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Research Article

Pharmocologic Inhibitors of the Mitogen Activated Protein Kinase Cascade Have the Potential to Interact with Ionizing Radiation Exposure to Induce Cell Death in Carcinoma Cells by Multiple Mechanisms

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Ionizing radiation, MAP kinase, MAPK, Apoptosis, Survival, Caspase

ABSTRACT

Recent studies have shown that inhibition of stress-induced signaling via the mitogen activated protein kinase (MAPK) pathway can potentiate the toxic effects of chemotherapeutic drugs and ionizing radiation. Because of these observations, we have further investigated the impact upon growth and survival of mammary (MDA-MB-231, MCF7, T47D), prostate (DU145, LNCaP, PC3) and squamous (A431) carcinoma cells following irradiation and combined long-term exposure to MEK1/2 inhibitors. Exposure of carcinoma cells to ionizing radiation resulted in MAPK pathway activation initially (0-4h) and modestly enhanced MAPK activity at later times (24h-96h). Inhibition of radiation-induced MAPK activation using MEK1/2 inhibitors potentiated radiation-induced apoptosis in two waves, at 21-30h and 96-144h after exposure. The potentiation of apoptosis was not observed in MCF7, LNCaP, or PC3 cells. At 24h, the potentiation of apoptosis was independent of radiation dose whereas at 108h, apoptosis correlated with increasing dose. Removal of the MEK1/2 inhibitor either 6h or 12h after exposure abolished the potentiation of apoptosis at 24h. At this time, the potentiation of apoptosis correlated with cleavage of pro-caspases -8, -9 and -3, and with release of cytochrome c into the cytosol. Inhibition of caspase function using a pan-caspase inhibitor ZVAD blocked the enhanced apoptotic response at 24h. Selective inhibition of caspase 9 with LEHD or caspase 8 with IETD partially blunted the apoptotic response in MDA-MB-231, DU145 and A431 cells, whereas inhibition of both caspases reduced the response by >90%. Removal of the MEK1/2 inhibitor either 24h or 48h after exposure abolished the potentiation of apoptosis at 108h. Incubation of cells with ZVAD for 108h also abolished the potentiation of apoptosis. In general agreement with the finding that prolonged inhibition of MEK1/2 was required to enhance radiation-induced apoptosis at 108h, omission of MEK1/2 inhibitor from the culture media during assessment of clonogenic survival resulted in either little or no significant alteration in radiosensitivity. Collectively, our data show that combined exposure to radiation and MEK1/2 inhibitors can reduce survival in some, but not all, tumor cell types. Prolonged blunting of MAPK pathway function following radiation exposure is required for MEK1/2 inhibitors to have any effect on carcinoma cell radiosensitivity.

INTRODUCTION

Mammary and prostate carcinomas are two of the leading causes of death for American women and men, respectively. A variety of modalities including surgery, chemotherapy and ionizing radiation have been used to treat these diseases with variable success at preventing re-occurrence. In vitro, carcinoma cells are often found to be relatively resistant to the toxic effects of ionizing radiation and thus further investigations designed to manipulate carcinoma cell survival are warranted.

The mechanisms by which radiation can either increase cell death or alter the proliferative rate of surviving cells are not fully understood. Recently, radiation has been shown to activate multiple signaling pathways within cells which can alter cell survival or proliferation depending upon the radiation dose, the cell type, and the culture conditions.[1-4] Several groups have shown that the epidermal growth factor receptor (EGFR) is activated in response to irradiation of carcinoma cells.[1,5-8] Radiation exposure, via activation of the EGFR, can activate the Mitogen Activated Protein kinase (MAPK) pathway to a level similar to that observed by physiologic, growth stimulatory, EGF concentrations.[5,6,9-11]

The ability of the "classical" mitogen activated protein kinase (MAPK) pathway to regulate proliferation versus differentiation and survival appears to depend upon the cell type examined, as well as upon the amplitude and duration of MAPK activation. A short activation of the MAPK cascade by growth factors has been correlated with increased proliferation, via both increased Cyclin D1 expression and an increased ability to progress

through the G1-S transition.[12] In contrast, prolonged elevation of MAPK activity has been demonstrated to inhibit DNA synthesis, via super-induction of the cyclin dependent kinase inhibitor proteins p16 and p21.[13] In addition to a role for MAPK signaling during G1/S phase, it has also been argued that MAPK signaling is involved in the ability of cells to progress through G2/M phase, particularly in cells following drug-induced growth arrest.[14,15]

The proliferation of many carcinoma cells in vitro and in vivo is in part regulated by the synthesis and autocrine actions of ligands such as transforming growth factor α(TGFα).[8,11] Irradiation of tumor cells can increase expression of TGFα, and this has been proposed to increase the proliferative rate of surviving cells.[16,17] Increased proliferative rates and poor prognosis of carcinomas in vivo are also correlated with increased expression of the EGFR.[18] MAPK signaling has also been linked to increased expression of other mitogens such as VEGF.[19-21] Growth factors such as VEGF and TGFα, in addition to a growth promoting role in vitro, may also play an important role in the development of tumors in vivo due to their abilities in the promotion of angiogenesis. Collectively, these findings argue that radiation may have a self-limiting effect on its toxicity via increased autocrine growth factor function and the activity of downstream signaling modules, including the MAPK pathway.

We and others have recently demonstrated that increased signaling by the EGFR and the MAPK pathway can be cytoprotective versus ionizing radiation and various cytotoxic drugs in diverse cancer cell lines.[1,8,10,11,22] Of note, several other groups have not observed MAPK signaling to be protective, which in part may be explained by both cell line differences and the fact that the ability of MAPK inhibition to sensitize DU145 cells in colony formation assays required a prolonged >24h blunting of MAPK function.[11,23-25] Despite the interest in MEK1/2 inhibitors as an anti-neoplastic agents by promoting growth arrest, the mechanisms by which they can induce cell death after irradiation remain incompletely understood. The studies in this report were designed to determine the mechanisms by which MEK1/2 inhibitors enhance radiosensitivity in carcinoma cells.

MATERIALS AND METHODS

Agarose conjugated anti-$p42^{MAPK}$ antibody (sc-154-AC) was from Santa Cruz Biotechnology (Santa Cruz Biotechnologies, CA). Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1000, rabbit polyclonal, NEB, Beverly, MA), anti-human Bcl-2 (1:2000, mouse monoclonal, Dako, Carpinteria, CA), Bax (N-20, 1:2000, rabbit polyclonal, Santa Cruz Biotechnology Inc.), Bcl-XS/L (S-18, 1:500, rabbit polyclonal, Santa Cruz Biotechnology Inc.), anti-human/mouse XIAP (1:500, rabbit polyclonal, R&D System, Minneapolis, MN), anti-cytochrome c (1:500, mouse monoclonal, Pharmingen), anti-caspase-3 (1:1000, rabbit polyclonal, Pharmingen), cleaved-caspase-3 (17kDa) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology), anti-caspase-9 (1:1000, rabbit polyclonal, Pharmingen), anti-caspase-8 (1:1000, rabbit polyclonal, Pharmingen), anti-PARP (1:2500, mouse monoclonal, Calbiochem), and cleaved PARP (89kDa) antibody (1:1000, rabbit polyclonal, Cell Signaling Technology). Radiolabelled [γ-^{32}P]-ATP was from NEN. Selective MEK1/2 inhibitors (PD184352, PD98059, and UO126) were supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile DMSO, and stored frozen under light-protected conditions at -20°C. In all experiments, the final concentration of DMSO did not exceed 0.1%. The pan-caspase inhibitor (Z-VAD-FMK), the caspase 8 inhibitor (Z-IETD-FMK), and the caspase 9 inhibitor (Z-LEHD-FMK) were purchased from Enzyme System Products (Livermore, CA), dissolved in DMSO and stored at 4 ºC. Western immunoblotting was performed using the Amersham Enhanced Chemi-Luminescence (E.C.L.) system. Other reagents were as in references 8 and 26.

