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Increased Radiation-Induced Apoptosis of Saos2 Cells via Inhibition of NFκB: A Role for c-Jun N-Terminal Kinase

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Abstract To elucidate the possible effect of NFκB on radioresistance, we used the osteosarcoma cell line Saos2, stably expressing the NFκB constitutive inhibitor, mIκB (Saos2-mIκB) or stably transfected with the empty vector (Saos2-EV). Ionizing radiation induced ''intrinsic'' apoptosis in Saos2-mIκB cells but not in Saos2-EV control cells, with intact NFκB activity. We find as expected, that this NFκB activity was enhanced following irradiation in the Saos2-EV control cells. On the other hand, inhibition of NFκB signaling in Saos2-mIκB cells led to the upregulation of the pro-apoptotic systems, such as Bax protein and c-Jun N-terminal Kinase (JNK)/c-Jun/AP1 signaling. Inhibition of NFκB resulted in decreased expression of the DNA damage protein GADD45β, a known inhibitor of JNK. Subsequently, JNK activation of c-Jun/AP-1 proteins increased radiation-induced apoptosis in these mutants. Radiation-induced apoptosis in Saos2-mIκB cells was inhibited by the JNK specific inhibitor SP600125 as well as by Bcl-2 over-expression. Furthermore, release of cytochrome-*c* from mitochondria was increased and caspase-9 and -3 were activated following irradiation in Saos2-mIκB cells. Antisense inhibition of GADD45β in Saos2-EV cells significantly enhanced apoptosis following irradiation. Our results demonstrate that radioresistance of Saos2 osteosarcoma cells is due to NFκB-mediated inhibition of JNK. Our study brings new insight into the mechanisms underlying radiation-induced apoptosis of osteosarcoma, and may lead to development of new therapeutic strategies against osteosarcoma. J. Cell. Biochem. 96: 1262–1273, 2005.

Key words: NFκB; radiation; apoptosis; Bax; JNK; GADD45β

Amongst the most devastating primary malignant bone cancers are osteosarcomas [Dorfman and Czerniak, 1998; Unni, 1998]. These tumors are highly aggressive and mostly affect children and young adults. The treatment of osteosarcomas is limited to chemotherapy as these tumors remain a poor candidate for radiotherapy due to their high resistance to radiation [Fuchs and Pritchard, 2002]. Understanding the mechanisms that govern cell survival versus programmed cell death of osteosarcoma will delineate new therapeutic strategies that may increase the effectiveness of radiation treatment of these tumors. In this study, we aimed to determine the molecular pathways underlying radioresistance of the human osteosarcoma cell line Saos2 through transcriptional regulation.

The cellular response to ionizing radiation commonly involves activation of the anti-oncogene p53 [Oren, 2003], which recognizes radiation-induced DNA damage and triggers a cascade of events leading to apoptosis. However, in various cancer cells including the osteosarcoma cell line Saos2, p53 is mutated [Vogelstein et al., 2000]. In addition, stress-responsive protein cJun N-terminal kinase, JNK [Kuwabara et al., 2003, Dent et al., 2003] is known to be involved in the cellular response to ionizing radiation yet may be insufficient to overcome the anti-apoptotic defense systems present in these cells. The JNK signal transduction pathway involves a series of phosphorylation events that result in activation of the transcription factor cJun, which translocates into the nucleus and homodimerizes with other Jun family members or heterodimerizes with Fos family

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members generating the activator protein-1 (AP-1) transcription complex [Angel and Karin, 1991]. AP-1 regulates an array of genes important for osteoblast biology, including osteopontin and osteocalcin [Stein et al., 1996].

The cJun N-terminal Kinase signaling pathway was previously shown to participate in osteoblast differentiation and apoptosis [Liu and Shuai, 2001], and is regulated by the transcription factor NFκB. Furthermore, NFκB has been established as a major inducer of malignancy [Barnes, 1997]. The mechanism by which NFκB signaling regulates tumor progression and apoptosis is still under heavy investigation. This pro-survival protein is constitutively activated in various cancer cell lines, thereby, leading to disruption of their apoptotic programs [Rayet and Gelinas, 1999]. NFκB upregulates Bcl-2-mediated anti-apoptotic mechanisms, while it downregulates pro-apoptotic mechanisms involving Bax protein [Chen et al., 1999]. Additionally, NFκB has been shown to regulate radiosensitivity in several cancer cell lines [Chendil et al., 2004; Egan et al., 2004; Guo et al., 2004]. De Smaele et al. [2001] demonstrated that JNK pathway inhibition by NFκB is a possible mechanism for NFκB-mediated suppression of TNF-induced apoptosis. Furthemore, NFκB upregulates the expression of the DNA damage protein GADD45β, which binds to MKK7/JNKK2 activator of JNK leading to inhibition of the JNK pathway [Papa et al., 2004]. Thus, we hypothesized that radioresistance of Saos2 osteosarcoma cells may be due to NFκB mediated inhibition of the JNK signaling and that interfering with NFκB signaling alters the radiosensitivity of osteosarcoma cells.

Here we report that inhibition of NFκB in human osteosarcoma Saos2 cells over-expressing NFκB constitutive supressor mIκB, results in increased sensitivity of these cells to ionizing radiation. We show an increase in the cytosolic fraction of cytochrome-*c* and activation of caspase-9 and -3 in irradiated Saos2-mIκB cells. These apoptotic markers were absent in irradiated control Saos2 cells (Saos2-EV). Furthemore, inhibition of NFκB in Saos2-mIκB cells was associated with increased levels of Bax protein and increased activity of the JNK/cJun/AP1 system, when compared to Saos2-EV cells. Overall, our current study demonstrates that the radioresistance of Saos2 osteosarcoma cells results from NFκB-mediated inhibition of JNK.

MATERIALS AND METHODS

Materials

Most chemicals were from Sigma unless otherwise noted. Cell culture media and components were from Gibco. All oligonucleotides were custom made by IDT. Primary antibodies were from Santa Cruz Biotechnology (anti-IκBα, anti-pIκBα, anti-p65, anti-lamin, anti-GADD45β, and anti-p-cJun), Trevigen (anti-holocytochrome-*c*), Sigma (anti-βactin), or Epitomics (anti-Bcl2 and anti-Bax). HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (anti-goat) or BioRad (anti-mouse and anti-rabbit). Caspase-3, -9, and -8 fluorogenic substrates were from Calbiochem, Hoechst33342 stain was from Molecular Probes. JNK inhibitor SP600125 was from Calbiochem.

