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Apoptotic Regression of MCF-7 Xenografts in Nude Mice Treated with the Vitamin D_3 Analog, EB1089*

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ABSTRACT

1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and its synthetic analog EB1089 induce characteristic morphological features of apoptosis in MCF-7 cells *in vitro* that coincide with up-regulation of clusterin and cathepsin B, proteins associated with apoptosis in the mammary gland, and with down-regulation of Bcl-2, an antiapoptotic protein. To determine whether vitamin D_3 compounds could mediate apoptosis of breast tumors *in vivo*, we treated nude mice carrying established MCF-7 xenografts with the low calcemic vitamin D_3 analog EB1089 via daily injection or sustained release pellets for up to 5 weeks. The volume of tumors from mice treated with 45 pmol/day EB1089 was 4-fold lower than that of tumors from vehicle-treated control mice after 5 weeks. The reduced growth of tumors from EB1089-treated mice was associated with characteristic apoptotic morphology and a marked reduction in the proportion of epithelial cells to stroma. After 5 weeks of treatment with EB1089, MCF-7 tumors exhibited a 6-fold increase in DNA fragmentation (as measured by *in situ* end labeling) relative to that in control tumors. The enhanced rate of apoptosis in tumors from EB1089-treated mice was coupled to a 2-fold reduction in proliferation (as measured by expression of proliferating cell nuclear antigen) compared with that in tumors from control mice. The antitumor effects of EB1089 were evident at doses that had minimal effects on serum calcium and body weight. EB1089 treatment did not alter the growth of xenografts derived from a vitamin D_3-resistant variant of MCF-7 cells (MCF-7^{D3Res} cells), which display resistance to EB1089 *in vitro*, indicating that resistance to EB1089 is maintained *in vivo*. Tumors derived from both MCF-7 and MCF-7^{D3Res} cells underwent apoptotic regression upon estradiol withdrawal, indicating comparable estrogen dependence of tumors with differential sensitivity to vitamin D_3 compounds. These are the first studies to demonstrate apoptotic morphology and regression of human breast tumors in response to treatment with a vitamin D_3 analog *in vivo* and support the concept that vitamin D_3 compounds can effectively target human breast cancer. (*Endocrinology* **139:** 2102–2110, 1998)

DESPITE decades of progress in diagnosis, research, and treatment, breast cancer remains the leading cause of cancer death in women. Antiestrogen treatment represents the most effective available endocrine therapy, but is limited to the one third of patients with breast tumors that are estrogen dependent at the time of diagnosis (1). Treatment with antiestrogens in these patients is often followed by the development of a hormone-resistant phenotype (2). Hormone resistance may in part be caused by the selective deletion of hormone-responsive cells by antiestrogen-induced apoptosis in a heterogeneous tumor. It is clear that effective breast cancer therapies must target both estrogen-dependent and -independent cells to minimize the development of hormone-independent tumors with increased metastatic potential.

1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the biologically active form of vitamin D_3 (cholecalciferol), is a potent negative growth regulator of both estrogen-dependent and -independent breast cancer cells *in vitro* (3–7). The vitamin D_3 receptor, like the estrogen receptor, is a member of the steroid/thyroid/retinoic acid family of nuclear receptors. Although the estrogen receptor is present in only two thirds of breast tumors, the vitamin D_3 receptor is present in over 80% of tumors and does not necessarily colocalize with the estrogen receptor (4, 8). Vitamin D_3-based therapeutics thus offer promise as either adjunctive agents for estrogen-dependent tumors or alternative agents for estrogen-independent tumors.

Although it is clear that vitamin D_3 compounds inhibit the growth of both estrogen receptor-positive and estrogen receptor-negative breast cancer cells (3–7), the precise mechanism of its effects is unclear. We initially demonstrated that 1,25-$(OH)_2D_3$ induces characteristic features of apoptosis, such as chromatin condensation, nuclear matrix degradation, and DNA fragmentation, in MCF-7 cells *in vitro* (9, 10). Subsequently, we and others reported that apoptosis in breast cancer cells treated with 1,25-$(OH)_2D_3$ or its synthetic analogs is associated with up-regulation of proteins linked to apoptosis in the mammary gland (such as clusterin and cathepsin B) and down-regulation of Bcl-2, an antiapoptotic protein (11–14).

Although 1,25-$(OH)_2D_3$ exerts potent antiproliferative effects *in vivo*, chronic administration induces undesirable hypercalcemic side-effects (4, 15). For this reason, synthetic vitamin D_3 compounds have been developed that mimic the antiproliferative effects of 1,25-$(OH)_2D_3$ with less calcemic activity (12, 13, 16–18). Previous studies have demonstrated the efficacy of the synthetic vitamin D_3 analog EB1089 (Leo Pharmaceuticals, Ballerup, Denmark) in reducing the growth

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of breast cancer cells and tumors *in vitro* and *in vivo* (12, 17–19). Among several vitamin D_3 analogs investigated, EB1089 exhibited the best profile for inhibition of nitrosomethylurea-induced rat mammary tumors in the absence of hypercalcemia (20). The first objective of the studies described here was to determine whether EB1089 could modulate the growth of human breast tumors using a nude mice xenograft model. Our second objective was to determine whether the antitumor effects of EB1089 in human breast tumors involve activation of apoptosis. Our data demonstrate that EB1089 significantly reduces the growth of established human breast tumors by enhancing apoptosis and reducing proliferation of tumor epithelial cells. These data emphasize the potential effectiveness of vitamin D_3-based therapeutics for induction of apoptosis in human breast cancer.

Materials and Methods

Cell culture

MCF-7 human breast cancer cells (obtained from American Type Culture Collection, Rockville, MD) as well as the vitamin D_3-resistant variant (MCF-7^{D3Res} cells), which has been previously described (21), were cultured in αMEM containing 5% FBS (Life Technologies, Grand Island, NY). Both cell lines were grown in T-150 flasks and yielded 5–10 × 10^6 cells/flask depending on confluence. For inoculation into nude mice, cells were washed with PBS, trypsinized, resuspended in αMEM, and pooled. After centrifugation, cells were resuspended in Matrigel (Collaborative Biomedical, Waltham, MA)-αMEM (4:1).

Nude mouse xenograft model

Three series of studies were conducted to examine the effects of EB1089 on growth and apoptosis of MCF-7 xenografts. In all studies, ovariectomized Ncr *nu/nu* mice (Taconic Farms, Germantown, NY) were implanted sc with 17β-estradiol-sustained release pellets (Innovative Research, Sarasota, FL). The mice were fed a low calcium (0.1%), purified rodent chow (Purina Test Diets, Richmond, IN) for the duration of the study to minimize the calcemic effects of vitamin D_3 analog treatment. Mice were inoculated sc with approximately 5 × 10^6 MCF-7 or MCF-7^{D3Res} cells suspended in 0.3 ml Matrigel-αMEM. The tumor take rate ranged from 95–100%. Tumor volumes were monitored weekly by caliper measurement of the length, width, and height and were calculated using the formula for a semiellipsoid ($4/3\pi r^3/2$). After 3 weeks, mice bearing tumors with volumes averaging approximately 200 mm^3 were randomized for treatment. Because of the variations in tumor take and initial tumor growth as well as the removal of mice for analysis at various time points, the number of mice at each time point varied from experiment to experiment. The number of mice analyzed is reported in the text or figure legends.

