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Inhibition of Development of *N,N'*-Dimethylhydrazine-induced Rat Colonic Aberrant Crypt Foci by Pre, Post and Simultaneous Treatments with 24*R*,25-Dihydroxyvitamin D_3

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It has recently been reported that new vitamin D_3 derivatives can exert inhibitory effects on colon carcinogenesis in rats. In the present study the chemopreventive potential of 24*R*,25-dihydroxyvitamin D_3 (24*R*,25(OH)$_2$vitamin D_3) was assessed in a murine model of colon carcinogenesis. In experiment 1, male 6-week-old F344 rats were administered *N,N'*-dimethylhydrazine (DMH) 20 mg/kg s.c. once a week 4 times. The rats were fed 24*R*,25(OH)$_2$vitamin D_3 at 10 ppm in the diet prior to (pre), together with (simultaneous) or after (post) DMH treatment. Modifying effects were assessed using aberrant crypt foci (ACF), putative preneoplastic lesions, as the end point markers in this model of colon carcinogenesis. After 8 weeks, pre and more markedly simultaneous administration of 24*R*,25-(OH)$_2$vitamin D_3 was found to have reduced the total numbers of ACF and significantly inhibited the development of foci. After 16 weeks, numbers of foci with $\geq$4 crypts, which are more likely to progress to tumors, were significantly reduced. The most pronounced inhibition of ACF development was noted in rats fed the 24*R*,25(OH)$_2$vitamin D_3 after DMH administration. The reduction was particularly marked in the proximal colon. Blood levels of calcium were not significantly increased over the control levels in groups administered DMH and the vitamin. Immunohistochemical staining showed numbers of proliferating cell nuclear antigen-positive cells to be lower in the colonic epithelia of rats fed the vitamin D_3 metabolite than in the controls. In experiment 2, the effect of 24*R*,25-(OH)$_2$vitamin D_3 on the alterations in c-*fos*, c-*myc* and c-*jun* oncogene expression in response to DMH administration was examined by northern blot analysis. The early increase in expression of ornithine decarboxylase (ODC) activity was not altered by 24*R*,25(OH)$_2$vitamin D_3. The results suggest that 24*R*,25(OH)$_2$vitamin D_3 is a cancer chemopreventive agent which may suppresses DMH induction of lesions and their subsequent development via an antiproliferative action.

Key words: 24*R*,25-Dihydroxyvitamin D_3 — Chemoprevention — ACF — Colon — Rat

The effectiveness of vitamin D_3 and its metabolites for prevention of neoplasia is currently being evaluated in humans and in animal models.[1] Substantial efforts are being made to elucidate the underlying mechanisms of inhibition and to find the most effective analogs.[2] It has been established that 1,25(OH)$_2$vitamin D_3, the primary steroid hormonal derivative of vitamin D_3 and 24*R*,25-dihydroxyvitamin D_3, another active metabolite, are both hydroxylation products of 25-hydroxyvitamin D_3 (25-OH-D_3).[3] 1,25(OH)$_2$vitamin D_3, a hormone regulating calcium homeostasis,[4] has been proven also to modulate the proliferation and differentiation of normal and cancer cells *in vitro*[5] and *in vivo*.[6] 24*R*,25(OH)$_2$Vitamin D_3, like the other steroid hormones, acts by binding to specific cytoplasmic and nuclear receptors in target cells.[7] In addition to its classical stimulation of parathyroid hormone secretion, intestinal calcium mobilization,[8] cartilage development[9] and bone mineralization,[10] it appears to have other unique biological effects.[11,12] 24*R*,25-(OH)$_2$Vitamin D_3 can antagonize the suppressive effect of 1,25(OH)$_2$vitamin D_3 on intracellular exchangeable calcium and on proline incorporation[13] and also exerts a significant antagonistic effect against the hypercalcemic action of 1,25(OH)$_2$vitamin D_3 in thyroparathyroidectomized rats.[14] However, 24*R*,25(OH)$_2$vitamin D_3 has until recently received relatively little attention concerning its significance for cancer development.

Many compounds, including vitamins, terpenoids, indoles and flavones,[15,16] have been shown to prevent cancer induction. Previously,[17] we reported chemopreventive effects of 22-oxa-calcitriol (OCT), a synthetic analog of vitamin D_3, on rat colon carcinogenesis. Recently, Ikezaki *et al.*[18] found that 24*R*,25(OH)$_2$vitamin D_3 can inhibit glandular stomach carcinogenesis. Therefore, its effects on a murine model of colon carcinogenesis were evaluated in the present study.

Aberrant crypt foci (ACF), first observed in the colons of carcinogen-treated rodents,[19] are considered to

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be one of the earliest hallmarks of colon carcinogenesis.[20] They can be readily identified topographically in methylene blue-stained whole colons of rodents administered carcinogenic agents. Their dysplastic appearance, increased cell proliferation and high frequency of K-*ras* and *p53* mutations,[21,22] point to a preneoplastic role during colorectal carcinogenesis in humans and in rodents, and they are now generally accepted as intermediate biomarkers for colon cancer prevention studies.[23,24] In man, ACF found in the colons of patients with familial adenomatous polyposis have been reported to be histologically more dysplastic than those in subjects with benign diseases.[25]