Cell Culture and Treatment

Human primary osteosarcoma Saos2 cells were cultured in DMEM media supplemented with 10% fetal bovine albumin and 1% penicillin/streptomycin mixture. Our laboratory previously created Saos2 cell lines stably expressing mIκB (Saos2-mIκB cells) or an empty vector (Saos2-EV cells) [Andela et al., 2002]. These stably transfected cells were maintained in the presence of 200 μg/ml of G418. Cells were irradiated with 10 Gy of γ-radiation using a cesium source. Some Saos2-mIκB cells were transiently transfected with the pIRESneo-Bcl2 construct [Eliseev et al., 2003] or with the empty pIRESneo vector (Clontech) as a control starting at 48 h before irradiation. Some Saos2-mIκB cells were continuously treated with 100 nM JNK inhibitor SP600125 or DMSO as a vehicle control starting at 3 h before irradiation. A subset of Saos2-EV cells was incubated with 5 μg/ml of GADD45β antisense (5′- GTC ATC CTC CTC CTC CTC GT -3′) or sense (5′- ACG AGG AGG AGG AGG ATG AC -3′) oligonucleotides starting at 48 h before irradiation. In all cases, DNA was introduced into cells using Fugene-6 liposome reagent (Roche) according to manufacturer's instructions.

Cell Fractions and Whole Cell Lysates

Cytosolic fractions were prepared as in Eliseev et al. [2003] by incubating at 2.5×10^7 cells per ml of "permeabilization buffer" (195 mM mannitol, 95 mM sucrose, 2 mM HEPES, pH 7.4, 0.1 mM EGTA, 0.01% digitonin) for 5 min on ice, spinning down at 10,000*g* for 5 min, and

collecting the supernatant. Nuclear fractions were prepared using the NE-PER nuclear extraction kit (Pierce) according to the manufacturer's protocol. Whole cell lysates were prepared by washing cells in PBS at room temperature, resuspending them at 2.5×10^7 cells/ml in lysis buffer (20 mM Tris-HCl, pH 7.9, 137 mM NaCl, 1 mM EDTA, 1% Triton X100, 10% glycerol) supplemented with protease inhibitors on ice for 30 min and spinning down cell debris at 10,000*g* for 15 min at +4°C. The concentration of protein in cell fractions and lysates was measured using Coomassie Plus Protein Assay Reagent (Pierce) in a Beckman Coulter DU640 spectrometer. Cell fractions and lysates were stored at −80°C until needed.

Caspase-3, -9, and -8 Activities Assay

Caspase-3, -9, and -8 activities were measured using fluorogenic substrate cleavage assay [Nicholson, 1999]. The reaction mixture contained 200 μl of PBS, cell lysates at a concentration of 10 μg of total protein per reaction and 20 μM of either caspase-3 fluorogenic substrate Ac-DEVD-amc, caspase-9 fluorogenic substrate Ac-LEHD-afc, or caspase-8 fluorogenic substrate Z-IETD-afc. The reactions were loaded into 96-well plates and incubated at 37°C for 30 min. Fluorescence at corresponding wavelength was measured using a Hitachi plate reader according to the manufacturer's protocol.

Hoechst Staining

Cells were trypsinized, washed in PBS, and resuspended in fresh DMEM media in 6-well plates at 10^5 cells per ml. A nuclear stain Hoechst33342 was added to cell suspensions to a final concentration of 1 μM. After 5 min incubation at room temperature, plates were mounted on a stage of Zeiss AxioVert inverted fluorescence microscope. Nuclear morphology and condensation of chromatin were assessed and the number of condensed, that is, apoptotic nuclei versus total nuclei were counted. Pictures of stained nuclei were taken using AxioCam CCD camera.

Trypan Blue Staining

Plasma membrane integrity was assayed by staining cells with Trypan Blue as follows: cells plated in 6-well plates and treated in various ways as described above were incubated with 1% Trypan Blue in DMEM media at room temperature for 5 min. Plates were mounted on a microscope stage and the number of Trypan Blue positive cells versus total number of cells was counted.

Clonogenic Survival

Irradiated and non-irradiated cells were trypsinized, resuspended in fresh media, and counted. An equal proportion of each culture was diluted and plated in 6-well plates. Plates were incubated for 10 days at 37°C in 5% CO_2 humidified incubator before counting colonies. Colonies were visualized by staining with 1% Toluidin Blue. Numbers of colonies in irradiated cultures were compared to non-irradiated cultures and expressed as percent of survival.

Western Blot

Twenty-five micrograms of total protein in whole cell lysates or fractions per sample were mixed with Laemli sample buffer, boiled for 5 min, loaded into 4–20% Tris/Glycine precast gels (Gradipore), electrophoresed at 150 V, and then electroblotted onto PVDF membranes at 100 V for 1 h on ice. Blots were blocked in 5% BSA in PBST for 1 h, probed with primary antibody diluted in 2.5% BSA/PBST to 1 μg/ml, washed 5×5 min in PBST, incubated with corresponding HRP-conjugated secondary antibody diluted in 2.5% BSA/PBST to 0.2 μg/ml, washed 5×5 min in PBST, developed using SuperSignal WestPico chemiluminescent substrate (Pierce), and photographed. Blots were stripped in Re-Blot Plus buffer (Chemicon) for 15 min at room temperature and reprobed with anti-lamin (nuclear fractions) or anti-βactin antibody (whole cell lysates and cytosolic fractions) to verify equal loading. Blots were scanned and band intensities were measured using Adobe Photoshop software.

Heterologous Promoter-Reporter Luciferase Assay

To assay the transcriptional activity of NFκB, cells were co-transfected with the NFκB-firefly luciferase reporter vector pNFκB-Luc (Invitrogen) and the renilla luciferase vector pRL-TK (Promega). To assay the transcriptional activity of AP1, cells were co-transfected with the AP1-firefly luciferase reporter vector pAP1-Luc (Stratagene) and the renilla luciferase vector pRL-TK. Firefly luciferase activity was measured using a Dual Luciferase Reporter Assay

System (Promega) according to the manufacturer's protocol as previously described [Andela et al., 2002], normalized to renilla luciferase and expressed as relative luminescence units (RLU).

