

Inhibition of constitutively activated nuclear factor- κ B radiosensitizes human melanoma cells

Anupama Munshi,¹ John F. Kurland,¹
Takashi Nishikawa,¹ Paul J. Chiao,²
Michael Andreeff,³ and Raymond E. Meyn¹

Departments of ¹Experimental Radiation Oncology, ²Surgical Oncology, and ³Blood and Marrow Transplantation, University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

Melanoma tumors and cultured cell lines are relatively resistant to the cytotoxic effects of ionizing radiation, thereby limiting the use of radiotherapy for the clinical treatment of melanoma. New strategies for sensitizing melanoma cells therefore deserve examination. In an attempt to identify and target signaling pathways that contribute to radioresistance, we investigated the role of nuclear factor- κ B (NF- κ B), a transcription factor known to inhibit apoptosis induced by a variety of stimuli and promote radioresistance. Two human metastatic melanoma cell lines, A375 and MeWo, were used to examine the radiosensitizing effects of inhibitors of the NF- κ B pathway. Nuclear extracts from these cell lines were tested for active NF- κ B using the electrophoretic mobility shift assay. Both melanoma cell lines had constitutively activated NF- κ B as observed by electrophoretic mobility shift assay. In an attempt to reverse NF- κ B activity, cells were treated either with vehicle alone (DMSO) or with a proteasome inhibitor Z-Leu-Leu-Leu-H (MG132; 10 μ M/L for 2 hours prior to irradiation) that inhibited both constitutive and radiation-induced NF- κ B activity. The clonogenic cell survival assay showed that pretreatment with MG132 enhanced tumor cell radiosensitivity with the survival factor at 2 Gy being reduced from $48 \pm 0.8\%$ and $48 \pm 1.6\%$ in vehicle-treated cells to $27.7 \pm 0.32\%$ and $34.3 \pm 0.7\%$ in MG132-treated MeWo and A375 cells, respectively. To test the role of NF- κ B in radioresistance more

directly, MeWo cells were stably transfected with a dominant-negative mutant I κ B α construct, which led to the inhibition of both constitutive and radiation-induced NF- κ B activity. A modest restoration of radiosensitivity was also observed in the stably transfected MeWo cells with survival factor at 2 Gy values being reduced from $47 \pm 0.8\%$ in parental MeWo cells to $32.9 \pm 0.7\%$ in stable transfectants. Because constitutively activated mitogen-activated protein kinase kinase (MEK) pathway has been shown to lead to activated NF- κ B, we wanted to determine the relative contribution of activated MEK in the human melanoma cells. To test this, MeWo and A375 melanoma cells were exposed to the MEK inhibitor PD184352. Treatment with PD184352 partially reversed NF- κ B activity but did not impart radiation sensitivity to these cells. Our results indicate that activated NF- κ B may be one of the pathways responsible for the radioresistance of melanoma cells and that strategies for inhibiting its influence may be useful in restoring the radioresponse of melanomas. [Mol Cancer Ther 2004;3(8):985–92]

Introduction

Melanoma cells are well known to be resistant to radiation and commonly used chemotherapy agents, and this resistance is associated with a poor prognosis for this metastatic disease. As such, radiotherapy is not commonly used as a primary treatment for melanoma. Instead, melanoma is typically treated with surgery, but adjuvant therapies such as IFN, combination chemotherapy, and the use of other biological response modifiers have begun to have major impact on the success of treatment (1). The incidence of melanoma is on the rise worldwide, with 43,000 new patients diagnosed in the United States. Therefore, improving the treatment of melanoma could benefit many patients. For example, the use of small molecules or gene therapy strategies that overcome the relative radioresistance of melanoma tumors could prove to be useful. To improve radiotherapy, however, it is important to understand the biological response these cells have to radiation, and any new strategies for sensitizing cells to radiation should target those pathways in the cell that contribute to radiation resistance. One such pathway may involve nuclear factor- κ B (NF- κ B).

Constitutive activation of NF- κ B has been showed in a variety of human malignancies including pancreatic cancer, colon cancer, breast cancer, T-cell leukemias, and lymphomas. Several reports have also showed that NF- κ B is constitutively activated in human melanoma cells (2, 3), and it has recently been shown that this constitutive activity is a result of elevated I κ B kinase (IKK) activity arising from aberrant NF- κ B-inducing kinase activation (4). Recent studies have shown NF- κ B to be a critical regulator

Received 7/24/03; revised 5/12/04; accepted 5/14/04.

Grant support: National Cancer Institute grants P01 CA78778, P01 CA06294, and P30 CA 16672.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: A. Munshi and J.F. Kurland contributed equally to this work. J.F. Kurland is currently at Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724.

Requests for reprints: Raymond E. Meyn, Department of Experimental Radiation Oncology, University of Texas M.D. Anderson Cancer Center, Box 066, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-3424; Fax: 713-794-5369.

E-mail: rmeyn@mdanderson.org

Copyright © 2004 American Association for Cancer Research.

of apoptosis by controlling the transcription of genes with products that block cell death. It has been implied that constitutive activation of NF- κ B induces overexpression of its downstream targets such as bcl-x_L, bcl-2, vascular endothelial growth factor, and interleukin-8, which may in turn mediate resistance to apoptosis induced by chemotherapy and radiation. Several genes involved in tumor initiation, promotion, and metastasis are also regulated by NF- κ B, suggesting that NF- κ B could mediate tumorigenesis and can thus be used as target for chemoprevention and treatment of cancer. Agents that suppress NF- κ B activation have been shown to suppress the expression of genes involved in carcinogenesis and tumorigenesis *in vivo* (5).

The role of NF- κ B as a modifier of radiosensitivity has only been analyzed in limited studies. Regulation of NF- κ B is mainly controlled by the inhibitory I κ B proteins, which include I κ B α . On stimulation, I κ B is rapidly phosphorylated by IKK and degraded via the ubiquitin-proteasome pathway, permitting nuclear import of NF- κ B. There is sufficient evidence in the literature demonstrating the ability of proteasome inhibitors such as Z-Leu-Leu-Leu-H (MG132), lactacystin, and PS-341 in blocking NF- κ B activation in cultured cells by preventing the degradation of I κ B without interfering with its phosphorylation (6). Russo et al. (7) showed that inhibition of proteasome function dramatically enhanced the apoptotic response of colorectal cells to radiation both *in vitro* and in an *in vivo* xenograft model. In their study, treatment of colorectal cancer cells with the proteasome inhibitor PS-341 decreased radiation-induced NF- κ B activation and resulted in an enhanced apoptotic response to radiation. Other studies have also showed an enhanced antitumor effect when PS-341 was combined with cytotoxic agents (8) or ionizing radiation (9). Dominant-negative mutant I κ B α (I κ B α M) has been engineered, which cannot be phosphorylated and degraded; thus, the nuclear importation and DNA binding of NF- κ B complexes are repressed constitutively if cells are stably transfected with I κ B α M. The stable expression of I κ B α M has been shown to inhibit the activation of NF- κ B in several cell types (10).

