The Combination of a Potent Vitamin D_3 Analog, EB 1089, with Ionizing Radiation Reduces Tumor Growth and Induces Apoptosis of MCF-7 Breast Tumor Xenografts in Nude Mice[1]

Sujatha Sundaram,[2] Andrea Sea, Stephanie Feldman, Rendall Strawbridge, P. Jack Hoopes, Eugene Demidenko, Lise Binderup, and David A. Gewirtz

Departments of Surgery [S. S., A. S., S. F., R. S., P. J. H.] and Community and Family Medicine [E. D.], Dartmouth Medical School, Lebanon, New Hampshire 03756; Leo Pharmaceuticals, Ballerup, Denmark [L. B.]; and Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia 23298 [D. A. G.]

ABSTRACT

Purpose: **The purpose of this research was to evaluate the influence of the combination of the vitamin D_3 analogue EB 1089 with fractionated radiation on growth and apoptosis of MCF-7 tumor xenografts in athymic mice.**

Experimental Design: **Four to six-week-old ovariectomized mice were injected s.c. with MCF-7 tumor cells suspended in Matrigel. When tumors reached a size of approximately 150–200 mm^3, animals were exposed to EB 1089 (45 pmols/day) for 8 days, whereas mice that were to be irradiated in the absence of EB 1089 received solvent (Solutol HS15). After the termination of EB 1089 and solvent administration, tumors were irradiated (3 × 5 Gy) over a period of 3 days using a 300 KV Pantax Therapax irradiator. Tumor growth was monitored for 25–30 days after the last dose of irradiation in a double-blind manner; tumor cellularity was assessed by H&E and trichrome staining, cell proliferation by Ki-67 staining, and apoptosis by terminal deoxynucleotidyltransferase-mediated nick end labeling assay. Rates of tumor regression were assessed using a mixed effects statistical model.**

Results: **A significantly higher rate of decline in tumor volume (7.5% per day) was observed in mice exposed to radiation subsequent to EB 1089 compared with animals treated with radiation alone (5.6% per day). Final tumor volumes in animals irradiated after EB 1089 were ~50% lower than in the group that received radiation alone. Loss of cellularity, a marked reduction in the fraction of proliferating cells, and the promotion of apoptosis confirmed that the combination of EB 1089 with radiation was significantly more effective than radiation alone in blocking tumor cell growth and promoting tumor cell death.**

Conclusions: **This work demonstrates that EB 1089 can improve local tumor control by fractionated radiation, in part through the promotion of apoptotic cell death.**

INTRODUCTION

Breast cancer is the most common malignant disease of middle-aged women in the United States, with the expectation that ~200,000 women will be diagnosed with invasive breast cancer this year (1, 2). Radiotherapy has been used extensively in the treatment of breast cancer, both as a neoadjuvant for the reduction of breast tumors preoperatively and to reduce the local recurrence of microtumors postoperatively, as well as in combination with chemotherapy for nonresectable tumors (3, 4).

The development of combined modality treatments for breast cancer has relied largely on empirical approaches using drugs with established effectiveness against advanced disease in combination with external beam radiation therapy. More recent approaches that have been developed for the selective enhancement of tumor cell killing with sparing of normal tissue have emphasized mechanism(s) of interaction or cooperation between radiation and drugs (5). Targets for development of new therapeutic approaches have included inhibition of growth factor receptors, blocking of tumor angiogenesis, modulation of apoptosis, and inhibition of invasion and metastasis (6).

Vitamin D has demonstrated *in vitro* differentiating activity against a broad range of tumor cell types (7, 8). While differentiation-inducing agents have sometimes proven effective for the treatment of leukemias (9, 10) such compounds have displayed little clinical activity against other malignancies. The clinical utility of differentiating agents alone against solid tumors has additionally been confounded by their limited therapeutic efficacy and high toxicity. To develop an approach that might harness differentiating agents for the treatment of solid malignancies, we have combined a relatively noncalcemic vitamin D_3 analogue, EB 1089, with radiation (11) and with Adriamycin (12).

EB 1089 (Seocalcitol) is a novel vitamin D analogue where the side chain has been altered by the addition of two double bonds (13). EB 1089 is 50–200-fold more active than vitamin D_3 in inhibiting cell proliferation and inducing differentiation, while having a 3-fold lower calcemic effect (13, 14). A number of clinical trials have demonstrated that EB 1089 is well tolerated at doses ranging from 5 to 40 μg/day (13–15).