Culture of Carcinoma Cells. Asynchronous cells (MCF7; MDA-MB-231; PC3; LNCaP; T47D; DU145; A431) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal calf serum at 37°C in 95% (v/v) air/5% (v/v) CO_2. Cells were plated at a density 3.2 x 10^4 cells/cm^2 plate area and grown for 24-36h prior to further experimentation.

Exposure of Cells to Ionizing Radiation and Cell Homogenization. Cells were cultured in DMEM + 5% (v/v) fetal calf serum as above. U0126/PD98059/PD184352 treatment was from a 100 mM stock solution and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). Cells were irradiated to a total of 2 Gy using a ^{60}Co source at dose rate of 2.1 Gy/min. Cells were maintained at 37°C throughout the experiment except during the ~1 min irradiation itself. Zero time is designated as the time point at which exposure to radiation ceased. After radiation-treatment cells were incubated for specified times followed by aspiration of media and snap freezing at 70°C on dry ice. Cells were homogenized in 1 ml ice cold buffer A [25 mM β-glycerophosphate, pH 7.4 at 4°C, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM phenylmethyl sulphonylfluoride, 1 μg/ml soybean trypsin inhibitor, 40 μg/ml pepstatin A, 1 μM Microcystin-LR, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 0.05% (w/v) sodium deoxycholate, 1% (v/v) Triton X100, 0.1% (v/v) 2-mercaptoethanol], with trituration using a P1000 pipette to lyse the cells. Homogenates were stored on ice prior to clarification by centrifugation (4°C).

Immunoprecipitations from Lysates. Fifty microliters of Protein A agarose (Ag) slurry (25 μl bead volume) was washed twice with 1 ml PBS containing 0.1% (v/v) Tween 20, and resuspended in 0.1 ml of the same buffer. Antibodies (2 μg, 20 μl), serum (20 μl) were added to each tube and incubated (3h, 4°C). For pre-conjugated antibodies, 10 μl of slurry (4 μg antibody) was used. Clarified equal aliquots of lysates (0.25 ml, ~100 μg total protein) were mixed with Ag-conjugated antibodies in duplicate using gentle agitation (2.5h, 4°C). Ag-antibody-antigen complexes were recovered by centrifugation, the supernatant discarded, and washed (10 min) sequentially with 0.5 ml buffer A (twice), PBS and buffer B [25 mM Hepes, pH 7.4, 0.1 mM Na_3VO_4].

Assay of p42MAPK Activity. Immunoprecipitates were incubated (final volume 50 μl) with 50 μl of buffer B containing 0.2 mM [g-^{32}P]ATP (5000 cpm/pmol), 1 μM Microcystin- LR, 0.5 mg/ml myelin basic protein (MBP), which initiated reactions at time = 0. After 20 min, 40 μl of the reaction mixtures were spotted onto a 2 cm circle of P81 paper (Whatman, Maidstone, England) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid, and once with acetone, and ^{32}P-incorporation into MBP was quantified by liquid scintillation spectroscopy.

SDS Poly-Acrylamide Gel Electrophoresis (SDS PAGE) and Western Blotting. Cells were irradiated and at specified time points/treatments media aspirated and the plates snap frozen. Cells were lysed with homogenization buffer and subjected to immunoprecipitation. Immunoprecipitates were solubilized with 100 μl 5X SDS PAGE sample buffer (10% (w/v) SDS), diluted to 250 μl with distilled water, and placed in a 100?C dry bath for 15 min. One hundred microliter aliquots of each time point were subjected to SDS PAGE on 10% (w/v) acrylamide gels. Gels were transferred to nitrocellulose by the Method of Towbin and Western blotting using specific antibodies performed as indicated. Blots were developed using Enhanced Chemi-Luminescence (Amersham) using Fuji RX x-ray film. Blots were digitally scanned using Adobe Photoshop 5.5, their color removed, and Figures created in Microsoft PowerPoint.

Terminal Uridyl-Nucleotide End Labeling (TUNEL) for Apoptosis. Cells were treated with or without varying concentrations of U0126/PD184352/PD98059/DMSO control 30 min prior to irradiation and irradiated (2 Gy). Cells were isolated 24h after irradiation by trypsinization followed by centrifugation onto glass slides (cytospin). Terminal Uridyl-Nucleotide End Labeling (TUNEL) was performed on these cells as described in references 8, 26, 28. Randomly selected fields of fixed cells (~150 cells per field, n=5 per slide) were counted initially using propidium iodide counter stain, followed by examination and counting of TUNEL positive staining cells of the same field under FITC/fluorescence light.

Analysis of Cytosolic Cytochrome C. At various times after irradiation/MEK1/2 inhibitor treatment, 2 x 10^6 cells were washed in PBS and lysed by incubating for 30 seconds in lysis buffer (75mM NaCl, 8mM Na_2HPO_4, 1mM NaH_2PO_4, 1mM EDTA, and 350 μg/ml digitonin). The lysates were centrifuged at 12,000g for 1 min, and supernatant was collected and added to equal volume of 2X sample buffer. The protein samples were quantified, separated by 15% SDS-PAGE, and subjected to immunoblot analysis as described above. Anti-cytochrome c (mouse monoclonal, Pharmingen) was used as primary antibody at a dilution of 1: 500.

Analysis of Mitochondrial Membrane Potential (mm). At various times after irradiation/MEK1/2 inhibitor treatment, 2 x 10^5 cells were incubated in situ with 40nM 3,3-dihexyloxacarbocynine (DiOC6, Molecular Probes Inc. Eugene, OR) in PBS at 37ºC for 20 min, isolated by trypsinization, and then analyzed by flow cytometry as described in references 26 and 28. The percentage of cells exhibiting low level of DiOC6 up-take, which reflects loss of mitochondrial membrane potential, was determined using a Becton-Dickinson FACScan analyzer.

Colony Forming (Clonogenic) Assay. Cells were pre-treated with MEK1/2 inhibitor as indicated, 2h prior to exposure. Cells were irradiated (2 Gy). After 48h, cells were isolated by tryptic digestion and re-plated as single cell suspensions plated on Linbro® plates at densities of 500 cells/well and 1000 cells/well. Colony formation was defined as a colony of 50 cells or greater, 10-14 days after plating.

Data Analysis. Comparison of the effects of treatments was done using one way analysis of variance and a two tailed t-test. Differences with a *p*-value of <0.05 were considered statistically significant. Experiments shown, except where indicated, are the means of multiple individual points from multiple separate experiments (±SEM). Statistical analyses were made using SigmaPlot and SigmaStat.

