Preventive Effects of Calcitriol on the Development of Capsular Invasive Carcinomas in a Rat Two-Stage Thyroid Carcinogenesis Model

Sayaka KEMMOCHI^{1,2)}, Hitoshi FUJIMOTO³⁾, Gye-Hyeong WOO³⁾, Masao HIROSE⁴⁾, Akiyoshi NISHIKAWA³⁾, Kunitoshi MITSUMORI¹⁾ and Makoto SHIBUTANI^{1)*}

¹⁾Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509,

²⁾Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193,

³⁾Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501 and ⁴⁾Food Safety Commission, Akasaka Park Bld. 22nd F. 5-2-20 Akasaka, Minato-ku, Tokyo 100-8989, Japan

(Received 27 September 2010/Accepted 17 December 2010/Published online in J-STAGE 28 December 2010)

ABSTRACT. We have shown phosphoinositide 3-kinase (PI3K)/Akt signaling activation in thyroid capsular invasive carcinomas (CICs), which are highly induced by promotion with sulfadimethoxine (SDM) in a rat 2-stage thyroid carcinogenesis model. To examine the potency of calcitriol, a synthetic vitamin D₃ analog, on the development or progression of CICs, male F344 rats were injected with calcitriol (0.1 µg/kg body weight) three times a week intraperitoneally, during an entire period of SDM-promotion for 13 weeks (Experiment 1) or during the last 2 weeks of a 15-week SDM-promotion (Experiment 2). Initiation with *N*-bis(2-hydroxypropyl)nitrosamine preceded all treatments. In Experiment 1, long-term calcitriol treatment reduced the multiplicity of CICs, while cell proliferation activity, estimated by Ki-67 cell index in the induced CICs, was unchanged with SDM-promotion alone. Considering the strong dependency of promotion with SDM during the early stages on thyroid-stimulating hormone, the reduced multiplicity in Experiment 1 may be due to the effect on an early stage of neoplastic proliferation. Although the magnitude was mild, cell proliferation activity was decreased in existing CICs after short-term calcitriol treatment in Experiment 2, which was associated with a mild decrease in cyclin-dependent kinase-2-positive cells, cytoplasmic immunolocalization of phosphorylated, inactive, Rb protein and a mild increase in nucleocytoplasmic expression of p27^{Kip1}. Although the effect was mild at the late stage of SDM-promotion in this hypothyroidism-related thyroid carcinogenesis model, our results suggest that calcitriol targets cell proliferation via inhibition of a molecular cascade downstream of PI3K/Akt signaling, controlling G1/S transition.

KEY WORDS: calcitriol (1α, 25-dihydroxyvitamin D₃), p27^{Kip1}, phosphoinositide 3-kinase/Akt signaling, sulfadimethoxine, thyroid carcinogenesis.

J. Vet. Med. Sci. 73(5): 655–664, 2011

Recently, Imai *et al.* [14, 15] have shown a rapid, high frequency induction model of follicular cell carcinomas invasive into fibrously thickened capsule, in contrast to the low frequency induction of parenchymally developed carcinomas, in a rat 2-stage thyroid carcinogenesis model by promotion with the anti-thyroidal, antimicrobial sulfonamide, sulfadimethoxine (SDM). Capsular invasive carcinomas (CICs) are characterized by atypical neoplastic cells having large nuclei with prominent nucleoli and mixtures of micro-follicular and solid patterns, growing in the capsule or extrathyroidal adjacent tissues accompanied with extracellular matrix formation, sometimes without any fibrous thickening of the capsular layer [14]. It is still unclear whether CICs are derived from migrated follicular cells during capsular formation or from parenchymal proliferative lesions, i.e., focal follicular cell hyperplasias (FFCHs), adenomas, or parenchymal carcinomas (PCas). In our previous study, we found that CICs induced by SDM, exclusively altered the expression of phosphoinositide 3-kinase (PI3K) signaling molecules [20]. An important role of PI3K signaling in tumor progression has been demonstrated [17].

Mechanisms of activation of this pathway include loss of tumor suppressor function of phosphatase and tensin homolog (PTEN), amplification or mutation of PI3K and Akt, and activation of growth factor receptors [23]. Interestingly, SDM-induced thyroid CICs exhibited PTEN inactivation via increased expression of the phosphorylated, inactive isoform, which may be a unique mechanism for the activation of downstream signaling including Akt [22]. Furthermore, we found that thyroid proliferative lesions induced by promotion with 6-propyl-2-thiouracil (PTU), another anti-thyroid agent, increased the expression of cyclins, in addition to cyclin-dependent kinase (Cdk) 2, phosphorylated glycogen synthase kinase (p-GSK)-3β and phosphorylated, inactive isoform of tumor suppressor retinoblastoma (p-Rb) protein in association with the development of lesion stages [2]. GSK-3 is a critical downstream element of the PI3K/Akt pathway that plays an important role in cell proliferation by suppressing cyclin D, which ends when GSK-3 is phosphorylated [6, 30]. PTEN/Akt signaling is linked to cell cycle control [28] via the inactivation of Rb protein to progress cell cycle [10, 24].