Previous studies from this laboratory have demonstrated

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[2] To whom requests for reprints should be addressed, at Department of Surgery, Dartmouth Medical School, One Medical Center Drive, HB 7850, Lebanon, NH 03756. Phone: (603) 650-5008; Fax: (603) 650-4928; E-mail: Sujatha.Sundaram@dartmouth.edu.

significant growth inhibition in several human breast tumor cell lines in culture by the combination of EB 1089 with radiation (11). In addition, a significant increase in TUNEL-positive (*i.e.*, apoptotic) cells was detected when EB 1089 was combined with either radiation (11) or Adriamycin (12). Whereas our previous studies were performed with a single dose of radiation (10 Gy), breast cancer patients typically receive up to 60 Gy in fractions of 2–2.5 Gy. Gupta *et al.*[4] have extended and substantiated these findings *in vitro* using lower doses of fractionated radiation (5 × 2 Gy) administered at daily intervals subsequent to EB 1089 treatment. In the current work, we report that EB 1089 in concert with fractionated radiation also effectively induces apoptosis and reduces the number of proliferating cells in an experimental model system of MCF-7 xenografts in nude mice, additionally supporting the potential utility of vitamin D_3 analogues in the treatment of breast cancer.

MATERIALS AND METHODS

Cells. Studies were performed using MCF-7 human breast tumor cells that were obtained from the National Cancer Institute tumor repository (Frederick, MD). Twenty-four h before inoculation in nude mice, subconfluent cultures grown in RPMI 1640 at 37°C were fed with fresh medium, washed with PBS, trypsinized, resuspended in medium, and pooled. After centrifugation, cells were resuspended in Matrigel (Collaborative Biomedical, Waltham, MA) and cold RPMI 1640 (4:1) for s.c. inoculation in mice.

Animals. Ovariectomized NCr (*nu/nu*) mice of 4–6 weeks of age (National Cancer Institute) were implanted s.c. with 17β-estradiol release pellets (90-day release, 3.0-mm size pellets with a total of 0.72 mg; Innovative Research, Sarasota, FL) followed by inoculation of $\sim 5 \times 10^6$ cells in 0.1 ml volume of Matrigel/medium mix into the right flank of the animal. Animals were fed a vitamin D-deficient diet with 1.0% calcium (Purina Test Diets, Indianapolis, IN) throughout the study to minimize the hypercalcemic effects of EB 1089. The tumor take rate was ~90%. Animals were examined twice weekly for the development of palpable tumors at the site of injection.

Treatments. When tumor volumes reached approximately 150–200 mm^3, tumor-bearing mice were randomly assigned to treatment groups receiving either solvent (consisting of disodium phosphate dihydrate, sodium dihydrate phosphate dihydrate, sodium chloride, sodium, and Solutol HS-15), EB 1089 alone (45 pmol/24 h for 8 days), radiation alone (3 × 5 Gy), or EB 1089 followed by radiation. EB 1089 was delivered via continuous infusion using Alzet osmotic pumps (model No: 1002) at a rate of 0.25 μl/h. Pumps were primed in saline at 37°C before implantation in animals in order that the drug could be infused from the time of implant. After solvent or EB 1089 treatment, a subset of animals was subjected to fractionated doses of radiation for 3 consecutive days. Radiation was administered to two of the tumor-bearing mice at one time (anesthetized by halothane/Isoflurane) using a 300 KV Pantax Therapax irradiator with a source-to-skin distance of 30 cm and using a 2-cm cone at a dose rate of 330 rad/min.

Analysis of Tumor Growth. Tumor length and width were measured using calipers, and tumor volume was calculated using the formula L × W × (1/2 of the greater of length or width). Tumor volumes were determined at least twice weekly for ~30 days after radiation treatment. The rate of change in tumor volumes was determined using the linear mixed effect model for logarithmic tumor volume of Vonesh and Chinchilli (16). Unlike the standard statistical assumption where all of the parameters are assumed to be constant, this model assumes that each mouse tumor has a subject-specific initial volume but a constant rate of decline within the same treatment group, making the assumption a more realistic representation of the data. The significance of differences in tumor volumes between treatment groups was analyzed using the statistical package S-Plus-6 (Insightful Inc., Seattle, WA).