There are several other kinases known to act upstream of IKK. These include members of the mitogen-activated protein kinase (MAPK) kinase (MEK) kinase (MEKK) family such as MEKK-1, MEKK-2, and MEKK-3 (11–15). MEKK-1 activity has been associated with pancreatic cancer (16); MEKK-3 activity has been associated with hepatocellular carcinoma (17); and Tpl-2 activity has been associated with breast, colon, and gastric cancers (18, 19). Thus, cancers that have aberrant kinase activity upstream of IKK would also be expected to have constitutively active nuclear NF- κ B, and this activity could in turn contribute to the expression of genes important to the survival of those cancers.

The present study was designed to investigate whether melanoma cell lines that are normally resistant to radiation could be made susceptible by inhibiting the activation of NF- κ B. The inhibition of NF- κ B, either by treatment with MG132 or by stable expression of I κ B α M, led to down-

regulation of the antiapoptotic proteins bcl-x_L, bcl-2, and survivin and decreased clonogenic survival in response to ionizing radiation. However, exposure of melanoma cells to the MEK inhibitor PD184352 inhibited constitutive extracellular signal-regulated kinase (ERK) activity determined based on phospho-p44/p42 expression and partially suppressed NF- κ B activation but was unable to induce radiosensitivity as assessed by clonogenic cell survival assay. Therefore, approaches that inhibit the nuclear translocation or function of NF- κ B, including proteasome inhibitors or I κ B α M gene therapy, may be beneficial to the treatment of melanoma when combined with standard anticancer therapies such as radiation, but MEK inhibitors may not necessarily act in a synergistic manner to restore sensitivity to radiation in this particular setting.

Materials and Methods

Cells

Two different human melanoma cell lines were used to study the radiosensitizing effect of inhibition of NF- κ B: A375 and MeWo. Cells were obtained from American Type Culture Collection (Manassas, VA) and routinely maintained in recommended media supplemented with 10% fetal bovine serum, 10,000 units/mL penicillin-streptomycin, and 2 mmol/L L-glutamine.

Chemicals

The proteasome inhibitor MG132 was obtained from Sigma-Aldrich Co. (St. Louis, MO). For all *in vitro* assays, a 10 mmol/L stock solution in DMSO was prepared and stored at -20°C until use. The MEK inhibitor PD184352 was prepared as a 10 mmol/L stock in DMSO and stored at -20°C until use.

Transfection

The I κ B α M has been engineered to not be phosphorylated or degraded through the introduction of mutations in Ser³² and Ser³⁶ (20). The plasmid was a generous gift of Dr. Paul Chiao (Department of Surgical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX). Semiconfluent cells were transfected with the plasmid construct using FuGENE (Life Technologies, Inc., Rockville, MD). Stable clones were selected in geneticin (Life Technologies)-containing media. Mock-transfected cells were used as controls.

Clonogenic Survival

The effectiveness of the combination of proteasome inhibitors/I κ B α M or the MEK inhibitor PD184352 and ionizing radiation was assessed by clonogenic assays. Melanoma cells were either mock treated with vehicle alone (DMSO) or pretreated with MG132 (10 $\mu\text{mol/L}$) for 2 hours. Then, cells were irradiated, drug was washed off, and cells were harvested for clonogenic assay. For experiments comparing the mock-transfected MeWo with those carrying I κ B α M, cells were irradiated at various doses and processed for clonogenic cell survival assay. To study the effect of the MEK inhibitor PD184352, cells were exposed to 1 and 2 $\mu\text{mol/L}$ concentrations for 24 hours and were irradiated and further processed for clonogenic cell

survival assay. Cells were irradiated with a high-dose rate ^{137}Cs unit (4.5 Gy/min) at room temperature. Following treatment, cells were trypsinized and counted. Known numbers were replated in triplicate and returned to the incubator to allow macroscopic colony development. Colonies were stained with 0.5% gentian violet solution and counted after ~14 days. The percentage plating efficiency and fraction surviving a given treatment was calculated based on the survival of nonirradiated cells treated with the agent in question.

Western Blot Analysis

Cells were harvested after treatment, rinsed in ice-cold PBS, and lysed in lysis buffer containing 50 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 2 $\mu\text{g}/\text{mL}$ leupeptin, 2 $\mu\text{g}/\text{mL}$ aprotinin, 5 $\mu\text{g}/\text{mL}$ benzamide, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1% NP40. The lysed cells were centrifuged at 14,000 rpm to remove any cellular debris. Protein concentrations of the lysates were determined by the Dc protein assay system (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by 12% SDS-PAGE, transferred to Immobilon (Millipore, Bedford, MA), and blocked with 5% nonfat dry milk in TBS-Tween 20 (0.05% v/v) for 1 hour at room temperature. The membrane was incubated with the respective primary antibody overnight. Antibody to human bcl-2 was obtained from DAKO (Carpinteria, CA), bcl-x_L and survivin were from Santa Cruz Biotechnology (Santa Cruz, CA), and actin was from Chemicon (Temecula, CA). After washing, the membrane was incubated with the appropriate horseradish peroxidase secondary antibody (1:2,000 dilution; Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 hour. Following several washes, the blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech) on a STORM 860 PhosphorImager (Molecular Dynamics, Heyward, CA).