Real-Time RT-PCR

Total cellular RNA was prepared using RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. The concentration of prepared RNA was assayed by UV absorption at 260 nm. Five hundred nanograms of total RNA was reverse transcribed into cDNA using SuperScript First-Strand Synthesis Kit (Invitrogen) and Oligo-dT primers, according to the manufacturer's protocol. One microliter of the cDNA was subjected to real-time PCR using following sets of primers: GADD45β (5′-TGT ACG AGT CGG CCA AGT TGA TGA-3′ and 5′-TGT GAG GGT TCT GTA GGA AGG GAA-3′) or GAPDH (5′- GAG TCA ACG GAT TTG GTC GT - 3′ and 5′- GAC AAG CTT CCC GTT CTC AG - 3′).Real-time PCR was performed as previously described [Andela et al., 2002] using the RotorGene real-time DNA amplification system (Corbett Research) and the double-strand-specific fluorescent dye SYBR Green (Bio-Rad) to monitor DNA synthesis. PCR products were subjected to a melting curve analysis and the data were analyzed and quantified with the RotorGene analysis software. The expression of GADD45β was normalized to the expression of GAPDH.

Statistical Analysis

Experiments were repeated three to five times, mean values and standard deviations were calculated, and the statistical significance was determined using a standard *t*-test. Data with $P < 0.05$ were considered statistically significant.

RESULTS

NFκB Activity is Inhibited in Saos2 Cells Stably Over-Expressing mIκB

We determined the effect of NFκB on the sensitivity of human primary osteosarcoma cells Saos2 to ionizing radiation. We compared radiation effects between the osteosarcoma cell line Saos2 stably expressing the NFκB constitutive inhibitor, mIκB (Saos2-mIκB) and Saos2 cells stably transfected with the empty vector (Saos2-EV). Figure 1A shows strong overexpression of IκBα in the Saos2-mIκB cells in comparison to the empty vector infected controls (top panel). This mIκB protein cannot be phosphorylated (middle panel) rendering it non-degradable. This stabilization of mIκB induced the total abrogation of the p65 (RelA) regulatory subunit of NFκB in the nuclear fraction (Fig. 1B) as assessed by Western blot analyses. These results suggest that mIκB

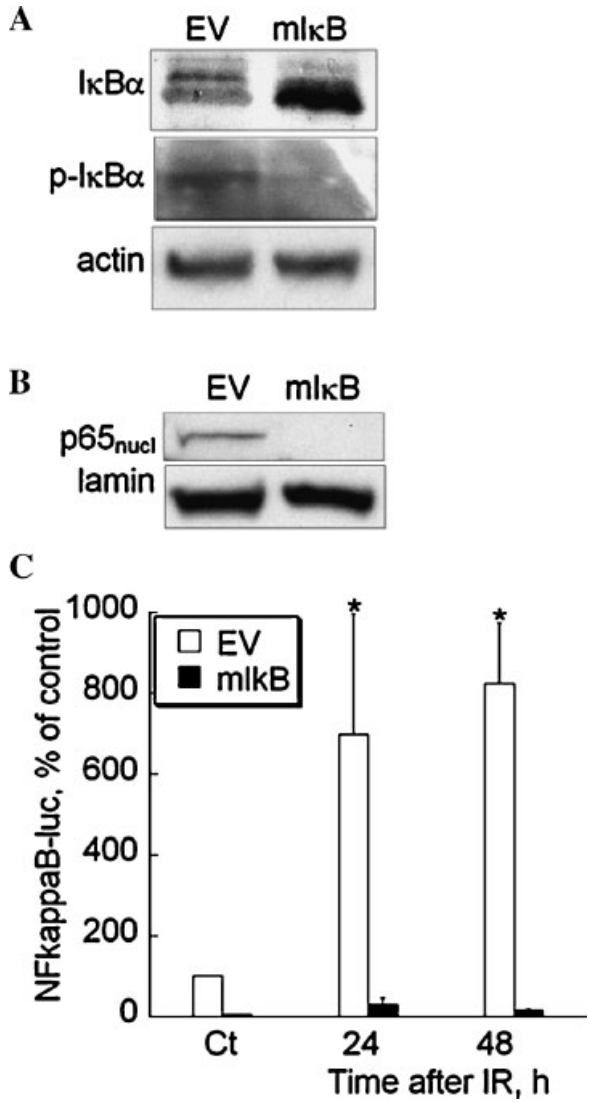

Fig. 1. Expression of IκBα and activity of NFκB in Saos2-EV and Saos2-mIκB cells. **A**: A representative (n = 3) Western blot for total (top blot) or phosphorylated (middle blot) IκBα in whole cell lysates of Saos2-EV (EV) or Saos2-mIκB (mIκB) cells. Equal loading was verified by reprobing for β-actin; (**B**) A representative (n = 3) Western blot for the p65 subunit of NFκB in nuclear fractions. Equal loading was verified by reprobing for lamin; (**C**) The heterologous promoter-reporter luciferase assay of NFκB in non-irradiated control and irradiated Saos2-EV or Saos2-mIκB cells was performed as described in Materials and Methods. Data are Means ± SD (n = 4). * indicates $P < 0.05$ when compared to control level in Saos2-EV cells.

prevents the translocation of p65 into the nuclei of Saos2-mIκB cells, thereby effectively inhibiting NFκB signaling in these mutant cells. We further confirmed the inhibition of NFκB signaling in Saos2-mIκB cells using the NFκB-luc reporter. Figure 1C shows that this reporter displays an 80% inhibition of reporter gene expression in Saos2-mIκB cells in comparison to Saos2-EV control cells. In addition, NFκB activity in Saos2-mIκB cells irradiated with 10 Gy of γ irradiation was also assessed after 24 and 48 h in culture. Figure 1C shows that NFκB activity is dramatically induced in Saos2-EV control cells after irradiation at both time points (sevenfold at 24 h and eightfold at 48 h). However, in Saos2-mIκB cells, this irradiation-induced upregulation of promoter activity is abrogated. These results indicate that by over-expressing mIκB, successful inhibition of NFκB signaling is achieved in Saos2-mIκB cells when compared to the empty vector infected controls.

Molecular Inhibition of NFκB Leads to Increased Radiation-Induced Apoptosis in Saos2 Cells

To determine the effects NFκB signaling inhibition on irradiation-mediated cell death, we exposed Saos2-mIκB cells and Saos2-EV controls to 10 Gy of ionizing radiation and assessed cell death markers after 24 and 48 h of culture. Figure 2A shows that Saos2-mIκB cells displayed a characteristic apoptotic phenotype including rounding and shrinkage of cell bodies after irradiation. Condensation of nuclei from irradiated Saos2-mIκB cells was also observed after staining with Hoechst33342 (Fig. 2B). These apoptotic features were absent in irradiated Saos2-EV cells (data not shown) demonstrating that inhibition of NFκB signaling in Saos2-mIκB cells correlates with increased apoptosis following irradiation. We further determined the number of apoptotic cells 24 and 48 h after irradiation. Figure 2C shows that in Saos2-EV cells, very few apoptotic cells were found at all time points following irradiation.

