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Chemopreventive Effects of 24*R*,25-Dihydroxyvitamin D_3, a Vitamin D_3 Derivative, on Glandular Stomach Carcinogenesis Induced in Rats by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine and Sodium Chloride[1]

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ABSTRACT

The modifying effects of 24*R*,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$], a vitamin D_3 derivative, on glandular stomach carcinogenesis were investigated in male Wistar rats by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and sodium chloride exposure during the postinitiation phase. A total of 130 male 6-week-old rats was divided into five groups. Groups 1–3 (consisting of 30 rats/group) were given MNNG in drinking water at a concentration of 100 ppm and were simultaneously fed a diet supplemented with 10% NaCl for 8 weeks. They were fed a diet containing either 5.0 ppm (group 1) or 2.5 ppm (group 2) 24R,25$(OH)_2D_3$ or were kept on the basal diet alone (group 3) for the following 57 weeks. Rats in groups 4 and 5 were given 24R,25$(OH)_2D_3$, as were animals in groups 1 and 3, but did not receive the MNNG + NaCl treatment. The total incidence of combined atypical hyperplasias and adenocarcinomas in the glandular stomachs was significantly lower in group 1 (24%) than in group 3 (70%; $P < 0.01$). The mean numbers of atypical hyperplasias or adenocarcinomas of the glandular stomachs in groups 1 (0.31) and 2 (0.66) were also significantly decreased ($P < 0.01$ and $P < 0.05$, respectively) as compared to the group 3 value (1.21). Thus, the development of cancerous and precancerous lesions in the glandular stomach was decreased by exposure to 24R,25$(OH)_2D_3$ in a dose-dependent manner. Urinary calcium levels were increased by this vitamin D_3 derivative (in line with the applied dose) when assayed at 10, 30, and 62 weeks, regardless of the MNNG + NaCl treatment. The present results clearly indicate that 24R,25$(OH)_2D_3$ exerts chemopreventive effects, possibly by influencing calcium pharmacodynamics, when given during the postinitiation phase of glandular stomach carcinogenesis in rats.

INTRODUCTION

Epidemiological studies have suggested that elevated calcium intake and dietary or endogenously synthesized vitamin D may decrease the risk of gastrointestinal cancer (1). In fact, calcium supplementation has been shown to inhibit colon carcinogenesis in rats (2, 3). We have also shown that calcium chloride ingestion has a dose-dependent chemopreventive effect on glandular stomach carcinogenesis in rats when given during the postinitiation phase (4). In addition, 1,25$(OH)_2D_3$,[3] a major active form of vitamin D, has been proven to inhibit colon (5), skin (6, 7), and mammary (8) carcinogenesis in rats or mice, although this vitamin D derivative has been reported to cause excessive hypercalcemia (5, 9). Another active form of vitamin D, 24R,25$(OH)_2D_3$, has been reported to cause milder hypercalcemia than 1,25$(OH)_2D_3$ (9, 10). It is known that 24R,25$(OH)_2D_3$ induces some histopathological as well as pharmacological changes in bone tissue (11–14), but no information on the action of cancer prevention with this chemical has yet been available.

MNNG is a well-known carcinogen applied to induce gastric carcinomas in rodents (15). Based on the enhancing effects of NaCl on glandular stomach tumorigenesis in rats, an initiation-promotion carcinogenesis model has been established in our laboratory, using simultaneous administration of MNNG and NaCl (16). In the present study, the modifying effects of 24R,25$(OH)_2D_3$ on stomach carcinogenesis were investigated by exposure during the postinitiation phase in the two-stage model.

MATERIALS AND METHODS

Chemicals and Animals. MNNG was a commercially available preparation obtained from Aldrich Chemical Co. (Milwaukee, WI). NaCl (purity >99.5%) was purchased from Wako Pure Chemicals, Inc. (Osaka, Japan). 24R,25$(OH)_2D_3$ was generously donated by Kureha Chemical Co. Ltd. (Tokyo, Japan). Male 6-week-old Wistar rats (Japan SLC Inc., Shizuoka, Japan) were housed five animals per polycarbonate cage and were maintained under standard laboratory conditions (room temperature, 23 ± 2°C; relative humidity, 60 ± 5%; a 12 h/12 h light/dark cycle). They were fed a basal diet, Oriental MF (Oriental Yeast Co. Ltd., Tokyo, Japan), supplemented with or without the test chemical.