Nuclear Extract Preparation

Cells were rinsed twice with ice-cold PBS, harvested by scraping with a cell scraper, and centrifuged at 800 rpm for 10 minutes at 4°C. The cell pellet was resuspended in 400 μL of cold lysis buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 2 $\mu\text{g}/\text{mL}$ leupeptin, 2 $\mu\text{g}/\text{mL}$ aprotinin, and 0.5 mmol/L phenylmethylsulfonyl fluoride] and incubated on ice for 10 minutes; 10% NP40 (12.5 μL) was added and the mixture was vortexed for 5 seconds. The lysate was centrifuged for 5 minutes at 4°C (14,000 rpm). The supernatant was removed and stored as cytosolic extract. The pellet was resuspended in 30 μL of extraction buffer [20 mmol/L HEPES (pH 7.9), 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 2 $\mu\text{g}/\text{mL}$ leupeptin, 2 $\mu\text{g}/\text{mL}$ aprotinin, and 0.5 mmol/L phenylmethylsulfonyl fluoride], mixed thoroughly, and incubated on ice for 30 minutes. The pellet was vortexed every 10 minutes. At the end of 30 minutes, the extract was centrifuged for 10 minutes at maximum speed (14,000 rpm) in a microcentrifuge. The supernatant was designated as nuclear extract, aliquoted, and stored at -70°C until used in the electrophoretic mobility shift assays (EMSA).

Electrophoretic Mobility Shift Assay

To measure suppressed activation of NF- κ B in response to treatment with MG132, PD184352, or I κ B α M and radiation, EMSA was carried out. Nuclear extracts from these cells were run on EMSA to determine the best dose and timing that leads to maximum inhibition of NF- κ B. Basically, nuclear extracts (15 μg) were incubated with poly(deoxyinosinic-deoxycytidylic acid) (1 μg) in binding buffer containing 10 mmol/L Tris, 50 mmol/L NaCl, 20% glycerol, 0.5 mmol/L EDTA, and 1 mmol/L DTT. [^{32}P]-labeled probe containing the NF- κ B site was added and allowed to bind for 15 minutes. The complexes were separated on a 4% polyacrylamide gel and visualized by phosphorimaging.

Statistical Analysis

Data were analyzed using the paired *t* test (Microsoft Excel). Data are presented as means \pm SE. A difference was regarded as significant if *P* < 0.05.

Results

NF- κ B Nuclear DNA Binding Is Constitutively Active in Human Melanoma Cells and Is Enhanced by Ionizing Radiation

It has been shown previously that NF- κ B is constitutively activated in tumors of different origins including melanomas. We investigated NF- κ B activity in two human melanoma cell lines, A375 and MeWo, by EMSA and compared it with the activity in nuclear extracts prepared from a lung cancer cell line, H1299, that does not express NF- κ B. NF- κ B was constitutively activated in both melanoma cell lines when compared with the H1299 cell line, which lacked any detectable activity (Fig. 1A). Further induction of NF- κ B in response to ionizing radiation was observed in MeWo cells 2 hours following exposure to 4 Gy dose (Fig. 1B).

The Proteasome Inhibitor MG132 Blocks Constitutive and Radiation-Induced Activation of NF- κ B

To measure suppressed activation of NF- κ B in response to the proteasome inhibitor MG132, EMSA was done. Nuclear extracts from A375 and MeWo cells were analyzed by EMSA to determine the best dose and timing that leads to maximum inhibition of NF- κ B. When the cells were pretreated with a relatively low dose (0.5 $\mu\text{mol}/\text{L}$) of the proteasome inhibitor MG132 for 24 hours, neither constitutive nor radiation-induced NF- κ B activity was affected (data not shown). In both cell lines, MeWo (Fig. 2A) and A375 (Fig. 2B), we observed an induction of NF- κ B activity by ionizing radiation that was blocked by a 2-hour preincubation with 10 $\mu\text{mol}/\text{L}$ MG132. Therefore, this treatment with MG132 was picked for all future experiments.

Treatment with Proteasome Inhibitors Enhances Radiosensitivity in Human Melanoma Cells in an *In vitro* Clonogenic Survival Assay

To determine if inhibition of activated NF- κ B can reverse the radioresistance of melanoma cells, MeWo and A375 cells were pretreated with 10 $\mu\text{mol}/\text{L}$ MG132 for 2 hours, and the cells were irradiated and plated for clonogenic cell

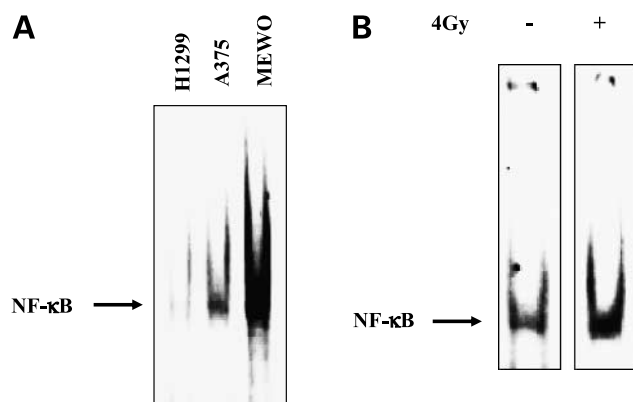


Figure 1. Human melanoma cells have constitutive NF- κ B DNA binding activity, which is enhanced by ionizing radiation. **A**, nuclear extracts from A375, MeWo, and H1299 cell lines were prepared and assessed for NF- κ B activity by EMSA. **B**, MeWo cells were exposed to a 4 Gy dose of radiation and assessed after a 2-hour incubation by EMSA.

survival. Figure 3 shows that MG132 suppressed the clonogenic survival of both melanoma cell lines: MeWo (representing the mean of four independent experiments; Fig. 3A) and A375 (representing the mean of three independent experiments; Fig. 3B). Survival at 2 and 4 Gy was reduced from $48 \pm 0.65\%$ and $11.6 \pm 0.33\%$ in the control MeWo cells to $27.7 \pm 0.32\%$ and $6.25 \pm 0.23\%$ in MG132-treated MeWo cells, respectively (Fig. 3A). The observed sensitization was statistically significant with $P < 0.005$ and 0.0006 at 2 and 4 Gy, respectively. Similar results were obtained on exposure of A375 cells to MG132 with survival at 2 and 4 Gy being reduced from $48 \pm 1.6\%$ and $12.1 \pm 0.7\%$ in the control cells to $34.3 \pm 0.7\%$ and $7.6 \pm 0.22\%$, respectively (Fig. 3B). The observed sensitization was statistically significant with $P < 0.004$ and 0.025 at 2 and 4 Gy, respectively. Survival enhancement ratios were calculated at the 50% cell survival by dividing radiation dose of the radiation-only survival curve with that of the corresponding MG132 plus radiation curve. Survival enhancement ratio was 1.47 for the MeWo cells and 1.3 for the A375 cells. Therefore, NF- κ B activation may be one of the pathways responsible for radioresistance in melanoma cells. However, because inhibition of the proteasome does not specifically target just NF- κ B, it was necessary to test the role of NF- κ B by an independent and more specific means.

