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Potentiation of cell killing by fractionated radiation and suppression of proliferative recovery in MCF-7 breast tumor cells by the Vitamin D_3 analog EB 1089☆

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Abstract

A senescence-like growth arrest succeeded by recovery of proliferative capacity was observed in MCF-7 breast tumor cells exposed to fractionated radiation, 5 × 2 Gy. Exposure to EB 1089, an analog of the steroid hormone 1α, 25 dihydroxycholecalciferol (1α, 25 dihydroxy Vitamin D_3; calcitriol), prior to irradiation promoted cell death and delayed both the development of a senescent phenotype and the recovery of proliferative capacity. EB 1089 also reduced clonogenic survival over and above that produced by fractionated radiation alone and further conferred susceptibility to apoptosis in MCF-7 cells exposed to radiation. In contrast, EB 1089 failed to enhance the response to radiation (or to promote apoptosis) in normal breast epithelial cells or BJ fibroblast cells. EB 1089 treatment and fractionated radiation additively promoted ceramide generation and suppressed expression of polo-like kinase 1. Taken together, these data indicate that EB 1089 (and 1α, 25 dihydroxycholecalciferol or its analogs) could selectively enhance breast tumor cell sensitivity to radiation through the promotion of cell death, in part through the generation of ceramide and the suppression of polo-like kinase.

Keywords: Breast cancer; Radiation; Vitamin D; Apoptosis; Ceramide

1. Introduction

The growth of various tumor cell lines, both in cell culture and in the form of xenografts, have been shown to be sensitive to the metabolically active form of Vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$ D_3; 1α, 25 dihydroxycholecalciferol) [1–3]. Because of limitations to the concentrations of 1,25$(OH)_2$ D_3 that can be achieved in the clinic before hypercalcemia is evident [4], a number of 1,25$(OH)_2$ D_3 analogs, such as EB 1089 (seocalcitol; 1(*S*),3(*R*)-dihydroxy-20(*R*)-(5′-ethyl- 5′ - hydroxy - hepta - 1′(*E*),3′(*E*)-dien-1′-yl)-9,10-secopregna-5(*Z*),7(*E*),10(19)-triene), have been synthesized with reduced hypercalcemic activity [5].

Vitamin D_3 and its analogs may exert their antiproliferative effects via multiple pathways which include cross-talk with the estrogen signaling pathway [6], modulation of growth factor responses [7], differentiation induction [8], induction of cell cycle regulatory proteins [9–13], as well as

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the promotion of apoptosis [14,15]. Studies in this laboratory have shown that MCF-7 breast tumor cells are relatively refractory to apoptosis in response to clinically relevant concentrations of adriamycin [16] as well as to ionizing radiation, even at relatively high doses [17]. However, apoptosis was evident in MCF-7 cells exposed to either adriamycin or radiation when exposure to these agents was preceded by incubation of the cells in the presence of Vitamin D_3 analogs, indicating that these compounds may selectively manipulate the signaling pathway that is permissive for apoptotic cell death in MCF-7 breast tumor cells [18–21].

Previous work from this laboratory describing the interaction of Vitamin D_3 and its analogs with ionizing radiation [18,20] involved a single dose of radiation, generally focusing on a supraclinical (10 Gy) dose. However, sensitization was most pronounced at lower doses in the range of 2 Gy. As breast cancer is routinely treated using multiple fractionated low doses of radiation in the range of 2 Gy to minimize normal tissue toxicity [22], the present studies were designed to extend the previous findings by assessing the interaction of the 1,25$(OH)_2$ D_3 analog, EB 1089, with a cumulative dose of 10 Gy administered in 2 Gy fractions. The time course of the response was evaluated in order to distinguish between effects on growth arrest and cell death, as well as to assess the influence of EB 1089 on radiation-induced senescence arrest. The question of therapeutic ratio was also addressed by evaluating the effects of this combination in two normal cell lines, human breast epithelial cells and human fibroblasts.

In order to evaluate possible mechanisms underlying the ability of EB 1089 to promote radiation-induced cell death, the generation of ceramide was assessed, as several groups have demonstrated the association of ceramide with radiation-induced cell death [23–25]. Effects on expression of Polo-like kinase 1 (Plk1) were examined since depletion of Plk1 is associated with tumor cell apoptosis [26], while the mechanism of tumor cell response to radiation involves activation of the cell cycle G_2 checkpoint through inhibition of the Polo-like kinase 1 [27].

2. Materials and methods

2.1. Materials

The p53 wild-type MCF-7 human breast tumor cell line was obtained from NCI, Frederick, MD. EB 1089 was provided by Dr. Lise Binderup, Leo Pharmaceuticals, Denmark. Normal human mammary epithelial cells were obtained from Clonetics, San Diego, CA; human foreskin BJ fibroblasts, originally generated by Dr. JR Smith at the Baylor College of Medicine, were obtained from Dr. Jerry Shay at the University of Texas Southwestern Medical Center [28]. Human cDNA clones for PLK and CCNB1 were obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany). The human cDNA probe for cyclin B1 was a gift from Dr. B. Vogelstein (The John Hopkins University School of Medicine, Baltimore, MD), whereas the human cyclin D1 cDNA kindly was provided by Dr. D. Beach (Howard Hughes Medical Institute, Cold Spring Harbor, NY). RPMI 1640 and supplements were obtained from GIBCO Life Technologies, Gaithersburg, MD. Reagents used for the TUNEL assay (terminal transferase, reaction buffer, and Fluorescein-dUTP) were purchased from Boehringer Manheim, Indianapolis, IN. X-gal was obtained from Gold Biotechnology, St. Louis, MO. All other reagents used in the study were analytical grade.