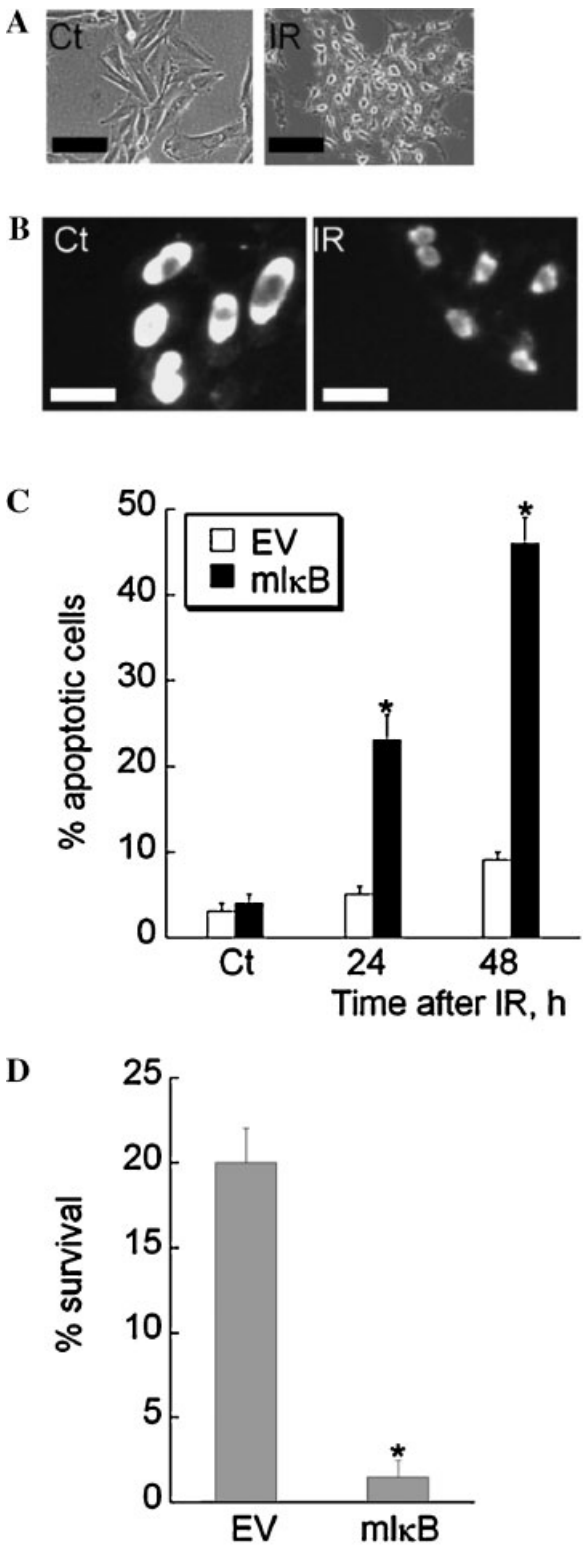

Fig. 2. Inhibition of NFκB in Saos2-mIκB cells leads to increased radiosensitivity. Cells were exposed to 10 Gy of ionizing radiation and various assays were performed at the indicated time-points: (**A**) Light microscopy of non-irradiated control (Ct) or irradiated (IR) Saos2-mIkB cells after 48 h in culture; (**B**) Hoechst333442 staining of control or irradiated Saos2-mIkB cells after 48 h in culture; (**C**) The percentage of apoptotic cells was determined by counting nuclei with condensed chromatin stained with Hoechst33342; (**D**) Clonogenic survival assay of irradiated Saos2-EV or Saos2-mIκB cells was performed as described in Materials and Methods. Data in C and D are Means ± SD (n = 4). * indicates $P < 0.05$ when compared to corresponding levels in Saos2-EV cells.

However, in Saos2-mIκB cells, irradiation led to a fivefold increase in apoptotic cell number at 24 h, and 10-fold increase at 48 h in comparison to non-irradiated cells. Neither irradiated Saos2-EV nor irradiated Saos2-mIκB cells displayed any necrosis as measured by Trypan Blue staining (data not shown). Cell survival was also determined via a clonogenic survival assay 10 days following irradiation. Figure 2D shows that inhibition of NFκB in Saos2-mIκB cells decreased post-irradiation colony forming ability by 10-fold when compared to Saos2-EV cells. Our results demonstrate that inhibition of NFκB signaling in Saos2-mIκB cells leads to increased sensitivity of these cells to ionizing radiation. Furthermore, irradiated Saos2-mIκB cells display strong characteristics of apoptosis.

Radiation-Induced Apoptosis in Saos2-mIκB Cells Progresses via the "Intrinsic" Pathway

Previous studies defined death receptor ligand-independent cell death as "intrinsic" apoptosis. This intrinsic pathway involves release of cytochrome-*c* from mitochondria, activation of caspase-9 and then caspase-3 [Earnshaw et al., 1999]. In order to understand the mechanism by which radiation induces apoptosis through NFκB-dependent pathways in osteosarcoma cells, we assessed the effects of radiation on cytochrome-*c* release and caspase-9 activation in Saos2-mIκB cells. Additionally we also evaluated the extrinsic apoptosis marker caspase-8 [Ashkenazi and Dixit, 1998], and the common caspase-3 marker for both intrinsic and extrinsic apoptosis in these irradiated cells. Figure 3A shows that irradiation led to activation of caspase-3 and -9 in Saos2-mIκB cells. Caspase-3 activity increased sixfold over control 24 h following irradiation and then slightly decreased to fourfold over control 48 h following irradiation (top panel). Caspase-9 activity increased fourfold over control at both time points (middle panel). We did not observe any activation of caspase-8 (bottom panel) following irradiation. In Saos2-EV control cells, there was no caspase activation 24 and 48 h after exposure to ionizing radiation. Western blot analysis of cytosolic fractions from non-irradiated and irradiated cells showed a significant increase