Many factors play essential roles in cell proliferation and differentiation, and participate in macromolecular synthesis. The induction of ornithine decarboxylase, a key regulatory enzyme for polyamine metabolism, is considered to be important for carcinogenesis and it has been applied as an intermediate biomarker of cell proliferation in cancer chemoprevention studies.[26] Similarly, the proto-oncogenes c-*fos*, c-*myc* and c-*jun* have been implicated in cell growth, differentiation and development, and show a pronounced tissue, cell-type and stage specific expression.[27,28] Induction of the c-*myc* and c-*fos* genes takes place when cells transit from G_0 to G_1 suggesting that they may have a role in regulating the cell cycle.[29] The c-*jun* protein interacts with the product of c-*fos* to form a heterodimer that is able to recognize and bind to a specific sequence of nucleotides in the promoter sites of a family of genes.[28] It has also been proposed that such protooncogenes cooperate in the induction of competence with other factors.[30]

The proliferating cell nuclear-antigen (PCNA) is an auxiliary protein for DNA-polymerase delta (δ) in S phase, expressed in genomic DNA during cell growth and division.[31,32] It has been confirmed to be a useful parameter for evaluating cell proliferative activity in an azoxymethane-induced rat colon carcinogenesis model.[33]

In the present study, we evaluated the chemopreventive activity of 24*R*,25-dihydroxyvitamin D_3 in *N*,*N'*-dimethylhydrazine dihydrochloride (DMH)-induced rat colon carcinogenesis by means of two experiments. The first was designed to elucidate the effect of feeding the vitamin at different time periods on the induction and development of preneoplastic lesions in the colonic epithelium and to determine proliferative activity of target cells by measuring PCNA. The second was conducted to assess the activity of ODC, and the early response of the protooncogenes c-*fos*, c-*myc* and c-*jun* to DMH administration, in the colons of rats fed 24*R*,25$(OH)_2$vitamin D_3.

MATERIALS AND METHODS

Chemicals and diet DMH (purity 99%) was obtained from Wako Chemicals Co., Osaka. 24*R*,25$(OH)_2$Vitamin D_3 was obtained from Kureha Chemical Ind. Co., Ltd., Tokyo, and administered at a concentration of 10 ppm in CE-2 powdered basal diet (Clea Japan Inc., Tokyo).

Animals Male F344 rats were obtained at 5 weeks of age (Charles River Japan Inc., Hino) and housed in plastic cages with wood chips for bedding in an air-conditioned animal room at a temperature of 22 ± 1°C and a relative humidity of 55.5 ± 1%, with a 12 h light-12 h dark cycle. Diet and water were available *ad libitum*, and body weights and food intake were measured weekly during the experiments.

Experiment 1 (experimental protocol: Fig. 1): One hundred and twenty-five rats at 6 weeks of age after a 1 week

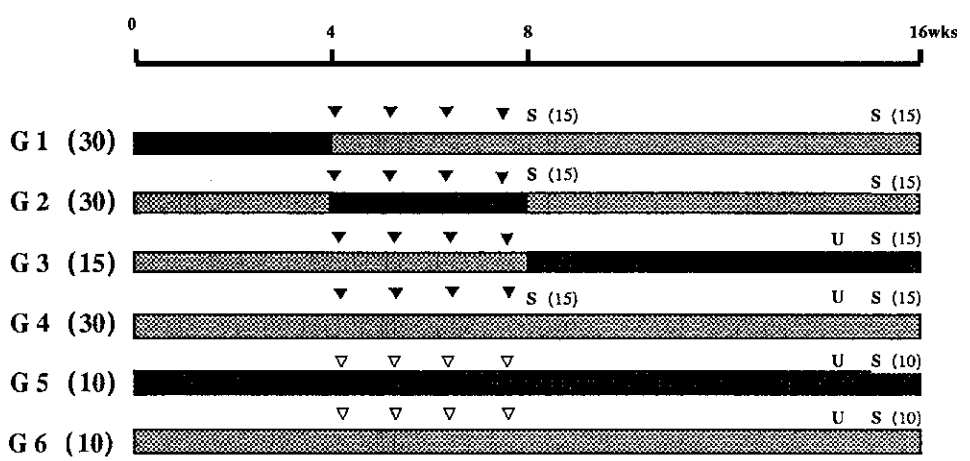

Fig. 1. Experimental protocol (1). ▼ DMH 20 mg/kg s.c. ▽ 0.09% saline 20 mg/kg s.c. U: urinalysis. ■ 24,25$(OH)_2D_3$ 10 ppm in CE2 diet. ▨ No treatment, basal diet (CE2). S: animals killed and blood analyzed.

acclimation period, were divided randomly into 6 groups. From week 5 after the start, those in groups 1–4 were given s.c. injections of DMH (20 mg/kg body wt.) once a week for 4 weeks. Rats in groups 5 and 6 were injected with 0.9% saline (vehicle) at the same time, in the same manner. Animals of group 1 (30 rats) were administered the 24*R*,25(OH)$_2$vitamin D_3 diet from week 1 to week 4 (pre DMH). Group 2 (30 rats) received the vitamin D_3 diet simultaneously with DMH administration (weeks 5–8), and group 3 (15 rats) was given the vitamin D_3 diet post DMH treatment (weeks 9 to 16). Group 5 (10 rats) was offered the 24*R*,25(OH)$_2$vitamin D_3 diet from the first week to the end of the experiment. Animals of groups 4 (30 rats) and 6 (10 rats) were given the basal powdered CE-2 diet without vitamin D_3 supplementation. The total period of the experiment was 16 weeks.