Experimental Protocol. As shown in Fig. 1, rats in groups 1–3 (each group consisted of 30 animals) were given MNNG in drinking water at a concentration of 100 ppm and were simultaneously fed a diet supplemented with 10% NaCl for 8 weeks. They were then given a diet supplemented with 24R,25$(OH)_2D_3$ at doses of 5.0 ppm (group 1), 2.5 ppm (group 2), or 0 ppm (group 3) for the following 57 weeks. The doses of 24R,25$(OH)_2D_3$ were chosen based on the experiment in rats (12). The 20 rats of group 4 were fed 5.0 ppm 24R,25$(OH)_2D_3$ alone during the 57 weeks (similar to group 1) but did not receive the MNNG + NaCl treatment. Group 5 (20 animals) served as a nontreatment control. The animals were observed daily for toxicological symptoms and were weighed once a month. At the end of experiment (65 weeks), all surviving animals were killed and autopsied. At autopsy, lung, liver, kidney, and heart were excised and weighed. The stomachs were subjected to a particularly careful macroscopic examination before fixation in 10% buffered formalin and then were cut into longitudinal strips (3-mm wide) for examination of the entire gastric mucosa. After processing for histology by routine methods, sections were stained with H&E. Calcium and phosphorus levels in urine samples collected over 24-h periods at weeks 10, 30, and 62 were analyzed using a Hitachi 736–60E autoanalyzer (Hitachi Ltd., Tokyo, Japan). Serum biochemical parameters were also determined (using a Hitachi 736–60E autoanalyzer) in blood collected from the abdominal aorta immediately before autopsy.

Statistical Analysis. The data for lesion incidences were analyzed using Fisher's exact test, and the data for lesion multiplicities, organ weights, and biochemical quantitation were examined using Student's *t* test.

RESULTS

Consumption of 24R,25$(OH)_2D_3$ and Mortality. The mean daily intake of 24R,25$(OH)_2D_3$ in groups 1–5 (calculated from food consumption and mean body weight values) was 25.5, 13.1, 0, 23.2, and 0 μg/100 g body weight, respectively. The intake of 24R,25$(OH)_2D_3$ correlated well with the dose levels used in the present study. One animal in each of groups 1–3 was found dead at weeks 60, 52, and 63,

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[3] The abbreviations used are: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24R,25$(OH)_2D_3$, 24*R*,25-dihydroxyvitamin D_3; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 1(OH)D_3, 1-hydroxyvitamin D_3.

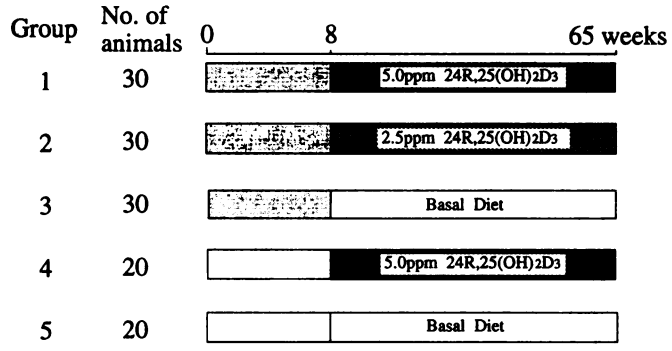

Fig. 1. Experimental design. ☐, 100 ppm MNNG in drinking water + 10% NaCl in diet.

respectively. All surviving rats were included in the effective numbers.