Biochemical/Histopathological Analyses. Blood was drawn during the course of the study, and plasma was separated and analyzed for calcium by colorimetry (Sigma Diagnostic kit). Tumors were excised and fixed in 10% buffered formalin and processed for histopathological analyses. Tumors were embedded in paraffin, sectioned at 5-μm thickness, and stained with H&E and Masson's trichrome stain for morphological assessment. The mitotic index and apoptotic index, respectively, were assessed by histomorphometric analyses of the two established markers, Ki- 67 expression and *in situ* terminal transferase-mediated fluorescein dUTP nick end labeling or TUNEL (Boehringer Mannheim, Indianapolis, IN) in accordance with the manufacturers' protocols. Ki-67 expression was analyzed using a Biogenex I6000 immunostainer with a multilink, horseradish peroxidase detection system from Biogenex (San Ramon, CA). The formalin-fixed tissue was pretreated with citra buffer in a steam chamber before immunostaining with the primary antibody Ki-67 (clone Mib-1; Immunotech, Miami, FL) at a dilution of 1:1000 for a 25-min incubation. After Ki-67 staining, an average of 7–10 digital images from different fields of the tissue sections were captured, and the number of positively stained brown cells was counted using NIH Imaging software in a double-blind manner.

RESULTS AND DISCUSSION

Growth Inhibitory Effects of EB 1089 and Radiation in MCF-7 Breast Tumor Xenografts. Fig. 1*A* shows the growth of untreated tumors, tumors exposed to radiation preceded by solvent, and tumors exposed to radiation subsequent to EB 1089 treatment. Day 0 represents the termination of the EB 1089 or solvent exposure period and the initiation of radiation treatment. All of the treatment groups were compared for the post-treatment rate of tumor volume regression, assuming exponential growth/regression after day 0. The slopes of the bold lines in Fig. 1*A* represent the average rates of tumor growth (controls) or regression of tumor volume for the treatment groups. Before day 0 (initiation of radiation treatment), the average rate of tumor growth in the EB 1089 treatment group (4.9% per day) was significantly lower ($P < 0.05$; $n = 6–10$) than in the solvent control group (6.7% per day;

[3] The abbreviations used are: TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; IR, ionizing radiation.

[4] M. S. Gupta, G. DeMasters, H. Wang, C. Gennings, M. Cabot, and D. A. Gewirtz. Influence of the vitamin D3 analog, EB 1089 on senescence, apoptosis, and sensitivity to fractionated radiation in MCF-7 breast tumor cells, manuscript in preparation.