In the first series of studies, MCF-7 tumor-bearing mice were treated with EB1089 at a dose of 60 pmol/day. This dose was chosen based on preliminary studies in nontumor-bearing BALB/c mice, which indicated that doses up to 90 pmol/day could be tolerated with little weight loss or elevation of serum calcium when animals were fed the low calcium diet (data not shown). EB1089 was suspended in 80% propylene glycol-20% PBS and administered daily via sc injection. Control mice received daily injections of the vehicle alone. In a third group of tumor-bearing mice, which served as a positive control, estradiol pellets were removed to induce apoptotic tumor regression, as reported by Kyprianou *et al.* (22). Body weights and tumor volumes were monitored weekly, and mice were killed after 2–5 weeks of treatment. At the termination of treatment, mice were anesthetized with sodium pentobarbital, and blood was collected by cardiac puncture for serum calcium determination. Tumors were removed, weighed, and fixed in 4% formalin for histological analysis.

In the second series of studies, the dose of EB1089 was lowered to 45 pmol/day, because mice given 60 pmol EB1089/day experienced weight loss and hypercalcemia. In this experiment, mice bearing MCF-7 tumors were randomized into control (n = 14), EB1089-treated (n = 16), and estradiol withdrawal (n = 3) groups. To determine whether EB1089 induced nonspecific or indirect effects on tumor growth kinetics, tumors were also established from a vitamin D_3-resistant variant of MCF-7 cells, termed MCF-7^{D3Res} cells. We have previously demonstrated that MCF-7^{D3Res} cells are resistant to the growth inhibitory effects of EB1089 *in vitro* (21). Mice bearing MCF-7^{D3Res} tumors were randomized into control (n = 8) and EB1089-treated (n = 6) groups in one experiment and into control (n = 3) and estradiol withdrawal (n = 3) groups in another trial. The experimental designs for these studies were otherwise identical to those described for studies employing the 60 pmol/day dose.

To investigate whether EB1089 could be administered via sustained release pellets similar to those used for estrogen supplementation, a preliminary study was conducted with custom-made pellets (Innovative Research) designed to continuously release 30, 45, or 60 pmol EB1089/day for 5 weeks. MCF-7 xenografts were established from MCF-7 cells, and EB1089 or placebo pellets were implanted ip under sodium pentobarbital anesthesia 3 weeks after inoculation. Tumor volumes were measured, and treatment was terminated after 5 weeks. Due to the small numbers of mice in each group (EB1089 pellets, n = 3 for 30 pmol and n = 1 each for 45 or 60 pmol; control, n = 4), the data for all three doses were combined in the analysis.

Histological analysis of tumors

Tumors were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin (Gill's formulation 3, Fisher Scientific, Fairlawn, NJ) and eosin Y (Sigma Chemical Co., St. Louis, MO). The epithelial nature of the tumors was verified by immunostaining with antibodies directed against epithelia-specific antigen and cytokeratin 18 (data not shown). The mitotic index and apoptotic index were assessed by quantitative morphometric analysis of proliferating cell nuclear antigen (PCNA) expression and *in situ* terminal transferase-mediated fluorescein deoxy-UTP nick end labeling (TUNEL), two established markers of proliferation and apoptosis. For PCNA localization, formalin-fixed, paraffin-embedded sections were incubated for 30 min with a mouse monoclonal anti-PCNA (Nova Castra Laboratories, Newcastle Upon Tyne, UK) at a 1:100 dilution in 1% BSA-PBS. A biotin-conjugated antibody to mouse IgG (Vector Laboratories, Burlingame, CA) was applied at a 1:200 dilution for 30 min in 1% BSA-PBS. The ABC technique was used (avidin and biotinylated horseradish peroxidase complex, Vector) followed by diaminobenzidine (Sigma) to localize peroxidase in the sections, and the sections were counterstained with hematoxylin (Harris modified, Fisher). DNA fragmentation was assessed by TUNEL, using the commercially available assay according to manufacturer's directions (Boehringer Mannheim, Indianapolis, IN). In these sections, nuclei were counterstained with Hoechst 33258 dye (Sigma).

Quantitation of apoptosis and proliferation

PCNA expression and TUNEL were quantitated by viewing and photographing random fields of each tissue section on a Nikon Optiphot-2 microscope and Nikon Microflex UFX-IIA photomicrographic attachment (Nikon Corp., Melville, NY), using a ×40 objective. The photographs were scanned and analyzed with the University of Texas Health Science Center at San Antonio Image Tool program, and the percentage of cells staining positively for PCNA or TUNEL was calculated. For both TUNEL and PCNA, 2–6 fields of view (containing at least 250 cells) were quantitated on each section, with 4–8 samples evaluated for each treatment per time point.

Statistical analyses

Statistical comparisons were performed using Student's unpaired *t* test (for two groups) or one-way ANOVA for more than two groups. Data are expressed as the mean ± SE, and differences between means were considered significant at $P < 0.05$.

Results

Effect of 60 pmol EB1089 on MCF-7 tumor growth kinetics, body weight, and serum calcium

Tumor growth kinetics in ovariectomized mice treated with 60 pmol EB1089/day or vehicle in the presence of es-

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tradiol supplementation are presented in Fig. 1. In control mice, average tumor volume increased rapidly over the 3-week treatment period. In EB1089-treated mice, tumor volume increased during the first week of treatment and then plateaued, with no further increase in tumor volume between weeks 2–3. In control mice, the mean change in tumor volume between the first and third weeks was 272.3 ± 113.2 mm^3 (n = 4) compared with 3.6 ± 15.6 mm^3 (n = 5) for tumors from EB1089-treated mice ($P < 0.05$). Tumor volume after 2 weeks was significantly lower in the EB1089 group (351.2 ± 80.4 mm^3; n = 10) than in the control group (631.1 ± 65.9 mm^3; n = 7). In mice subjected to removal of the estradiol supplementation, tumors regressed rapidly, becoming undetectable within 3 weeks, demonstrating the estrogen dependence of the MCF-7 xenografts.

In contrast to our preliminary data, which indicated that nontumor-bearing nude mice could tolerate doses of EB1089 up to 90 pmol/day, tumor-bearing mice treated with 60 pmol/day EB1089 experienced weight loss and elevated serum calcium levels despite being maintained on the low calcium diet (Table 1).

Effect of 45 pmol EB1089 on growth of tumors derived from MCF-7 and MCF-7^{D3Res} cells, body weight, and serum calcium

Although significant effects of EB1089 on MCF-7 tumor growth kinetics were observed in our initial studies, the low tolerance of the tumor-bearing mice to the 60 pmol/day dose precluded definitive conclusions regarding the specificity of this effect. Therefore, a second series of studies using a lower (45 pmol) dose of EB1089 was conducted. In these studies, the effects of EB1089 on tumors derived from a vitamin D_3-resistant variant of MCF-7 cells (MCF-7^{D3Res} cells) were also investigated.