Epidemiological studies have suggested that vitamin D attained through nutrition and sun exposure is associated with reduced cancer risk [12]. In addition, the hormonally active form of vitamin D, 1,25-dihydroxyvitamin D₃ (cal-

* CORRESPONDENCE TO: SHIBUTANI, M., Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan.
e-mail: mshibuta@cc.tuat.ac.jp

citriol), has been identified in playing a role in modulating proliferation and differentiation of several malignancies including thyroid cancers [13, 21, 27]. Mechanisms of the anti-cancer effect of vitamin D include inhibition of proliferation associated with cell cycle arrest, induction of differentiation, reduction of invasiveness and angiogenesis, and enhanced apoptosis [4]. Furthermore, prevention of metastasis in implanted breast, prostate, and thyroid cancer models has been demonstrated [8, 9, 22]. Calcitriol induces G0/G1 arrest, modulates p27 and p21, the cdk inhibitors implicated in G1 arrest, and induces cleavage of caspase 3, poly-(ADP-ribose) polymerase and the mitogen-activated protein kinase in a caspase-dependent manner [7]. Calcitriol also decreases phosphorylated isoform of both Erk and Akt and up-regulates mitogen-activated protein kinase kinase 1 [7].

The purpose of this study was to evaluate the potency and the potential molecular targets of calcitriol on tumor invasion in a rat two-stage thyroid carcinogenesis model, inducing CICs with SDM as a promoter. We examined the hypothesis that calcitriol has suppressive effects on cell cycle progression by modifying the molecules downstream of the PTEN/Akt signaling pathway.

MATERIALS AND METHODS

Chemicals, animals and experimental design: *N*-bis(2-hydroxypropyl)nitrosamine (DHPN), sulfadimethoxine (SDM), and calcitriol (1 α , 25-dihydroxyvitamin D₃) were purchased from Nacalai Tesque (Kyoto, Japan), Sigma (St. Louis, MO, U.S.A.) and Enzo Life Science Inc. (Plymouth Meeting, PA, U.S.A.), respectively.

Animal studies consisted of three experiments analyzing the effect of calcitriol during the entire promotion period (Experiment 1), during the late stage of tumor promotion (Experiment 2) and on serum thyroid-related hormones (Experiment 3). For all experiments, 6-week-old male F344/NSlc rats (Japan SLC, Inc., Hamamatsu, Japan) were purchased. All animals were on a basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan), allowed water *ad libitum* during the acclimation week and were kept in polycarbonate cages with white wood chips for bedding, in an air-conditioned animal room (24 $\pm$ 1°C, 55 $\pm$ 5% relative humidity; 12 hr light and dark cycle).

In Experiment 1, a total of 52 animals were initiated with a single subcutaneous injection of DHPN at a dose of 2,800 mg/kg body weight after 1 week of acclimation. One week after the initiation treatment, they were divided into 2 groups and were provided drinking water containing 0.1% SDM in distilled water for 13 weeks. During the promotion with SDM, half of the animals were injected intraperitoneally, three times a week for 13 weeks, with calcitriol, which was dissolved in 80% polyethylene glycol in phosphate-buffered saline, at a dose of 0.1 μ g/kg body weight/ml. This treatment regimen has been shown to exert effective suppression of tumor growth in a mouse model of orthotopically implanted thyroid cancers [8]. Body weight, food

intake and water consumption were measured once a week. After 13 weeks of treatment, all animals were anesthetized with ether and blood samples were collected from the abdominal aorta for serum calcium (Ca) level measurements and then euthanized by exsanguination from the posterior vena cava and abdominal aorta. Thyroids were collected from all animals, weighed and fixed in phosphate-buffered 10% formalin, pH 7.4 and routinely embedded in paraffin.

In Experiment 2, using the same SDM-induced rat thyroid carcinogenesis model, the half of a total of 32 rats were injected with calcitriol in the same way as in Experiment 1, but only for the last 2 weeks of the 15-week SDM treatment. At necropsy, thyroid tissues were processed similarly to Experiment 1. In Experiment 3, 32 animals were initiated with DHPN as above and then promoted with 0.1% SDM for 10 weeks. Half of the animals were injected with calcitriol in the same way as in Experiment 1 at a dose of 0.1 μ g/kg body weight, three times a week, for 2 weeks before being sacrificed on week 10. At the end of the experiment, all animals were anesthetized with ether and blood samples were collected from the abdominal aorta and then euthanized by exsanguination from the posterior vena cava and abdominal aorta.

The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan (Experiment 1), and the Faculty of Agriculture, Tokyo University of Agriculture and Technology (Experiment 2 and 3).

Histopathology: Histopathological assessment was performed on thyroid tissues obtained in Experiment 1 and 2. One coronal slice each from the left and right thyroid lobes embedded in paraffin were sectioned at 3 μ m and stained with hematoxylin and eosin (HE) for microscopic examination. Microscopic counts of CICs were made in thyroid sections of both lobes from each animal. Then, the multiplicity of CICs was calculated as the average number per animal.

Immunohistochemistry: Immunohistochemical analysis was performed on formalin-fixed thyroid tissues with regard to molecules related to the PI3K-signaling pathway using the following antibodies:

In both of Experiment 1 and 2, the Ser380 phosphorylated form of PTEN (p-PTEN; rabbit polyclonal antibody; 1:50; Cell Signaling Technology, Inc., Danvers, MA, U.S.A.; Catalog No. #9551) and phosphorylated Akt substrate peptides (Ser/Thr)(p-Akt substrates; rabbit polyclonal antibody, 1:50; Cell Signaling Technology, Inc.; Catalog No. #9611) were examined. In Experiment 2, immunohistochemistry was also performed for p27^{kip1} (rabbit polyclonal antibody; 1:100; Cell Signaling Technology, Inc.; Catalog No. #2552), cyclin-dependent kinase 2 (Cdk2; mouse monoclonal antibody; mouse IgG_{2a} clone55, 1:100; BD Biosciences, San Diego, CA, U.S.A.; Catalog No. 610145), phosphorylated retinoblastoma (p-Rb) protein at Ser780 (rabbit polyclonal antibody, 1:100; Abcam, Cambridge, UK; Catalog No. ab47763) and Ki-67 nuclear antigen (mouse monoclonal antibody; mouse IgG clone MIB-5, 1:50; DAKO Japan Inc., Kyoto, Japan; Catalog No.