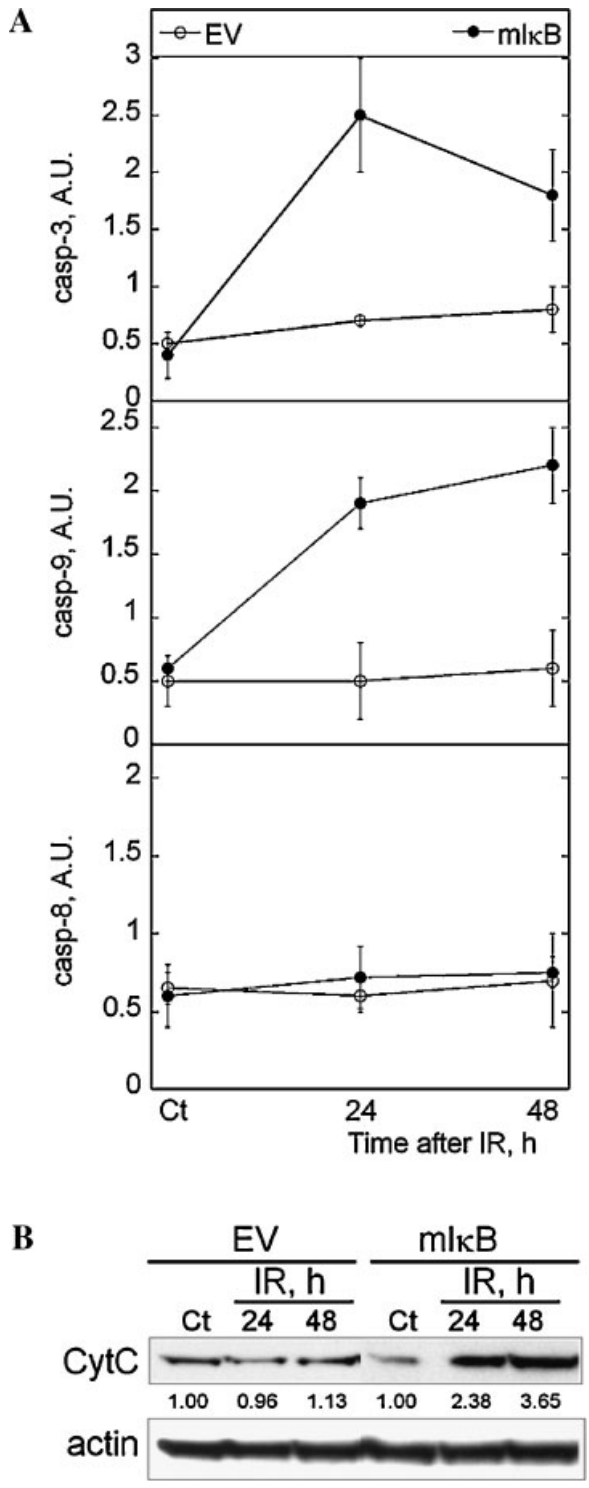

Fig. 3. Radiation-induced apoptosis in Sasos2-mIκB cells proceeds via the "intrinsic" pathway. **A**: Caspase-3 (casp-3), caspase-9 (casp-9), and caspase-8 (casp-8) activities were measured in lysates from control or irradiated Saos2-EV or Saos2-mIκB cells using Ac-DEVD-amc, Ac-LEHD-afc, or Z-IETD-afc substrates respectively as described in Materials and Methods. Data are Means ± SD (n = 5); (**B**) A representative (n = 3) Western blot for cytochrome-*c* (**CytC**) in cytosolic fractions from control or irradiated Saos2-EV or Saos2-mIκB cells. The numbers indicate relative intensities of each band. Each blot was reprobed for β-actin to verify equal loading.

in cytosolic cytochrome-*c* in Saos2-mIκB cells 24 and 48 h following irradiation in comparison to control (Fig. 3B). In irradiated Saos2-EV cells, the cytosolic fraction of cytochrome-*c* was not significantly higher than that in non-irradiated controls. These results indicate that radiation-induced apoptosis in Saos2-mIκB cells progresses via the intrinsic pathway that involves release of cytochrome-*c* into the cytosol and activation of caspase-9 and -3 but not caspase-8.

NFκB Regulation of Bcl-2 to Bax Protein Ratio in Saos2-mIκB Cells

The Bcl-2 family members are established regulators of apoptosis, particularly via the intrinsic pathway [Adams and Cory, 1998]. NFκB has been previously shown to have both direct and indirect effects on Bcl-2 family members' expression including Bcl-2 and Bax [Pahl, 1999]. We compared the protein levels of the anti-apoptotic protein Bcl-2 to the pro-apoptotic protein Bax in Saos2-EV and Saos2-mIκB with and without irradiation. Western blot analysis (Fig. 4A) shows that Bcl-2 levels did not significantly change in non-irradiated and irradiated Saos2-mIκB cells when compared to Saos2-EV cells. However, Bax protein levels were increased by twofold in both non-irradiated and irradiated Saos2-mIκB cells in comparison to Saos2-EV controls. As we have shown earlier in Figure 3, ionizing radiation induced the intrinsic pathway of apoptosis, which involves mitochondria. This pathway was previously shown to be regulated by the Bcl-2 to Bax ratio [Reed, 1997]. Figure 4B shows that Bcl-2 to Bax expression ratio is significantly reduced in Saos2-mIκB cells in comparison to Saos2-EV cells.