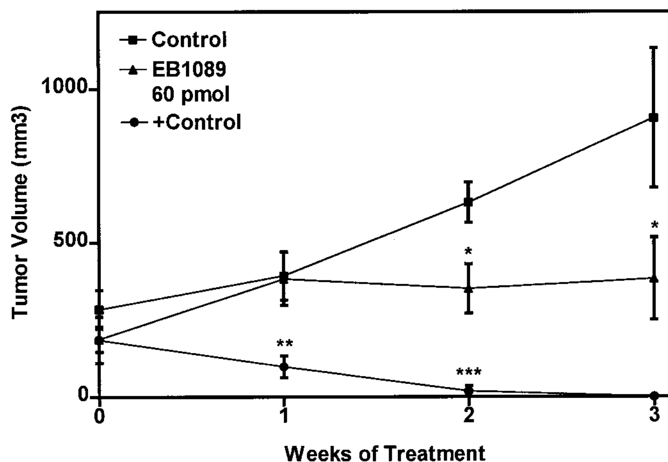

FIG. 1. Effect of 60 pmol/day EB1089 and estradiol withdrawal on growth of MCF-7 tumors. Ovariectomized nude mice supplemented with estradiol and bearing MCF-7 xenografts were given daily sc injections of 60 pmol EB1089 or vehicle beginning 3 weeks after tumor cell inoculation. In a separate group of mice, estradiol pellets were removed at time zero, and no further treatment was given. Tumor volumes were monitored weekly and calculated as described in *Materials and Methods*. Each *point* represents the mean ± SE of four control mice, five EB1089-treated mice, and three mice subjected to estradiol withdrawal. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 1. Effect of 60 pmol/day EB1089 on serum calcium and body weight in nude mice bearing MCF-7 tumors

	Vehicle	EB1089
2 Weeks of treatment		
Serum Ca (mg/dl)	8.59 ± 0.21 (2)	12.87 ± 0.05 (2)[a]
BW (g)	21.23 ± 0.84 (4)	19.08 ± 1.00 (5)
3 Weeks of treatment		
Serum Ca (mg/dl)	9.41 ± 0.08 (5)	11.75 ± 1.23 (2)[b]
BW (g)	20.20 ± 0.31 (4)	16.56 ± 1.23 (5)[a]

Body weights and serum calcium were measured after 2 or 3 weeks of treatment with 60 pmol/day EB1089 or vehicle. Data are expressed as the mean ± SE, with the number of samples analyzed in *parentheses*.

[a] $P < 0.05$.

[b] $P < 0.01$.

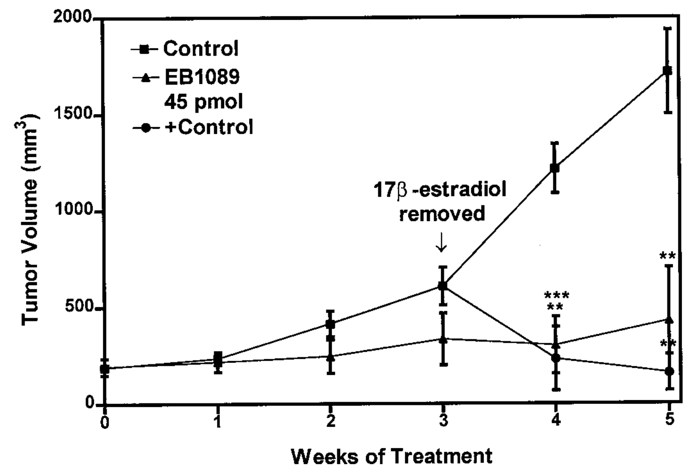

FIG. 2. Effect of 45 pmol/day EB1089 and estradiol withdrawal on growth of MCF-7 tumors. Ovariectomized nude mice supplemented with estradiol were inoculated with MCF-7 cells and allowed to grow for 3 weeks. Mice bearing established tumors were then given daily sc injections of 45 pmol EB1089 or vehicle for 5 weeks. In a subset of vehicle-treated mice, estradiol pellets were removed at week 3, and no further treatment was given. In all animals, tumor volumes were monitored weekly. Each *point* represents the mean ± SE of four control mice, five EB1089-treated mice, and three mice subjected to estradiol withdrawal. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Similar results were obtained in two independent trials.

Temporal changes in tumor volume for animals bearing MCF-7 tumors and treated with 45 pmol/day EB1089 or vehicle are shown in Fig. 2. This graph shows mean tumor volumes for all animals that completed the 5-week study protocol (*i.e.* not including tumors that were removed for histological analysis at various times). Consistent with the data shown in Fig. 1 for the 60 pmol/day dose, the growth of MCF-7 tumors in mice treated with 45 pmol/day EB1089 was slower than that of tumors in vehicle-treated control animals from 1 week on. The mean change in tumor volume between the first and fifth weeks was 366.6 ± 53.6 mm^3 (n = 6) in control mice compared with 53.2 ± 56.9 mm^3 (n = 6) in EB1089-treated mice ($P < 0.01$). Tumor volume after 5 weeks was significantly ($P < 0.01$) lower in the EB1089-treated group (428.6 ± 274.0 mm^3, n = 6) than in the control group (1716.0 ± 217.7 mm^3, n = 6). Final tumor weight was significantly ($P < 0.01$) lower in EB1089-treated mice (0.43 ± 0.27 g; n = 6) than in control mice (1.52 ± 0.19 g; n = 6). These data indicate a good correlation between tumor volume as-

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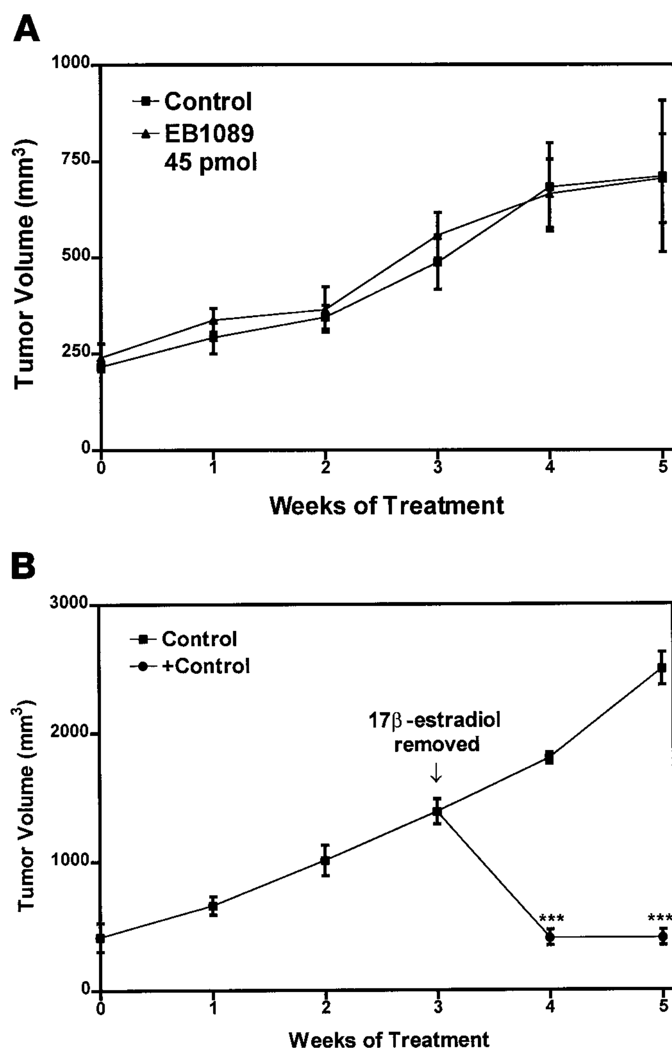