To determine the modifying effects of vitamin D_3 in a short-term assay, 15 rats each from groups 1, 2 and 4 were killed by over-anesthesia at the end of the 8th week. Colons were quickly excised, flushed with saline, and inflated by intraluminal injection of 10% phosphate-buffered formalin solution, slit along the longitudinal median axis from cecum to anus, and fixed flat between two pieces of filter papers in 10% phosphate-buffered formalin. At the termination of the experiment (the end of week 16), all remaining animals were killed, and the colons were again prepared as mentioned above.

Analysis of blood and urine Fresh urine samples were collected from all animals by forced urination in the morning one day before the termination. The pH was measured (Horiba model F-15 pH meter, Kyoto) and the urine samples were analyzed for calcium, sodium, potassium and phosphorus (Hitachi 710 electrolyte analyzer, Tokyo).

Blood was collected at autopsy using 10 ml heparinized syringes and immediately centrifuged at 4000 rpm for 15 min at 4°C. Supernatant plasma was collected and preserved frozen at −80°C before measurement of calcium (Hitachi 7150 electrolyte analyzer, Tokyo), total protein, albumin (Hitachi 736) and globulin (Aloka ARC 950, Tokyo).

ACF count After fixation for at least 24 h at 4°C, colons were stained in 0.2% methylene blue (in H_2O) for 3–5 min. After staining, they were divided into proximal, intermediate, and distal segments and examined for ACF by light microscopy at 40× and 100× magnification using the following criteria for identification: a) increased size comparing to normal crypts, b) enlarged pericryptal zone, c) slight elevation above the surrounding mucosa, and d) frequently more elongated shape of the luminal opening. The number of ACF in each segment, as well as the number of aberrant crypts in each focus, the "crypt multiplicity," were counted.

Tissue processing Directly after scoring ACF, samples collected from the proximal, intermediate and distal colons were embedded in paraffin and sectioned at 3–4 μm.

Immunohistochemical staining Anti-PCNA antibody (Dako Co., Ltd., Kyoto) was used with the avidin-biotin complex method.[34] Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series, and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. They were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining, and overnight at 4°C with mouse monoclonal antibody to PCNA (Dako-PCNA, PC 10, Denmark) diluted 1 : 500. After exposure for 30 min at room temperature to biotinylated horse anti-mouse IgG (Vector Lab. Inc., Burlinghame, CA), sections were incubated with avidin-biotin-peroxidase complex at 1 : 25 dilution. Each step was followed by washing with TBS. Peroxidase activity was visualized by treatment with 0.02% diaminobenzidine tetrahydrochloride containing 0.05% hydrogen peroxide. The nuclei were counterstained with hematoxylin or methyl green. At least 10 sections from different parts of the colons of all animals were stained for PCNA. Numbers of positively stained nuclei in 20 complete crypts from each section were counted and divided by the total number of nuclei of the crypts to generate the PCNA-positive index.

Experiment 2 (experimental protocol: Fig. 2): Thirty rats were randomized at 6 weeks of age into two groups; group 1 (15 rats) was fed 24*R*,25(OH)$_2$vitamin D_3 10 ppm in the diet for 4 weeks. Group 2 (15 rats) was offered the basal diet CE-2 for the same time period. At the end of the 4th week, both groups received one s.c. injection of DMH (20 mg/kg body wt.). Three animals from each group were killed at 0 h, 2 h, 6 h, 12 h and 48 h after injection. Whole colon scrapings were prepared for northern blot analysis and frozen at −80°C for subsequent measurement of ODC activity.

ODC assay The activity of ODC was assayed by a radiometric technique which measures the release of

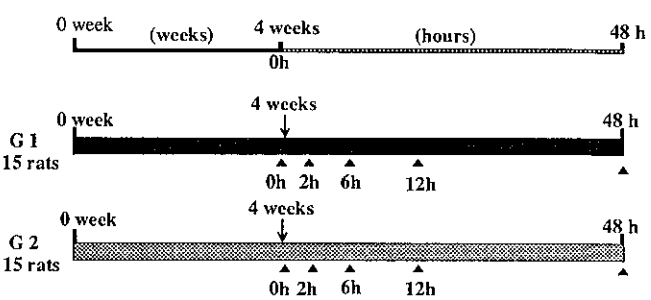

Fig. 2. Experimental protocol (2). ■ 24,25(OH)$_2$D$_3$ 10 ppm in CE2 diet. ▨ basal diet; (CE2). ↓ DMH 20 mg/kg s.c. ▲ animals killed: 3 rats at 0, 2, 6, 12 and 48 h.

$^{14}CO_2$ from L-[1-^{14}C]ornithine as previously described.[35] Frozen mucosal samples were suspended in 0.5 ml of 50 m*M* Tris-HCl, pH 7.5, 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 activity.