2.2. Cell culture and treatment protocols

All cell lines were grown from frozen stocks in basal RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin at 37 °C under a humidified, 5% CO_2 atmosphere. In all studies, MCF-7 cells were exposed either to EB 1089 (100 nM) alone for 72 h, five fractions of 2 Gy gamma radiation administered on three consecutive days (using a cesium irradiator at a dose rate of 3 Gy/m)[1] or EB 1089 for 72 h followed by fractionated radiation (again, five fractions administered on three consecutive days). For the studies in which EB 1089 preceded irradiation, the EB 1089 was removed and the cells washed twice with phosphate buffered saline prior to irradiation. This sequence of exposure was based on the studies by Wang et al. [29] which have demonstrated a requirement for prolonged incubation with Vitamin D_3 or its analogs to promote sensitivity to adriamycin, as well as our own previous work [19–21].

2.3. Cell viability and clonogenic survival

Cell viability was determined by trypan blue exclusion at various time points starting 6 h after the last dose of radiation. Cells were harvested using trypsin, stained with 0.4% trypan blue dye, and counted using phase contrast microscopy.

For clonogenic survival studies, cells were trypsinized under sterile conditions following EB 1089 and/or radiation and plated in triplicate in six well tissue culture dishes at the appropriate density for each condition. After 14 days, the cells were fixed with 100% methanol, air-dried for 1–2 days and stained with 0.1% crystal violet. For computing the survival fraction, groups of 50 or more cells were counted as colonies. Data were normalized relative to untreated controls which were taken as 100% survival.

2.4. TUNEL assay for apoptosis

The method of Gavrielli et al. [30] was utilized as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and non-adherent cells.

[1] In some experiments, radiation was administered daily at doses of 2 Gy over a period of 5 days. The response of the cells was essentially identical for both patterns of irradiation.

Cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using the In Situ Cell Death Detection Kit (Boehringer-Manheim), where strand breaks are end labeled with fluorescein dUTP by the enzyme terminal transferase. Cells were then washed, mounted in Vectashield and photographed using a Nikon fluorescent microscope.

2.5. Propidium iodide staining and flow cytometry

After treatment with EB 1089 and/or fractionated radiation, cells were harvested using trypsin, pelleted by centrifugation and washed twice with PBS. Cellular DNA was labeled by resuspending 1×10^6 cells in 1 ml propidium iodide staining solution (3.8 mM NaCitrate, 0.05 mg/ml propidium iodide, 0.1% triton X-100, 9 K units/ml RNase B). Cells were analyzed for DNA content with a Beckman Coulter XL-MCL flow cytometer. A minimum of 25,000 events were collected for each sample.

2.6. Alkaline unwinding assay

The induction of DNA fragmentation was substantiated using the alkaline unwinding assay [31] as described previously [18]. Briefly, this involved determination of the ratio of double stranded and single stranded DNA after exposure to the various agents. *F*-values [31] were converted to radiation equivalence based on standardization of the assay using graded doses of radiation.

2.7. Beta-galactosidase histochemical staining

At the appropriate times after treatment, cells were washed twice with PBS and fixed with 2% formaldehyde, 0.2% glutaradehyde for 5 min. The cells were then washed again with PBS and stained with a solution of 1 mg/ml 5-bromo-4-chloro-3-inolyl-β-galactosidase in dimethylformamide (20 mg/ml stock), 5 mM potassium ferrocyanide, 5mM potassium ferricyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0, and 2 mM $MgCl_2$ [32]. Following overnight incubation at 37 °C, the cells were washed twice with PBS, and photographed with a light microscope.

2.8. Ceramide analysis

Cell radiolabeling with [^{3}H]palmitic acid, lipid extraction, and analysis of [^{3}H]ceramide by thin layer chromatography were conducted as described previously [33]. Lipids were visualized in iodine vapor. Commercial standards were co-chromatographed and spots were scraped and analyzed for tritium by liquid scintillation counting.

2.9. Northern blot analysis

Total RNA was extracted and analyzed by standard Northern blotting technique. Samples of 10 μg RNA were resolved by gel electrophoresis before transfer onto Hybond-N+ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The cDNA probes were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by random priming technique, and standard Church hybridization conditions were used. To evaluate the amounts of RNA loaded, the filters were rehybridized to a kinase-labeled oligonucleotide probe complementary to nucleotides 287–305 of human 18S rRNA.