M7248). Signal detection was performed using VECTASTAIN® Elite ABC kit for rabbit IgG or mouse IgG (Vector Laboratories Inc., Burlingame, CA, U.S.A.) with 3, 3'-diaminobenzidine/H₂O₂ as the chromogen. Antigen retrieval was carried out in an autoclave for 10 min at 120°C in 10 mM citrate buffer, pH 6.0, for Cdk2, Ki-67, p-Akt substrates or in a microwave for 1 min at 100°C in the same buffer for p27^{kip1}. No antigen retrieval was performed for p-PTEN and p-Rb protein.

TUNEL staining: To examine the effect of calcitriol on apoptosis induction in existing CICs, the thyroid tissue sections obtained from Experiment 2 were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-staining, using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, U.S.A.) according to the manufacturer's protocol. Before staining, deparaffinized sections were treated with 20 µg/ml proteinase K for 15 min at room temperature. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide. Color development and counter staining were as described above for immunohistochemistry.

Quantitative immunohistochemical analysis: Because immunoexpression of p-PTEN and p-Akt substrates were selectively and diffusely observed in CICs in the capsular area, immunoreactivity was evaluated by scoring the staining intensity in each CIC in both Experiment 1 and 2. Scores and criteria were as follows: 0, no signals or unchanged signal intensity with surrounding non-proliferating follicular cells; 1, slightly increased signal intensity from surrounding follicular cells; 2, moderately increased signal intensity; 3, highly increased signal intensity.

In Experiment 2, the cell ratios for Ki-67, Cdk2, p27^{kip1} and p-Rb protein in CICs were determined by counting positive cells in total neoplastic cells, counted in 1–3 fields/lesion at × 400 magnification. Ki-67 showed only nuclear localization, therefore, the nuclear positive cell ratio was estimated in each lesion. With regard to Cdk2, nuclear and cytoplasmic expression were simultaneously observed within the positive cells, hence, the nuclear positive cell ratio was estimated for this molecule in each lesion. On the other hand, the subcellular immunolocalization pattern of p27^{kip1} was either cytoplasmic or both cytoplasmic and nuclear, thus the ratio of each pattern in total cells counted was estimated. For p-Rb protein, the subcellular localization was found to be either nuclear or both nuclear and cytoplasmic, thus the ratio of each pattern in total cells counted was estimated.

In Experiment 2, TUNEL-positive apoptotic cells were also counted in CICs and positive cell ratios in total neoplastic cells counted in 1–3 fields/lesion were estimated at × 400 magnification.

Measurement of serum levels of Ca and thyroid-related hormones: To examine long-term treatment effects of calcitriol on Ca disposition, serum Ca concentrations were measured in Experiment 1 at SRL Inc. (Tokyo, Japan). Similarly, serum levels of triiodothyronine (T₃), thyroxine (T₄) and thyroid stimulating hormone (TSH) were measured in

Experiment 3, using a chemiluminescent immunoassay method at Mitsubishi Chemical Medience Corp. (Tokyo, Japan).

Statistical analysis: Numerical data including body weight and water consumption during experiments, multiplicity of CICs per animal, body and thyroid weights, serum parameters and immunohistochemical data of positive cells for p27^{kip1}, p-Rb protein, Cdk2 and Ki-67 in CICs, and TUNEL-positive cells in CICs were compared between the SDM-alone group and the SDM+calcitriol group, using the Student's *t*-test when the variance was proven to be homogeneous among the groups by the equal variance test. When a significant difference in the variance was observed, Aspin Welch's *t*-test was performed. For the categorical data regarding scores of immunohistochemical intensity of p-PTEN and p-Akt substrates, Mann-Whitney's *U*-test was applied for comparison between the groups.

RESULTS

Body weight and SDM-consumption (Experiment 1 and 2): In Experiment 1, significantly lower body weight was observed in the SDM+calcitriol group from week 3 until the end of the experiment (Fig. 1). On the other hand, significantly higher SDM consumption was observed in this group from week 1 to week 10 and during week 11 compared with the SDM-alone group. In Experiment 2, calcitriol treatment during the last 2 weeks of the SDM-promotion did not alter the body weight and SDM-consumption compared with the SDM-alone group (Fig. 1).

Final body and thyroid weights (Experiment 1–3): In Experiment 1, the final body weight was significantly decreased in the SDM+calcitriol group compared with the SDM-alone group (Table 1). Although no statistically significant, absolute thyroid weight was also decreased in the SDM+calcitriol group as compared with the SDM-alone group. However, relative thyroid weight was unchanged between the 2 groups.

In Experiment 2 and 3, calcitriol treatment during the last 2 weeks of SDM treatment did not alter the final body and thyroid weights (Table 1 and 2).