Body and Organ Weights. Although body weight gain was remarkably suppressed in animals given the MNNG + NaCl treatment as compared to rats without the treatment, especially in the beginning of experiment, the body weights had recovered by the termination of the experiment (Fig. 2). Regardless of administration of 24R,25$(OH)_2D_3$, rats in the groups exposed to MNNG + NaCl (groups 1–3) showed body weight curves similar to those of the groups without exposure (groups 4 and 5) toward termination of the experiment, indicating that even the high dose of 24R,25$(OH)_2D_3$ did not affect body weight gain. In group 1, the absolute heart weights were significantly depressed ($P < 0.01$), and the relative kidney weights were significantly elevated ($P < 0.01$) as compared to the respective values of the control (group 3). There were no statistical differences among groups in the lung and liver weights, with or without the MNNG + NaCl or the 24R,25$(OH)_2D_3$ treatment.

Effects of 24R,25$(OH)_2D_3$ on Glandular Stomach Carcinogenesis. Cancerous and precancerous lesions in the glandular stomach were all diagnosed as adenocarcinomas and atypical hyperplasias (Fig. 3), respectively, as described previously (4, 16, 17). As shown in Table 1, the incidences of atypical hyperplasias of glandular stomachs in groups 1 (21%) and 2 (38%) were significantly lower ($P < 0.01$ and $P < 0.05$) than in group 3 (66%). The total incidence of combined atypical hyperplasias and adenocarcinomas in group 1 (24%) was also significantly lower ($P < 0.01$) than that of group 3 (70%), although the development of adenocarcinomas was not frequent in MNNG-treated rats, regardless of 24R,25$(OH)_2D_3$ treatment. The total incidence of combined proliferative lesions was dose-dependently decreased by 24R,25$(OH)_2D_3$ treatment in both fundic and pyloric mucosa and was statistically significant in the former case ($P < 0.01$). No rats in groups 4 and 5 developed adenocarcinomas or atypical hyperplasias. As shown in Table 2, the mean numbers of atypical hyperplasias plus adenocarcinomas per animal, *i.e.*, multiplicities, in the glandular stomachs of groups 1 (0.31) and 2 (0.66) were significantly decreased ($P < 0.01$ and $P < 0.05$, respectively) as compared to the group 3 value (1.21). The multiplicities of atypical hyperplasias in groups 1 (0.24) and 2 (0.59) were also significantly smaller ($P < 0.01$ and $P < 0.05$, respectively) than that of group 3 (1.14). Accordingly, the treatments of 24R,25$(OH)_2D_3$ significantly reduced the development of stomach neoplasia and preneoplasia in a dose-dependent manner.

Urinary Excretion of Calcium and Phosphorus and Serum Biochemistry. Urinary calcium levels were significantly increased by the administration of 24R,25$(OH)_2D_3$ at all three time points, regardless of the MNNG + NaCl treatments (Fig. 4). The calcium level was highest at week 30 in all groups. No apparent changes in the urinary phosphorus levels were detected, although dose-independent decreases or increases were infrequently noted (data not shown). Serum phosphorus levels were comparable among the groups, with the exception of the group 4 value, which was significantly higher ($P < 0.05$) than the control value. The levels of blood urea nitrogen were slightly but significantly higher ($P < 0.05$) in group 1 than in group 3. There were no significant changes in the other serum parameters including calcium (data not shown).

Histopathology of the Other Organs. No apparent toxic damage was recognized histopathologically in the liver, kidney, lung, and heart.

DISCUSSION

The results of the present study clearly indicate that 24R,25$(OH)_2D_3$ has a chemopreventive action against glandular stomach carcinogenesis by MNNG and NaCl in rats. Because atypical hyperplasias are generally accepted as preneoplastic lesions (4, 17) and the inhibitory effect of this agent was confirmed in the early lesions toward stomach cancers, additional studies regarding: (*a*) comparison with the other active metabolites of vitamin D like 1,25$(OH)_2D_3$; (*b*) reversibility of the observed inhibitory effect; and (*c*) the effects during the initiation phase could warrant our results. It has been well documented that vitamin D is a promising chemopreventive agent against colon carcinogenesis (1, 2). The underlying mechanisms remain to be elucidated, but several hypotheses have been proposed. For example, in addition to an increase in calcium intake (2, 18), other physiological activities of active vitamin D, such as regulation of terminal cell differentiation (19, 20), influence on immune systems (21, 22), and inhibition of ornithine decarboxylase induction (23, 24), may contribute to the chemopreventive action. Furthermore, it has