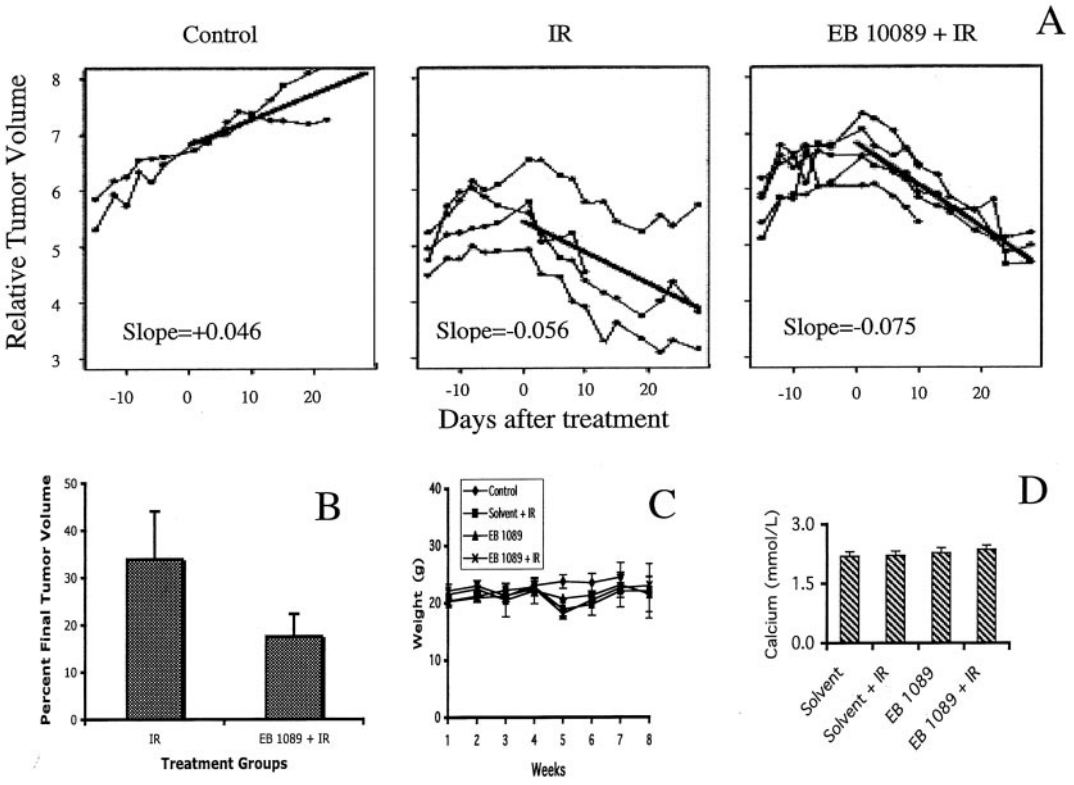

Fig. 1 *A,* influence of EB 1089 and radiation on the growth of MCF-7 tumor xenografts. Time zero represents the termination of the EB 1089 or solvent exposure and the initiation of radiation treatments (3 × 5 Gy) for the groups receiving radiation. Relative tumor volumes of each animal over the course of the study are plotted individually against time. It is assumed that tumor growth/regression follow an exponential function of time. The *bold line* shows the average pattern of change in tumor volume for the particular treatment group. The maximum regression rate occurs for the treatment group where EB 1089 and radiation are combined; 7.5% ($P < 0.05$) volume decrease per day after treatment. *B,* percentage of final tumor volumes for EB 1089 + radiation compared with radiation alone treatments. *C,* mean body weights of animals in all treatment groups during the course of the entire study. *D,* serum calcium levels of animals in all treatment groups at the termination of the experiment; *bars,* ±SD.

data not shown). This reduction in tumor growth rate by EB 1089 is consistent with the work of vanWeelden *et al.* (17).

Exposure to fractionated radiation produced a decline in tumor cell volume both in the absence and presence of EB 1089. However, the rate of tumor volume decline was greater in the group that received EB 1089 followed by radiation than in the group receiving radiation (+ solvent) alone (Fig. 1*A*). In animals exposed to solvent before radiation, the decline in tumor volume was 5.6% per day, resulting in a final tumor volume that was ~35% of the initial tumor volume. In contrast, when radiation was administered to animals subsequent to treatment with EB 1089, the tumors regressed at the rate of 7.5% per day, resulting in a final tumor volume that was ~18% of the initial tumor volume (Fig. 1*B*).

Animal Toxicity. Toxicity of the treatment protocols was evaluated by monitoring animal weights and serum calcium. No significant or irreversible weight loss or increase in serum calcium levels was detected under any of the treatment conditions (Fig. 1, *C* and *D*). Serum calcium levels appeared to be within the normal range under the different treatment conditions (Fig. 1*D;* 2.18–2.36 mmol/liter), which is consistent with other published studies (18–20). Although a transient increase in serum calcium levels in the EB 1089-treated animals may not have been detected with the serum calcium levels measured from a single time point, the data suggest that any elevation of serum calcium that might have been induced by EB 1089 during the treatment period was reversible and did not result in any permanent damage.

Influence of EB 1089 and Radiation on Tumor Histomorphometry. The influence of EB 1089, radiation, and EB 1089 + radiation on the occurrence of apoptotic cell death was determined using the TUNEL assay. Fig. 2 indicates minimal apoptosis with either radiation alone (3 × 5 Gy) or EB 1089 alone; in contrast, a wide field of apoptotic cells (bright green) is detected with the combination of EB 1089 followed by radiation. Apoptotic cells present in multiple images were quantified by a distribution function of the bits in the digitized images after conversion to gray scale. A lower distribution function seen in the combination group indicate a lighter image because of the presence of a higher fraction of apoptotic cells. As evident from the graph in Fig. 2, the combination group indicates a significant apoptotic effect ($P < 0.001$; Kolmogorov-Smirnov test; Ref. 21). This marked increase in the number of positively stained apoptotic cells observed in tumors exposed to EB 1089 and radiation suggests that this could be a mechanism for the potentiation of the rate of tumor regression observed when radiation exposure is preceded by EB 1089.