Multiplicity of CICs (Experiment 1 and 2): Within the capsular fibrous tissue, multiple CICs were induced in all animals in Experiment 1 and 2, showing either solid or diffusely invasive growth pattern within capsular connective tissue. In Experiment 1, the multiplicity of CICs after long-term co-administration of SDM and calcitriol for 13 weeks was significantly lower than that of SDM treatment alone, although histological cellular feature of CICs did not differ between the 2 groups (Fig. 2). On the other hand, in Experiment 2, calcitriol treatment in the last 2 weeks during the 15-week SDM treatment did not change the multiplicity of CICs compared with the SDM treatment alone. Histological cellular feature of CICs was also unchanged between the two groups in Experiment 2.

Multiplicity of parenchymal proliferative lesions (Experiment 1): With regard to the parenchymal proliferative

Fig. 1. Body weight and sulfadimethoxine (SDM)-consumption curves during Experiment 1 and 2. Square dots represent the SDM-alone group and triangular dots represent the SDM+calcitriol group in both Experiment 1 and 2. In Experiment 2, the diamond dots represent values before dividing into SDM-alone and SDM+calcitriol groups. * and ** $P < 0.05$, 0.01 vs. SDM-alone (Student's or Aspin-Welch's t -test).

Table 1. Final body weight, absolute thyroid weight and relative thyroid weight in Experiment 1 and 2

Experiment	Experiment 1		Experiment 2	
Treatment type	SDM-alone	SDM+calcitriol	SDM-alone	SDM+calcitriol
No. of animals examined	26	26	16	16
Body Weight (g)	269.8 ± 19.4	$231.9 \pm 26.1^{**}$	301.8 ± 10.9	295.8 ± 10.9
Thyroid weight				
Absolute (g)	0.248 ± 0.028	0.211 ± 0.035	0.236 ± 0.017	0.244 ± 0.026
Relative (mg/g body weight)	0.92 ± 0.14	0.91 ± 0.22	0.78 ± 0.07	0.82 ± 0.09

** $P < 0.01$ vs. SDM-alone (Student's or Aspin-Welch's t -test).

Table 2. Final body weight, absolute thyroid weight, relative thyroid weight and serum level of thyroid-related hormones in Experiment 3

Treatment type	SDM-alone	SDM+calcitriol
No. of animals examined	16	16
Body Weight (g)	308.8 ± 10.9	301.4 ± 10.6
Thyroid weight		
Absolute (g)	0.236 ± 0.018	0.244 ± 0.025
Relative (mg/g body weight)	0.77 ± 0.07	0.81 ± 0.07
T_3 (ng/dl)	74.13 ± 9.71	$66.86 \pm 9.58^{**}$
T_4 (μ g/dl)	2.27 ± 0.48	$1.27 \pm 0.74^{**}$
TSH (ng/ml)	0.018 ± 0.009	$0.046 \pm 0.037^{**}$

** $P < 0.01$ vs. SDM-alone (Student's or Aspin-Welch's t -test).

lesions in Experiment 1, calcitriol treatment non-significantly decreased the multiplicity of adenomas but significantly increased the multiplicity of FFCHs as compared to the SDM-alone group (Fig. 3). The multiplicity of PCas was

lower than that of CICs, and was unchanged between the SDM-alone group and SDM+calcitriol group.

Serum concentrations of Ca (Experiment 1) and thyroid-related hormones (Experiment 3): In Experiment 1, the

serum concentration of Ca in the SDM+calcitriol group (10.49 ± 0.38 mg/dl, $n=15$ animals) did not differ from the SDM-alone group (10.35 ± 0.22 mg/dl, $n=15$ animals).

In Experiment 3, serum concentrations of T_3 and T_4 in the SDM+calcitriol group was slightly, but significantly, lower

than that in the SDM-alone group (Table 2). In conjunction with the decrease in serum thyroid hormones, serum concentration of TSH was significantly increased after calcitriol treatment compared with that in the SDM-alone (Table 2).

PTEN/Akt-signaling molecules (Experiment 1 and 2): As shown in Fig. 4, increased staining intensities of p-PTEN and p-Akt substrates were observed in CICs compared with the surrounding tissue. Comparison of staining intensity scores showed that calcitriol treatment did not change the distribution of p-PTEN and p-Akt substrates in CICs in both Experiment 1 and 2.

Distribution of Ki-67-positive cells (Experiment 1 and 2): Strong immunoexpression of Ki-67 nuclear antigen was observed in CICs, though the number of positive cells varied among CICs. In Experiment 1, the ratio of Ki-67-positive cells was not significantly different between the groups of SDM-alone ($22.4 \pm 10.0\%$; $n=36$ CICs/12 animals) and SDM+calcitriol ($22.0 \pm 10.3\%$; $n=35$ CICs/12 animals). On the other hand, after 2 weeks of calcitriol treatment, the number of Ki-67-positive cells slightly, but significantly, decreased compared with the SDM-alone group (Fig. 5).

TUNEL-positive apoptotic cell ratio (Experiment 2): Very few TUNEL-positive cells were found in CICs in both SDM-alone and SDM+calcitriol groups and no significant difference was observed between the groups (Fig. 5).

Fig. 3. Multiplicity of parenchymal carcinomas (PCas), adenomas (Ads) and focal follicular cell hyperplasias (FFCHs) in the sulfadimethoxine (SDM)-alone group (open column) and the SDM+calcitriol group (black column) in Experiment 1. 26 animals/group were examined. * $P<0.05$ vs. SDM-alone (Student's or Aspin-Welch's t -test).