Northern blot assay Total RNA was extracted from mucosal samples by the guanidinium thiocyanate-phenol-chloroform method.[36] For RNA hybridization, aliquots of 20 μg of total RNA were fractionated on 1% agarose gels and transferred to nylon membranes (Hybond N; Amersham Int., Buckinghamshire, UK). The membranes were prehybridized and then hybridized with probes for c-*fos* 4th exon (Cat. No. 7025, Takara Shuzo Co., Shiga), c-*myc* 3rd exon (Cat. No. P2110, Oncor, Gaithersburg, MD), c-*jun* cDNA, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, labeled with [α-^{32}P]dCTP by using a Rediprime labeling system (Amersham). c-*jun* cDNA was kindly supplied by the Japanese Cancer Research Resources Bank, and GAPDH cDNA was a generous gift from Dr. Ph. Fort (Université des Sciences et Techniques du Languedoc Laboratoire de Biologie Moleculaire, Montpellier, France). Signals were detected by autoradiography and quantified using an image analyzer (BAS 2000 II, Fuji, Tokyo). To normalize the amounts of RNA applied to the gel, the amounts of c-*fos*, c-*myc* or c-*jun* mRNA relative to those of GAPDH mRNA were calculated.

Statistical analysis The significance of differences between mean values for lesions and immunohistochemistry data were analyzed by using Duncan's new multiple range analysis, super ANOVA; (1989-1991 Abacus Concepts, Inc., USA). The significance of differences between groups was assessed by using Cochran's two-tailed Student's *t* test.

RESULTS

Body weight and food consumption The data for the body weights of rats killed at weeks 8 and 16 are shown in Table I. At week 16, average body weights of animals treated with the carcinogen did not significantly differ among groups 1–4. However, animals of group 5 showed significant body weight loss as compared to group 6. Food consumption was similar among groups and the mean daily intake of 24*R*,25(OH)$_2$vitamin D_3 was essentially the same in all cases (data not shown).

Number and multiplicity of the DMH-induced ACF Tables II and III summarize data for the effects of 24*R*,25(OH)$_2$vitamin D_3 on induction or development of

Table I. Body Weights of Rats Killed at Weeks 8 and 16 (Experiment 1)

Group	No. of rats	Treatment	Body weight (g)			
			Rats killed at week 8		Rats killed at week 16	
			No. of animals	Body wt.	No. of animals	Body wt.
1	30	Vit. D_3 (pre) +DMH	15	262.9±9.7	15	300.6±11.9
2	30	Vit. D_3 (simult.) +DMH	15	258.3±9.3	15	294.3±15.9
3	15	Vit. D_3 (post) +DMH	—	—	15	296.9±20.3
4	30	Basal diet+DMH	15	261.5±14.5	15	300.7±17.0
5	10	Vit. D_3+Saline	—	—	10	291.5±9.4*
6	10	Basal diet + Saline	—	—	10	315.8±24.6

Mean±SD.
* Significantly different from group 6 at $P<0.02$; Cochran's two-tailed *t* test.

Table II. Effects of 24*R*,25(OH)$_2$Vitamin D_3 on DMH-induced Colon ACF Formation in F344 Rats Killed after 8 Weeks (Experiment 1)

Group	Treatment	No. of rats	Total No. of ACF	No. of foci containing:			
				1 crypt	2 crypts	3 crypts	≧4 crypts
1	Vit. D_3 (pre)+DMH	15	157.9±63.4*	133.4±55.9*	21.8±8.9*	2.5±2.3*	0.2±0.6*
2	Vit. D_3 (simult.)+DMH	15	140.4±58.9*	126.7±51.5*	12.2±7.8*	1.5±1.8*	0*
4	Basal diet+DMH	15	254.9±81.2	214.3±62.6	34.3±14.0	9.0±1.7	1.9±1.7

Means±SD.
* All values of ACF in groups 1 and 2 were significantly different from the respective group 4 values at $P<0.01$ (super ANOVA; Duncan's new multiple range analysis).

Table III. Effects of 24*R*,25(OH)$_2$Vitamin D$_3$ in DMH-induced Colon ACF Formation in F344 Rats Killed after 16 Weeks

Group	Treatment	No. of rats	Total No. of ACF	No. of foci containing:			
				1 crypt	2 crypts	3 crypts	≧4 crypts
1	Vit. D$_3$ (pre)+DMH	15	295.7±37.7	97.1±28.6[b]	119.1±41.4	55.6±17.9	18.3±11.8[a]
2	Vit. D$_3$ (simult.)+DMH	15	304.7±66.7	87.4±30.4[b]	122.5±43.5	67.1±27.1	27.7±14.3[a]
3	Vit. D$_3$ (post)+DMH	15	269.5±48.0[b]	103.5±33.3[b]	96.6±19.7	48.4±10.2[b]	17.3±6.7[a]
4	Basal diet+DMH	15	352.0±103.2	133.9±55.4	98.3±28.9	72.1±26.9	47.7±14.6

Mean±SD.
Significantly different from group 4 at: *a*) $P<0.01$, *b*) $P<0.05$ (super ANOVA; Duncan's new multiple range analysis).

Table IV. Blood Concentration Levels of Calcium, Total Protein and Albumin, and A/G Ratio after 16 Weeks

Group	Treatment	No. of rats	Ca (mg/dl)	Total protein	Albumin	A/G ratio
1	Vit. D$_3$ (pre)+DMH	10	10.45±0.13	6.25±0.12	2.53±0.02	0.67±0.02
2	Vit. D$_3$ (simult.)+DMH	10	10.32±0.36	6.06±0.23	2.54±0.15	0.72±0.04
3	Vit. D$_3$ (post)+DMH	10	11.37±0.13	6.04±0.12	2.6±0.05	0.75±0.01
4	Basal diet+DMH	10	10.91±0.72	6.01±0.28	2.5±0.12	0.71±0.03
5	Vit. D$_3$+Saline	10	12.01±0.41*	6.24±0.15	2.67±0.08	0.75±0.03
6	Basal diet+Saline	10	10.41±0.15	6.1±0.18	2.6±0.11	0.72±0.03

Mean±SD.
* Significantly different from group 6 at $P<0.001$; Cochran's two-tailed *t* test.