Expression of I κ B α M Blocks Constitutive and Radiation-Induced NF- κ B Activity and Enhances Radiosensitivity in MeWo Cells

Because the proteasome selectively eliminates many cellular proteins, the effects observed on treating cells with proteasome inhibitors may not be specifically targeted toward inhibition of NF- κ B. Therefore, a specific inhibitor of NF- κ B activation, I κ B α M, was introduced into MeWo cells to create a stable cell line. Figure 4A shows the expression of the I κ B α M in the stably transfected clone by Western blot analysis. To determine if the activation of

NF- κ B in response to ionizing radiation was suppressed, nuclear extracts from I κ B α M-transfected MeWo cells were run on EMSA. We observed that the induction of NF- κ B activity by ionizing radiation (4 Gy) and the constitutive activation of NF- κ B were completely abolished in the stably transfected cell line (Fig. 4B). These cells were further used to determine the role of NF- κ B in mediating radioresistance in melanoma cells. We carried out clonogenic cell survival assay on MeWo I κ B α M-transfected cells (representing the mean of four independent experiments) and observed a modest sensitization to ionizing radiation. Surviving fraction at both 2 and 4 Gy was decreased from $47 \pm 0.8\%$ to $32.9 \pm 0.7\%$ and $10.4 \pm 0.4\%$ to $7.4 \pm 0.3\%$ in the control versus the I κ B α M cells (Fig. 5). The observed sensitization was statistically significant at 6 Gy with $P = 0.045$. The P values at 2 and 4 Gy were 0.12 and 0.14, respectively. Survival enhancement ratio at the 50% cell survival was found to be 1.28. These findings confirmed that activated NF- κ B can be one of the possible mechanisms to explain the radioresistance of melanoma cells.

Effect of Inhibition of Activated NF- κ B on Its Antiapoptotic Transcriptional Targets

We determined the effects of NF- κ B inhibition on some of its downstream antiapoptotic targets such as bcl-2, bcl-x_L, and survivin, a unique member of the inhibitor of apoptosis protein family of proteins, by Western blot analysis. Treatment with MG132 (10 μ mol/L for 2 hours) and transfection of the I κ B α M into MeWo cells caused a down-regulation of bcl-2, bcl-x_L, and survivin proteins (Fig. 6A and B). Combination of these treatments with a 4 Gy dose of radiation did not further suppress the levels of bcl-2 or bcl-x_L. Radiation alone slightly suppressed the levels of bcl-2 and survivin but enhanced bcl-x_L. However, combination treatments of MG132 or I κ B α M with ionizing radiation suppressed survivin to a greater degree than either treatment alone (Fig. 6A and B).

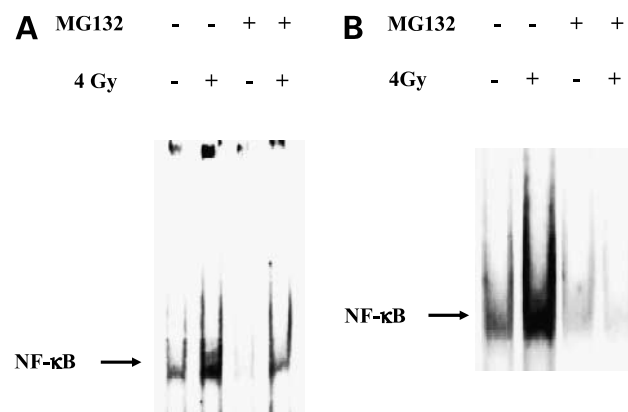


Figure 2. The proteasome inhibitor MG132 blocks constitutive and radiation-induced NF- κ B activity in melanoma cells. A375 (**A**) and MeWo (**B**) cells were left untreated, exposed to 4 Gy, and treated with 10 μ mol/L MG132 for 2 hours or a combination of MG132 and radiation. Cells were harvested 2 hours following irradiation for analysis by EMSA.

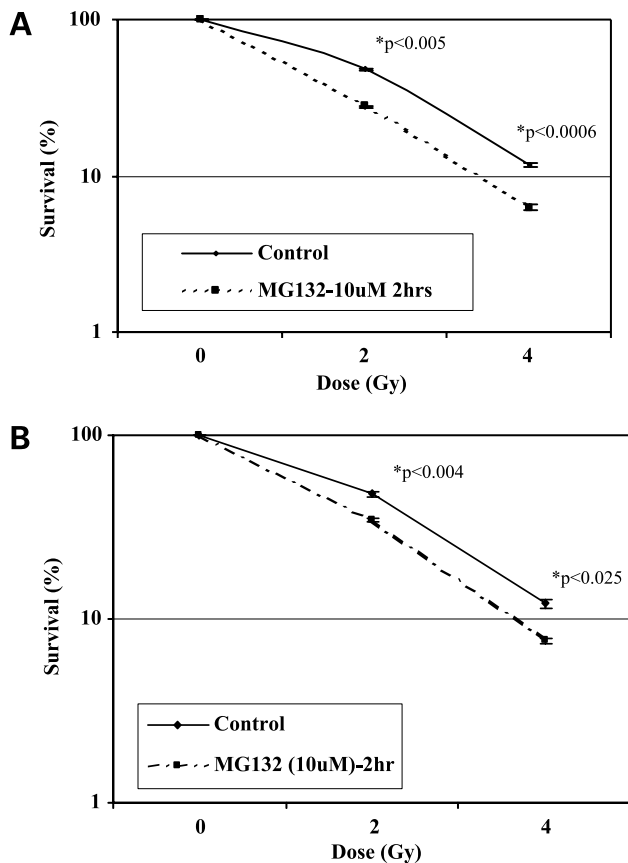


Figure 3. Treatment with MG132 sensitizes melanoma cells to ionizing radiation. Radiosensitization by MG132 was based on clonogenic cell survival assays. MeWo (A) and A375 (B) cells were pretreated with MG132 (10 $\mu\text{mol/L}$ for 2 hours), and the drug was washed off and cells were irradiated at various doses and plated for clonogenic cell survival assay. Points, average of three (A375) and four (MeWo) independent experiments plated in triplicate; bars, SE.