FIG. 3. Effect of 45 pmol/day EB1089 and estradiol withdrawal on growth of MCF-7^{D3Res} tumors. A, Ovariectomized nude mice supplemented with estradiol and bearing tumors derived from MCF-7^{D3Res} cells were given daily sc injections of 45 pmol EB1089 or vehicle for 5 weeks. Tumor volumes were monitored as described in Fig. 1. Each *point* represents the mean ± SE of eight control mice and six EB1089-treated mice. Similar results were obtained in three independent treatment trials. B, Ovariectomized nude mice supplemented with estradiol and bearing tumors derived from MCF-7^{D3Res} cells were subjected to estradiol withdrawal as described in Fig. 2. In control mice, estradiol pellets were left intact. Tumor volumes were monitored as described in Fig. 1. Each *point* represents the mean ± SE of three control mice and three mice subjected to estradiol withdrawal. ***, Significantly different, control *vs.* estradiol withdrawal, $P < 0.001$. Similar results were obtained in two independent treatment trials.

sessed by caliper measurement in live mice and actual tumor size measured after death. The antitumor effect of EB1089 persisted over the entire 5-week experiment. In addition, although not readily evident from the graph (which shows mean tumor volume), two MCF-7 tumors completely regressed in response to EB1089 treatment. Tumors that regressed were monitored for up to 2 months in the absence of EB1089 treatment, and no regrowth was observed (data not shown). In contrast, spontaneous regression was never observed in tumors from control mice.

TABLE 2. Effect of 45 pmol/day EB1089 on serum calcium and body weight of nude mice bearing MCF-7 tumors

5 Weeks of treatment	Vehicle	EB1089
Serum Ca (mg/dl)	9.70 ± 0.26 (9)	10.88 ± 0.67 (6)[a]
Corrected BW (g)	23.68 ± 0.39 (6)	22.81 ± 0.52 (6)

Body weights and serum calcium were measured after 5 weeks of treatment with 45 pmol/day EB1089 or vehicle. Because of the significant differences in tumor weights between control and EB1089-treated mice, corrected body weights (total body weight minus tumor weight) are reported. Data are expressed as the mean ± SE, with the number of samples analyzed in *parentheses*.
[a] $P < 0.05$.

In contrast to tumors derived from MCF-7 cells, tumors derived from MCF-7^{D3Res} cells failed to respond to EB1089 treatment (Fig. 3a). There were no significant differences in MCF-7^{D3Res} tumor volumes of control or EB1089-treated mice at any time point throughout the 5-week experiment. As demonstrated in Fig. 3b, MCF-7^{D3Res} tumors underwent rapid regression in response to estradiol withdrawal. Thus, despite resistance to the antitumor effects of EB1089, tumors derived from the MCF-7^{D3Res} cells retained the ability to undergo regression in response to estradiol deprivation, which is known to induce apoptosis in MCF-7 tumors (22).

As indicated in Table 2, although serum calcium was elevated in mice treated for 5 weeks with 45 pmol/day EB1089 compared with that in control mice, the levels were not as high as those in mice receiving 60 pmol/day (Table 1). Furthermore, the significant loss of body weight that was evident with the 60 pmol/day treatment was not observed when EB1089 was given at the 45 pmol/day dose. Corrected final body weight (mouse body weight minus tumor weight) was similar for EB1089-treated and control mice. There were no significant differences in body weight or serum calcium in mice bearing tumors derived from MCF-7 cells compared with those derived from MCF-7^{D3Res} cells regardless of treatment with EB1089 (data not shown). As tumors derived from MCF-7^{D3Res} cells did not respond to EB1089 treatment, the antitumor effect of EB1089 in MCF-7 tumors is probably not due to indirect effects of the vitamin D_3 analog on body weight or calcium homeostasis.

Effect of EB1089 on morphology, apoptosis, and mitosis of MCF-7 tumors

Sections from MCF-7 tumors taken from mice treated with 45 or 60 pmol/day EB1089 or vehicle for 2–5 weeks were stained with hematoxylin and eosin for assessment of general morphology. The histological appearances of tumors from mice treated with 45 and 60 pmol/day were similar, and representative micrographs from the 2 week point are presented in Fig. 4. Tumors from vehicle-treated mice were primarily composed of tumor epithelial cells, with small amounts of mouse-derived stroma and frequent blood vessels. The majority of epithelial cells in control tumors were quiescent, although mitotic figures were visible in most sections. Tumors from mice treated with 60 pmol/day EB1089 were composed primarily of quiescent epithelial cells, with few mitotic figures. In many areas, epithelial cells with classic apoptotic morphology (condensed cells with pyknotic nuclei) were frequent. Many EB1089-treated tumors displayed

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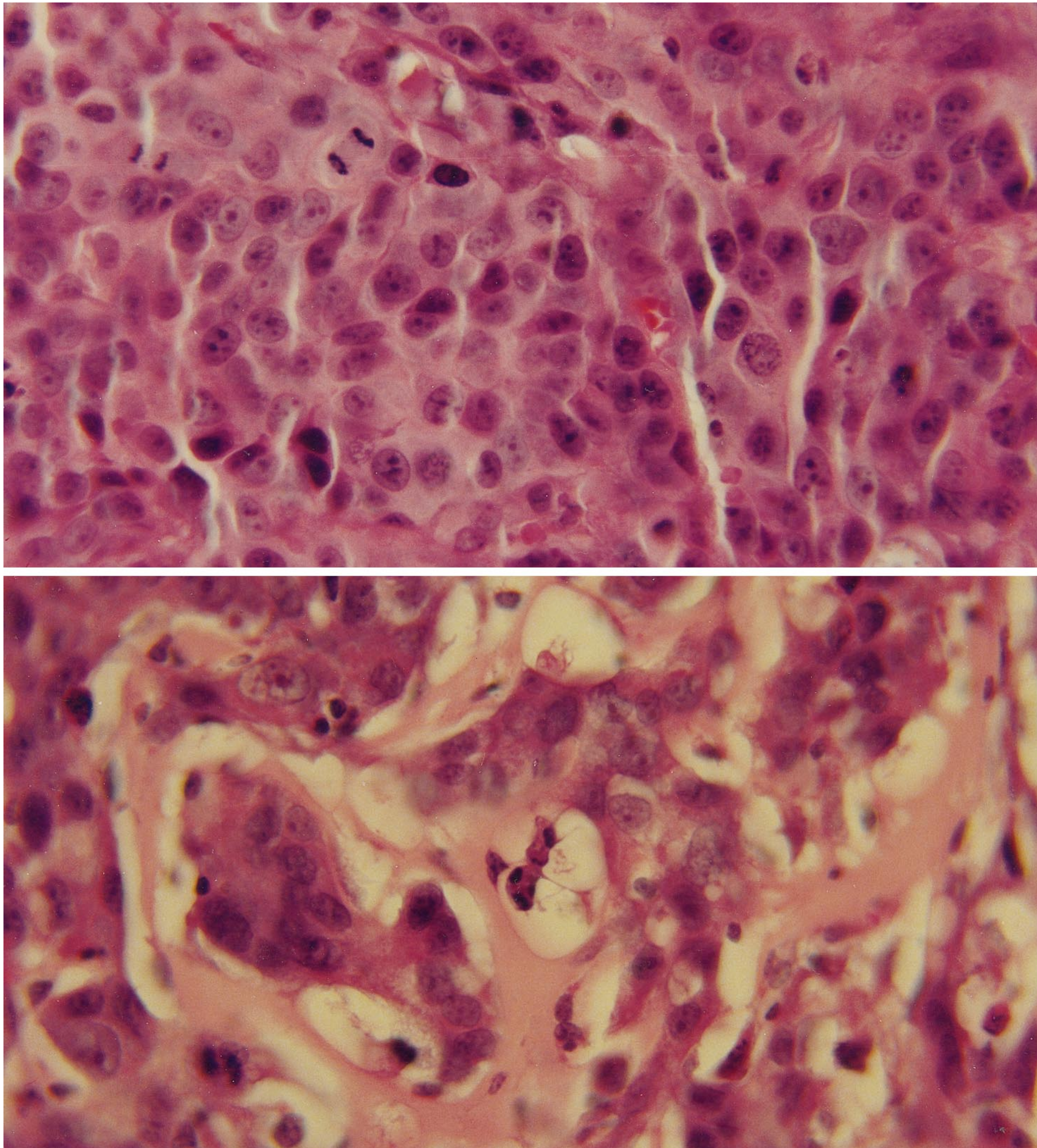