Table V. Urinary Levels of Calcium, Sodium, Potassium and Phosphorus and pH after 16 Weeks

Group	Treatment	No. of rats	Ca (mg/dl)	Na (mg/dl)	K (mg/dl)	P (mg/dl)	pH
1	Vit. D$_3$ (pre)+DMH	10	5.58±1.6	125.0±58.2	320.8±75.0	228.04±43.1	–
2	Vit. D$_3$ (simult.)+DMH	10	5.3±2.5	91.4±46.2	344.5±75.9	220.6±38.4	–
3	Vit. D$_3$ (post)+DMH	10	61.7±14.2[a]	32.0±30.5[a]	92.2±34.5[a]	236.6±37.04	6.5±0.3[b]
4	Basal diet+DMH	10	5.75±0.96	142.8±57.6	353.84±51.8	258.02±51.2	6.8±0.3
5	Vit. D$_3$+Saline	10	46.7±13.2[d]	47.6±8.9[c]	117.06±13.8[c]	183.9±36.4	6.6±0.3[c]
6	Basal diet+Saline	10	14.9±8.8	116.3±26.8	334.2±52.8	187.1±55.1	7.4±0.18

Significantly different from group 4 at; *a*) $P<0.001$ and *b*) $P<0.05$.
Significantly different from group 6 at; *c*) $P<0.001$ and *d*) $P<0.01$.
Cochran's two-tailed *t* test.

ACF by DMH in experiment 1. The rats treated with DMH (groups 1, 2, 3 and 4) showed 100% incidence of ACF, in contrast to the complete lack of such lesions without DMH treatment (groups 5 and 6). In rats killed after 8 weeks, average total numbers of ACF/colon, as well as the numbers of foci containing 1, 2, 3 and ≧4 crypts, were significantly decreased by pre and simultaneous feeding of 24*R*,25(OH)$_2$vitamin D$_3$ (Table II). The inhibition was marked along the length of the colons and was not restricted to a certain region only. Concurrent feeding of the vitamin with DMH administration caused the more pronounced inhibition. After 16 weeks, large ACF containing ≧4 crypts, which have been reported to be more likely to develop into tumors,[37] were significantly inhibited by the pre, simultaneous and post initiation regimens (Table III). Numbers of foci containing 1 crypt were also significantly inhibited by the three treatment regimes, though only post initiation treatment significantly inhibited total numbers of ACF and foci containing 3 crypts.

Urine electrolyte concentration levels and plasma biochemistry Rats which were fed the 24*R*,25(OH)$_2$vitamin D$_3$ and administered DMH (groups 1–4), showed no significant increase in plasma calcium (Table IV), although slight elevation was observed for group 3, given the vitamin at termination. However, animals of group 5

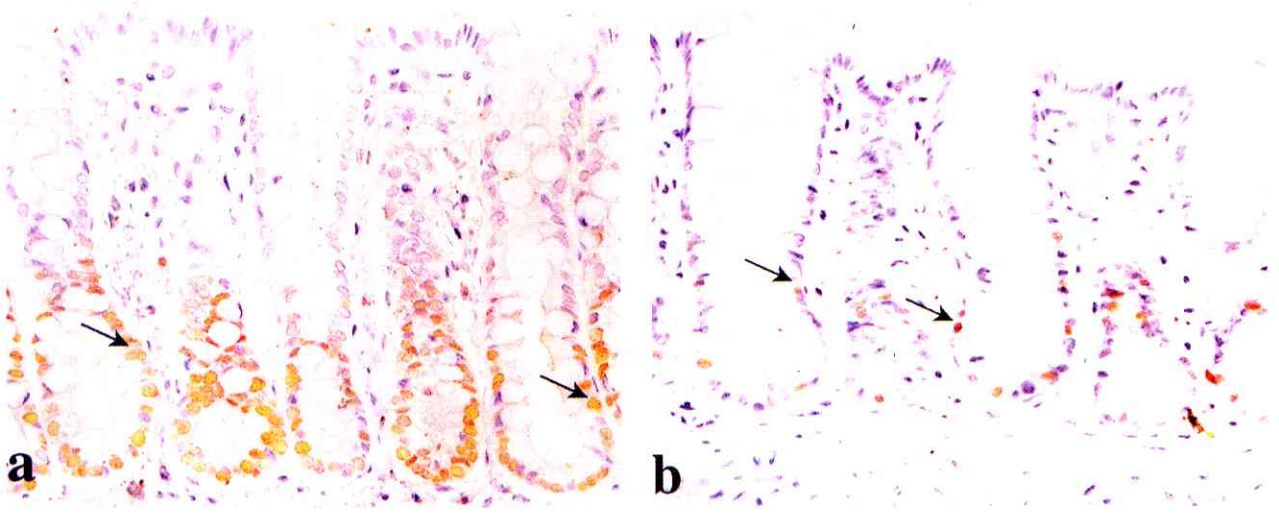

Fig. 3. a, PCNA immunohistochemical staining of DMH-treated rat colonic epithelium showing high numbers of positive nuclei (arrows) (×1000). b, PCNA immunohistochemical staining of the colonic epithelium of a rat fed 24*R*,25$(OH)_2$vitamin D_3 post DMH administration, showing a reduced number of positive nuclei (arrows) (×1000).