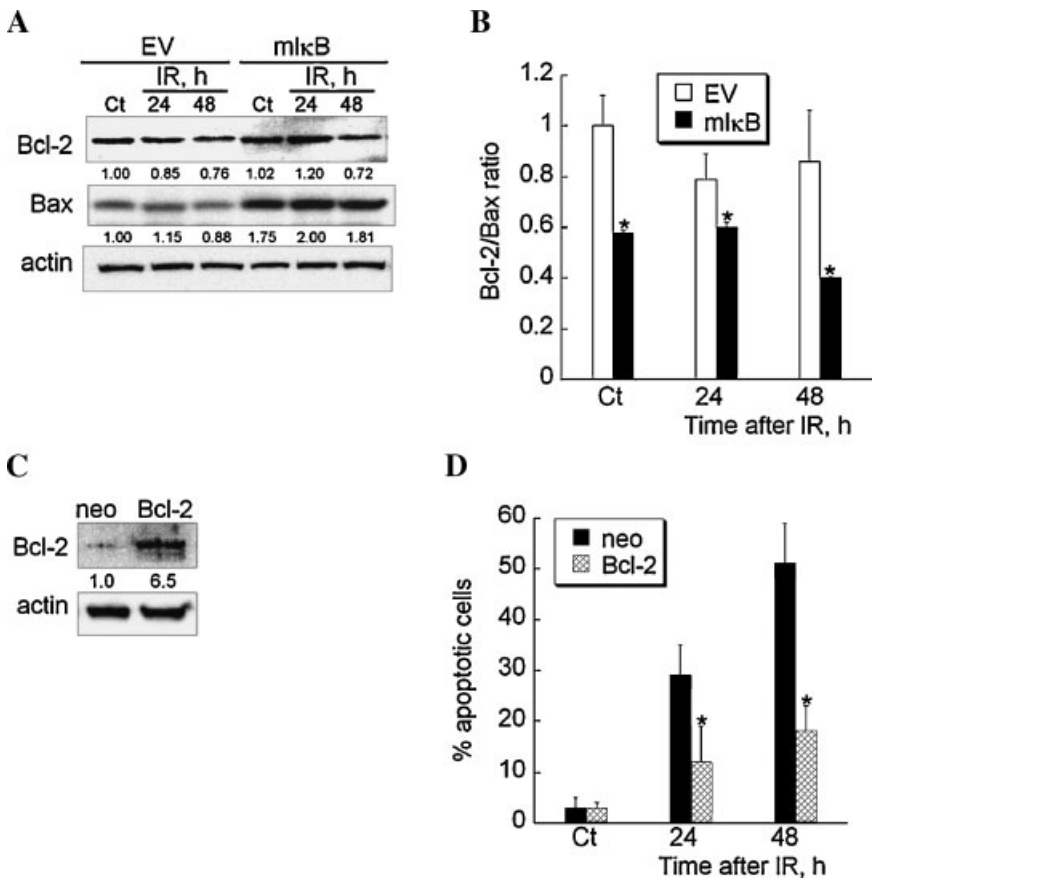

Fig. 4. Inhibition of NFκB in Saos2-mIκB cells leads to upregulation of Bax and decrease in Bcl-2/Bax ratio. Cells were exposed to ionizing radiation and analyzed at the indicated time-points. **A**: A representative (n = 3) Western blot for Bcl-2 in cell lysates. Blots were reprobed for Bax and then for β-actin to verify equal loading. The numbers indicate relative intensities of each band; (**B**) Bcl-2 to Bax ratio was calculated from densitometric analysis of Western blots; (**C**) A representative (n = 3) Western blot for Bcl-2 in lysates from pIRESneo (neo) or pIRESneo-Bcl-2 (Bcl-2) transfected Saos2-mIκB cells. Blots were reprobed for β-actin to verify equal loading; (**D**) The number of apoptotic cells was assayed in *neo* or *bcl-2*-transfected non-irradiated or irradiated Saos2-mIκB cells using Hoecsht33342 staining. Data in B and D are Means ± SD (n = 3). * indicates $P < 0.05$ when compared to corresponding values in Saos2-EV cells (B) or neo cells (D).

To assess whether the observed upregulation of Bax protein and corresponding decrease in Bcl-2/Bax ratio in Saos2-mIκB cells was responsible for increased sensitivity of these cells to ionizing radiation, we transfected Saos2-mIκB cells with a vector carrying *bcl-2* or with the empty vector (*neo*) as control. The transfection with *bcl-2* resulted in 6.5-fold over-expression of Bcl-2 protein when compared to *neo* cells as evident from a representative Western blot shown in Figure 4C. We then exposed transfected cells to ionizing radiation and measured apoptosis using Hoechst33342 staining. Figure 4D shows that over-expression of Bcl-2 protein inhibited the radiation-induced apoptosis in Saos2-mIκB cells. These data indicate that the inhibition of NFκB in Saos2-mIκB cells results in upregulation of the pro-apoptotic Bax and corresponding decrease in Bcl-2 to Bax ratio and that over-expression of Bcl-2 partially rescues Saos2-mIκB cells from radiation-induced apoptosis.

JNK Signaling is NFκB-Dependent and Regulates Radiation-Induced Apoptosis

NFκB has been reported to repress Bax expression [Chen et al., 1999]; however, the mechanism underlying this regulation is not fully understood. One possibility is that NFκB inhibits a transcriptional regulator of the *Bax* gene such as AP1 [Lei et al., 2002]. Furthermore, AP1 is a downstream component of the JNK/c-Jun/AP1 cascade, a stress response system that can be activated by ionizing radiation and also can be regulated by NFκB [De Smaele et al., 2001; Kuwabara et al., 2003].

To determine whether NFκB has an effect on the JNK cascade in our model system, we compared JNK/c-Jun/AP1 signaling in Saos2-EV and Saos2-mIκB cells. We first performed Western blot analysis of the phosphorylated form of c-Jun (p-cJun). Figure 5A shows a significant increase in p-cJun levels in both non-irradiated and irradiated Saos2-mIκB cells when compared to Saos2-EV cells. Phosphorylation of cJun was reversed in the presence of a specific JNK inhibitor, SP600125. Additionally, using the AP1-luciferase reporter assay we observed an eightfold increase in JNK/cJun/AP1 basal activity in control Saos2-mIκB cells when compared to Saos2-EV cells (Fig. 5B). The AP1-luciferase reporter activity slightly decreased in Saos2-mIκB cells after irradiation due likely to increasing rate of cell death but