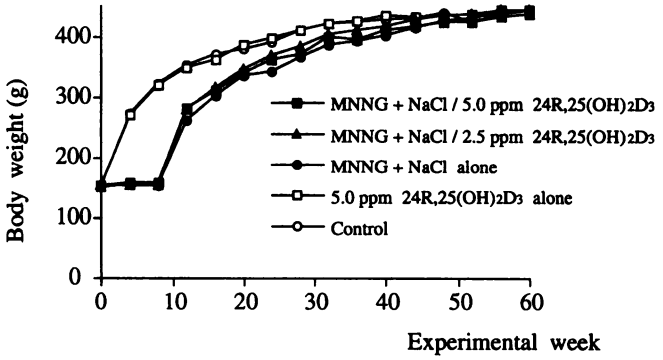

Fig. 2. Growth curves for rats receiving MNNG + NaCl and/or 24R,25$(OH)_2D_3$ treatment.

Fig. 3. A photomicrograph showing a fundic atypical hyperplasia in a rat from group 3. H&E, ×90.

Table 1 *Incidences of gastric proliferative lesions*

Group	Effective no. of rats	No. of rats with gastric lesions (%)								
		Fundus			Pylorus			Total		
		ADC[a]	AH	Total	ADC	AH	Total	ADC	AH	Total
After MNNG + NaCl										
1. 5.0 ppm 24R,25$(OH)_2D_3$	29	0	5 (17)[b]	5 (17)[b]	2 (7)	2 (7)	3 (10)	2 (7)	6 (21)[b]	7 (24)[b]
2. 2.5 ppm 24R,25$(OH)_2D_3$	29	1 (3)	10 (35)	11 (38)	1 (3)	3 (10)	4 (14)	2 (7)	11 (38)[c]	13 (45)
3. Nontreatment	29	0	16 (55)	16 (55)	2 (7)	4 (14)	5 (17)	2 (7)	19 (66)	20 (70)
Without MNNG + NaCl										
4. 5.0 ppm 24R,25$(OH)_2D_3$	20	0	0	0	0	0	0	0	0	0
5. Nontreatment	20	0	0	0	0	0	0	0	0	0

[a] ADC, adenocarcinoma; AH, atypical hyperplasia.
[b] Significantly different from group 3 at $P < 0.01$.
[c] Significantly different from group 3 at $P < 0.05$.

Table 2 *Multiplicities of gastric proliferative lesions*

Group	Effective no. of rats	No. of gastric lesions per rat (mean ± SD)								
		Fundus			Pylorus			Total		
		ADC[a]	AH	Total	ADC	AH	Total	ADC	AH	Total
After MNNG + NaCl										
1. 5.0 ppm 24R,25$(OH)_2D_3$	29	0	0.17 ± 0.38[b]	0.17 ± 0.38[b]	0.07 ± 0.26	0.07 ± 0.26	0.14 ± 0.44	0.07 ± 0.26	0.24 ± 0.51[b]	0.31 ± 0.60[b]
2. 2.5 ppm 24R,25$(OH)_2D_3$	29	0.03 ± 0.19	0.45 ± 0.69	0.48 ± 0.69[c]	0.03 ± 0.19	0.14 ± 0.44	0.17 ± 0.47	0.07 ± 0.26	0.59 ± 0.87[c]	0.66 ± 0.86[c]
3. Nontreatment	29	0	0.90 ± 1.05	0.90 ± 1.05	0.07 ± 0.26	0.24 ± 0.69	0.31 ± 0.76	0.07 ± 0.26	1.14 ± 1.16	1.21 ± 1.15
Without MNNG + NaCl										
4. 5.0 ppm 24R,25$(OH)_2D_3$	20	0	0	0	0	0	0	0	0	0
5. Nontreatment	20	0	0	0	0	0	0	0	0	0