2.10. Measure of synergism versus additivity

The predicted response for the cell viability assay was determined using the following model: $y = \gamma \exp(-\exp(-(\beta_0 + \beta_1 x_1 + \beta_2 x_2))) + \varepsilon$, where y is the predicted response (% reduction in cell viability), x_1 is concentration of EB 1089 (nM), x_2 is the fractionated radiation dose (Gy), γ is an unknown parameter associated with the maximum effect response, β_0 is an unknown parameter associated with the intercept, is β_1 an unknown parameter associated with the slope of x_1, and β_2 is an unknown parameter associated with the slope of x_2. ε is an unobserved random error term assumed to have mean 0 and constant variance. Parameter estimates were found using a generalized least squares criterion for nonlinear models. A constant variance was assumed across the concentration range of the drug/radiation. The Gauss–Newton iterative algorithm was used in PROC NLIN in SAS (version 8.01) to find parameter estimates. An overall test for additivity [34] was based on testing the hypothesis that the mean response under the hypothesis of additivity is the true mean response at the observed mixture points. The estimated responses for the true means were provided by the sample means at each mixture group.

3. Results

3.1. Effects of EB 1089 and fractionated radiation on cell viability and clonogenic survival of MCF-7 cells

Previous studies from this laboratory have demonstrated that the Vitamin D_3 analog EB 1089 enhances the response of both MCF-7 and ZR-75-1 breast tumor cells to ionizing radiation [18,20]. Effects on the response of MCF-7 cells to adriamycin were also reported [19]. In the current studies, radiation was administered in 2 Gy fractions, an experimental condition that more closely reflects the manner in which radiation is administered to patients in the clinical setting. As shown in Fig. 1A, fractionated radiation (five daily doses of 2 Gy) and EB 1089 (100 nM for 72 h) each alone respectively produced a 75% and 84% reduction in cell number compared to the untreated control, while treatment with EB 1089 prior to fractionated radiation reduced viable cell number by 98%.

In separate studies, prior exposure to EB 1089 failed to modify the response to fractionated radiation in either normal breast epithelial cells or normal human fibroblasts (data

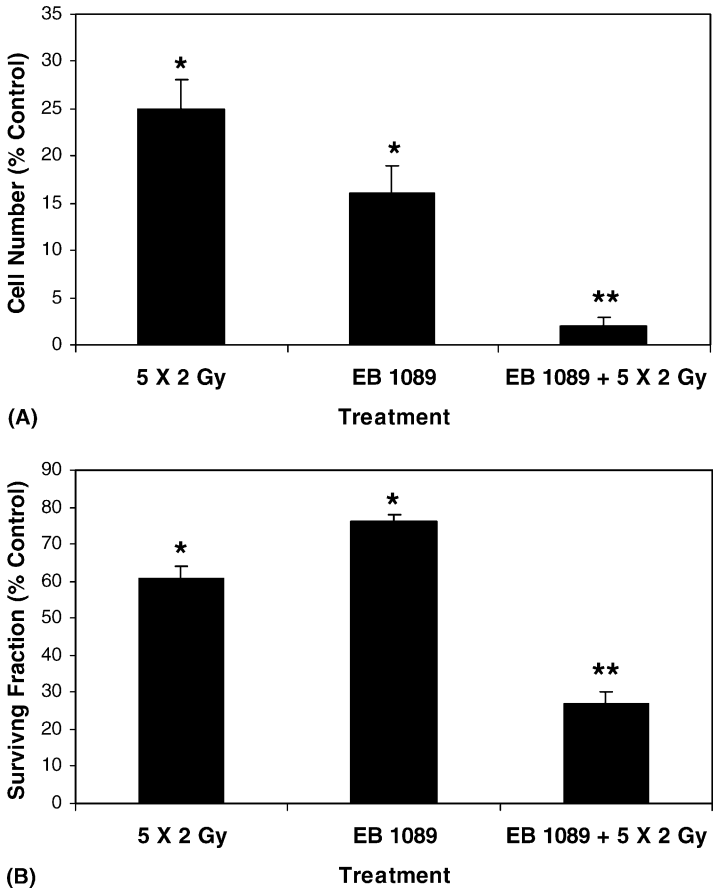

Fig. 1. (A) Influence of EB 1089 on the antiproliferative effects of fractionated radiation in MCF-7 cells. Cells were treated with EB 1089 (100 nM) for 72 h followed by fractionated radiation (5×2 Gy). Six hours following the 5th dose of radiation, all cells were harvested by trypsinization and viable cell number was determined by trypan blue exclusion assay. Data presented are mean ± S.E.M. of three experiments. (*) Significantly different than control ($p > 0.05$); (**) significantly different than fractionated radiation or EB 1089 alone ($p < 0.05$). (B) Clonogenic survival of MCF-7 cells after exposure to either 100 nM EB1089, 5×2 Gy fractionated irradiation, or EB 1089 followed by 5×2 Gy IR. Data represent mean ± range from two independent experiments. (*) Significantly different than control ($p > 0.05$); (**) significantly different than fractionated radiation or EB 1089 alone ($p < 0.05$).

not shown). These findings are similar to those reported previously, demonstrating a lack of potentiation of the response to radiation by the Vitamin D_3 analog, ILX23-7553 (1,25-dihydroxy-16-ene-23-yne Vitamin D_3), in human fibroblasts [21].