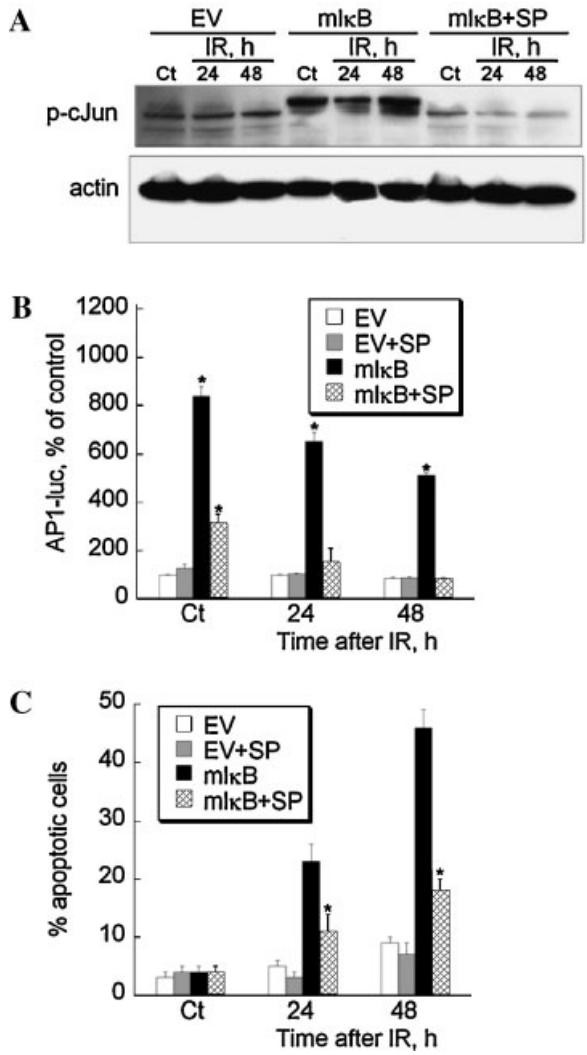

Fig. 5. JNK/cJun/AP1 pathway is activated in Saos2-mIκB cells. **A**: A representative (n = 3) Western blot for activated, phosphorylated cJun (**p-cJun**) in cell lysates. Some Saos2-mIκB cells were incubated in the presence of a specific JNK inhibitor SP600125 (**mIκB + SP**). Blots were reprobed for β-actin to verify equal loading; (**B**) AP1 luciferase reporter assay in cells irradiated in the absence or presence of SP600125 was performed as described in Materials and Methods; (**C**) Hoechst33342 apoptosis assay of cells irradiated in the absence or presence of SP600125 was performed as described in Materials and Methods; Data in B and C are Means ± SD (n = 3). * indicates $P < 0.05$ when compared to control level in Saos2-EV cells in (**B**) or to corresponding values in the absence of SP600125 in (**C**).

remained significantly higher than that in Saos2-EV cells. The JNK specific inhibitor SP600125 did not alter AP1-luciferase reporter activity in Saos2-EV cells but had a significant inhibitory effect on AP1-luciferase reporter activity in Saos2-mIκB cells. To determine a possible JNK signaling-mediated effect on radiation-induced apoptosis in our model

system, we irradiated cells in the presence of SP600125 and measured apoptosis 24 and 48 h following irradiation using Hoechst33342 staining. Figure 5C shows that in Saos2-mIκB cells, inhibition of JNK with SP600125 induced a twofold inhibition of apoptotic cell number 24 h following irradiation and threefold decrease in the number of apoptotic cells 48 h following irradiation. Thus, inhibition of NFκB results in activation of JNK signaling in Saos2-mIκB cells. JNK/cJun/AP1 cascade regulates cellular apoptotic response to ionizing radiation in our model system.

NFκB Controls the Expression of the JNK Inhibitor, Gadd45β in Saos2 Cells

One mechanism by which NFκB suppresses JNK signaling was previously reported and involved the DNA damage protein GADD45β [De Smaele et al., 2001]. GADD45β was recently identified as an inhibitor of MKK7/JNKK2 activator of JNK [Davis, 2000; Tournier et al., 2001]. To determine whether NFκB has an effect on the expression of GADD45β in the osteosacroma cell line Saos2, we compared the mRNA and protein levels of GADD45β in Saos2-EV and Saos2-mIκB cells. Real-time RT-PCR analysis of GADD45β mRNA shows that in Saos2-mIκB cells, GADD45β transcripts are decreased by fivefold when compared to Saos2-EV controls (Fig. 6A). Western blot analysis of GADD45β protein showed a total repression of GADD45β levels in lysates from Saos2-mIκB cells in comparison to an equal amount of proteins isolated from Saos2-EV controls (Fig. 6B). Our results demonstrate that mIκB over-expression dramatically inhibits the JNK signaling regulator, GADD45β. In order to confirm that GADD45β is a mediator of NFκB-driven suppression of JNK signaling in Saos2

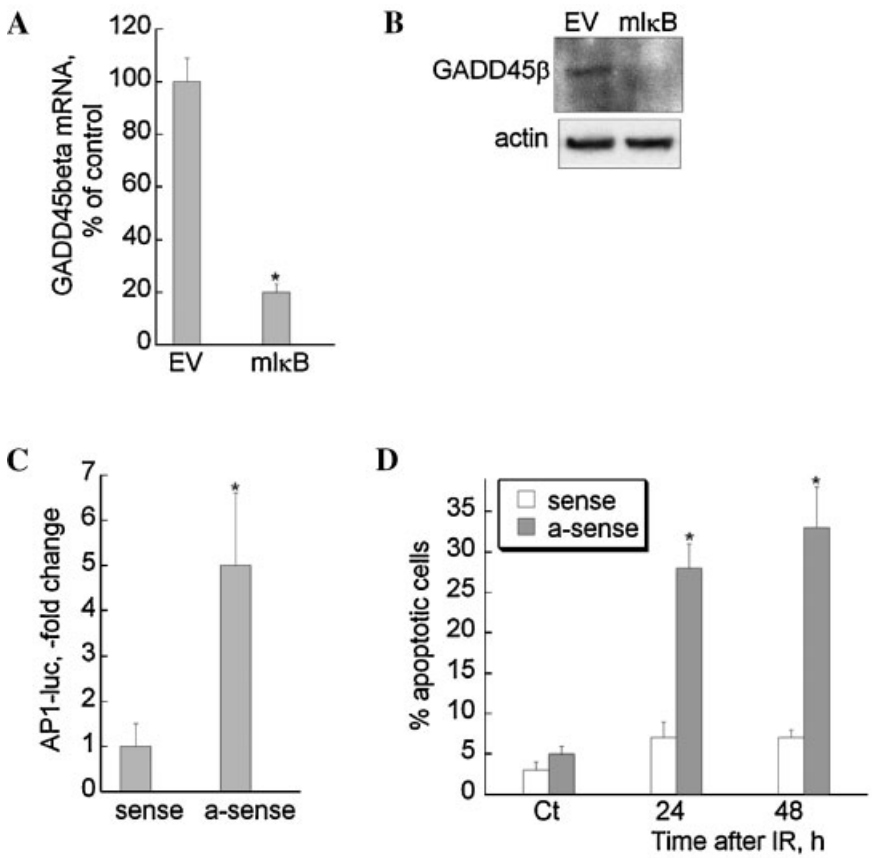

Fig. 6. GADD45β is NFκB dependent and regulates JNK signaling and radiation-induced apoptosis in Saos2 cells. **A**: A real-time RT-PCR assay of mRNA for GADD45β normalized to mRNA for GAPDH; (**B**) A representative (n = 3) Western blot for GADD45β in lysates from Saos2-EV or Saos2-mIκB cells. Blots were reprobed for actin to verify equal loading; (**C**) AP1 luciferase reporter assay in Saos2-EV cells exposed to sense or anti-sense GADD45β oligonucleotides; (**D**) Hoechst33342 apoptosis assay of Saos2-EV cells exposed to sense or anti-sense GADD45β oligonucleotides and then irradiated. Data in A, C, and D are Means ± SD (n = 3). * indicates $P < 0.05$ when compared to control.

cells, we knocked down GADD45β protein in Saos2-EV cells using specific antisense oligonucleotides. Seventy percent inhibition of GADD45β in Saos2-EV cells was confirmed by Western blotting (data not shown) and led to a fivefold activation of JNK signaling as shown by AP1-luciferase reporter assay (Fig. 6C). Antisense inhibition of GADD45β also significantly increased the number of Saos2-EV undergoing apoptosis following irradiation (Fig. 6D). Our results suggest that in Saos2 osteosarcoma cells, NFκB suppresses JNK signaling by upregulating the JNK inhibitor GADD45β; and inhibition of NFκB in Saos2-mIκB cells may lead to downregulation of GADD45β and subsequent activation of JNK signaling.

