each of groups 1–3 died during the observation period. One rat in group 2 died at week 25 and had a colon adenocarcinoma and one rat in group 3 died at week 28 and had a thymoma. The tumor data for animals dying during the observation period were not available. Final average body weights of the animals did not differ significantly among groups 1–3, but the final average body weights of animals in groups 1–3 were significantly lower than in groups 4 and 5. The left kidney weight in group 1 was significantly different from group 3 because there were two rats with large tumors in the left kidney in group 1.

The colon tumor (adenoma and/or adenocarcinoma) incidence and multiplicity were significantly lower in the 5 p.p.m. 24R,25(OH)2D3 group (group 1) than in the control group (Figure 4). However, an effect of 24R,25(OH)2D3 on formation of GST-P-positive foci in the liver was not observed (Figure 5) and in other organs there were no significant differences
in incidences of tumors or preneoplastic lesions between 24R,25(OH)2D3-treated and control groups (groups 1–3) (Table III). Thus 24R,25(OH)2D3 clearly inhibited colonic tumor development but exerted no effects on tumor induction in other organs.

**Experiment 5**

The level of ODC activity in the colonic epithelium of the 24R,25(OH)2D3 group was significantly higher (0.90 ± 0.48 pmol/h/mg protein) than in the control group (0.20 ± 0.30 pmol/h/mg protein), but the level of SAT activity in the colonic epithelium of the 24R,25(OH)2D3 group was significantly lower (2.17 ± 0.66 fmol/mg protein) than in the control group (3.5 ± 1.01 fmol/mg protein).

**Discussion**

The present study has demonstrated that dietary 24R,25(OH)2D3 has a chemopreventive action against colon carcinogenesis in rats induced by DMH in the post-initiation phase but not during the initiation phase. Administration of 24R,25(OH)2D3 during the post-initiation phase reduced the number of DMH-induced ACF in colonic epithelium in a dose-dependent manner and 24R,25(OH)2D3 significantly inhibited the number and incidence of colon tumors induced by the DMBDD protocol.

The inhibitory mechanism of 24R,25(OH)2D3 is not likely through modification of the metabolic pathway of DMH, since it did not influence colon carcinogenesis on simultaneous treatment with DMH. However, it cannot be excluded that the inhibitory effect of 24R,25(OH)2D3 is mediated by calcium. Several investigators have reported that calcium and vitamin D play an important role in the inhibition of colon cell proliferation and colon cancer development (26–29). It has also been shown that the ratios of vitamin D3 metabolites in the kidney depend on the serum calcium levels (30). 24R,25(OH)2D3 and 1,25(OH)2D3 are metabolites of 25(OH)2D3 in the kidney. 24R,25(OH)2D3 is predominantly produced when serum calcium levels are >9 mg/dl, whereas production of 1,25(OH)2D3 is increased when serum calcium levels are <9 mg/dl. In the present study, serum calcium levels were increased dose-dependently in rats given 24R,25(OH)2D3, but the increase was mild in the low dose groups. Nevertheless, colonic ACF were decreased dose-dependently in rats given 24R,25(OH)2D3. Therefore, inhibition of colon carcinogenesis by 24R,25(OH)2D3 appears to depend on the serum calcium concentration. Moreover, measurement of calcium at the colonic mucosal surface might be revealing in this context.

In the present experiment, 24R,25(OH)2D3 inhibited cellular proliferation as indicated by the BrdU labeling index. However, ODC activity, a marker of increased cell proliferation (31), was elevated after 24R,25(OH)2D3 treatment. Thus, there was a discrepancy between the results for two cell proliferation markers on the effect of 24R,25(OH)2D3 on the colon mucosa. However, recently it was reported that ODC may contribute to cell differentiation (32), so that the effect of 24R,25(OH)2D3 may be partially explained by induction of colon cellular differentiation in addition to growth suppression. BrdU labeling index is a more specific measure of DNA synthesis and cell proliferation, since ODC appears to have several effects not directly related to cell proliferation. Vitamin D3 was initially reported to induce tumor cell differentiation in leukemic cell lines (33) and, furthermore, vitamin D3 inhibited growth of a colon cancer cell line and induced its differentiation (34).

Ikezaki et al. (10) reported chemopreventive effects of 24R,25(OH)2D3 on N-methyl-N’-nitro-N-nitrosoguanidine-induced glandular stomach carcinogenesis in rats during the post-initiation phase. In the present study, however, glandular stomach tumors were not significantly decreased by treatment with 24R,25(OH)2D3. Rats treated with the DMBDD protocol and with 24R,25(OH)2D3 (5 p.p.m.) had no tumors in the glandular stomach, whereas two rats treated with DMBDD without 24R,25(OH)2D3 had gastric dysplasias and one an adenoma. Since the initiating action of the DMBDD treatment in the present study appears to be weak, an inhibitory effect on glandular stomach carcinogenesis might be masked.

A chemical may act as a tumor inhibitor in one organ and as a tumor promoter in other organs (18,35,36). It is therefore important to examine the modification potential not only in a single organ, but rather in the whole body. Recently, multi-organ carcinogenesis models have been developed for the detection of carcinogens, promoters or inhibitors of the whole body in individual rats subjected to a wide-spectrum initiation protocol (18–20,37–40). The present multi-organ carcinogenesis model has already proved useful. The present study clearly indicates that 24R,25(OH)2D3 does not promote carcinogenesis in any of the organs examined. The lack of any possible interaction with carcinogen initiation allows assessment in all organs. The present study demonstrates that 24R,25(OH)2D3 inhibits colon carcinogenesis when administered in the post-initiation phase without any enhancement of carcinogenesis in other organs.

**Acknowledgement**

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

**References**


Received December 31, 1998; revised August 30, 1999; accepted September 27, 1999