FIG. 4. Effect of EB1089 treatment on MCF-7 tumor morphology. Representative tumor sections from mice treated with vehicle (*top*) or 60 pmol/day EB1089 (*bottom*) for 2 weeks were formalin fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin as described in *Materials and Methods*.

large areas of stroma where deletion of epithelial cells had occurred. The extent of vascularization appeared equivalent in tumors from EB1089-treated and control mice.

To determine whether the changes in MCF-7 tumor morphology induced by EB1089 were associated with changes in the apoptotic or mitotic index, we quantitated the extent of DNA fragmentation (assessed as TUNEL-positive cells) as a marker of apoptosis and expression of PCNA as a marker of proliferation. Representative sections stained with Hoechst dye to visualize nuclear morphology and processed for TUNEL are presented in Fig. 5. Nuclei of epithelial cells in MCF-7 tumors from control mice exhibited normal morphology and were generally negative for DNA fragmentation. In contrast, sections of tumors from EB1089-treated mice exhibited a high prevalence of condensed, irregularly shaped nuclei that were positive for DNA fragmentation. Tumors from mice that had been subjected to estradiol withdrawal also exhibited irregularly shaped, condensed nuclei that were positive for DNA fragmentation. The morphology of tumors from estradiol-deprived mice was similar to that of tumors from EB1089-treated mice, although the extent of apoptosis was higher after estradiol deprivation than after EB1089 treatment (Fig. 5). This finding is consistent with the more pronounced tumor regression in mice subjected to estradiol withdrawal compared with that in mice given EB1089 treatment (Figs. 1–3). Quantitative analysis indicated an increase in the percentage of TUNEL-positive cells in tumors from EB1089-treated mice compared with that in tumors from control mice at all time points examined (Table 3). After 2 weeks of treatment, the higher dose of EB1089 (60 pmol/day) was associated with a more pronounced increase in apoptotic index than the 45 pmol/day dose. After 5 weeks of

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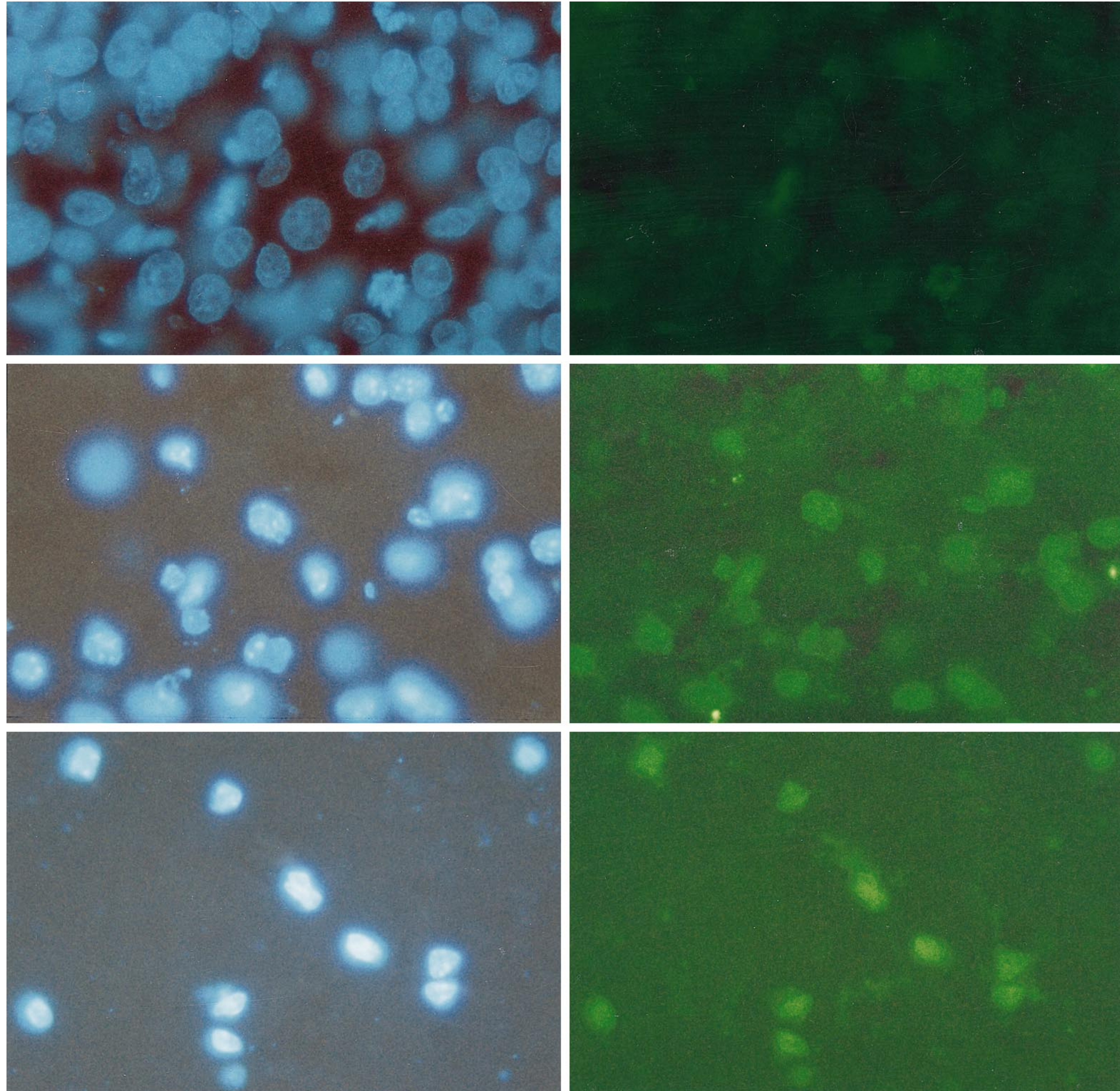

FIG. 5. Effect of EB1089 treatment and estradiol withdrawal on nuclear morphology and DNA fragmentation of MCF-7 tumors. Representative MCF-7 tumor sections from mice treated with vehicle (*top panels*) or 60 pmol/day EB1089 (*middle panels*) or subjected to removal of estradiol supplementation (*bottom panels*) were stained with Hoechst dye to visualize nuclear morphology (*left panels*) and processed for TUNEL (*right panels*) as described in *Materials and Methods*.

treatment, however, both doses of EB1089 were equivalent, with tumors from treated mice exhibiting a 6-fold increase in the percentage of cells positive for DNA fragmentation compared with that in control tumors.