Fig. 2. The morphological features of capsular invasive carcinomas (CICs) induced by promotion with sulfadimethoxine (SDM) in Experiment 1 and 2. (A) High magnification view of a CIC. HE stain. Magnification: $\times 200$. (B) Low magnification view of the thyroid in Experiment 1. CICs are indicated with arrowheads. Note that multiple CICs are induced by SDM-promotion, while fewer CICs are evident after calcitriol treatment. HE stain. Magnification: $\times 12.5$. Graphs show multiplicity of CICs/section in the SDM-alone group (open column) and the SDM+calcitriol group (black column). In Experiment 1, 26 animals/group were examined and in Experiment 2, 16/group. ** $P<0.01$ vs. SDM-alone (Student's or Aspin-Welch's t -test).

Fig. 4. Immunohistochemical distribution of the phosphorylated isoform of phosphatase tensin homolog (p-PTEN) (Ser380) and phosphorylated Akt substrate peptides (Ser/Thr) (p-Akt substrates) in capsular invasive carcinomas (CICs) induced by promotion with sulfadimethoxine (SDM) in Experiment 1 and 2. Photomicrographs of representative immunoreactivity patterns in the SDM-alone group and the SDM+calcitriol group are shown. Magnification: $\times 200$. Graphs show immunohistochemical staining intensity scores for p-PTEN (Ser380) and p-Akt (Ser/Thr) substrates in CICs in the SDM-alone group (open column) and the SDM+calcitriol group (black column). 26 animals/group were examined in Experiment 1 and 15/group in Experiment 2. In Experiment 1, p-PTEN-immunoreactivity was examined with 193 and 90 CICs in the SDM-alone group and SDM+calcitriol group, respectively. Immunoreactivity for p-Akt substrates was examined with 197 and 98 CICs in the SDM-alone group and SDM+calcitriol group, respectively. In Experiment 2, p-PTEN-immunoreactivity was examined with 38 and 42 CICs in the SDM-alone group and SDM+calcitriol group, respectively. Immunoreactivity for p-Akt substrates was examined with 40 and 42 CICs in the SDM-alone group and SDM+calcitriol group, respectively.

Distribution of Cdk2, p27^{kip1} and p-Rb protein-positive cells (Experiment 2): With regard to p27^{kip1}, scattered distribution in CICs was observed with either nucleocytoplasmic or cytoplasmic expression (Fig. 6). After 2 weeks of calcitriol-treatment, positive cell ratio showing nucleocytoplasmic expression was significantly increased, whereas that showing cytoplasmic expression alone was unaffected by calcitriol.

Similar to Ki-67, Cdk2 showed slight, but statistically significant, reduction in the positive cell ratio after calcitriol treatment compared with SDM-alone (Fig. 6).

In the case of p-Rb protein, both nuclear alone and nucleocytoplasmic distribution patterns were observed in CICs. Calcitriol treatment slightly, but significantly, reduced the cell population showing nucleocytoplasmic expression, whereas it did not change the nuclear alone population (Fig. 6).

DISCUSSION

In the present study, we investigated the effects of calcitriol on the development/progression of CICs, using a rat thyroid carcinogenesis model. We found that treatment with calcitriol under SDM-promotion for 13 weeks reduced the multiplicity of CICs in Experiment 1, while it did not affect the serum Ca level. However, cell proliferation activity, estimated by the number of Ki-67-positive cells in CICs, did not differ between the SDM-alone and SDM+calcitriol groups. It is possible that neoplastic cells acquire a resistance phenotype against calcitriol during the course of treatment. Vitamin D anti-tumor effect is often found reduced in the late stages of cancer. In a calcitriol-resistant human prostate cancer LNCap cell line (LNCap-R), nuclear factor-kappaB signaling was found to be critical for calcitriol sensitivity, and dysregulation of this pathway would result in calcitriol resistance and disease progression [3].

On the other hand, following chronic administration of

Fig. 5. Distribution of proliferating cells and apoptotic cells in capsular invasive carcinomas (CICs) induced by promotion with sulfadimethoxine (SDM) in Experiment 2. Photomicrographs of representative immunoreactivity patterns of Ki-67-positive proliferating cells and TUNEL-positive apoptotic cells are shown in the SDM-alone group and the SDM+calcitriol group. Magnification: $\times 200$. Graphs show ratios of Ki-67-positive cells and TUNEL-positive cells in CICs in the SDM-alone group (open column) and the SDM+calcitriol group (black column). Numbers of animals examined were 10 with SDM-alone and 12 with SDM+calcitriol for Ki-67 immunohistochemistry and 11/group for the TUNEL assay. * $P < 0.05$ vs. SDM-alone (Student's or Aspin-Welch's t -test). For Ki-67-immunohistochemistry, 58 and 54 CICs were examined in the SDM-alone group and SDM+calcitriol group, respectively. For TUNEL assay, 32 and 34 CICs were examined in the SDM-alone group and SDM+calcitriol group, respectively.