MEK Inhibitors Partially Block Constitutive and Radiation-Induced Activation of NF- κ B but Do Not Impart Radiation Sensitivity

There is a close relationship between NF- κ B and MEK pathways, and MEK activation has been linked to activation of NF- κ B. As shown in Fig. 7, MEK is constitutively active in MeWo and A375 cells as detected based on phospho-p44/p42 expression. Therefore, we used the MEK1 inhibitor PD184352 to test whether the MEK/ERK pathway was acting upstream of NF- κ B activation. Treatment with 1 and 2 $\mu\text{mol/L}$ doses of PD184352 for 24 hours down-regulated phospho-ERK in both A375 and MeWo melanoma cells (Fig. 7A). MeWo cells were treated with PD184352 (1 and 2 $\mu\text{mol/L}$) for 24 hours, and nuclear extracts were prepared and run on EMSA to determine its inhibitory effects on NF- κ B activation. PD184352 when used at the above-mentioned concentrations suppressed both constitutive and ionizing radiation-induced NF- κ B activation (Fig. 7B) but not to the extent seen previously with either MG132 or I κ B α M. To determine the ability of

PD184352 to produce a radiosensitizing effect, melanoma cells were exposed to 1 and 2 $\mu\text{mol/L}$ doses of PD184352 for 24 hours, and cells were irradiated and harvested for clonogenic assays. In spite of its ability to partially suppress NF- κ B activation, PD184352 was unable to mediate any sensitization to ionizing radiation in the MeWo cells within experimental error (Fig. 8).

Discussion

NF- κ B is a transcription factor that is activated by various stress stimuli including oxidative stress and DNA-damaging agents such as ionizing radiation. In general, activation of NF- κ B leads to induction of antiapoptotic genes such as bcl-2 and bcl-x_L and members of the inhibitor of apoptosis protein family, thereby promoting cell survival. NF- κ B has also been implicated in resistance to radiation-induced cytotoxicity, and its role in radiation response has been addressed in several studies showing that NF- κ B activation mediates radiation-induced gene expression in both normal and tumor cells (21–23). Because NF- κ B was reported to be constitutively activated in melanoma cells (2), we investigated the effect of modulation of the NF- κ B DNA binding activity on the intrinsic radiosensitivity of human melanoma cell lines. Both melanoma cell lines used in this study, MeWo and A375, showed constitutively activated NF- κ B (Fig. 1). In addition to melanomas, constitutive activation

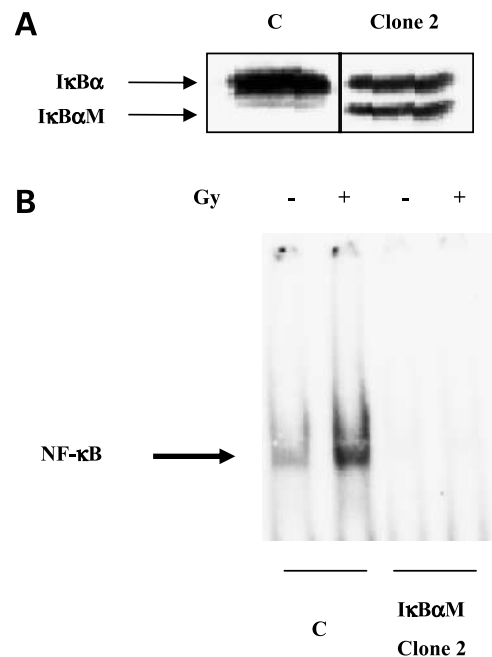


Figure 4. I κ B α M blocks constitutive and radiation-induced NF- κ B activity. A, MeWo cells were transfected with the I κ B α M plasmid, and clones were selected in G418-containing media. Western blot analysis shows the presence of I κ B α M in control mock-transfected (C) MeWo cells versus one of the clones selected in G418 (Clone 2). B, control MeWo cells (C) or MeWo cells stably transfected with I κ B α M (Clone 2) were left untreated or exposed to 4 Gy dose of radiation and incubated for 2 hours, and cells were harvested and nuclear extracts were prepared and analyzed by EMSA.

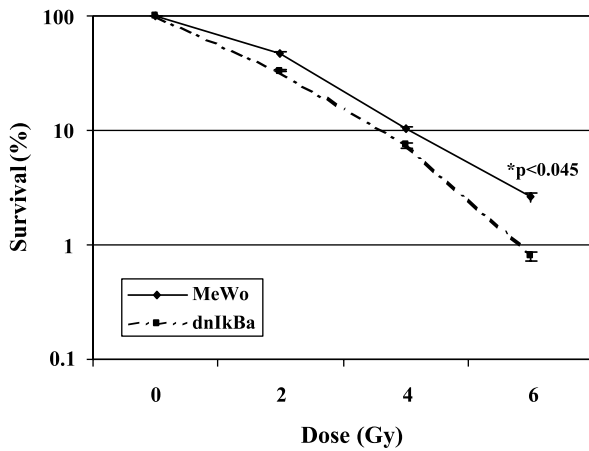


Figure 5. Expression of $I\kappa B\alpha M$ sensitizes MeWo cells to ionizing radiation. Radiosensitization by expression of $I\kappa B\alpha M$ was based on clonogenic cell survival assays. $I\kappa B\alpha M$ -expressing MeWo (clone 2) cells were exposed to various doses of radiation prior to plating for clonogenic cell survival. Points, average of four independent experiments plated in triplicate; bars, SE.

of NF- κB is a common feature in several other tumor types including glioblastomas and tumors of the pancreas, colon, and breast where it apparently influences oncogenesis in several ways including enhancement of cell survival, metastasis, and angiogenesis (24–27). Blockade of basal NF- κB activity through the use of inhibitors efficiently reduces chemoresistance of human tumor cells and offers the potential for improved therapeutic strategies. For example, it has been shown that selective inhibition of constitutive NF- κB activity with various NF- κB inhibitors (MG132, gliotoxin, sulfasalazine, and transfection with the $I\kappa B\alpha$ superrepressor) renders pancreatic cancer cell lines more sensitive to the chemotherapeutic drugs VP16 and doxorubicin (28). Therefore, we hypothesized that strategies that selectively inhibit NF- κB activity in melanomas could produce a radiosensitizing effect.