Expression of PCNA was examined as a marker of proliferation in MCF-7 tumor sections derived from control and EB1089-treated mice (Fig. 6). In control tumors, PCNA expression was detected in the nucleus of approximately half of all epithelial cells. A decrease in PCNA expression was detected in tumors from EB1089-treated mice at all time points examined. Quantitation of PCNA expression after 2 weeks indicated a 2.5-fold reduction in PCNA staining in EB1089-treated tumors compared with control tumors (Table 3). Treatments with 45 and 60 pmol/day EB1089 were equally effective in down-regulation of PCNA expression at both time points. The reduced expression of PCNA in EB1089-treated tumors compared with that in control tumors was maintained throughout the 5 weeks of treatment.

Effect of EB1089 sustained release pellets on growth of MCF-7 tumors

In a third preliminary study, we examined whether EB1089 could be administered via sustained release pellets similar to those used for estradiol supplementation. Pellets designed to continuously release 30, 45, or 60 pmol EB1089/day or placebo pellets were implanted into mice bearing established MCF-7 tumors. Due to the small number of animals (four control and five EB1089-treated mice) used in this trial, data from the three EB1089 treatment groups were pooled for analysis, as there were no obvious differences in responses to the three doses. As presented in Fig. 7, administration of EB1089 via sustained release pellets elicited a similar antitumor response, as observed in Figs. 1 and 2, for 60 and 45 pmol sc injections. Tumors from mice implanted with EB1089 pellets grew at a slower rate than tumors from mice implanted with placebo pellets from 1 week on. The

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TABLE 3. Quantitation of PCNA expression and DNA fragmentation in MCF-7 tumors

	% PCNA	% TUNEL
2 Weeks treatment		
Vehicle	64.52 ± 4.96	6.80 ± 2.61
EB1089 (60 pmol/day)	23.20 ± 9.35[a]	57.03 ± 3.73[b]
Vehicle	60.11 ± 4.53	8.10 ± 1.29
EB1089 (45 pmol/day)	26.66 ± 6.23[a]	32.42 ± 2.11[b]
5 Weeks treatment		
Vehicle	69.99 ± 6.68	8.69 ± 2.07
EB1089 (60 pmol/day)	38.47 ± 1.04[c]	63.50 ± 3.89[b]
Vehicle	56.20 ± 3.72	7.45 ± 0.51
EB1089 (45 pmol/day)	24.51 ± 7.29[a]	54.37 ± 3.21[a]

Tumor sections from mice treated with EB1089 or vehicle were processed for PCNA immunohistochemistry or TUNEL positivity and quantitated as described in *Materials and Methods*. Data are expressed as the mean ± SE of at least 500 cells counted in a minimum of two different fields of view as described in *Materials and Methods*.
[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.05$.

mean change in tumor volume between the first and fifth weeks was 1594.3 ± 418.7 (n = 4) in mice bearing placebo pellets compared with 285.8 ± 164.3 (n = 5) in mice bearing EB1089 pellets ($P < 0.01$), including one EB1089-treated tumor that regressed completely. Final tumor volume at 5 weeks was significantly ($P < 0.05$) lower in the EB1089 pellet group (619.7 ± 256.0 mm^3; n = 5) than in the placebo group (2189.6 ± 554.8 mm^3; n = 4). There were no significant differences in body weights [control, 24.6 ± 0.2 g (n = 5); EB1089 pellets, 23.6 ± 1.5 g (n = 4)] or serum calcium [control, 9.5 ± 0.2 (mg/dl) (n = 5); EB1089 pellets, 9.6 ± 0.2 (mg/dl) (n = 4)] between mice bearing placebo or EB1089 pellets after 5 weeks.

Discussion

In this series of *in vivo* studies we have demonstrated that the vitamin D_3 analog EB1089 significantly reduces the growth of estrogen-dependent MCF-7 human breast tumors. In our studies, tumor volumes from nude mice treated with EB1089 by daily sc injection at a dose of 45 pmol/mouse·day (~0.8 μg/kg BW) were 4-fold less than those of vehicle-treated mice. At this dose of EB1089, serum calcium was minimally elevated, and no weight loss was observed. These findings complement those of Colston's group (17–20), who demonstrated that oral administration of EB1089 at doses up to 1 μg/kg BW daily slowed the growth of established nitrosomethylurea-induced rat mammary tumors without induction of hypercalcemia. In our nude mice studies, treatment with 60 pmol/mouse·day EB1089 had a more pronounced antitumor effect than treatment with 45 pmol/day, but the higher dose was associated with hypercalcemia, weight loss, and mortality. Preliminary studies using sustained release pellets designed to continuously release EB1089 at doses up to 60 pmol/day indicated that this mode of administration induced antitumor effects similar to those achieved with daily injections. These preliminary data indicated that pellet delivery of EB1089 for 5 weeks was not associated with hypercalcemia or weight loss. As delivery of EB1089 via pellets offers obvious advantages over daily injections (especially when working with nude mice), additional studies to document the actual release rate of EB1089 from pellets and directly assess the efficacy of pellet administration relative to sc injections are warranted.

Histological examination indicated that the decreased size of tumors from EB1089-treated mice, compared with that in control mice, was associated with a reduction in the epithelial component and an increase in the stroma. The 4-fold reduction of tumor volume in mice treated with 45 pmol/day EB1089 for 5 weeks could reflect a decreased rate of cell proliferation, an increased rate of cell death, or both. Our analyses confirmed that EB1089 mediates tumor regression by modulation of both apoptosis and proliferation of tumor epithelial cells.