The results of staining the tumor tissue sections with the

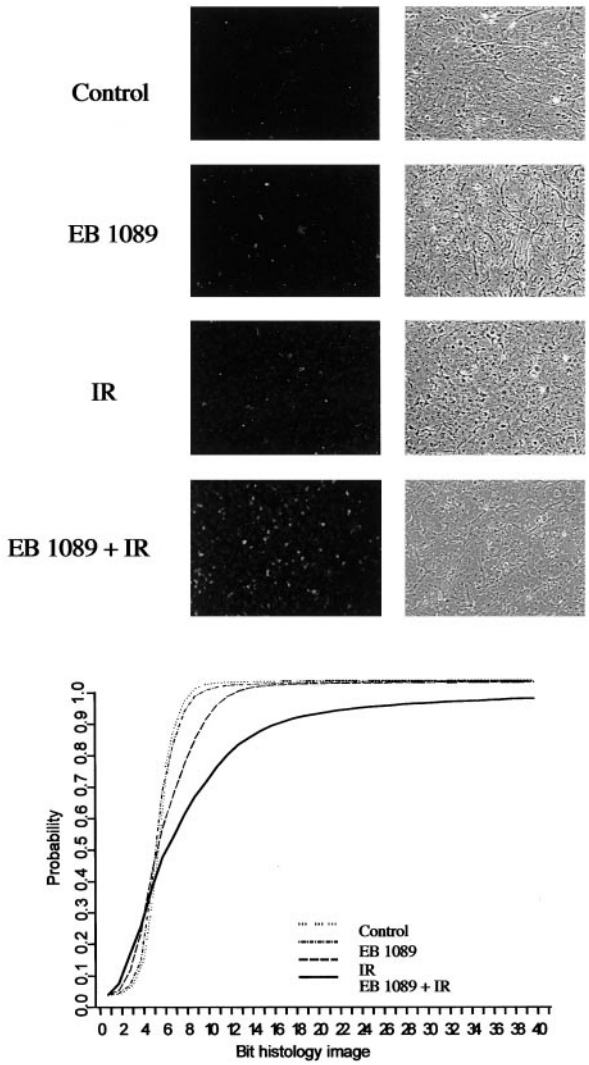

Fig. 2 Influence of EB 1089 and radiation on promotion of apoptosis. The distribution of apoptotic cells in tumor tissue sections was determined by the TUNEL assay using fluorescent microscopy as well as analyses of the digitized histological images. Apoptotic cells are bright green, permitting quantification by a distribution function of the bits in the digitized images after conversion to gray scale. A lower distribution function indicates a lighter image and, therefore, a higher fraction of apoptotic cells after staining. As evident from this graph, the combination of EB 1089 and radiation has a significant apoptotic effect ($P < 0.001$; Kolmogorov-Smirnov test). The *right panel* shows bright field images of the same field of tissue section after fluorescent imaging.

proliferation marker, Ki-67, are presented in Fig. 3. An approximately 8–10-fold reduction in Ki-67 positively stained brown cells was evident in the EB 1089 + radiation (14 ± 1) group compared with either radiation alone (160 + 14) or EB 1089 alone (111 ± 13). A strong correlation was noted between the decline in the number of positively stained Ki-67 proliferative cells and the rates of change in tumor volumes after day 0 in all of the treatment groups ($R^2 = 0.903$; data not shown). These studies additionally substantiate the enhanced antiproliferative effects of the combination of EB 1089 with radiation in MCF-7 breast tumor cell xenografts.

Histological analyses by H&E staining provided additional confirmation that the combination of EB 1089 with radiation has a marked impact on the cellular content of the tumor cell xenografts. Tumor sections from the untreated group consisted of tightly packed cells (typical of adenocarcinomas) interspersed with many blood sinuses, whereas the sections from the combination treatment group were considerably less cellular (Fig. 4*A*). This histomorphometric analysis is additionally consistent with a reduced tumor burden in animals treated with EB 1089 + radiation.

The relatively acellular nature of the tumors exposed to the combination treatment group is additionally supported by the results of tissue staining with Massan's trichrome stain (Fig. 4*B*). In this protocol, the cytoplasm, keratin, muscle fiber, and intracellular fiber stain red, whereas the collagen and mucus stain blue. The percentage of blue staining was found to be significantly higher in the tissue taken from tumors exposed to EB 1089 + radiation group compared with tissue from tumors that were either untreated or exposed to either radiation alone or EB 1089 alone.