The proteasome has recently been proposed to be a molecular target for cancer therapy (29). Proteasome inhibitors have been shown to promote apoptosis (30, 31). Several proteasome inhibitors have been described such as MG132, lactacystin, and PS-341. The effects of proteasome inhibition on NF- κB activation have been well established (32). Typically, the use of proteasome inhibitors has been shown to decrease NF- κB DNA binding activity, thereby leading to induction of a more radioresponsive and chemosensitive phenotype in cancer cells. In the present study, we have shown that the proteasome inhibitor MG132 can inhibit NF- κB activity in melanoma cell lines as determined by EMSA. Suppression of NF- κB DNA binding activity by PS-341 has been shown previously to affect the radioresponse of human prostate cancer cells (9, 10). This earlier report is confirmed by our *in vitro* clonogenic assays, which revealed that melanoma cells were significantly sensitized to subsequent doses of radiation when pretreated with MG132 to reverse NF- κB activity (Fig. 3).

Activation of NF- κB requires that $I\kappa B$ is phosphorylated at two serine residues (Ser³² and Ser³⁶) by IKKs, polyubiquitinated, and subsequently degraded by the 26S proteasome. This process frees NF- κB for translocation to the nucleus and activation of its target genetic programs (33). $I\kappa B\alpha M$, which cannot be phosphorylated, has been constructed and used to alter the radiation response of tumor cells. In a previous report by Pajonk et al. (10), PC3 prostate and HD-MyZ Hodgkin's lymphoma cells were transfected with an adenovirus carrying a dominant-negative $I\kappa B\alpha$ (AdCMV- $I\kappa B\alpha$). An increase in radiation-induced apoptosis was observed, but there was no substantial effect on clonogenic survival. These authors concluded that radiosensitivity of the surviving cells was not significantly altered in PC3 cells transduced with AdCMV- $I\kappa B\alpha$, although the constitutive DNA binding activity of NF- κB was dramatically decreased and the clonogenicity was decreased to 19.6% of untreated control cells. This report is in contrast to our results (conducted on cells having 80% survival for both untreated controls), which show that inhibition of constitutive NF- κB by proteasome inhibitors or $I\kappa B\alpha M$ restores some radiosensitivity to human melanoma cells. However, due to the toxicity of their vector, Pajonk et al. (10) would have carried out the radiation survival experiments on relatively small subsets of surviving cells, whereas their biochemical assays were

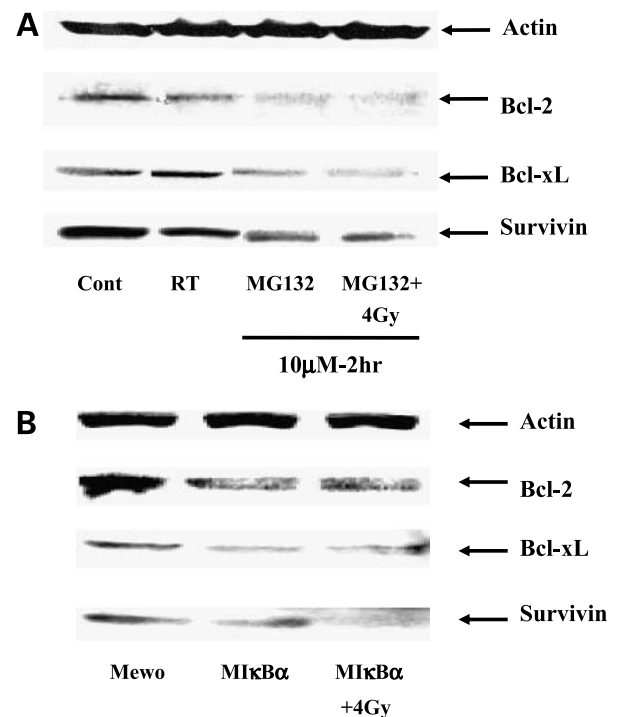


Figure 6. Decrease in levels of bcl-2, bcl-x_L, and survivin in MeWo cells following treatment with MG132 or $I\kappa B\alpha M$. Western blot analysis for survivin, bcl-2, and bcl-x_L in MeWo cells treated with 10 μ M/L MG132 for 2 hours (A) or stably transfected with $I\kappa B\alpha M$ plasmid DNA construct (B). Blots are representative of at least two independent experiments. Actin was used as a loading control.

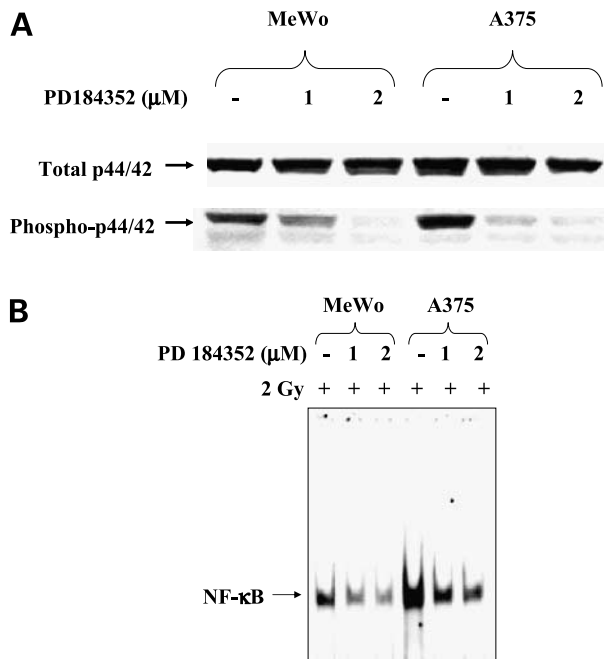


Figure 7. Treatment of melanoma cells with MEK1 inhibitor PD184352 leads to decreased phospho-p42/p44 and partially blocks activated NF- κ B. **A**, MeWo and A375 cells were incubated for 24 hours in the presence of 1 or 2 $\mu\text{mol/L}$ PD184352, and Western blot analysis for phospho-p42/p44 and total p42/p44 was done using 30 μg of protein from whole cell lysates. **B**, EMSA was done using nuclear extracts prepared from MeWo and A375 cells incubated for 24 hours in the presence of 1 or 2 $\mu\text{mol/L}$ PD184352.

done using cellular extracts prepared from the total cell population. In our study, we did EMSA on nuclear extracts rather than on total cellular extracts. Our results revealed a substantial decrease in the amount of radiation-induced nuclear NF- κ B activation by MG132 or I κ B α M compared with appropriate controls, which correlated with a restoration with radiosensitivity.