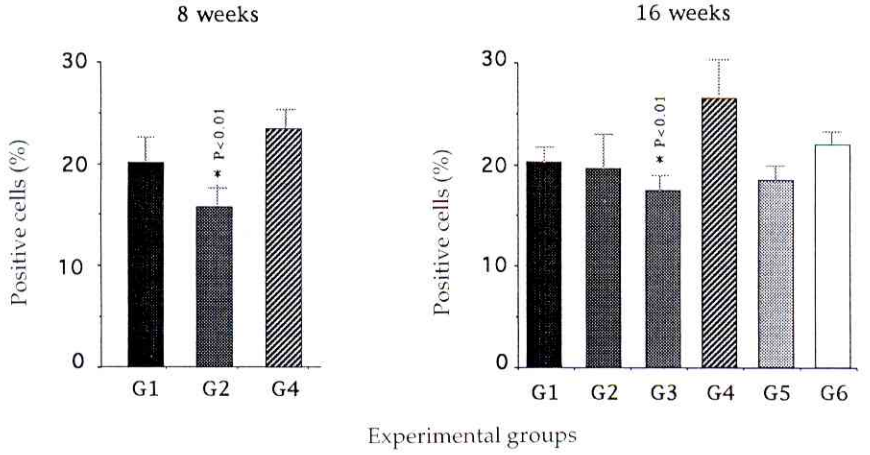

Fig. 4. PCNA-positive cell ratios in colonic crypts in rats treated with DMH and 24*R*,25$(OH)_2$vitamin D_3 (Experiment 1). * Significantly different from group 4 at $P<0.01$ (super ANOVA; Duncan's new multiple range analysis).

showed an increase in plasma calcium as compared to the controls (group 6). Urinary excretion of calcium was significantly increased in groups 3 and 5 (Table V). The levels of sodium and potassium in the urine were also decreased in groups 3 and 5, although values for phosphorus did not differ. Plasma values of total protein, albumin and albumin/globulin ratio were not changed among the groups.

Anti-PCNA staining Immunohistochemical examination showed PCNA-positive nuclei to be mostly located in the lower third of the crypts (Fig. 3, a and b). Fig. 4 illustrates differences in the ratios of PCNA-positive nuclei in rat colonic crypts. Simultaneous feeding of 24*R*,25-$(OH)_2$vitamin D_3 was found to reduce significantly the PCNA-positive nuclei ratio in the mucosal crypts at 8 weeks as compared to the group 4 value. Post treatment with the vitamin was shown to cause a significant reduction in the PCNA-index of the colonic epithelium of rats of group 3 at 16 weeks.

Sequential changes in ODC activity Fig. 5 summarizes

data for sequential changes in ODC activity. Basal levels of colonic mucosal ODC activity in the 24*R*,25(OH)$_2$-vitamin D$_3$-treated and control groups were similar (0 h). At 2 h after DMH injection, the mucosal ODC activity was increased approximately 2-fold in both groups, then it fell below the basal level at 6 h and 12 h. At 48 h, however, the ODC activity of the vitamin-treated group was about 65% of the control value. However, this decrease was not statistically significant.

Sequential changes in c-*fos*, c-*myc* and c-*jun* mRNA (Figs. 6 and 7) Northern blotting analysis data processed by image analyzer showed basal levels (0 h) of c-*jun* and c-*fos* expression to be similar in the two groups, while that of c-*myc* was higher in the 24*R*,25(OH)$_2$vitamin D$_3$ than in the control group. After DMH administration, the mRNA expression levels in the 24*R*,25-(OH)$_2$vitamin D$_3$ treated-group were maximal at 2 h for c-*jun* and c-*myc* and at 6 h for c-*fos*. Moreover, after 6 h, a dramatic down-regulation in the expression of the three

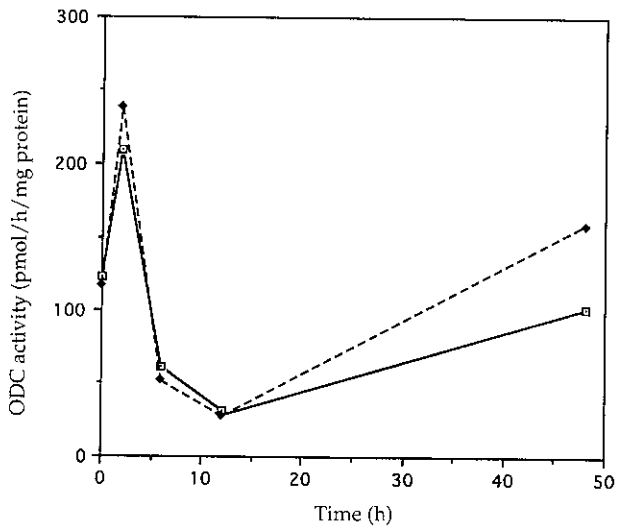

Fig. 5. Effects of 24*R*,25(OH)$_2$vitamin D$_3$ on the changes of ODC activity after DMH injection (Experiment 2). □ 24*R*,25(OH)$_2$vitamin D$_3$, ◆ control.