RESULTS

Radiation Induces Activation of the MAPK Pathway in Carcinoma Cells. The ability of ionizing radiation to modulate MAPK activity was investigated in carcinoma cells (Fig. 1 and Fig. 2). In the representative studies shown, radiation caused immediate primary activation of the MAPK pathway (0-10 min) followed by a later secondary activation (Fig. 1A). Incubation of cells with a relatively specific inhibitor of MEK1/2/5 (PD98059), blunted the ability of radiation to activate MAPK (Fig. 1A). In some cell types, e.g., LNCaP, radiation appeared to cause a reduction in MAPK activity (data not shown). Of note, basal MAPK activity varied greatly between the cell lines used in our study (Fig. 1B). Subsequent studies examined radiation-induced MAPK activity 24h-120h after exposure (Fig. 2). Radiation enhanced MAPK activity ~1.3 to 1.4-fold above basal levels 24h following exposure, which declined to control levels by 72h and remained at or near control levels until 120h after exposure. In contrast, radiation appeared to reduce MAPK activity in cells treated with a MEK1/2 inhibitor beyond that of MEK1/2 inhibitor alone, 48-120h after exposure (Fig. 2A and Fig. 2B).

Inhibition of Radiation-Induced MAPK Signaling Enhances Radiation-Induced Apoptosis at 24h. The impact of MEK1/2 inhibitors on radiation-induced apoptosis was initially determined 24h after treatment/exposure (Figs. 3A-3G). Inhibition of radiation-induced MAPK signaling had a variable effect on the levels of apoptosis observed 24h after exposure: for example, MCF7 and PC3 cells exhibited no potentiation in apoptotic cell killing whereas A431 cells showed a greater sensitivity to MEK1/2 inhibitors alone as well as a larger significant enhancement in double stranded DNA breaks by radiation. In the majority of cell lines examined, with the exception of T47D, the basal activity of ERK1/2 correlated with the ability of MEK1/2 inhibitors to enhance apoptosis (compare Fig. 1B to Fig. 3). Similar data were evident when plasma membrane integrity and staining of DNA was assessed using 7AAD (Figs. 4A-4D). Removal of the MEK1/2 inhibitor either 6h or 12h after irradiation abolished the potentiation of apoptosis at 24h (Fig. 5A), and increasing the radiation dose did not enhance the potentiation of apoptosis by MEK1/2 inhibitors at this time (Fig. 5B).

Simplistically, apoptosis is mediated via two inter-related cascades of

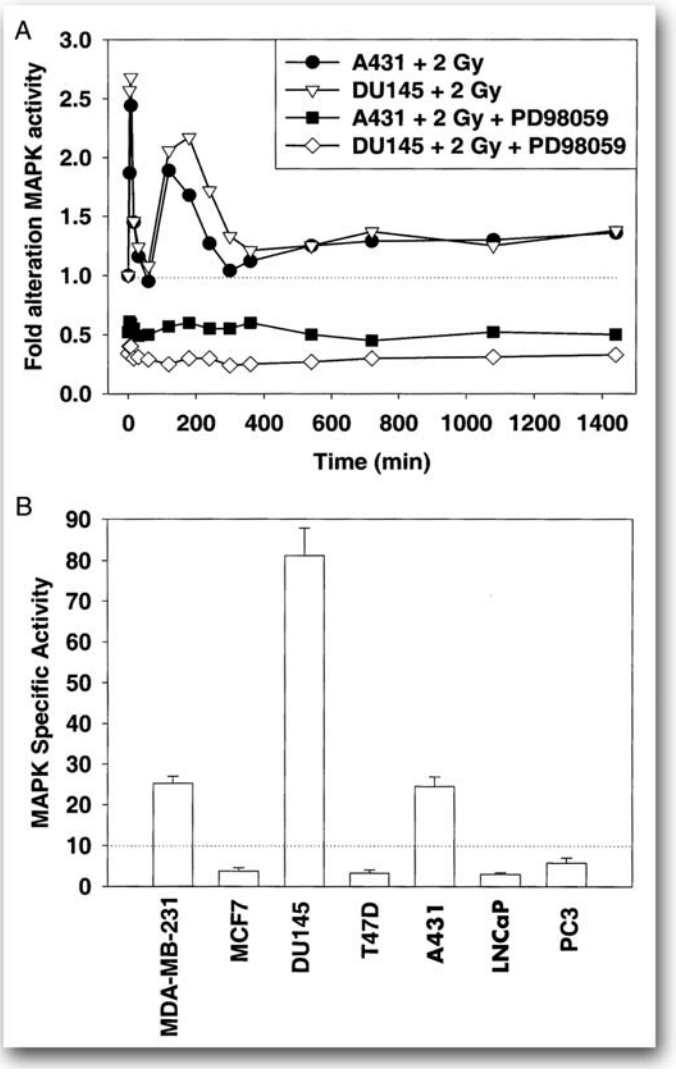

Figure 1. Ionizing radiation activates the MAPK pathway in carcinoma cells, which is blocked by inhibitors of MEK1/2. Cells were cultured as described in Methods. A. A431 and DU145 cells. B. Basal MAPK activity in MDA-MB-231, A431, DU145, MCF7, T47D, PC3 and LNCaP cells. Cells were pre-treated for 30 min with MEK1/2 inhibitor (PD98059; A431; 10 μM; DU145 25 μM) followed by irradiation (2 Gy) and MAPK activity determined over 0-1440 min as in Methods. Cells were lysed and portions (~100 μg) from each plate used to immunoprecipitate MAPK followed by immune-complex kinase assays as in Methods. In Panel A, MAPK activity data are duplicate values from a single representative experiment and are shown as -fold increases in ^{32}P-incorporation into MBP substrate. In Panel B, data are shown as specific MAPK activity (fmol/min/mg) ± SEM (n=3).

proteases (caspases); the intrinsic mitochondrial (caspase 9) pathway and the extrinsic death receptor (caspase 8) pathway. Caspase 3 can also mediate additional activation of caspase 9 via a feed-forward amplification loop involving it in caspase 8 processing, leading to BID cleavage and further cytochrome c release from the mitochondrion. We found that the potentiation of radiation-induced apoptosis at 24h was blocked by the pan-caspase inhibitor ZVAD and in MDA-MB-231, DU145 and A431 cells partially blunted by individual additions of either the caspase 8 inhibitor IETD or the caspase 9 inhibitor LEHD (Figs. 6A-6E). Combined use of both IETD and LEHD was required to completely prevent the apoptotic response in these cells. In contrast, IETD completely blocked the apoptotic response in T47D cells. In agreement with data arguing that both caspase 8 and caspase 9 played roles in the apoptotic process, enhanced apoptosis (Fig. 4 and Fig. 6) correlated with cleavage and reduced expression of pro-caspase 8, pro-caspase 9