Quantitative morphometric analysis of DNA fragmentation indicated that tumors from EB1089-treated mice exhibited apoptotic morphology and a 6-fold increase in the percentage of TUNEL-positive cells compared with tumors from control mice. Our studies also demonstrated that MCF-7 tumor regression and DNA fragmentation induced by EB1089 were morphologically similar to tumor regression resulting from estradiol withdrawal, which is known to induce apoptosis in estrogen-dependent MCF-7 tumors (22). Our data demonstrating induction of apoptosis in MCF-7 tumors *in vivo* correlate with earlier findings that demonstrated that 1,25-$(OH)_2D_3$ and its structural analogs induce apoptosis in MCF-7 cells *in vitro* (3, 4, 9–12). In addition to induction of apoptosis, EB1089-treated tumors exhibited a significant decrease in proliferation, as measured by PCNA expression, at all time points examined. These data are consistent with flow cytometric studies of MCF-7 cells *in vitro*, which indicated that 1,25-$(OH)_2D_3$ and EB1089 increase the percentage of cells in G_0/G_1 and reduce the percentage of cells in S phase (12). Thus, our *in vivo* results with EB1089 correlate well with the *in vitro* reports that vitamin D_3 compounds induce both growth arrest and apoptosis in estrogen-dependent breast cancer cells (5, 11, 12, 14).

The quantitative data indicate that effect of EB1089 on tumor cell proliferation (2- to 3-fold decrease) was less than the effect of EB1089 on apoptosis (4- to 8-fold increase). Although actual mean tumor volumes plateau rather than decrease in EB1089-treated mice, histological examination indicated a reduction in epithelial cells and replacement by stromal tissue in EB1089-treated tumors, supporting the concept that the epithelial cell compartment has regressed by apoptosis. An effect of EB1089 on tumor cell apoptosis is consistent with our observation in two studies that some EB1089-treated tumors regressed completely. As tumors that underwent complete regression in response to EB1089 were not available for analysis, the apoptotic index in some tumors treated with EB1089 may be even higher than that indicated by the quantitative data presented in Table 3. Our data support the hypothesis that EB1089 has a predominant effect on the apoptotic cell death pathway *in vivo*.

Studies with xenografts derived from MCF-7^{D3Res} cells that display resistance to EB1089 *in vitro* (21) demonstrate that resistance to EB1089 is maintained *in vivo*. Although the basis for vitamin D_3 resistance in these cells is unclear, MCF-7^{D3Res} cells (20) and tumors (data not shown) express the vitamin D_3 receptor protein at levels comparable to those in

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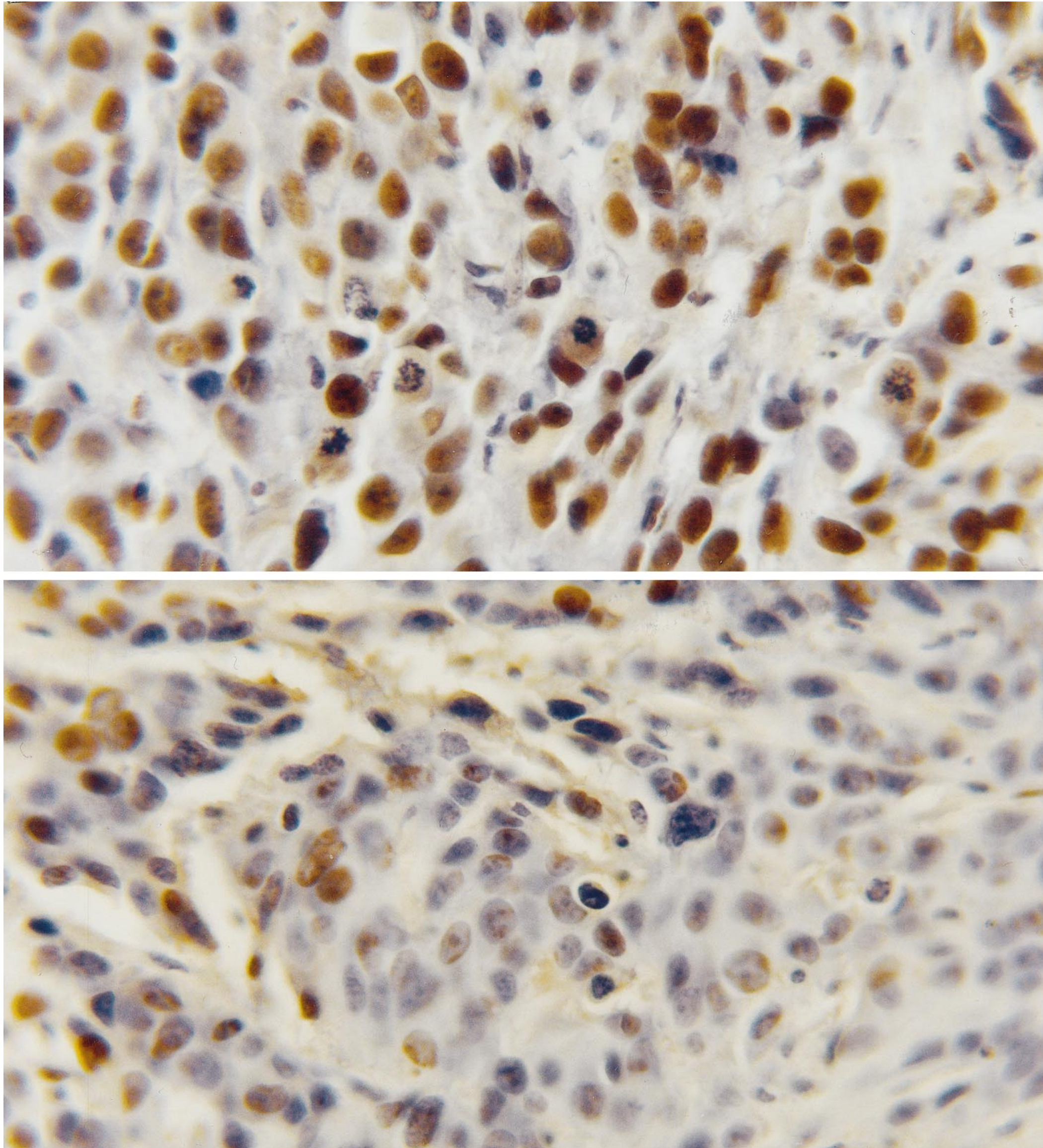

FIG. 6. Effect of EB1089 treatment on PCNA expression in MCF-7 tumors. Expression of PCNA in representative tumor sections from mice treated with vehicle (*top*) or 60 pmol/day EB1089 (*bottom*) for 2 weeks. Sections were incubated with a mouse monoclonal antibody directed against human PCNA and developed with the ABC technique as described in *Materials and Methods*. PCNA expression appears as brown nuclear staining.

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MCF-7 cells and tumors. The growth rates of tumors derived from MCF-7 and MCF-7^{D3Res} cells in the absence of treatment were comparable, indicating that tumors selected for vitamin D_3 resistance are unlikely to be more aggressive than tumors that are sensitive to vitamin D_3. Tumors derived from MCF-7^{D3Res} cells displayed comparable regression in response to estradiol withdrawal, suggesting a functional apoptotic pathway in these tumors that can be activated by other strategies that induce apoptosis. This finding is consistent with our *in vitro* work demonstrating that MCF-7^{D3Res} cells are resistant to EB1089 but sensitive to antiestrogens such as tamoxifen (21). We are currently examining whether tumors derived from MCF-7 and MCF-7^{D3Res} cells exhibit comparable sensitivity to antiestrogen-induced apoptosis *in vivo* to further test the hypothesis that antiestrogens and vitamin D_3 compounds act independently to induce apoptosis in breast cancer cells. Support for this hypothesis would suggest that for patients with mixed tumors containing estrogen-dependent and estrogen-independent cells, a distinct therapeutic advantage might be achieved by combining agents that activate vitamin D_3-mediated apoptosis with those that disrupt estrogen-mediated survival signals.