SDM during the promotion stage, serum TSH values return to normal or below normal after profound increases during the early stages [26]. Downregulation of TSH receptor mRNA was found to occur in FRTL-5 cells, a rat thyroid epithelial cell line, following TSH treatment [1]. The putative mechanism for thyroid carcinogenesis by anti-thyroid agents is believed to be through the decrease of serum levels of T_3 and T_4 , causing suppression of negative feedback via

the pituitary and increase in serum TSH. TSH then stimulates thyroid functions, including growth and proliferation of follicular cells [5, 29]. Therefore, proliferation activity in proliferative lesions at the late stage of SDM-promotion might be suppressed compared with the early stage of development due to a reduction in serum TSH levels and/or its receptor downregulation [2]. Because calcitriol exerts effective growth inhibition of neoplastic cells by increasing

Fig. 6. Immunohistochemical distribution of p27^{kip1}, cyclin-dependent kinase (Cdk) 2 and phosphorylated Rb (p-Rb) protein in capsular invasive carcinomas (CICs) induced by promotion with sulfadimethoxine (SDM) in Experiment 2. Magnification: p27^{kip1} and p-Rb protein, $\times 400$; Cdk2, $\times 200$. Graphs show ratios of cells positive for p27^{kip1} staining in the nucleus and cytoplasm (NC) or cytoplasm alone (C); ratios of cells showing nuclear Cdk2 immunoreactivity; and ratios of cells positive for p-Rb protein staining in the nucleus alone (N) or both in the nucleus and cytoplasm (NC) in the SDM-alone group (open column) and the SDM+calcitriol group (black column). Numbers of animals examined were 12/group for p27^{kip1} and 10/group for Cdk2 and p-Rb protein. * and ** $P < 0.05$, 0.01 vs. SDM-alone (Student's or Aspin-Welch's *t*-test). For p27^{kip1}-immunohistochemistry, 48 and 42 CICs were examined in the SDM-alone group and SDM+calcitriol group, respectively. For Cdk2-immunohistochemistry, 33 and 38 CICs were examined in the SDM-alone group and SDM+calcitriol group, respectively. For p-Rb protein, 56 and 58 CICs were examined in the SDM-alone group and SDM+calcitriol group, respectively.

p21^{CIP1/WAF1}, governing G1/S transition [16, 25], calcitriol may be rather effective in proliferative lesions showing TSH-dependent growth at earlier stages. Therefore, while we did not examine the effect of calcitriol at earlier stages of neoplastic proliferation and the TSH-dependency on the CIC-development in this study, the decreased multiplicity of CICs after long-term co-treatment of calcitriol and SDM may be the signature of the calcitriol effect at the earlier promotion phase.

In the present study, 13-week-treatment of calcitriol at 0.1 $\mu\text{g/kg}$ body weight in addition to SDM reduced the body weight of rats, suggestive of the development of a systemic

toxicity. It is well known that calcitriol is effective as an antitumor agent only at doses, which are highly toxic causing suppression of the body weight. Calcitriol-treatment at 0.5 $\mu\text{g/kg}$ body weight, three times a week for 22 days was effective for inhibition of tumor metastasis in rats but also resulted in body weight loss (20% of the initial weight) [22]. On the other hand, treatment-related body growth suppression is a well-known phenomenon caused by administration of anti-thyroid agents [26]. In this study, although the reason for the increased water consumption was unclear, SDM-consumption through drinking water was increased by calcitriol treatment in Experiment 1. The increase in SDM-

consumption may be responsible for the suppression of body growth. It is generally accepted that the carcinogenic responses are influenced by the body growth condition [17], therefore, calcitriol treatment may have indirectly affected the development of CICs. However, the effect of hypothyroidism in terms of serum levels of thyroid-related hormones was also stronger in our calcitriol-treated animals in Experiment 3. Moreover, a dose-dependent tumor-promoting effect on thyroid carcinogenesis, reflecting a proportional increase in serum TSH concentrations, was evident with anti-thyroid agents, irrespective of the dose-dependent suppression of body growth [26]. In Experiment 1, we observed that calcitriol-treatment increased SDM-intake for long-term through increased water consumption, suggesting an enhanced hypothyroidal TSH-stimulation of the thyroid by increasing the intake of SDM from the early stage. However, calcitriol might have exerted direct effect on suppression of neoplastic cell proliferation to result in decreased multiplicity of CICs.

In the present study, calcitriol-treatment did not change the thyroid weight as compared to the SDM-alone in Experiment 1, irrespective of the reduction in the number of CICs. In the SDM-promoted thyroid tissues in a rat two-stage thyroid carcinogenesis model, parenchymal proliferative lesions account for larger population than CICs as shown in Fig. 2 in the present study. By calcitriol-treatment, we found a significant increase in the multiplicity of FFCHs as compared to the SDM-alone group, and this increase might have offset the reduced number of CICs to result in unchanged thyroid weight between the two groups. Interestingly, we also found non-significant reduction in the multiplicity of adenomas by calcitriol-treatment, suggesting the inhibitory effect of calcitriol on the progression from FFCHs to adenomas. We recently found localized interstitial expression of tenascin C, a molecule that is expressed at the invasion front of cancer cell nests [11], in conjunction with increased proliferation activity in some FFCHs of SDM-promoted cases, suggesting that FFCHs might be the origin of CICs (data in preparation).