Our data from these *in vivo* studies closely parallel and support the recent findings by Gupta *et al.*[4] on the interaction of EB 1089 and fractionated radiation using MCF-7 cells in culture. In these studies, EB 1089 (100 nM) produced an ~6-fold reduction in the cumulative dose of fractionated radiation required to reduce clonogenic survival by 50% with an increase (6-fold) in DNA fragmentation in the irradiated cells quantified by alkaline unwinding. Furthermore, this work demonstrated selective enhancement of growth inhibition in the breast tumor cells, because no potentiation by EB 1089 was evident in either normal breast epithelial cells or normal human fibroblasts.

A number of research groups have shown EB 1089 alone to be effective in controlling growth of various types of tumors *in vivo*, at doses ranging from 15 pmol/day in human squamous carcinoma, 30 pmol/day in prostate tumor xenografts, and 45 pmol/day in breast tumor xenografts (17–20, 22). Additional preclinical evidence in the literature indicates that vitamin D_3 can potentiate the antitumor activity of agents such as cisplatin, dexamethasone, and paclitaxel in both murine squamous cell carcinoma and human prostate cancer model systems *in vivo* (23–26). However, to our knowledge, this is the first study to combine a vitamin D analogue with conventional radiotherapy to obtain enhanced tumor regression in an animal model system. Because radiation therapy continues to have a significant role in cancer treatment, identifying novel agents such as EB 1089 that will selectively enhance the antitumor efficacy of radiotherapy could have significant clinical potential.

This study demonstrates that combining EB 1089 with irradiation may enhance the control of breast tumor growth. Local recurrence of tumors in previously irradiated fields is known to occur in certain breast cancer patients and may be attributable, in part, to the presence of a subset of radioresistant tumor cells. In such cases, EB 1089 treatment could allow for lowered doses of radiation to be used for the treatment of these breast cancer patients who may now be more sensitive to the toxicity of radiation treatments. As indicated above, the dose of radiation required to obtain a 50% reduction in the number of surviving tumor colonies was significantly reduced by prior exposure EB 1089.[4]

The current work cannot provide complete mechanistic insights relating to the basis for the antitumor effects of the EB 1089-radiation combination in the MCF-7 breast tumor xe-