DISCUSSION

Radiosensitization achieved by inhibition of NFκB may be a general phenomenon as shown in previous studies with similar effects in different types of cancer. For instance, molecular or pharmacological inhibition of NFκB led to significant enhancement of radiation-induced apoptosis in colon cancer cells [Mukogawa et al., 2003] and fibrosarcoma cells [Wang et al., 1996]. Currently, treatment of osteosarcomas is limited to chemotherapy involving adriamycin or metothrexate followed by surgery; however, current chemotherapeutic pre-treatment protocols do not significantly increase the post-surgical 5-year survival rate of 50–60% [Goorin and Schwartzentruber, 2003]. Therefore, the inadequacy of current chemotherapeutic protocols requires new approaches. One of the most promising approaches is the sensitization of osteosarcomas to ionizing radiation followed by radiotherapy.

The inability of ionizing radiation to induce efficient cell death during the treatment of osteosarcoma by radiotherapy may arise from a mutation of a major anti-oncogene and DNA damage sensor protein, p53 [Vogelstein et al., 2000]. However, independently from the p53 pathway, additional stress response systems such as the JNK signaling were shown to respond to ionizing radiation-induced DNA damage and set forward the apoptotic machinery [Dent et al., 2003; Kuwabara et al., 2003]. We propose that osteosarcoma cells may possess a mechanism of supression of JNK signaling leading to their escape from radiation-induced apoptosis. We hypothesized that NFκB is responsible for this escape and that inhibition of NFκB signaling enhances responsiveness of osteosarcomas to ionizing radiation. We found that the molecular inhibition of NFκB with mIκB increased sensitivity of Saos2 cells to radiation-induced apoptosis proving that NFκB regulates apoptotic mechanisms activated by ionizing radiation. Radiation induced the intrinsic apoptotic pathways, which involve the release of cytochrome-*c* from the mitochondria and the activation of caspase-9 and -3. These events are regulated by a decreased Bcl-2 to Bax ratio in osteosarcoma cells in which NFκB signaling is inhibited. We found that radiation-induced apoptosis was sensitive to a specific JNK inhibitor, SP600125, in Saos2-mIκB cells. Inhibition of NFκB in Saos2-mIκB cells increased activity of JNK cascade via the JNK signaling inhibitor protein, GADD45β. Our study demonstrates for the first time that the radioresistance of Saos2 osteosarcoma cells is subsequent to NFκB-mediated inhibition of JNK signaling.

To verify our hypothesis, we first established that inhibition of NFκB signaling by over-expressing a constitutive non-degradable inhibitor mIκB in the human osteosarcoma cell line Saos2, which is p53 deficient, is a valid model to study irradiation-mediated apoptosis. Our results clearly show that over-expression of mIκB in Saos2 cells not only decreased basal NFκB activity but further prevented the radiation-induced activation of NFκB signaling observed in control cells. These results are in agreement with previously reported studies showing that specific over-expression of mIκB in various tumor-derived cells constitutes an effective model system for inhibiting NFκB signaling [Van Antwerp et al., 1996; Schwarz et al., 1998; Andela et al., 2002].

In our model system we observed cell shrinkage, chromatin and nuclear condensation, and activation of caspases, while the plasma membrane remained intact after irradiation. We, therefore, show that γ irradiation at a dose of 10 Gy induces effective programmed cell death without leading to cell necrosis. A moderate dose of γ irradiation ionizes water molecules surrounding DNA causing DNA damage. It has previously been reported that such DNA damage does not immediately kill the cell but activates the intrinsic pathway of apoptosis [Dent et al., 2003; Kuwabara et al., 2003]. We found that in Saos2-mIκB cells radiation-

induced apoptosis progressed via the intrinsic pathway as evidenced by elevation of cytochrome-*c* in cytosolic fractions and activation of caspase-9 and -3 but not caspase-8 in the Saos2-mIκB cells. Furthermore, the Bcl-2 to Bax ratio was decreased in Saos2-mIκB cells when compared to Saos2-EV controls subsequent to the significant elevation of Bax levels. The observed upregulation of Bax after inhibition of NFκB signaling is in agreement with earlier reports that showed Bax supression by NFκB [Pahl, 1999], although the mechanism of such supression was not fully elucidated. When we over-expressed Bcl-2 in our Saos2-mIκB cell model we effectively inhibited this radiation-induced apoptosis. The intrinsic pathway is known to be controlled by Bcl-2 to Bax ratio [Reed, 1997] and, therefore, the factor(s) that regulate the Bcl-2 to Bax ratio is likely to regulate the whole cell response to ionizing radiation. It is relatively accepted that Bax expression is controlled by the JNK signaling and JNK-activated AP1 factor [Lei et al., 2002] and previous studies reported that JNK signaling is a stress activated system that regulates cellular response to ionizing radiation [Dent et al., 2003; Kuwabara et al., 2003]. Our data demonstrate that inhibition of NFκB signaling in Saos2-mIκB cells results in activation of the JNK cascade and that radiation-induced apoptosis in these cells can be inhibited with a specific JNK inhibitor SP60025. Here we present strong evidence for the JNK/cJun/AP1 pathway as a major NFκB-dependent regulator of radiation-induced apoptosis in Saos2 osteosarcoma cells. One possible mechanism by which NFκB-mediates the downregulation of the JNK system includes the binding of the DNA damage protein GADD45β to the MKK7/JNKK2 activator of JNK. We found that inhibition of NFκB results in significant downregulation of GADD45β in Saos2 osteosarcoma cells and that antisense inhibition of GADD45β leads to activation of the JNK signaling and increased sensitivity to radiation-induced apoptosis. Overall, our results demonstrate that the radioresistance of Saos2 osteosarcoma cells is due to NFκB-mediated inhibition of JNK signaling via GADD45β; and that the molecular inhibition of NFκB causes downregulation of GADD45β followed by activation of JNK signaling and as a result, increased sensitivity of Saos2 cell to radiation-induced apoptosis. Therefore, the radiosensitization achieved by inhibition of NFκB followed by radiotherapy may represent a potential alternative to current protocols in treatment of osteosarcomas.

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