Similar to Experiment 1, there were no significant differences in the intensity scores of p-PTEN and p-Akt substrates in CICs between the SDM-alone and SDM+calcitriol groups in Experiment 2. Because of the mild decrease in positive cells for Ki-67 and Cdk2 after calcitriol treatment, it may be difficult to detect changes in immunoreactivity of p-PTEN and p-Akt substrates using the intensity scoring method. However, as with the mild decrease in the positive cells for Ki-67 and Cdk2, we also found changes in the number of positive cells for p27^{kip1} and p-Rb protein. PTEN/Akt signaling is linked to cell cycle control via the Rb protein [28]. Rb protein is a tumor suppressor that functions as a negative regulator of the cell cycle by interacting with transcription factors. Inactivation of Rb protein and subsequent cell cycle progression likely requires phosphorylation by cyclin D-Cdk4/6 [10, 24]. In this study, we found a mild decrease in cytoplasmic immunolocalization of phosphorylated, inactive Rb protein in CICs after calcitriol treatment.

Inactivation of Rb protein via aberrant nucleocytoplasmic transport may disrupt normal cell differentiation programs and accelerate cancer progression [19]. Therefore, decreased cytoplasmic localization of Rb protein in the CICs following calcitriol treatment may be the signature of suppression of tumor progression. Similarly, increase of p27^{kip1}-positive cells showing nucleocytoplasmic expression in CICs after calcitriol-treatment may be the reflection of arrest of G1/S phase transition via binding cyclin E-Cdk-2 complexes causing decrease of Rb protein-phosphorylation, since direct interaction of calcitriol on p27^{kip1} was observed in neoplastic thyroid follicular cells in both *in vitro* [21] and mouse implanted tumor model [8].

In conclusion, long-term treatment with calcitriol during the entire period of SDM-promotion reduced the multiplicity of CICs. This probably reflects the effect on the neoplastic cell population that is highly proliferative, depending on TSH-stimulation at an early stage of SDM-promotion. Although in this hypothyroidism-related thyroid carcinogenesis model, the calcitriol effect on existing CICs was mild at the late stage of SDM-promotion, inhibition of cell proliferation by inhibiting molecular cascades governing G1/S transition may be the main target of this treatment.

ACKNOWLEDGMENTS. We thank Miss Tomomi Morikawa for her technical assistance in conducting the animal studies. We also thank Mrs. Shigeko Suzuki and Miss Ayako Kaneko for their technical assistance in preparing the histological specimens. All of the authors disclose that there are no conflicts of interest that could inappropriately influence the outcomes of the present study.

REFERENCES

1. Akamizu, T., Ikuyama, S., Saji, M., Kosugi, S., Kozak, C., McBride, O. W. and Kohn, L. D. 1990. Cloning, chromosomal assignment, and regulation of the rat thyrotropin receptor: Expression of the gene is regulated by thyrotropin, agents that increase cAMP levels, and thyroid autoantibodies. *Proc. Natl. Acad. Sci. U.S.A.* **87**: 5677–16781.
2. Ago, K., Saegusa, Y., Nishimura, J., Dewa, Y., Kemmochi, S., Kawai, M., Harada, T., Mitsumori, K. and Shibutani, M. 2010. Involvement of glycogen synthase kinase-3 β signaling and aberrant nucleocytoplasmic localization of retinoblastoma protein in tumor promotion in a rat two-stage thyroid carcinogenesis model. *Exp. Toxicol. Pathol.* **62**: 269–280.
3. Bao, B. Y., Ting, H. J., Hsu, J. W., Yasmin-Karim, S., Messing, E. and Lee, Y. F. 2010. Down-regulation of NF-kappaB signals is involved in loss of 1 α ,25-dihydroxyvitamin D3 responsiveness. *J. Steroid. Biochem. Mol. Biol.* **120**:11–21.
4. Beer, T. M. and Myrthue, A. 2004. Calcitriol in cancer treatment: from the lab to the clinic. *Mol. Cancer Ther.* **3**: 373–381.
5. Brewer, C., Yeager, N. and Di Cristofano, A. 2007. Thyroid-stimulating hormone initiated proliferative signals converge *in vivo* on the mTOR kinase without activating AKT. *Cancer Res.* **67**: 8002–8006.
6. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**: 785–789.