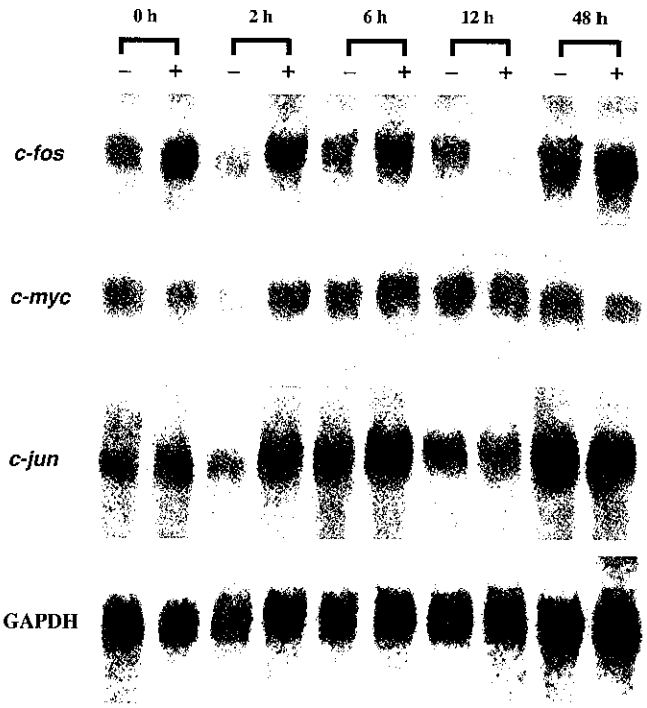

Fig. 6. Sequential changes in expression of c-*fos*, c-*myc* and c-*jun* mRNAs in rat mucosa (Experiment 2). Amounts of c-*fos*, c-*myc* and c-*jun* mRNAs applied to the gel were normalized relative to GAPDH mRNA.

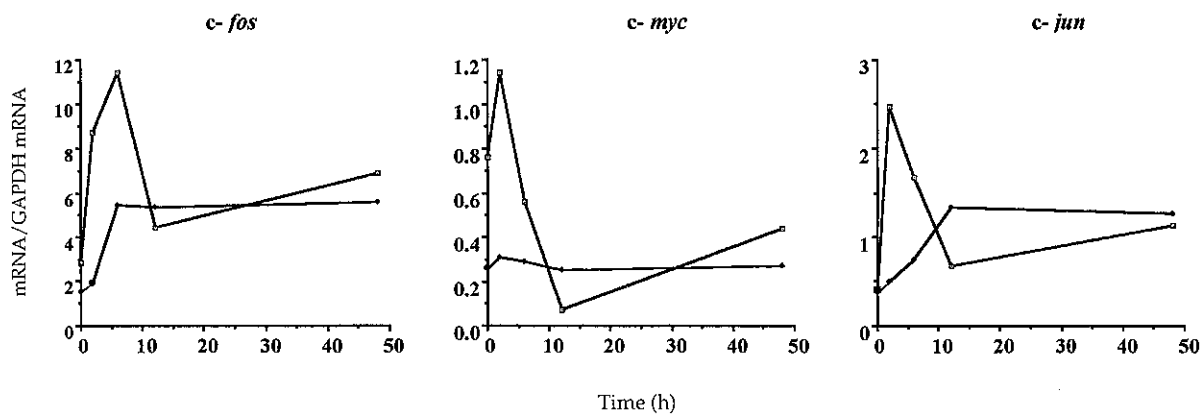

Fig. 7. Quantification of the sequential changes in c-*fos*, c-*myc* and c-*jun* mRNAs (Experiment 2). □, 24*R*,25(OH)$_2$vitamin D$_3$, ◆ control.

proto-oncogenes occurred. However, at 12 h, the expression of the oncogenes was almost the same as in the control.

DISCUSSION

The present results indicate that 24*R*,25$(OH)_2$vitamin D_3 can inhibit DMH-induced colon ACF when administered in the pre-initiation, initiation or post-initiation phase of carcinogenesis. The observed decrease in induction of ACF paralleled a decrease in the percentage of PCNA-positive cells in the colonic crypts especially in the cases of simultaneous treatment at 8 weeks and post initiation treatment at 16 weeks. The findings demonstrate an *in vivo* antiproliferative, chemopreventive effect of vitamin D_3 on pre-malignant lesions of the colon and show that the early response and behavior of the protooncogenes c-*fos*, c-*myc* and c-*jun* following administration of DMH is altered by 24*R*,25$(OH)_2$vitamin D_3.