Statistical analysis was performed on the data presented above to determine whether the observed effects of the combination of EB 1089 and fractionated radiation on cell viability were occurring through a synergistic or additive interaction between the two modalities. The study design included dose-effect data of fractionated radiation alone (5×0.5 Gy, 5×1.0 Gy, 5×2.0 Gy, and 5×3.0 Gy), concentration-effect data of EB 1089 alone (5, 10, 50, 100, 200 nM), and a mixture point of the sequence of (100 nM) EB 1089 followed by 5×2 Gy radiation. The endpoint of interest was percent reduction in cell viability 24 h after treatment. Table 1 indicates that the observed percent reduction in cell viability was not significantly different from that predicted by the model of additivity ($p = 0.917$).

The cell viability studies were substantiated by assessment of clonogenic survival. Fig. 1B indicates that while EB 1089 and fractionated radiation each alone reduced survival by approximately 20% and 40% respectively, the sequential exposure to EB 1089 followed by fractionated radiation reduced survival by almost 70%. In separate studies (not shown), exposure of the cells to EB 1089 for 72 h prior to radiation resulted in a six-fold reduction in the cumulative radiation dose which produces a 50% reduction in clonogenic survival (from 5×3 Gy to 5×0.5 Gy).

Table 1
Observed results from cell viability assay of EB 1089 in combination with radiation were compared to values predicted using a statistical model of additivity

EB 1089 (nM)	100
Radiation	2.0
Observed % reduction	0.82
Predicted % reduction under additivity	0.85
95% prediction interval under additivity	[0.41.1.19]

Observed and predicted values were not significantly different ($p = 0.917$); therefore the assumption of additivity is not rejected.

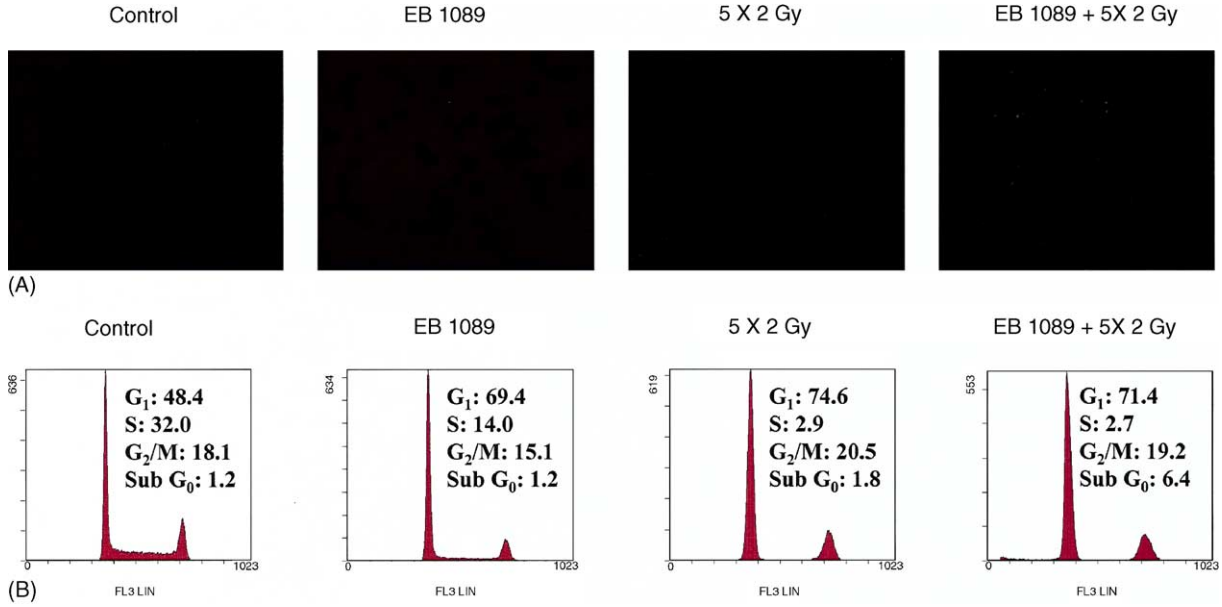

Fig. 2. Effects of EB 1089 on the promotion of fractionated radiation-induced apoptosis in MCF-7 cells. (A) DNA fragmentation in MCF-7 cells was determined by fluorescent end-labeling. Cells were isolated following the fifth dose of radiation, cytospun onto glass slides, and stained according to the TUNEL protocol as described in materials and methods. Magnification 20×. (B) The fraction of cells in different phases of the cell cycle (with a particular emphasis on cells with a sub G_0/G_1 DNA content indicative of DNA fragmentation associated with apoptosis) was also determined by propidium iodide staining of the DNA and quantitated by flow cytometry 24 h post-irradiation. The *X*-axis represents the degree of fluorescence (FL3 stands for fluorochrome 3) while the *Y*-axis reflects the number of cells (control—peak at 636; EB 1089—peak at 834; 5 × 2 Gy—peak at 619; EB 1089 + 5 × 2 Gy—peak at 563). Similar profiles were generated in duplicate experiments.

3.2. Effects of EB 1089 and fractionated radiation on apoptosis in MCF-7 cells

We have previously reported that while breast tumor cells are refractory to apoptosis in response to DNA damage by radiation [17], as well as clinically relevant doses of adriamycin [16,35], apoptosis is detected in response to adriamycin and (relatively high doses of) radiation in the presence of Vitamin D_3 analogs [18–20]. In order to determine whether EB 1089 might promote susceptibility to apoptosis in MCF-7 cells after the relatively low fractionated radiation doses used in the current studies, apoptosis was evaluated using three separate approaches, TUNEL analysis, propidium iodide (PI) staining/flow cytometry, and alkaline unwinding.