Apoptosis is regulated, in part, by members of the bcl-2 family. The relative abundance of proapoptotic and antiapoptotic members of this protein family determines whether a cell will undergo apoptosis or not. Apoptosis propensity is also regulated to a significant degree by the inhibitor of apoptosis protein family, which suppress apoptosis when expressed. The protein survivin is a member of this family. Thus, enhanced radiation sensitivity may be associated with a suppression of antiapoptotic proteins coupled with a restoration of apoptosis. Therefore, we examined the effect of inhibition of activated NF- κ B on its antiapoptotic transcriptional targets such as bcl-2, bcl-x_L, and survivin. These antiapoptotic proteins were found to be down-regulated in the I κ B α M transfectants and following treatment with MG132 (Fig. 6). However, in pilot experiments conducted as part of this study, these treatments did not seem to restore apoptosis propensity to these melanoma cells (data not shown), and this end point was not further pursued. Thus, we conclude that the inhibition of NF- κ B activation by MG132 and I κ B α M leads to a

suppressed expression of certain antiapoptotic proteins, but this is not sufficient to restore apoptosis in these cells. Moreover, restoration of radiosensitivity in this context is independent of the apoptotic pathway, suggesting that, in addition to antiapoptotic proteins, NF- κ B may target other genes that control radiation response.

Aberrant NF- κ B activation in human cancers has been attributed to activation of the signaling kinases acting upstream of IKK. Several MAPK family members have been found to act upstream of IKK. These kinases additionally activate members of the MAPK cascade, leading to the activation of Jun NH₂-terminal kinase, p38 MAPK, and/or ERK (34). In human melanoma cells, the NF- κ B activity downstream of NF- κ B-inducing kinase seems to be dependent not only on the activation of IKK but also on NF- κ B-inducing kinase-regulated activation of the MAPK/ERK cascade (4). Thus, several different MAPK proteins may be capable of activating NF- κ B independently of IKK. Apparently, this parallel pathway plays an important role for IKK-induced NF- κ B activity. Although previous studies have suggested that MEK inhibitors suppress the growth of various cancers and enhance the effectiveness of certain treatment modalities, some investigators have suggested that MEK inhibitors are not useful for sensitizing carcinoma cells to ionizing radiation (35, 36). Belka et al. (37) found that treatment with PD98059 did not alter radiation responses despite blocking MEK-1 kinase, indicating that MEK-1 and Erk1/2 are not involved in radiation resistance. However, Vrana et al. (38) used PD98059 in combination with ionizing radiation to treat HL-60 cells, which resulted in a large increase in apoptosis and a large decrease in clonogenicity when compared with either treatment alone. This study along with recent findings from Shonai et al. (39) implicate the MEK/ERK pathway in

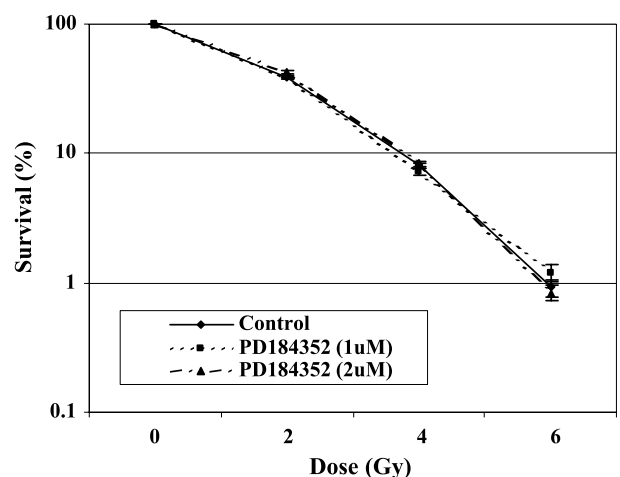


Figure 8. Treatment with the MEK inhibitor PD184352 does not radiosensitize MeWo cells. Radiosensitization by PD184352 was based on clonogenic cell survival assays. MeWo cells were exposed to 1 and 2 $\mu\text{mol/L}$ dose of PD184352 for 24 hours, and the drug was washed off and cells were irradiated at various doses and plated for clonogenic cell survival assay. Points, average of two independent experiments plated in triplicate; bars, SE.

the radioresistance of lymphocytic leukemia cells, suggesting that MEK inhibitors may be useful for radiosensitizing hematopoietic cancers. In the present study, the MEK inhibitor PD184352 was unable to sensitize melanoma cells to ionizing radiation in spite of its ability to partially block radiation-induced NF- κ B activation, suggesting that the MEK/ERK pathway may not be a key mediator of NF- κ B activation in melanoma cells and that other signaling pathways may mediate NF- κ B activation in these cells.

In summary, our results suggest that the NF- κ B pathway may play at least a partial role in mediating the radioresistance of human melanoma cells. Identification of upstream signaling pathways associated with its activity will shed additional light on its role in melanoma and could lead to novel strategies that reverse its effects, thereby enhancing response to therapeutics including radiation. Such strategies may also include small molecule proteasome inhibitors and gene therapy vectors that target NF- κ B activation.

Acknowledgments

The MEK inhibitor PD184352 was a kind gift from Dr. Eric Kaldjian (Pfizer, Inc., Ann Arbor, MI).