[a] ADC, adenocarcinoma; AH, atypical hyperplasia.
[b] Significantly different from group 3, $P < 0.01$.
[c] Significantly different from group 3, $P < 0.05$.

also been demonstrated that ornithine decarboxylase induction in the glandular stomach of rats by NaCl, a stomach tumor promoter, is inhibited by 1(OH)D_3 (24). Oral preadministration of 1(OH)D_3 provides a nonphysiological amount of 1,25$(OH)_2D_3$ because 25-hydroxylation of 1(OH)D_3 in the liver is not regulated by serum calcium levels (24, 25). Therefore, it is suggested that 1,25$(OH)_2D_3$ may have an antipromoting effect against gastric carcinogenesis as well as carcinogenesis in colon and skin (24). Nevertheless, dietary application of 1,25$(OH)_2D_3$ has been limited, partly because of the disadvantage of an excessive increase in serum calcium levels (5, 9).

Notwithstanding the presence of information for the influence on bone cartilage (11–14), the physiological effects of 24R,25$(OH)_2D_3$, a metabolite of 25(OH)D_3 in the kidney, are not well known. It has been shown that the ratios of vitamin D_3 metabolites in the kidney depend on the serum calcium levels. Namely, 1,25$(OH)_2D_3$ is predominantly produced when serum calcium levels are lower than 9 mg/dl, whereas production of 24R,25$(OH)_2D_3$ is increased when serum calcium levels are higher than this value (26). In relation to such data, 24R,25$(OH)_2D_3$ is known to induce little, if any, hypercalcemia (9, 10). In fact, 24R,25$(OH)_2D_3$ did not induce hypercalcemia at any dose in the present study, although the chemical generated a significant action for the prevention of stomach carcinogenesis. Increased urinary excretion of calcium might have contributed to maintaining physiological serum calcium levels. A possible acceleration by 24R,25$(OH)_2D_3$ in absorption as well as excretion of calcium may also play a role in the observed chemopreventive effects.

Currently, 1,25$(OH)_2D_3$ is considered to act through an intracellular receptor and to modulate transcription of specific genes in a manner similar to that of other steroid hormones (27). Conceivably, an alteration in 1,25$(OH)_2D_3$ receptor regulation occurs *in vivo* when a cell undergoes malignant transformation (28). Furthermore, the presence of 24R,25$(OH)_2D_3$ receptors is demonstrated in the chick parathyroid gland (29) and in the long bones of newborn rats (30). Although the question of whether a specific receptor for 24R,25$(OH)_2D_3$ is present in the rat stomach remains to be elucidated, this possibility and the mechanisms responsible for the chemopreventive effects of the analogue against glandular stomach carcinogenesis warrant further attention.

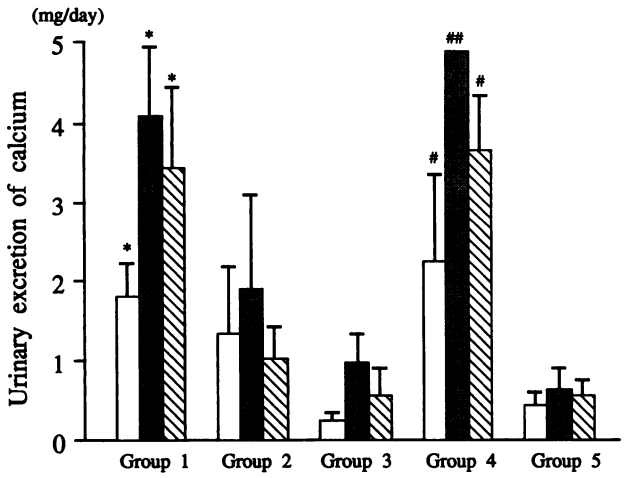

Fig. 4. Urinary excretion of calcium in rats receiving MNNG + NaCl and/or 24R,25$(OH)_2D_3$ treatment at weeks 10 (□), 30 (▨), and 62 (▧). Values represent the mean; *bars*, SD. *, significantly different from group 3 at $P < 0.01$. # and ##, significantly different from group 5 at $P < 0.05$ and $P < 0.01$, respectively.

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