The results of both the TUNEL assay presented in Fig. 2A and PI staining/flow cytometry presented in Fig. 2B indicate minimal evidence for apoptosis in MCF-7 cells treated with EB 1089 (100 nM) or fractionated radiation (5 × 2 Gy) alone. However, a low, but significant degree of apoptosis was clearly evident when radiation was preceded by exposure to EB 1089. The flow cytometry data indicate that the fraction of apoptotic cells (sub G_0 phase) was approximately 6%, a 3.5-fold increase over that observed with radiation or EB 1089 alone. A similar percentage (between 5 and 7%) of apoptotic cells was detected at 3 and 5 days after the last dose of radiation (data not shown). The flow cytometry data presented in Fig. 2B also show that both EB 1089 and radiation each alone promote a marked reduction in the S phase population, as well as an accumulation of cells in the G_1 phase of the cell cycle, consistent with the known modes of actions of these individual treatments.

Table 2
DNA fragmentation assay in MCF-7 cells

	RAD equivalents
Control	96 ± 2
EB 1089	187 ± 4
5 × 2 Gy	100 ± 3
EB 1089 + 5 × 2 Gy	1082 ± 8

Alkaline unwinding was utilized to quantify the extent of DNA fragmentation in MCF-7 cells 24 h after exposure to EB 1089, 5 × 2 Gy or EB 1089 + 5 × 2 Gy. Data are expressed as mean ± range of two independent experiments.

A quantitative increase in apoptosis by the combination of EB 1089 with fractionated radiation was confirmed by the alkaline unwinding assay shown in Table 2. DNA fragmentation did not exceed baseline levels for radiation alone, while a relatively small degree of fragmentation (equivalent to ∼1 Gy when corrected for baseline levels of fragmentation) was evident with EB 1089 alone. The combination of EB 1089 with fractionated radiation produced an approximately 10-fold increase in DNA fragmentation.[2] It should be emphasized that this assay is being utilized to measure (delayed) DNA fragmentation rather than direct and immediate DNA strand breaks produced by irradiation.

[2] Possible reasons for the discrepancy with Table 2 are addressed in the discussion.

3.3. Influence of EB 1089 on the temporal response to fractionated radiation on MCF-7 cells

Our previous studies have assessed single time point responses to radiation alone and radiation exposure subsequent to the Vitamin D_3 analogs. In order to distinguish between growth arrest and cell death responses, MCF-7 cells were treated with EB 1089 alone, 5×2 Gy, or EB 1089 followed by 5×2 Gy and cell viability was determined at different time points of up to 21 days post-irradiation.[3] Fig. 3A shows the fold increase in cell number at various times post-irradiation compared to the number of cells present at the initiation of the radiation treatment. Cells exposed to radiation alone continued to proliferate, albeit at a reduced rate, during the course of radiation exposure, and subsequently arrested for a period of 7–10 days. Pretreating MCF-7 cells with EB 1089 markedly alters the cellular response to subsequent radiation. Of note is the observation that the cells do not appear to proliferate during the radiation treatment,[4] and as shown in Fig. 3A, cell death appears to be the primary initial response to radiation in cells primed by exposure to EB 1089. By 24 h post-irradiation, approximately 20% of the original cell population has been lost, while by 5 days post-irradiation, almost 80% of the cells have died.

3.4. Induction of senescence by fractionated irradiation

As shown in Figs. 2B and 3A, growth arrest is evident after radiation alone and in cells which have been exposed to EB 1089 prior to irradiation. However, under the latter conditions, the growth arrest is delayed by cell death. Studies by Chang et al. [36], as well as our own published data [37] and unpublished work,[5] have identified a senescence arrest response to both adriamycin and high dose radiation. In order to determine whether the growth arrest associated with EB 1089 and/or fractionated irradiation in MCF-7 cells is characteristic of senescence, staining with the senescence marker Beta-galactosidase was evaluated. As shown in Fig. 3B, while cells treated with EB 1089 alone do not stain positive for beta-galactosidase, cells exposed to 5×2 Gy radiation alone show a strong beta-galactosidase signal by 72 h post-irradiation, suggesting that these cells are undergoing senescence arrest. Fig. 3B also indicates that EB 1089 treatment does not prevent radiation-induced senescence; however, the senescence response is delayed until 5–7 days after radiation, in large part because the primary response to the EB 1089-radiation combination is cell death.

In vivo studies using tumor cell xenografts exposed to irradiation generally show a transient growth inhibition followed by tumor regrowth (i.e. tumor growth delay) [38,39]. The temporal response studies presented in Fig. 3A also demonstrate "tumor cell regrowth" in the MCF-7 cell culture model system. In cells exposed to fractionated radiation alone, a cohort of cells demonstrated proliferative activity by 14 days. In contrast, in the cells exposed to EB 1089 prior to irradiation, proliferative recovery was significantly delayed. As a consequence, the number of viable cells remaining 14 days after treatment with EB 1089 followed by radiation is approximately 5% of that observed in cells exposed to fractionated radiation alone.