References

- Hall HI, Miller D, Rogers JD, Bewerse BB. Update on the incidence and mortality from melanoma in the United States. *J Am Acad Dermatol* 1999; 40:35–42.
- Yang J, Richmond A. Constitutive I κ B kinase activity correlates with nuclear factor κ B activation in human melanoma cells. *Cancer Res* 2001; 61:4901–9.
- Huang S, DeGuzman A, Bucana CD, Fidler IJ. Nuclear factor- κ B activity correlates with growth, angiogenesis and metastasis of human melanoma cells in nude mice. *Clin Cancer Res* 2000;6:2573–81.
- Dhawan P, Richmond A. A novel NF- κ B-inducing kinase-MAPK signaling pathway up-regulates NF- κ B activity in melanoma cells. *J Biol Chem* 2002;277:7920–8.
- Barkett M, Gilmore TD. Control of apoptosis by Rel/NF- κ B transcription factors. *Oncogene* 1999;18:6910–24.
- Bold RJ, Virudachalam S, McConkey DJ. Chemosensitization of pancreatic cancer by inhibition of the 26S proteasome. *J Surg Res* 2001;100: 11–7.
- Russo SM, Tepper JE, Baldwin AS, et al. Enhancement of radio-sensitivity by proteasome inhibition: implications for a role of NF- κ B. *Int J Radiat Oncol Biol Phys* 2001;50:183–93.
- Teicher BA, Ara G, Herbst R, Palombella VJ, Adams J. The proteasome inhibitor PS-341 in cancer therapy. *Clin Cancer Res* 1999;5:2638–45.
- Yang H, Hermann JL, Logothetis CJ, et al. Enhanced induction of prostate carcinoma cell death by inhibition of proteasomal activity using the novel dipeptidyl boronate PS-341 in combination with ionizing radiation; modulation by bcl-2. *Clin Cancer Res* 1999;5:3770.
- Pajonk F, Pajonk K, McBride WH. Inhibition of NF- κ B, clonogenicity, and radiosensitivity of human cancer cells. *J Natl Cancer Inst* 1999;91: 1956–60.
- Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. Identification and characterization of an I κ B kinase. *Cell* 1997;90:373–83.
- Lee FS, Hagler J, Chen ZJ, Maniatis T. Activation of the I κ B α kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* 1997;88:213–22.
- Nakano H, Shindo M, Sakon S, et al. Differential regulation of I κ B kinase α and β by two upstream kinases, NF- κ B-inducing kinase and mitogen-activated protein kinase/ERK kinase-1. *Proc Natl Acad Sci USA* 1998;95:3537–42.
- Zhao Q, Lee FS. Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate NF- κ B through IKK- α and IKK- β . *J Biol Chem* 1999;274: 8355–8.
- Lin X, Cunningham ET Jr, Mu Y, Geleziunas R, Greene WC. The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF- κ B acting through the NF- κ B-inducing kinase and I κ B kinases. *Immunity* 1999;10: 271–80.
- Hirano T, Shino Y, Saito T, et al. Dominant negative MEKK1 inhibits survival of pancreatic cancer cells. *Oncogene* 2002;21:5923–8.
- Tsou AP, Wu KM, Tsen TY, et al. Parallel hybridization analysis of multiple protein kinase genes: identification of gene expression patterns characteristic of human hepatocellular carcinoma. *Genomics* 1998;50: 331–40.
- Sourvinos G, Tsatsanis C, Spandidos DA. Overexpression of the Tpl-2/Cot oncogene in human breast cancer. *Oncogene* 1999;18:4968–73.
- Ohara R, Hirota S, Onoue H, Nomura S, Kitamura Y, Toyoshima K. Identification of the cells expressing Cot proto-oncogene mRNA. *J Cell Sci* 1995;108:97–103.
- Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF- α -induced apoptosis by NF- κ B. *Science* 1996;274:787–9.
- Dritschilo A. Radiosensitivity and transcription factor NF- κ B inhibition-progress and pitfalls. *J Natl Cancer Inst* 1999;91:1910–1.
- Hallahan DE, Clark ET. E-selectin gene induction by ionizing radiation is independent of cytokine induction. *Biochem Biophys Res Commun* 1995;217:784–95.
- Hallahan DE, Virudachalam S, Kuchibhotla J. Nuclear factor κ B dominant negative genetic constructs inhibit X-ray induction of cell adhesion molecules in the vascular endothelium. *Cancer Res* 1998;58: 5484–8.
- Liptay S, Weber CK, Ludwig L, Wagner M, Adler G, Schmid RM. Mitogenic and antiapoptotic role of constitutive NF- κ B/Rel activity in pancreatic cancer. *Int J Cancer* 2003;105:735–46.
- Lind DS, Hochwald SN, Malaty J, et al. Nuclear factor- κ B is upregulated in colorectal cancer. *Surgery* 2001;130:363–9.
- Bhat-Nakshatri P, Sweeney CJ, Nakshatri H. Identification of signal transduction pathways involved in constitutive NF- κ B activation in breast cancer cells. *Oncogene* 2002;21:2066–78.
- Schwartz SA, Hernandez A, Mark Evers B. The role of NF- κ B/I κ B proteins in cancer: implications for novel treatment strategies. *Surg Oncol* 1999;8:143–53.
- Arlt A, Vorndamm J, Breitenbroich M, et al. Inhibition of NF- κ B sensitizes human pancreatic carcinoma cells to apoptosis induced by etoposide (VP16) or doxorubicin. *Oncogene* 2001;20:859–68.
- Grimm LM, Osborne BA. Apoptosis and the proteasome. *Results Probl Cell Differ* 1999;23:209–28.
- Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective anti-tumor agents. *Cancer Res* 1999; 59:2615–22.
- You SA, Basu A, Haldar S. Potent antitumor agent proteasome inhibitors: a novel trigger for bcl-2 phosphorylation to induce apoptosis. *Int J Oncol* 1999;15:625–8.
- Sunwoo JB, Chen Z, Dong G, et al. Novel proteasome inhibitor PS-341 inhibits activation of NF- κ B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. *Clin Cancer Res* 2001;7:1419–28.
- Baeuerle PA, Baltimore D. NF- κ B: ten years after. *Cell* 1996;87:13–20.
- Hagemann C, Blank JL. The ups and downs of MEK kinase interactions. *Cell Signal* 2001;13:863–75.
- Gupta AK, Bakanauskas VJ, Cemiglia GJ, et al. The Ras radiation resistance pathway. *Cancer Res* 2001;61:4278–82.
- Grana TM, Rusyn EV, Zhou H, Sartor CI, Cox AD. Ras mediates radioresistance through both phosphatidylinositol 3-kinase-dependent and Raf-dependent but mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-independent signaling pathways. *Cancer Res* 2002;62:4142–50.
- Belka C, Knippers P, Rudner J, Faltin H, Bamberg M, Budach W. MEK1 and Erk1/2 kinases as targets for the modulation of radiation responses. *Anticancer Res* 2000;20:3243–9.
- Vrana JA, Grant S, Dent P. Inhibition of the MAPK pathway abrogates BCL2-mediated survival of leukemia cells after exposure to low-dose ionizing radiation. *Radiat Res* 1999;151:559–69.
- Shonai T, Adachi M, Sakata K, et al. MEK/ERK pathway protects ionizing radiation-induced loss of mitochondrial membrane potential and cell death in lymphocytic leukemia cells. *Cell Death Differ* 2002;9:963–71.