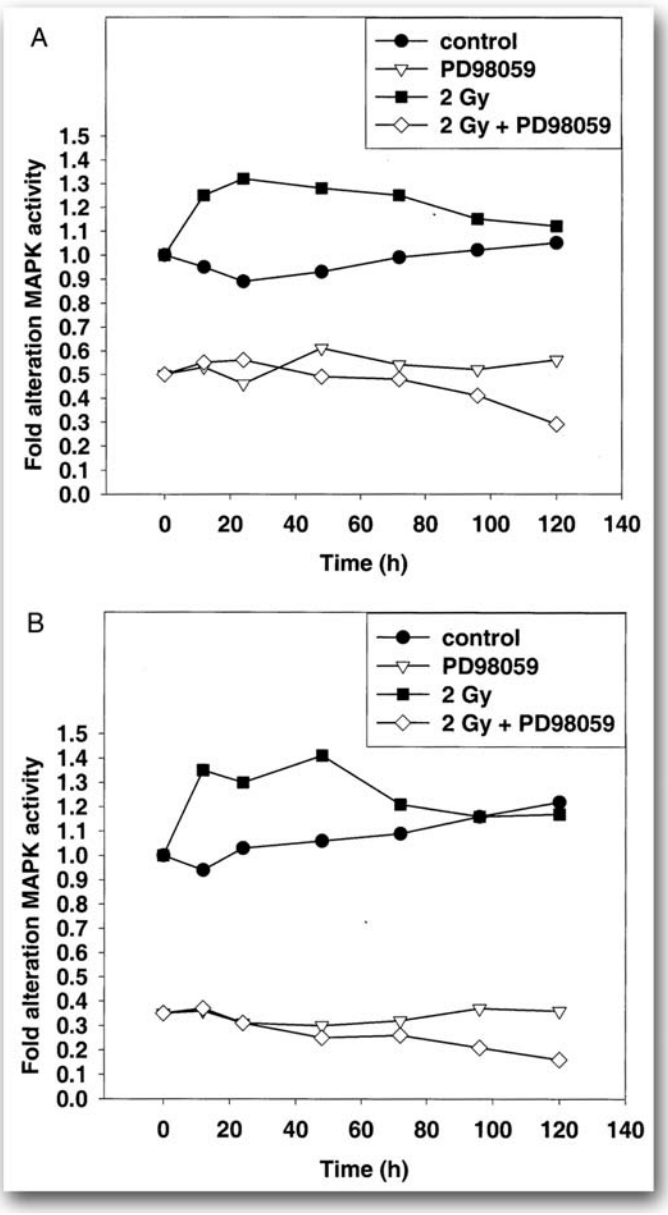

Figure 2. MAPK pathway activity in carcinoma cells 24-120h after exposure to ionizing radiation. Cells were cultured as described in Methods. A. A431 cells. B. DU145 cells. Cells were pre-treated for 30 min with MEK1/2 inhibitor (PD98059, 10 μM A431; 25 μM DU145) followed by irradiation (2 Gy) and MAPK activity determined over 24h-120h as in Methods. Cells were lysed and portions (~100 μg) from each plate used to immunoprecipitate MAPK followed by immune-complex kinase assays as in Methods. MAPK activity data are duplicate values from a single representative experiment (n=3) and are shown as -fold increases in ^{32}P-incorporation into MBP substrate.

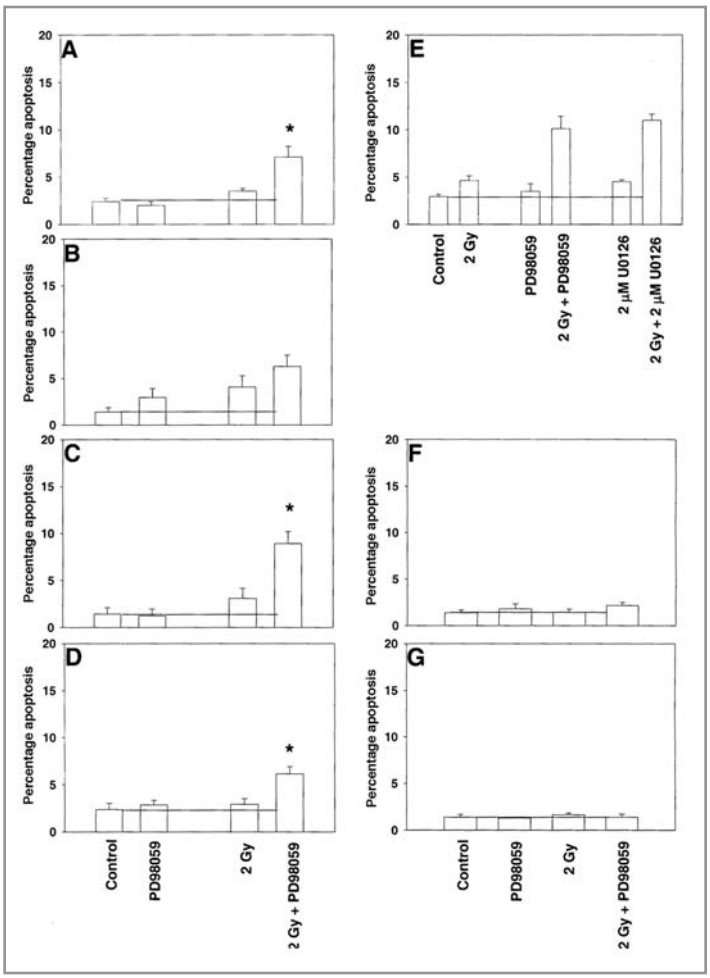

Figure 3. Combined exposure of carcinoma cells to ionizing radiation and MEK1/2 inhibitors causes apoptosis. Cells were incubated with matched vehicle control (DMSO) or with either 10 μM PD98059 (A431) or 25 μM PD98059 (MDA-MB-231, MCF7, T47D, DU145, LNCaP) alone. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. A. MDA-MB-231 cells. B. MCF7 cells. C. DU145 cells. D. T47D cells. E. A431 cells. F. LNCaP cells. G. PC3 cells. Data shown are the mean number of staining cells from randomly selected fields of fixed cells (n=5 per slide, ± SEM), which were examined and counted via fluorescent light microscopy. *p <0.05 greater than corresponding value in unirradiated cells.

and pro-caspase 3 in A431 and DU145 cells (Fig. 7A and Fig. 7B). Of note, in A431 cells, significant amounts of "active" p36 caspase 9 were observed in control cells. Combined exposure to radiation and MEK1/2 inhibitors also promoted release of cytochrome c from the mitochondria into the cytoplasm but surprisingly at this time did not alter the mitochondrial membrane potential (Fig. 7C). Loss of the mitochondrial membrane potential was variably observed within the following 6-12h (data not shown).

Because removal of the MEK1/2 inhibitor at 12h abolished the potentiation of apoptosis at 24h (Fig. 5), further studies were performed to examine the time course of pro-caspase cleavage. Reduced expression of p32 pro-caspase 3 and increased expression of p17 active caspase 3 was not observed 12h following combined radiation and MEK1/2 inhibitor exposure (Fig. 7D), indicating that at this time cells were not undergoing apoptosis (in agreement with Fig. 5A). However, at 18h, 21h and 24h after exposure loss of p32, and increased p17, expression was observed. The increase in p17 expression was greater in cells exposed to radiation and MEK1/2 inhibitor than to either radiation or MEK1/2 inhibitor alone. These findings suggest that MEK1/2 activity must be depressed for at least 18h to obtain a potentiation of radiation-induced apoptosis at 24h.

Inhibition of MAPK Signaling Enhances Radiation-Induced Apoptosis 96-144h After Exposure, Which is Dependent Upon Prolonged Inhibition of the MAPK Pathway. In a previous study, we had argued that inhibition of the MAPK pathway enhanced radiation-induced apoptosis in two waves, one at ~24h and a second ~96h after exposure. To further investigate these observations, we examined both the percentage apoptosis and the expression and integrity of pro-caspases in A413 and DU145 cells 24h-120h after irradiation (Fig. 8 and Fig. 9). Sixty hours after exposure, apoptosis had returned to near basal levels under all conditions (data not shown).