3.5. Generation of ceramide by EB 1089 and ionizing radiation

Ceramide generation has been associated with promotion of apoptosis by ionizing radiation as well as chemotherapeutic agents such as taxol [23–25,40]. To evaluate whether ceramide was generated in association with EB 1089 and radiation-induced cell death, MCF-7 cells were treated with EB 1089, ionizing radiation, or EB 1089 followed by radiation and analyzed for ceramide generation. These studies were performed utilizing a 10 Gy dose of radiation (rather than fractionated doses of 5×2 Gy) in order to generate sufficient ceramide to be detectable within the sensitivity limits of this assay. We felt that this approach would establish "proof of principle" as the effects of EB 1089 on the response to radiation are similar with a single high dose of radiation and lower fractionated doses. Table 3 indicates that EB 1089 increases the ceramide levels within the cell to 132% of control values. A single dose of radiation (10 Gy) likewise elicited an increase (148% of control) in ceramide. The combination of EB 1089 with radiation resulted in an essentially additive increase in ceramide levels (172% of control).

3.6. EB 1089 promotes radiation-induced down-regulation of Polo-like kinase 1 in MCF-7 cells

We have recently reported that cell cycle arrest at the G_2/M boundary after radiation involves repression of the gene for Plk1, PLK [41]. In order to determine whether EB 1089 pretreatment could modify this response, PLK mRNA expression was assessed after radiation exposure of MCF-7 cells

Table 3
Ceramide generation in MCF-7 cells

Treatment	Ceramide (% of control)
Control	100 ± 5.5
EB 1089	132.2 ± 4.3
10 Gy IR	148.1 ± 6.7
EB 1089 + 10 Gy IR	171.8 ± 4.5

MCF-7 cells were treated with 100 nM EB 1089 for 72 h. EB 1089 was then removed, [3H]palmitic acid was added, and cells were irradiated (10 Gy). Data are expressed as mean ± range of two independent experiments.

[3] Cells transiently exposed to EB 1089 alone demonstrate a reduced rate of growth (approximately 50%), but recover quickly once removed from the presence of EB 1089. Untreated cells have a doubling time of 18–20 h (data not shown).

[4] We cannot exclude the possibility that some cell proliferation and cell death are occurring concomitantly after both radiation alone and the combination of EB 1089 with radiation.

[5] Manuscript in progress: ionizing radiation-induced senescence is associated with telomere dysfunction in p53 wild-type breast tumor cells.