Reduction of ACF number and multiplicity, as well as the relation of cancer chemoprevention in general to the initiation phase of carcinogenesis, has been discussed previously.[37–39] Belleli *et al.*[6] showed colon tumor numbers to be decreased by 50% in rats receiving 1,25-$(OH)_2$vitamin D_3 prior to carcinogen administration, but found that the same treatment during the initiation or post initiation phases did not change the tumor incidence. However, we found[17] clear chemoprevention of colon cancer and ACF development in rats by OCT, when administered in the post-initiation phase. Furthermore, our recent investigations (unpublished data), indicated that simultaneous or pre-treatment with OCT significantly inhibits ACF formation in the colon of rats treated with DMH. Recently, various doses of 24*R*,25-$(OH)_2$vitamin D_3,[18] were also shown to decrease the development of cancerous and precancerous lesions in the glandular stomach of rats when administered during the post-initiation phase. Previously, Wattenberg[40] classified carcinogenesis inhibitors according to the phase in which they show inhibitory effects in animal models of carcinogenesis: 1) inhibitors of the formation of the active carcinogen itself, 2) "blocking agents" which block the carcinogen at the initiation phase, preventing it from reaching or reacting with target sites, and 3) "suppressing agents" that suppress the formation of neoplasia when administered in the post initiation phase. In our model of administration, 24*R*,25$(OH)_2$vitamin D_3 inhibited the formation of ACF in all phases. Previous studies have demonstrated antiproliferative and inhibitory properties of vitamin D_3 and its analogs on colon carcinogenesis.[41,42] A decrease in numbers of PCNA-positive cells reflects a decrease in S phase cells and thus reduced proliferative activity.[43] It is noteworthy that the treatment regimes administered here reduced the proliferative activity in the target cells without any pathological features in the colon, and were not accompanied by any significant increase in single cell death as detected by nick-end labeling index staining in the colonic mucosa (data not shown). Such inhibition of cellular proliferative activity without cell death may correlate with suppression of the production of some autocrine growth factor(s) by target cells.[44]

ODC activity is known to be increased in tumor tissue compared with normal tissue,[45] and the aberrant regulation of ODC is reported to play a role in neoplastic transformation and tumor growth.[46] Recently, it was demonstrated that there is an intimate correlation between ODC and c-*myc* genes.[47,48] Here, the early response of ODC activity was very similar to that described by Luk *et al.*,[49] who measured changes in rat colonic mucosal ODC activity during azoxymethane induction of colonic carcinogenesis. While the present study showed that the early expression of ODC activity after DMH administration was not altered by 24*R*,25$(OH)_2$vitamin D_3, the possibility that 24*R*,25$(OH)_2$vitamin D_3 may exert effects on ODC activity over a longer time period, especially during tumor progression, may deserve further attention.

The early *in vivo* expression of proliferation and differentiation-related genes, c-*fos*, c-*myc* and c-*jun*, was examined at the molecular level after DMH administration. These genes were chosen because of their involvement in the molecular sequence of events leading to colon cancer.[50–52] It is well known that c-*myc* plays a pivotal role in the regulation of cell proliferation,[53] and that c-*jun* and c-*fos* expression is up-regulated in human colorectal adenomas and adenocarcinomas.[51] Reitsma *et al.*[54] showed that vitamin D_3 metabolites reduced c-*myc* transcription in HL-60 leukemia cells to 50% of the initial value within 4 h and to 20–30% by 12 h. Stopera and Bird[55] showed that inhibition of growth of ACF in rat colons by all-*trans* retinoic acid was accompanied by a reduction in c-*myc* expression and an increase in c-*fos* mRNA transcription in these lesions. Their interpretation was that c-*myc* is more related to cell proliferation, while c-*fos* correlates with cell differentiation. The increase in the expression of the protooncogenes in the first 2 hours after DMH administration that we observed is inconsistent with previous literature. This discrepancy could be due to the fact that the expression of the three protooncogenes was evaluated in non-involved crypts, while the previous data referred to the effect of chemopreventive agents on the expression of the protooncogenes in ACF and tumors. Furthermore, the decrease of Ca^{2+} concentration in the large intestinal lumen, which might be correlated to lower pH,[56] may have resulted in the increase of sensitivity to DMH-induced expression of oncogenes. Therefore, further measurements of Ca^{2+} concentration

in the lumen of the colon, as well as intracellular Ca^{2+} concentration, may be useful. The reduction in the PCNA-positive index here, correlate with the decrease in the ACF numbers in the colonic mucosa after the administration of the vitamin. Previously, it was shown that induction of c-*myc* and c-*fos* is a phenomenon that normally occurs during recruitment of quiescent cells *in vivo*.[29] If administration of 24*R*,25(OH)$_2$vitamin D_3 reduced the number of S phase cells, that might explain the effect of preinitiation phase administration.

The slight elevation in plasma calcium and significant increase in urinary calcium in group 3, which received the vitamin post DMH administration, supports the suggestion that the action of vitamin D_3 metabolites may be mediated by this cation. Sodium and potassium ions concentrations were also increased in the urine of groups 3 and 5, but phosphorus excretion was normal. There are several lines of evidence supporting a role for Ca^{2+} in the inhibition of colonic cell proliferation and in colon tumor prevention.[57] Colonic epithelial cells exhibit decreased proliferation and induction of differentiative characteristics with increasing levels of calcium *in vivo* and *in vitro*.[58] Moreover, raising the calcium concentration of the diet has been found to reduce mouse colonic mucosal cell proliferation and hyperplasia induced by a western diet.[59] Measurement of the intracytoplasmic Ca^{2+} concentration in colon mucosa thus appears warranted.

In summary, the present investigation of the effects of 24*R*,25(OH)$_2$vitamin D_3 has demonstrated inhibition of the development of ACF, as well as reduction in the proliferative activity of the colonic mucosa. Although the results require confirmation in a long-term experiment, they do provide support for human intervention trials of vitamin D as a cancer chemopreventive agent.

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