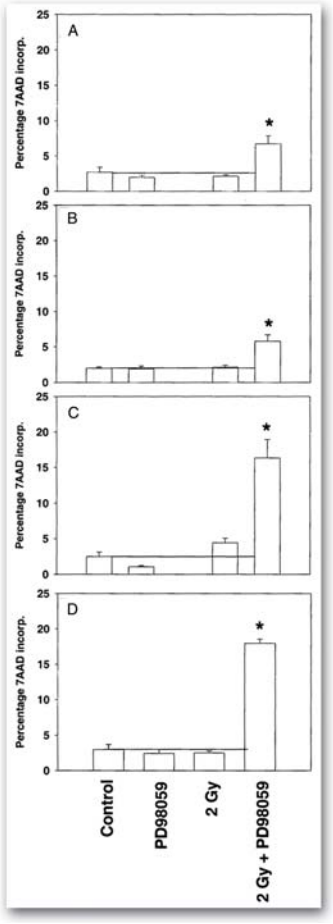

Figure 4. Combined exposure of carcinoma cells to ionizing radiation and MEK1/2 inhibitors increases cell death as judged by 7AAD staining. Cells were incubated with matched vehicle control (DMSO) or with either 10μM PD98059 (A431) or 25 μM PD98059 (MDA-MB-231, T47D, DU145) alone. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and incubated with 7AAD (10 μg/ml final) for 30 min at 37°C as in Methods. Cells were subjected to flow cytometric analysis to determine the percentage of cells incorporating 7AAD. A. MDA-MB-231 cells. B. DU145 cells. C. A431 cells. D. T47D cells. Data shown are the mean percentage of staining cells (3 parallel individual experiments ± SEM). *p <0.05 greater than corresponding value in unirradiated cells.

However, 108h after irradiation, the percentage of cells undergoing apoptosis in MEK1/2 inhibited and irradiated cells had again increased (Fig. 8A). The potentiation of apoptosis at 108h appeared to correlate with increasing radiation dose (Fig. 8B). Of note, removal of MEK1/2 inhibitor at either 24h or 48h after exposure abolished the increase in apoptosis at 108h (Fig. 8A, data not shown). Removal at 72h, however, still permitted a limited apoptotic response from these cells (data not shown). These findings correlated with modulation of cellular radiosensitivity by MEK1/2 inhibitors in clonogenic survival assays (Table 1): in some cell types e.g., DU145 and A431, removal of the inhibitor 48h after exposure during re-plating abolished any impact of MEK1/2 inhibitors on clonogenic radiosensitivity. In contrast, other cell types e.g. MDA-MB-231 appeared to be radiosensitized even when the inhibitor was removed after 48h. In agreement with the finding that apoptosis had again increased at later times, prolonged inhibition of MEK1/2 enhanced the radiation-induced cleavage of pro-caspases 96-144h after exposure (Fig. 9A and Fig. 9B).

Because apoptosis and caspase cleavage were observed at later times, we investigated whether the pan-caspase inhibitor ZVAD could block this process and whether enhanced apoptosis played an important role in the previously reported enhancement in radiosensitivity caused by MAPK inhibition. Small molecule inhibitors of caspases are relatively unstable, and in all of the following experiments ZVAD was re-supplemented into the media every 24h. Prolonged exposure to ZVAD blocked the potentiation of apoptosis at later times (Fig. 10) and also the cleavage of caspase 3 (data not shown). Collectively, these data argue that enhanced radiosensitivity caused by MEK1/2 inhibition is, in part, due to increased late phase apoptosis.

DISCUSSION

Previous investigations by this group have demonstrated that low dose radiation exposure of carcinoma cells, in combination with inhibition of MEK1/2, causes a small but statistically significant increase in radiation-induced apoptosis within 24h. Prolonged inhibition of MEK1/2 caused a second wave of apoptosis 4-6 days after exposure in DU145 cells. Removal of the MEK1/2 inhibitor at 24h was found to abolish the second apoptosis wave and any impact upon long-term clonogenic survival. The studies described in this manuscript were designed to determine the mechanisms by which MEK1/2 inhibitors enhance apoptosis at both 24h and in the second wave at 108h.

We initially compared a variety of carcinoma cell types in their abilities to be killed by radiation in the presence or absence of a MEK1/2 inhibitor. Low dose radiation as a single agent was relatively ineffective at enhancing apoptosis in all of the cell types examined, including MCF7 and LNCaP that express wild type p53. In all cell types that had elevated basal MAPK activity greater than 10 fmol/min/mg (A431, MDA-MB-231, DU145), MEK1/2 inhibitors weakly potentiated radiation-induced apoptosis to varying degrees. In cell types that had basal MAPK activity less than 10 fmol/min/mg (MCF7, PC3, LNCaP, T47D), with the exception of T47D, no potentiation of DNA strand breakage was observed using MEK1/2 inhibitors. Of note, serum starved/growt arrested cells also did not exhibit a potentiation of apoptosis (unpublished data). Within this group, it is also of note that LNCaP and PC3 cells do not express the inositol phospholipid phosphatase PTEN.[27] Loss of PTEN suggests these cells may utilize the PI 3-kinase signaling pathway, rather than the MAPK pathway, as a key growth and survival control signaling module. Collectively, these findings suggest that cells, which have elevated basal MAPK activity, are more responsive to MEK1/2 inhibitors as radiosensitizers than those with low basal MAPK activity.

Previously, we had discovered that removal of MEK1/2 inhibitor 6h following irradiation abolished the potentiation of apoptosis 24h afterwards. In the present study, we discovered that removal of the MEK1/2 inhibitor 12h-18h after exposure also abolished the potentiation of killing at 24h. Removal of the inhibitor at 18h had a partial blunting effect on the apoptotic response. These findings correlated with the cleavage of p32 pro-caspase 3: processing of pro-caspase 3 to p17 active caspase 3 was not observed until 18-21h after exposure. These findings suggest that prolonged blunting of MAPK signaling is required to enhance radiation-induced cell killing at 24h. The precise mechanisms that promote cell killing remain to be determined.

Two upstream pathways, the intrinsic and the extrinsic, mediate cleavage and activation of the effector pro-caspase 3. The intrinsic pathway utilizes the mitochondrion, cytochrome c and pro-caspase 9 and the extrinsic pathway that utilizes death receptors and pro-caspase 8. We found that combined exposure to radiation and MEK1/2 inhibitors caused release of cytochrome c from the mitochondria into the cytosol within 24h, but did not promote a large reduction in the mitochondrial membrane potential.

Induction of apoptosis, notably by chemotherapeutic drugs, has been linked to mitochondrial damage, including loss of the mitochondrial membrane potential or release of pro-apoptotic proteins from the mitochondria, such as cytochrome c. There is controversy regarding which of these events represents the central executioner of apoptosis, and the induction of apoptosis in both the presence or the absence of cytochrome c release has been described.[29] Release of cytochrome c is also often found to precede loss of the mitochondrial membrane potential, ψ_m.[30] The present findings suggest that cytochrome c release represents an important primary event in cells induced to undergo apoptosis when they are treated with MEK1/2 inhibitors and exposed to radiation.