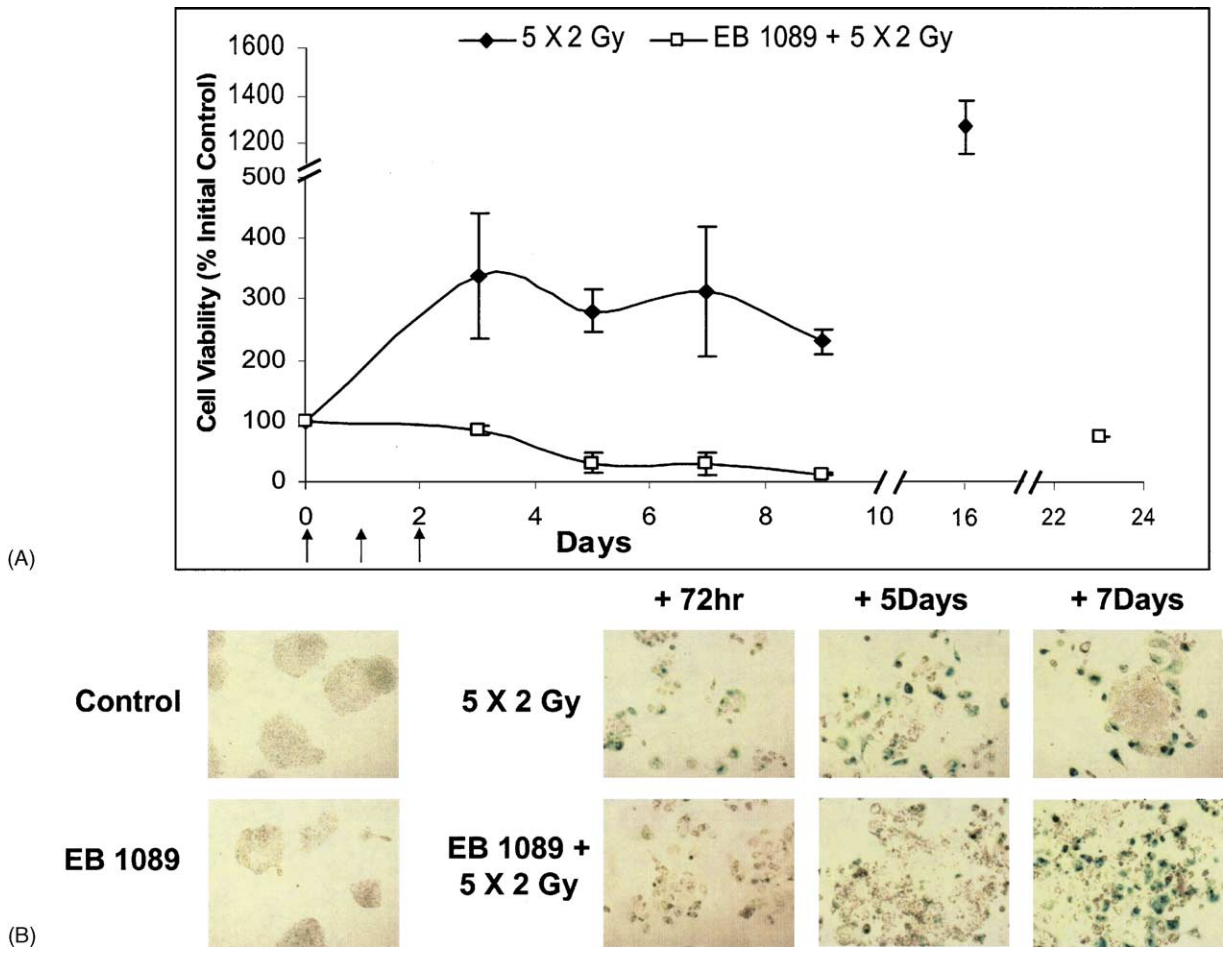

Fig. 3. (A) Proliferation time course of MCF-7 cells exposed to 5×2 Gy fractionated irradiation with and without prior treatment with 100 nM EB 1089. Proliferation was measured as a fold increase in viable cell number compared to the number of cells at the start of irradiation. Arrows indicate days of radiation exposure. Data represent mean ± range of two independent experiments. (B) Beta-galactosidase expression after EB 1089, 5×2 Gy IR, or EB 1089 followed by 5×2 Gy. Cells were treated and fixed after removal of EB 1089 or after indicated time following last dose of radiation.

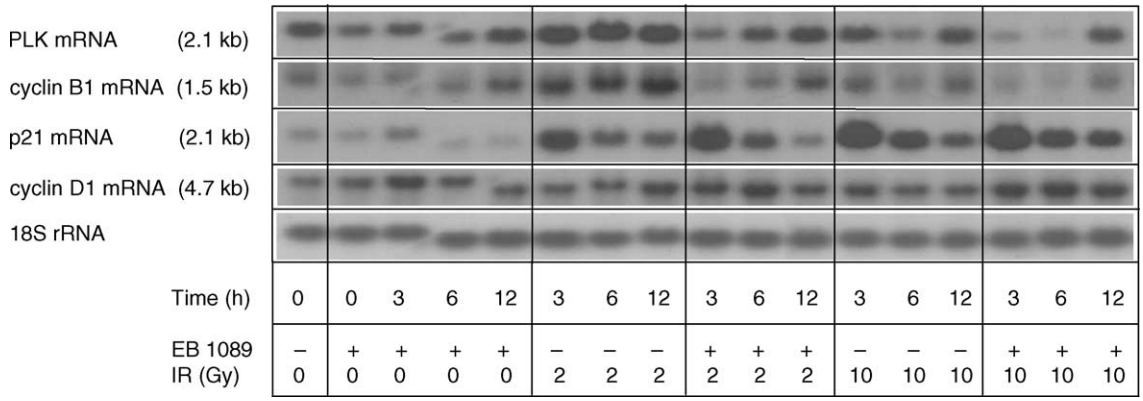

Fig. 4. Regulation of mRNA expression for cell cycle regulatory factors. MCF-7 cells were cultured in the absence (−) or presence (+) of EB1089 (100 nM) for 72 h before exposure to 2 Gy or 10 Gy of ionizing radiation (IR). Expression of mRNAs for PLK, cyclin B1, p21, and cyclin D1 was analyzed after the indicated time periods after IR, or removal of EB 1089 in studies with EB 1089 alone. 18S rRNA was measured as RNA loading control.

grown in the absence and presence of EB 1089. For comparison, the mRNA levels for cyclin B1, which is another radiation-sensitive regulatory factor in the G_2 phase of the cell cycle, as well as the G_1 phase regulatory factors were also analyzed (Fig. 4). Whereas the single radiation dose of 2 Gy was insufficient to cause any change in the level if PLK mRNA expression, a transient down-regulation of PLK mRNA was observed after radiation with 10 Gy. Interestingly, pretreatment with EB 1089 was clearly associated with a similar repression of PLK mRNA after exposure to 2 Gy, and also seemed to amplify the response of PLK mRNA to 10 Gy. The regulatory responses of cyclin B1 mRNA were essentially identical to those of PLK mRNA. Although EB 1089 transiently increased the expression of p21 mRNA, the radiation-induced mRNA for p21 was not modified by the EB 1089 pretreatment. Finally, the mRNA for the G_1 phase cyclin D1 was unaltered by radiation exposure as well as EB 1089 treatment.