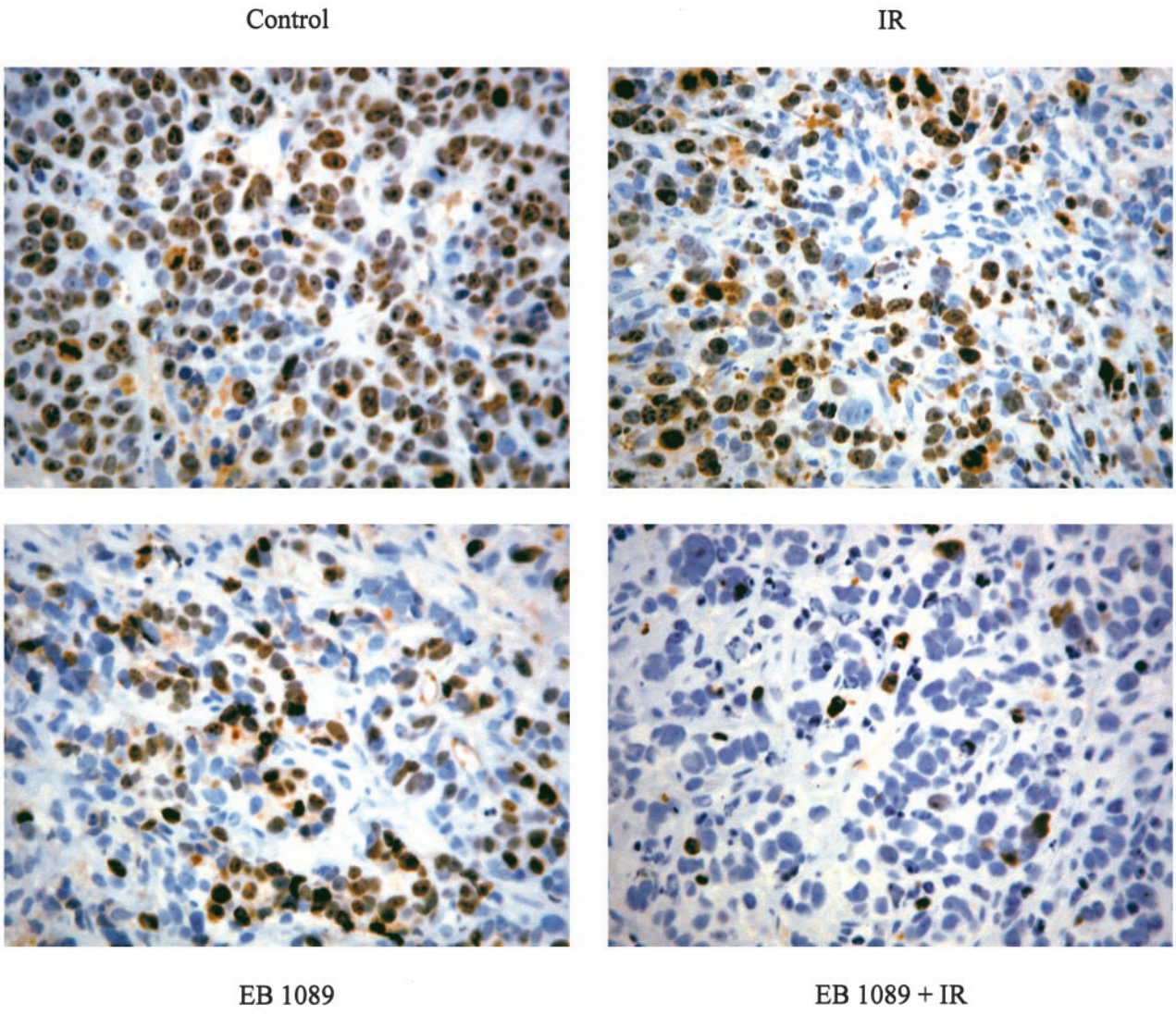

Fig. 3 Proliferative activity of tumors exposed to EB 1089, radiation, and EB 1089 + radiation by Ki67 staining of paraffin-embedded tumor tissue sections.

nografts. However, because the treatments of estrogen receptor-positive MCF-7 cells with EB 1089 or vitamin D_3 have been shown to cause a time- and dose-dependent decrease in the estrogen receptor at both protein and mRNA levels (27–29), the growth inhibition and promotion of apoptosis observed may perhaps be related, in part, to interference with the growth promoting and cytoprotective functions of estrogen. Evidence from the literature strongly indicates a requirement for sustained circulating estradiol levels by exogenous administration in ovariectomized athymic mice for optimal growth of this hormone dependent tumor (MCF-7; Refs. 17, 30, 31); therefore, estradiol supplementation is necessary to evaluate EB 1089-radiation interaction in this experimental model system.

It is imperative to acknowledge the limitations of studies using animal models of s.c. growing human tumors in immunodeficient mice. We recognize that these model systems are not truly representative of clinical cancer for a number of reasons. These include the use of a tumor that is not spontaneous or carcinogen induced, the implantation of a breast tumor in the flank of the animal rather than the site in which mammary tumors would normally develop, the fact that this is a human tumor growing in a mouse that requires immunosuppression and, therefore, negates the potential impact of the immune system, questions about whether the genetics of a cell line continuously passaged in culture is representative of human breast tumors, and reservations as to whether chemosensitivity or susceptibility to differentiation in such a model is predictive of the clinical situation (32–34). Nevertheless, this model has served to confirm and validate the interaction of EB 1089 with radiation in controlling MCF-7 tumor growth, thereby extending and substantiating the results of our previous studies in a cell culture system (11).

The results obtained using our current model provide quantitative as well as qualitative evidence that the combination of EB 1089 with radiation is effective in inducing apoptosis and