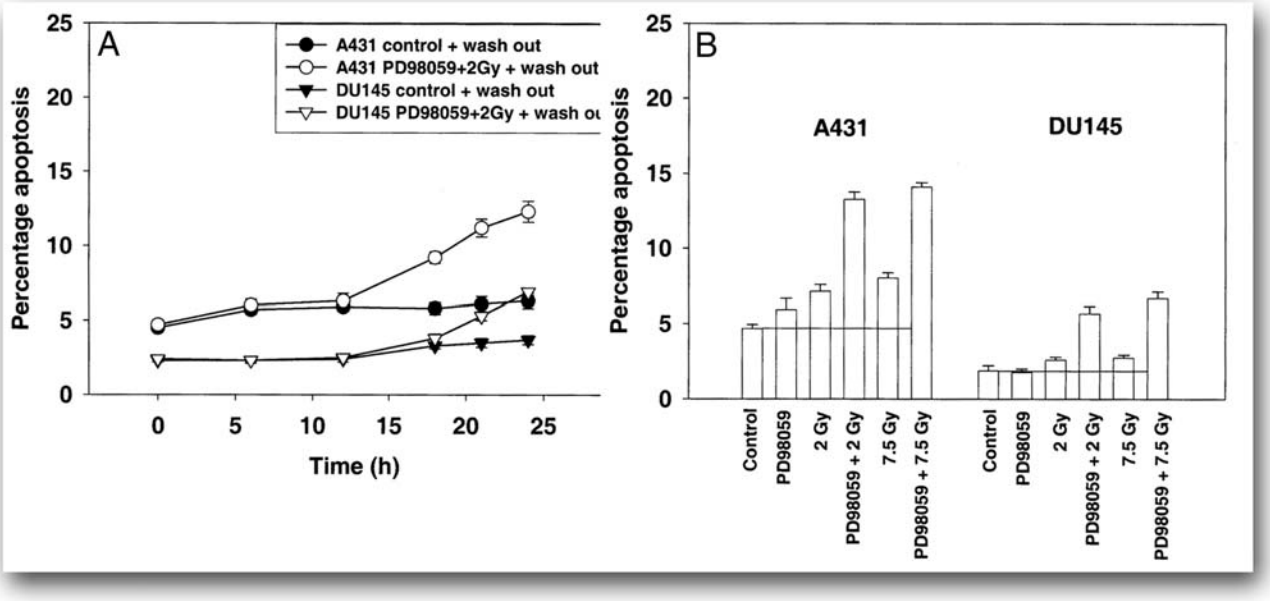

Figure 5. Combined exposure of A431 and DU145 carcinoma cells to ionizing radiation and MEK1/2 inhibitors causes apoptosis which requires >12h of incubation with MEK1/2 inhibitor. Cells were incubated with matched vehicle control (DMSO) or with either 10 μM PD98059 (A431) or 25 μM PD98059 (DU145) alone. Cells were either exposed to radiation (2 Gy, 7.5 Gy) or mock irradiated. Cells were either washed 6h, 12h, 18h and 21h following exposure with media containing either vehicle or PD98059. All cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. A. Cells with MEK1/2 inhibitor removed at 6h, 12h, 18h, and 21h after exposure. B. Cells without MEK1/2 inhibitor removal irradiated with either 2 Gy or 7.5 Gy. Percentage apoptosis determined at 24h. Data shown are the mean number of staining cells from randomly selected fields of fixed cells (n=5 per slide, 3 parallel individual experiments ± SEM) which were examined and counted via fluorescent light microscopy. *p <0.05 greater than irradiated value.

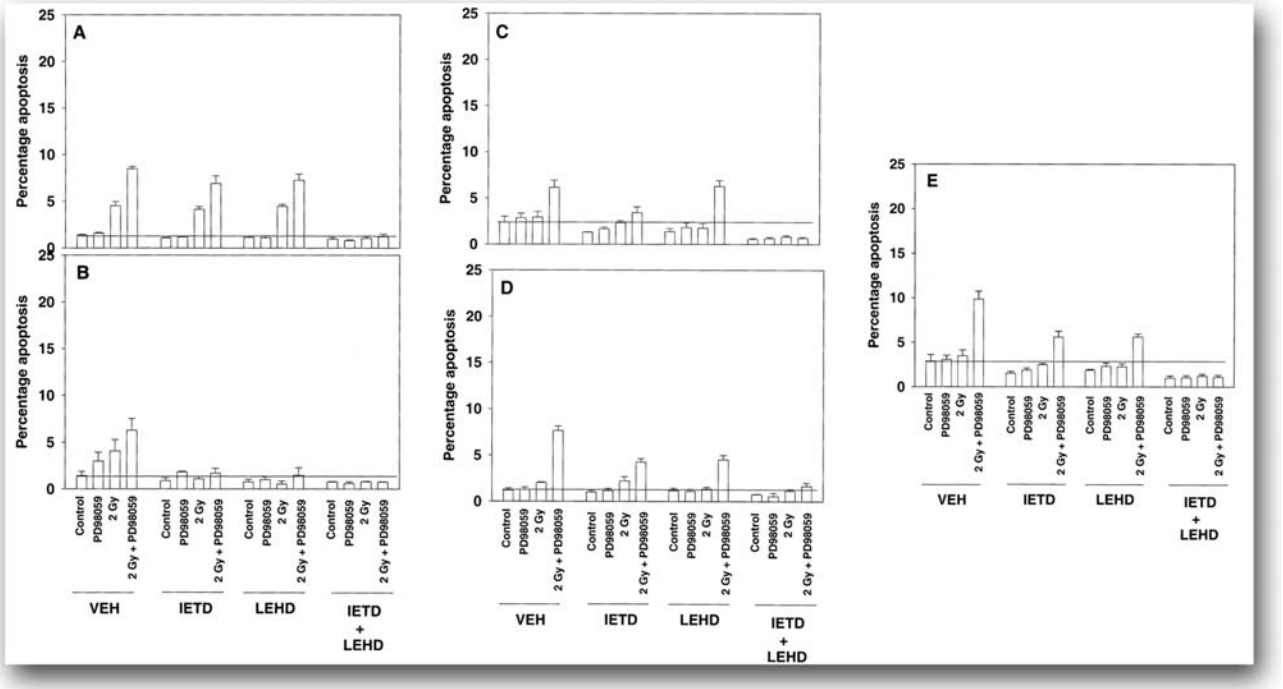

Figure 6. The potentiation of apoptosis by combined irradiation and MEK1/2 inhibition requires both caspase 9 and caspase 8 functions. Cells were either treated with either vehicle, with IETD (20 μM), with LEHD (20 μM) or with both IETD and LEHD. In parallel, cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. A. MDA-MB-231 cells. B. MCF7 cells. C. T47D cells. D. DU145 cells. E. A431 cells. Data shown are the mean number of staining cells from randomly selected fields of fixed cells (n=5 per slide, ± SEM) which were examined and counted via fluorescent light microscopy. #p <0.05 greater than corresponding value in unirradiated cells; *p <0.05 greater than control value;%p <0.05 less than corresponding value in cells not treated with caspase inhibitor. Cells treated with the pan-caspase inhibitor ZVAD (20 μM) exhibited little or no apoptosis (data not shown).