4. Discussion

A number of studies have demonstrated the utility of combining Vitamin D_3 analogs with conventional chemotherapeutic drugs such as tamoxifen, platinum compounds, and adriamycin against a variety of tumor cell lines [29,42–46]. In addition, phase I clinical trials have suggested that high dose intermittent therapy with 1α, 25 dihydroxycholecalciferol itself is potentially safe [47,48]. Breast tumor cells, in general, tend to be refractory to apoptotic cell death in response to modalities that induce DNA damage [17,35,49]. Our recent work [18–21] has focused on utilizing Vitamin D_3 analogs such as EB 1089 and ILX-23-7553 to confer susceptibility to apoptosis in response to adriamycin or ionizing radiation in the breast tumor cell. These studies have demonstrated that pretreatment with EB 1089 or ILX-23-7553 followed by an acute dose of adriamycin (1 μM) or a single (10 Gy) dose of radiation resulted in enhanced cell killing, as well as increased DNA fragmentation indicative of apoptosis [18–20]. Our recent short report indicated that an analog which is no longer under development, ILX23-7553, promotes apoptosis with fractionated radiation [21]. The current work extends these findings to a Vitamin D_3 analog in clinical trials, examines the temporal response to EB 1089 and fractionated radiation (a more clinically relevant approach), assesses effects on clonogenic survival, cell cycle and senescence, as well as addressing possible mechanisms of action of this sequence of treatment.

This work supports our previous observations indicating that EB 1089 enhances susceptibility to radiation and promotes apoptosis [18,20]. Three separate assays were used to assess the enhanced apoptotic response observed when radiation is preceded by exposure to EB 1089. The PI staining analyzed by flow cytometry yielded a 3.5-fold increase in apoptotic cells, while the alkaline unwinding assay showed a 10-fold increase in apoptotic cells. We believe that the discrepancy between the two analytical approaches may be the result of heavily fragmented DNA in the apoptotic cells. A 10-fold increase in overall DNA fragmentation could result from a 3.5-fold increase in the number of cells undergoing apoptosis as the DNA is being cleaved by apoptotic enzymes.

Whether the induction of apoptosis alone is sufficient to explain the observed cell killing effects of EB 1089 followed by radiation is not yet certain. Although we detect only 6% apoptotic cells after treatment, this reflects a snapshot of a single time point. Furthermore, a similar percentage of apoptotic cells was evident at other time points. Assuming that apoptotic cells do not survive for prolonged time periods, we hypothesize that cell death by apoptosis may represent a gradual process in this experimental model, with small cohorts of cells undergoing apoptosis over a period of 1–7 days until the bulk of the population has been decimated.

These findings in a cell culture system have also been substantiated in a breast tumor xenograft model system [50]. In this work, treatment with EB 1089 followed by fractionated radiation resulted in an accelerated decline in tumor volume and the promotion of apoptosis. It is of further importance to note that these studies showed no detectable toxicity to the experimental animals.

Although our initial studies with EB 1089 followed by fractionated radiation demonstrated a simple additive effect (Fig. 1 and Table 1), this conclusion was based on an evaluation of cell number shortly after the last dose of radiation. This approach appears to underestimate the potential value of this combination approach, since the extended time course study in Fig. 3A suggests a more pronounced impact on tumor cell growth. In addition to the finding that pretreatment of MCF-7 cells with EB 1089 converts the initial growth arrest response to cell death, resulting in a rapid and pronounced reduction in viable cell number within 3 days, the capacity of residual cells to recover proliferative capacity appears to be markedly attenuated (although not entirely eliminated). This observation could have clinical ramifications if Vitamin D_3 or Vitamin D_3 analogs prove to lower the remaining tumor volume post-irradiation and substantially increase the tumor growth delay after radiation therapy.

The dramatic effects observed in the time course study were not completely reflected in the clonogenic survival assay. Although clonogenic survival is the “gold standard” for radiation responsiveness, it is possible that the “regrowth” or “recovery” phenomenon is in some way dependent on the presence of a surrounding cell population. We know from empirical experience that the MCF-7 cells grow quite slowly at very low densities — and appear to require neighboring cells for optimal growth characteristics. It is possible that after irradiation, dead or dying cells that retain some residual metabolic capacity might provide factors that promote regrowth — which would not occur at the low density of plating for clonogenic survival assays. The identity of the “recovered” cells is under investigation, although studies from the laboratory of Roninson support the concept that these

recovered cells could be derived from the senescent cell population [51].

The mechanistic basis for the permissive effects of EB 1089 on the promotion of apoptosis, as well as enhancement of susceptibility to radiation in the breast tumor cell, remain to be fully elucidated. Our studies indicating that EB 1089 enhances radiation-induced ceramide generation further suggest that ceramide may have a distinct role in the action of EB 1089 in this experimental system. Although EB 1089 and radiation both promote ceramide generation in the breast tumor cell (Table 3), the levels achieved after either treatment alone may not be sufficient to promote apoptosis. The combined effects of EB 1089 and radiation may increase ceramide levels within the cell to a threshold level that is necessary to promote apoptosis.

Finally, while the suppression of polo like kinase message by the EB 1089-radiation combination appears to be a transient effect, it is becoming clear that the polo like kinases influence signaling pathways that could determine susceptibility to killing of the tumor cell by radiation [26,27]. Elevated Plk1 expression in the primary tumor is associated with an unfavorable clinical outcome [52,53]. Recent results have clearly shown that experimental depletion of Plk1 dramatically inhibits cellular proliferation and viability, possibly resulting from G_2 phase cell cycle arrest followed by apoptosis [26]. The finding that EB 1089 promotes the radiation-induced PLK mRNA repression may reflect the transient G_1 phase cell cycle arrest observed in Vitamin D_3-treated tumor cells harboring functional p53 [10,54], and is further consistent with the assumption that inhibition of Plk1 signaling may sensitize breast cancer cells to apoptosis in radiation therapy.

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