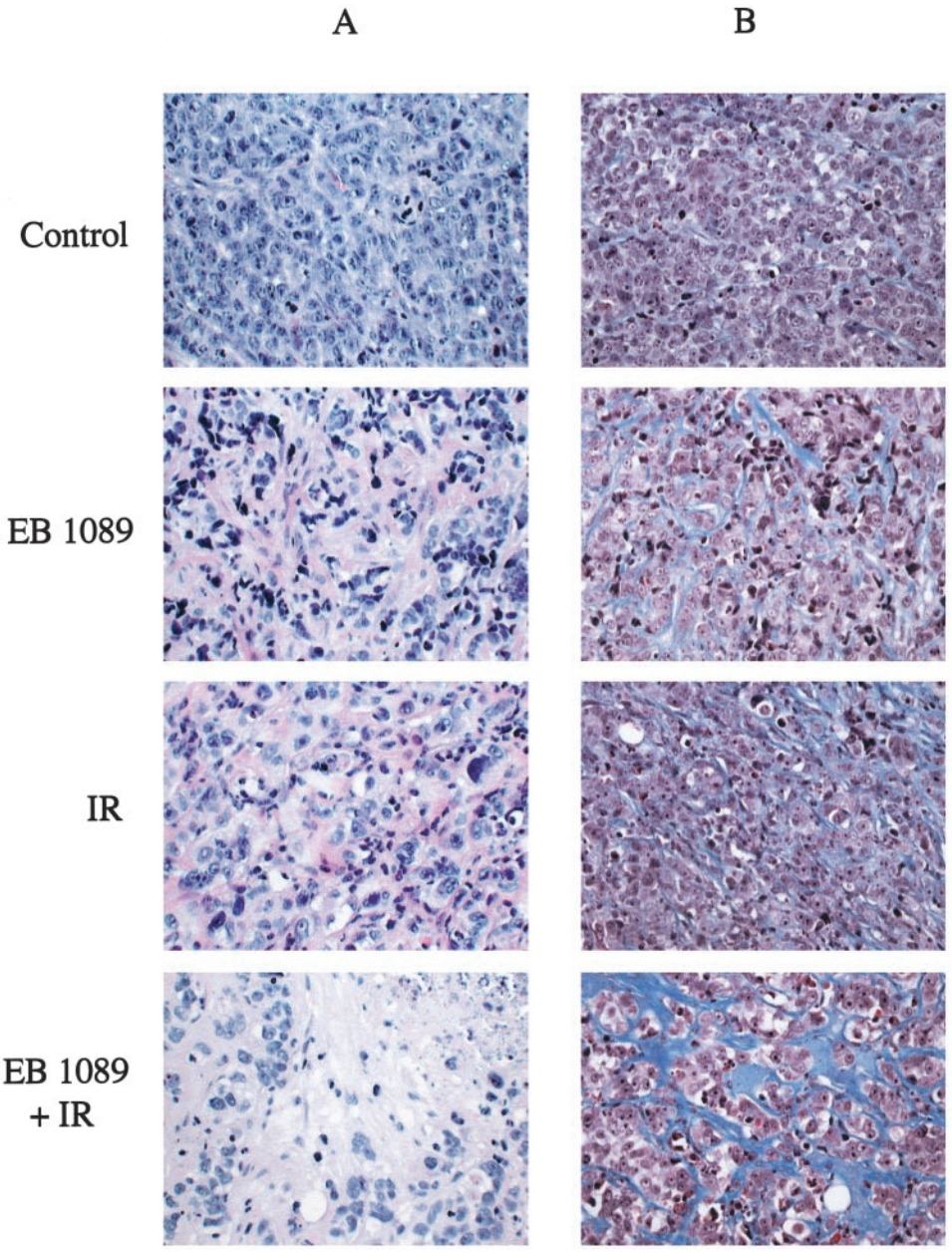

Fig. 4 Histomorphometry of tumor tissue sections after exposure to EB 1089, radiation, and EB 1089 + radiation. *A,* H&E staining denoting the increase of acellular material in the EB 1089 + radiation treatment group. *B,* Masson's trichrome staining of acellular material (*blue staining*) present in the tumor tissue sections.

enhancing the rate of tumor regression without evident toxicity. These findings could be extended using more clinically representative tumor xenograft models in which tumors have been transplanted into their original location, designated as the orthotopic site (35). Recent studies (36, 37) using this surgical orthotopic implantation model to transplant histologically intact fragments of human cancer, including tumors taken directly from the patient, to the corresponding organ of immunodeficient rodents, are proving to be quite useful in the development and evaluation of new anticancer drugs.

In conclusion, although EB 1089 and other vitamin D_3 analogues have previously been used alone and in combination with chemotherapeutic agents in several tumor models, to our knowledge, this is the first study to combine these compounds with radiation. Our work indicates that EB 1089 can be effective in promoting apoptosis in response to radiation therapy, which may result in an enhanced tumor regression rate. EB 1089 also holds promise for clinical use because of its ability to inhibit tumor growth without inducing hypercalcemia. Our findings additionally support the potential utility of combining vitamin D_3 analogues with conventional treatment protocols in the treatment of cancer.

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