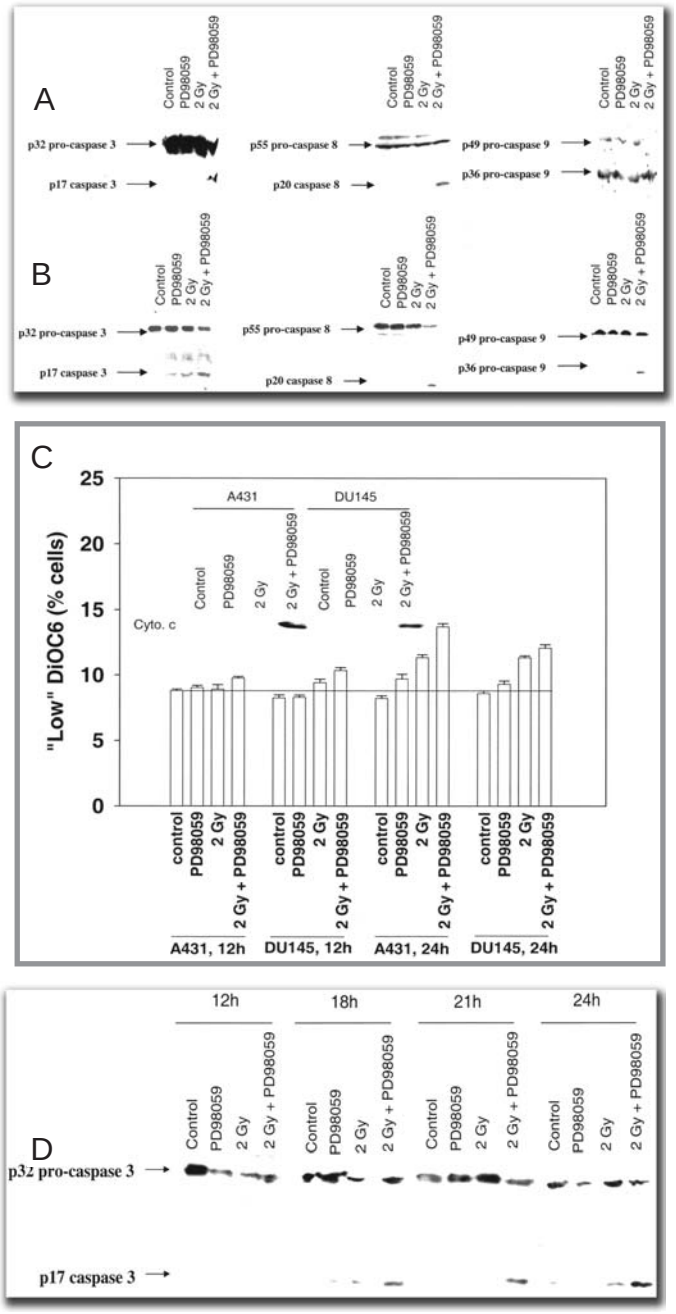

Figure 7. Combined treatment of A431 and DU145 cells with ionizing radiation and MEK1/2 inhibitors enhances the cleavage of pro-caspase 3. Cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24 hours post irradiation and lysed in SDS PAGE buffer containing bromophenol blue as described in Methods. Cells were subjected to SDS PAGE followed by immunoblotting to determine: A. In A431 cells at 24h, the expression/integrity of pro-caspases -3, -8 and -9. B. In DU145 cells at 24h, the expression/integrity of pro-caspases -3, -8 and -9. C. Mitochondrial membrane potential in A431 and DU145 cells 24h after exposure: inset panel, cytosolic cytochrome c levels 24h after exposure. D. A431 cells were irradiated and treated with 10 μM PD98059, and the integrity of pro-caspase 3 determined after 12h, 18h, 21h and 24h after a 2 Gy exposure. Data shown are from a representative experiment (n=3).

The apoptotic responses of the various cell lines examined, surprisingly, differed with respect to inhibition of caspase 8 and caspase 9. In MDA-MB-231, A431 and DU145 cells, inhibitors of caspase 8 and caspase 9 individually had partial effects at blunting the apoptotic response. In MCF7 cells, that had little apoptotic response, either inhibitor abolished increases in cell death. In contrast, caspase 9 inhibitors had little impact on the apoptotic response of T47D mammary carcinoma cells whereas inhibitors of caspase 8 completely blocked apoptosis in these cells. These findings suggest that the mechanism by which MEK1/2 inhibitors potentiate cell killing, 24h after exposure, is cell-type specific. Caspase 9 inhibitors had no effect on the apoptotic response in T47D cells suggesting that MEK1/2 inhibitors are promoting death receptor signaling, as has previously been demonstrated by this group in primary hepatocytes treated with bile acids.[28]

Previously, we have presented evidence that inhibition of MEK1/2 can radiosensitize cells when they are exposed repeatedly to low dose ionizing radiation. In these studies, cells were irradiated three times with 2 Gy per exposure and incubated with MEK1/2 inhibitors for 96 hours prior to re-plating.[8] In the present study we exposed cells to a single 2 Gy dose of radiation in the presence or absence of MEK1/2 inhibitor for 48h and examined apoptosis and clonogenic survival over the following week to fourteen days. Radiation, even several days after exposure, caused relatively little apoptosis in A431 and DU145 cells. However, the presence of MEK1/2 inhibitor, while by itself only marginally toxic, caused a significant enhancement in radiation-induced apoptosis. The pan-caspase inhibitor ZVAD blocked the increase in apoptosis. When the MEK1/2 inhibitor was removed 24h and 48h after irradiation, no enhancement in late phase apoptosis occurred, which correlated with negative data obtained when clonogenic survival was measured in the absence of this inhibitor. In contrast, when the MEK1/2 inhibitor was removed at 72h, a partial blunting effect on the potentiation of apoptosis at 108h became evident. Thus incubation of cells in the presence of a MEK1/2 inhibitor for ~72h, but not 48h, appears competent to produce a commitment for enhanced radiation-induced apoptosis at time periods distant to the initial exposure. These findings suggest that prolonged blunting of MAPK signaling is required to enhance radiation-induced cell killing at 108h. The precise mechanisms by which cell killing is promoted at this time point remain to be determined.

Surprisingly, in MDA-MB-231 and MCF7 cells, however, the MEK1/2 inhibitor PD98059 caused radio-sensitization in clonogenic survival assays, even though the drug was removed 48h after irradiation. The apoptotic responses of MCF7 cells at the 24h-48h time points were negligible, implying that in addition to potentiating radiation-induced apoptosis, MEK1/2 inhibition can also promote radiation-induced cell killing by other non-apoptotic mechanisms. Further studies will be required to determine: the mechanisms by which MEK1/2 inhibitors commit cells and then trigger an apoptotic response 18-24h after exposure, and the mechanisms by which MEK1/2 inhibitors commit cells and then trigger an apoptotic response 48-72h after irradiation.

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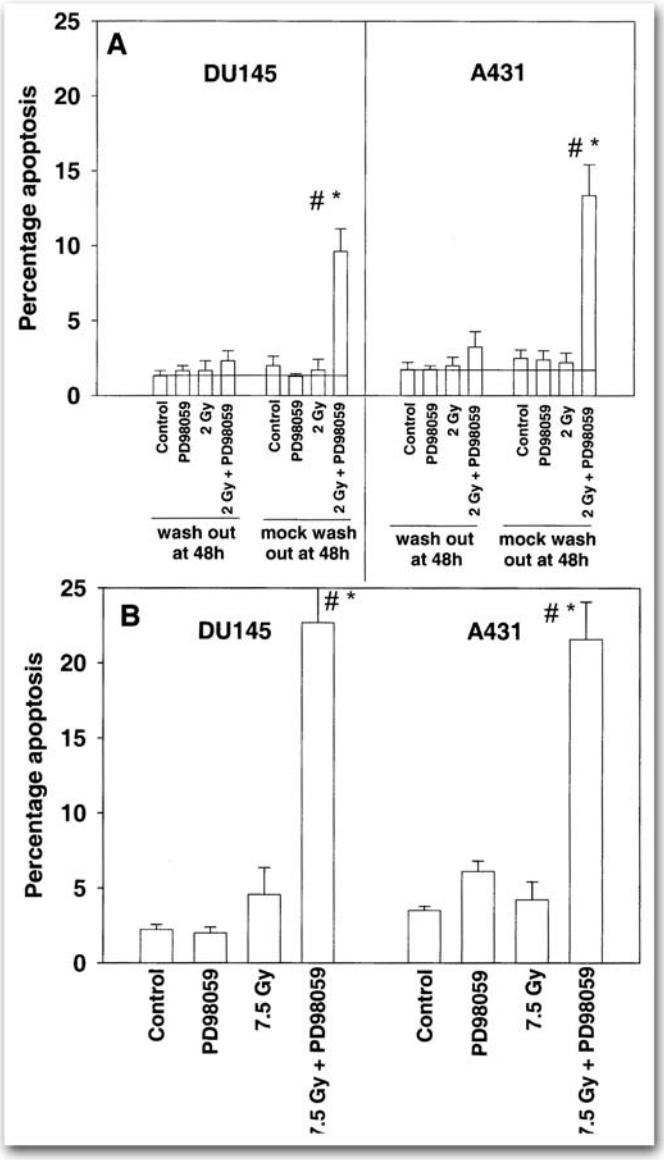