7. Johnson, C. S., Muindi, J. R., Hershberger, P. A. and Trump, D. L. 2006. The antitumor efficacy of calcitriol: preclinical studies. *Anticancer Res.* **26**: 2543–2549.
8. Dackiw, A. P., Ezzat, S., Huang, P., Liu, W. and Asa, S. L. Vitamin D₃ administration induces nuclear p27 accumulation, restores differentiation, and reduces tumor burden in a mouse model of metastatic follicular thyroid cancer. 2004. *Endocrinology* **145**: 5840–5846.
9. El Abdaimi, K., Dion, N., Papavasiliou, V., Cardinal, P. E., Binderup, L., Goltzman, D., Ste-Marie, L. G. and Kremer, R. 2000. The vitamin D analogue EB 1089 prevents skeletal metastasis and prolongs survival time in nude mice transplanted with human breast cancer cells. *Cancer Res.* **60**: 4412–4418.
10. Geng, Y., Yu, Q., Sicinska, E., Das, M., Bronson, R. T. and Sicinski, P. 2001. Deletion of the p27^{Kip1} gene restores normal development in cyclin D₁-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **98**: 194–199.
11. Gulubova, M. and Vlaykova, T. 2006. Immunohistochemical assessment of fibronectin and tenascin and their integrin receptors $\alpha 5 \beta 1$ and $\alpha 9 \beta 1$ in gastric and colorectal cancers with lymph node and liver metastases. *Acta Histochem.* **108**: 25–35.
12. Guyton, K. Z., Kensler, T. W. and Posner, G. H. 2003. Vitamin D and vitamin D analogs as cancer chemopreventive agents. *Nutr. Rev.* **61**: 227–238.
13. Hershberger, P. A., Modzelewski, R. A., Shurin, Z. R., Rueger, R. M., Trump, D. L. and Johnson, C. S. 1999. 1 α , 25-Dihydroxycholecalciferol (1 α , 25-D₃) inhibits the growth of squamous cell carcinoma and down-modulates p21^{Waf1/Cip1} *in vitro* and *in vivo*. *Cancer Res.* **59**: 2644–2649.
14. Imai, T., Onose, J., Hasumura, M., Ueda, M., Takizawa, T. and Hirose, M. 2004. Sequential analysis of development of invasive thyroid follicular cell carcinomas in inflamed capsular regions of rats treated with sulfadimethoxine after *N*-bis(2-hydroxypropyl)nitrosamine-initiation. *Toxicol. Pathol.* **32**: 229–236.
15. Imai, T., Hasumura, M., Onose, J., Ueda, M., Takizawa, T., Cho, Y. M. and Hirose, M. 2005. Development of invasive follicular cell carcinomas in a rat thyroid carcinogenesis model: biological impact of capsular inflammation and reduced cyclooxygenase-2 expression. *Cancer Sci.* **96**: 31–37.
16. Jensen, S. S., Madsen, M. W., Lukas, J., Binderup, L. and Bartek, J. 2001. Inhibitory effects of 1 α , 25-dihydroxyvitamin D₃ on the G1-S phase-controlling machinery. *Mol. Endocrinol.* **15**: 1370–1380.
17. Jiang, B. H. and Liu, L. Z. 2008. PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim. Biophys. Acta* **1784**: 150–158.
18. Jiang, W., Zhu, Z. and Thompson, H. J. 2003. Effect of energy restriction on cell cycle machinery in 1-methyl-1-nitrosourea-induced mammary carcinomas in rats. *Cancer Res.* **63**: 1228–1234.
19. Jiao, W., Lin, H. M., Datta, J., Braunschweig, T., Chung, J. Y., Hewitt, S. M. and Rane, S. G. 2008. Aberrant nucleocytoplasmic localization of the retinoblastoma tumor suppressor protein in human cancer correlates with moderate/poor tumor differentiation. *Oncogene* **27**: 3156–3164.
20. Kemmochi, S., Fujimoto, H., Woo, G. H., Inoue, K., Takahashi, M., Mitsumori, K., Hirose, M., Nishikawa, A. and Shibutani, M. 2010. Involvement of PTEN/Akt signaling in capsular invasive carcinomas developed in a rat two-stage thyroid carcinogenesis model after promotion with sulfadimethoxine. *J. Cancer Res. Clin. Oncol.* doi: 10.1007/s00432-010-0931-7 (in press).
21. Liu, W., Asa, S. L. and Ezzat, S. 2005. 1 α , 25-Dihydroxyvitamin D₃ targets PTEN-dependent fibronectin expression to restore thyroid cancer cell adhesiveness. *Mol. Endocrinol.* **19**: 2349–2357.
22. Lokeshwar, B. L., Schwartz, G. G., Selzer, M. G., Burnstein, K. L., Zhuang, S. H., Block, N. L. and Binderup, L. 1999. Inhibition of prostate cancer metastasis *in vivo*: a comparison of 1 α , 23-dihydroxyvitamin D (calcitriol) and EB1089. *Cancer Epidemiol. Biomarkers Prev.* **8**: 241–248.
23. LoPiccolo, J., Granville, C. A., Gills, J. J. and Dennis, P. A. 2007. Targeting Akt in cancer therapy. *Anticancer Drugs.* **18**: 861–874.
24. Lundberg, A. S. and Weinberg, R. A. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol. Cell Biol.* **18**: 753–761.
25. Moffatt, K. A., Johannes, W. U., Hedlund, T. E. and Miller, G. J. 2001. Growth inhibitory effects of 1 α , 25-dihydroxyvitamin D₃ are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. *Cancer Res.* **61**: 7122–7129.
26. Onodera, H., Mitsumori, K., Takahashi, M., Shimo, T., Yasuhara, K., Kitaura, K., Takahashi, M. and Hayashi, Y. 1994. Thyroid proliferative lesions induced by anti-thyroid drugs in rats are not always accompanied by sustained increases in serum TSH. *J. Toxicol. Sci.* **19**: 227–234.
27. Palmer, H. G., Sanchez-Carbajo, M., Ordonez-Moran, P., Larriba, M. J., Cordon-Cardo, C. and Munoz, A. 2003. Genetic signatures of differentiation induced by 1 α , 25-dihydroxyvitamin D₃ in human colon cancer cells. *Cancer Res.* **63**: 7799–7806.
28. Paramio, J. M., Navarro, M., Segrelles, C., Gomez-Casero, E. and Jorcano, J. L. 1999. PTEN tumour suppressor is linked to the cell cycle control through the retinoblastoma protein. *Oncogene* **18**: 7462–7468.
29. Smith, P., Williams, E. D. and Wynford-Thomas, D. 1987. *In vitro* demonstration of a TSH-specific growth desensitising mechanism in rat thyroid epithelium. *Mol. Cell Endocrinol.* **51**: 51–58.
30. Srivastava, A. K. and Pandey, S. K. 1998. Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. *Mol. Cell Biochem.* **182**: 135–141.