In summary, our studies demonstrate that the vitamin D_3 analog EB1089 induces human breast tumor regression by a mechanism that involves both activation of apoptosis and inhibition of proliferation. Our work also indicates that the pathways involved in vitamin D_3-mediated apoptosis of MCF-7 tumors are distinct from the pathways that trigger apoptosis in response to estradiol withdrawal. These results

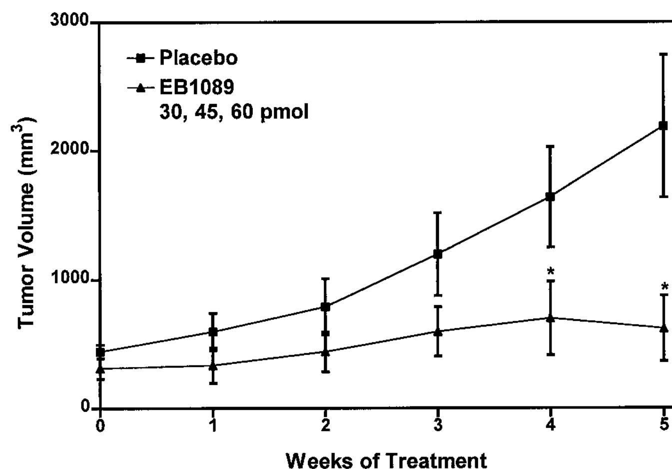

FIG. 7. Effect of EB1089 sustained release pellets on growth of MCF-7 tumors. Ovariectomized nude mice supplemented with estradiol and bearing MCF-7 xenografts were implanted ip with sustained release EB1089 pellets, and tumor volumes were monitored for 5 weeks. Data are expressed as the mean ± SE of four control and five EB1089-treated mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

support further clinical studies on the therapeutic efficacy of vitamin D_3 analogs such as EB1089 against human breast cancer.

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References

1. **Nandi S, Guzman R, Yang J** 1997 Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci USA 92:3650–3657
2. **Kardinal CG** 1995 Endocrine therapy of breast cancer. In: Donegan WL, Spratt JS (eds) Cancer of the Breast, ed 4. Saunders, Philadelphia, pp 534–580
3. **Chouvet C, Berger U, Coombes RC** 1986 1,25 Dihydroxyvitamin D_3 inhibitory effect on the growth of two human breast cancer cell lines (MCF-7, BT-20). J Steroid Biochem 24:373–376
4. **Colston K, Berger U, Coombes RC** 1989 Possible role for vitamin D in controlling breast cancer cell proliferation. Lancet 1:188–191
5. **Eisman J, Sutherland RL, McMenemy ML, Fragonas JC, Musgrove EA, Pang G** 1989 Effects of 1,25-dihydroxyvitamin D_3 on cell cycle kinetics of T47D human breast cancer cells. J Cell Physiol 138:611–616
6. **Elstner E, Linker-Israeli M, Said J, Umiel T, deVos S, Shintaku IP, Heber D, Binderup L, Uskovic M, Koeffler HP** 1995 20-Epi vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cells. Cancer Res 55:2822–2830
7. **Love-Schimenti C, Gibson D, Ratnam A, Bikle D** 1996 Antiestrogen potentiation of antiproliferative effects of vitamin D_3 anaolgues in breast cancer cells. Cancer Res 56:2789–2794
8. **Berger U, McClelland R, Wilson P, Greene G, Haussler M, Pike J, Colston K, Easton D, Coombes RC** 1991 Immunocytochemical determination of estrogen receptor, progesterone receptor and 1,25-$(OH)_2$-vitamin D_3 receptor in breast cancer and relationship to prognosis. Cancer Res 51:239–244
9. **Welsh JE** 1994 Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens. Biochem Cell Biol 72:537–545
10. **Welsh J, Simboli-Campbell M, Tenniswood M** 1994 Induction of apoptotic cell death by 1,25-$(OH)_2D_3$ in MCF-7 breast cancer cells. In: Norman AW, Bouillon R, Thomasett M (eds) Proceedings of the Ninth Workshop on Vitamin D. de Gruyter, Berlin, pp 526–527
11. **Simboli-Campbell M, Narvaez CJ, Tenniswood M, Welsh JE** 1996 1,25-$(OH)_2D_3$ induce morphological and biochemical indices of apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 58:367–337
12. **Simboli-Campbell M, Narvaez CJ, VanWeelden K, Tenniswood M, Welsh JE** 1997 Comparative effects of 1,25-$(OH)_2D_3$ and EB1089 on cell cycle kinetics and apoptosis in MCF-7 cells. Breast Cancer Res Treat 42:31–41
13. **Elstner E, Linker-Israeli M, Said J, Umiel T, deVos S, Shintaku IP, Heber D, Binderup L, Uskovic M, Koeffler HP** 1995 20-Epi vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and differentiation of human breast cancer cells. Cancer Res 55:2822–2830
14. **James SY, Mackay AG, Colston K** 1996 Effects of 1,25 dihydroxyvitamin D_3 and its analogues on induction of apoptosis in breast cancer cells. J Steroid Biochem Mol Biol 58:395–401
15. **Iino Y, Yoshida M, Sugamata N, Maemura M, Ohwada S, Yokoe T, Ishikita T, Ooriuchi R, Morishita Y** 1992 1α-Hydoxyvitamin D_3, hypercalcemia, and growth suppression of 7,12-dimethylbenz[a}anthracene-induced rat mammary tumors. Breast Cancer Res Treat 22:133–140
16. **Colston K, Chander SK, Mackay AG, Coombes RC** 1992 Effects of synthetic vitamin D analogs on breast cancer cell proliferation *in vivo* and *in vitro*. Biochem Pharmacol 44:693–702
17. **Colston K, Mackay AG, James SY, Binderup L** 1992 EB1089: a new vitamin D_3 analogue that inhibits the growth of breast cancer cells *in vivo* and *in vitro*. Biochem Pharmacol 44:2273–2280
18. **James SY, Mackay A, Binderup L, Colston K** 1994 Effects of a new synthetic vitamin D analogue, EB1089, on the oestrogen responsive growth of human breast cancer cells. J Endocrinol 141:555–563
19. **Colston K, Mackay AG, James SY** 1994 The role of $1\alpha25(OH)_2D_3$ and its analogs in breast cancer. In: Norman AW, Bouillon R, Thomasett M (eds) Proceedings of the Ninth Workshop on Vitamin D. de Gruyter, Berlin, pp 477–484
20. **Colston K, Mackay AG, James SY** 1995 Vitamin D_3 derivatives and breast cancer. In: Tenniswood M, Michna H (eds) Apoptosis in Hormone Dependent Cancers. Springer Verlag, Berlin, pp 201–224
21. **Narvaez CJ, VanWeelden K, Byrne I, Welsh JE** 1996 Characterization of a vitamin D_3 resistant MCF-7 cell line. Endocrinology 137:400–409
22. **Kyprianou N, English H, Davidson N, Isaacs J** 1991 Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res 51:162–166

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