Figure 8. Combined exposure of A431 and DU145 carcinoma cells to ionizing radiation and MEK1/2 inhibitors causes apoptosis 108h after exposure. Cells were incubated with matched vehicle control (DMSO) or with PD98059 alone. Cells were either exposed to radiation or mock irradiated. Portions of cells were taken 24, 60 and 108 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. A. A431 and DU145 cells irradiated with 2 Gy, either washed free of PD98059 at 48h or mock washed free at 48h. B. A431 and DU145 cells at 108h irradiated with 7.5 Gy. Data shown are the mean number of staining cells from randomly selected fields of fixed cells (n=5 per slide, ± SEM) which were examined and counted via fluorescent light microscopy. #p <0.05 greater than corresponding value in unirradiated cells; *p <0.05 greater than control value.

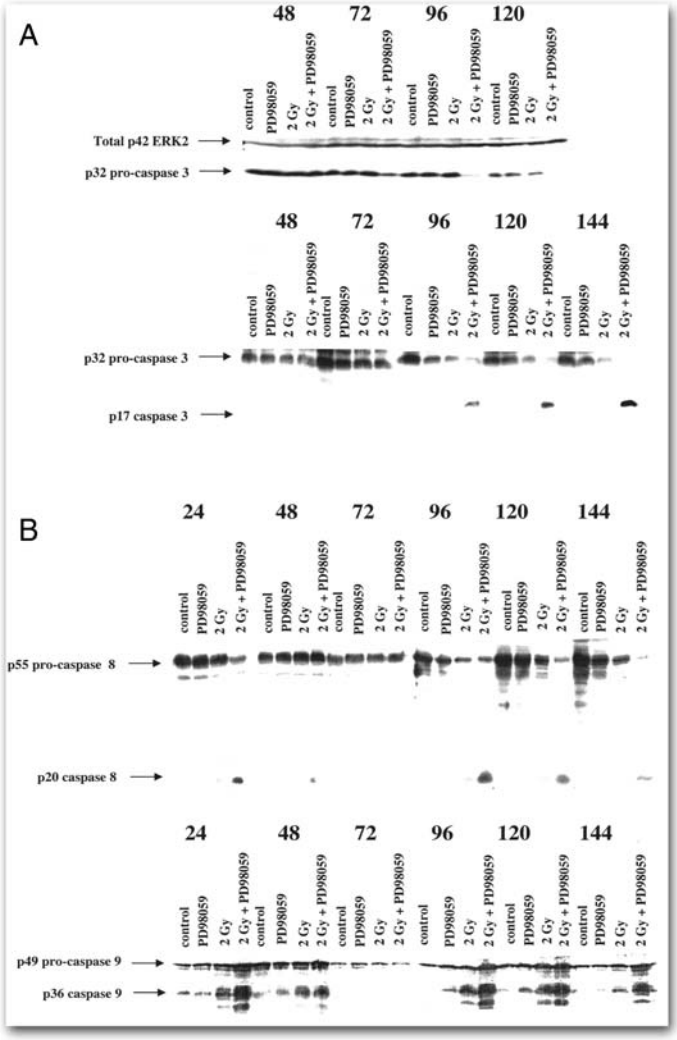

Figure 9. Combined treatment of A431 and DU145 cells with ionizing radiation and MEK1/2 inhibitors enhances the cleavage of pro-caspases 96-144h after exposure. Cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Portions of cells were taken 24-144 hours post irradiation and lysed in SDS PAGE buffer containing bromophenol blue as described in Methods. Cells were subjected to SDS PAGE followed by immunoblotting to determine: A. In A431 and DU145, the expression (A431)/integrity (DU145) of pro-caspase -3; B. In DU145, the expression and integrity of pro-caspases -8 and -9. Data shown are from a representative experiment (n=3).

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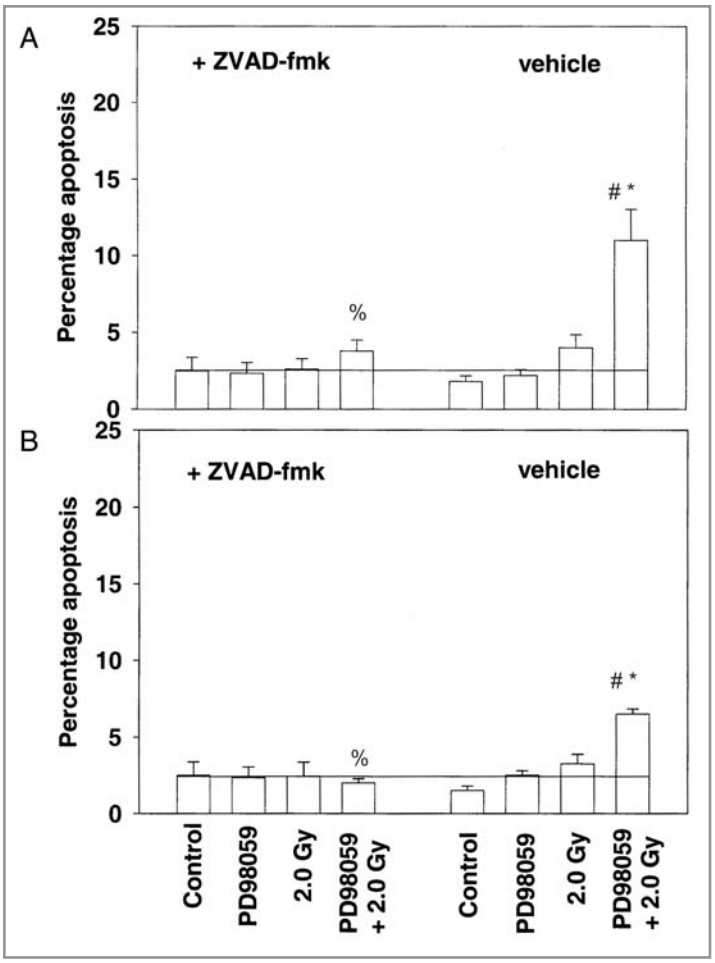

Figure 10. The potentiation of apoptosis 96-144h after exposure by combined irradiation and MEK1/2 inhibition is inhibited by the pan-caspase inhibitor ZVAD. Cells were either treated with either vehicle or with ZVAD (20 μM). In parallel, cells were incubated with matched vehicle control (DMSO) or with PD98059. Cells were either exposed to radiation (2 Gy) or mock irradiated. Vehicle or ZVAD was replenished in the media every 24h. Portions of cells were taken 108 hours post irradiation and fixed onto slides by cytocentrifuge followed by staining for double stranded DNA breaks as in Methods. A. A431 cells. B. DU145 cells. Data shown are the mean number of staining cells from randomly selected fields of fixed cells (n=5 per slide, ± SEM) which were examined and counted via fluorescent light microscopy. # $p < 0.05$ greater than corresponding value in unirradiated cells; * $p < 0.05$ greater than control value;% $p < 0.05$ less than corresponding value in cells not treated with caspase inhibitor.

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