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Curcumin Modulates the Radiosensitivity of Colorectal Cancer Cells by Suppressing Constitutive and Inducible NF-κB Activity

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Abstract

Purpose—Radiation therapy is an integral part of the preoperative treatment of rectal cancers. However, only a minority of patients achieve a complete pathological response to therapy due to resistance of these tumors to radiation therapy. This resistance may be mediated by constitutively active pro-survival signaling pathways or by inducible/acquired mechanisms in response to radiation therapy. Simultaneous inhibition of these pathways can sensitize these tumors to radiation therapy.

Methods and Materials—Human colorectal cancer cells were exposed to clinically relevant doses of gamma rays and the mechanism of their radioresistance was investigated. We characterize the transcription factor nuclear factor-κB (NF-κB) activation as a mechanism of inducible radioresistance in colorectal cancer and use curcumin, the active ingredient in the yellow spice turmeric to overcome this resistance.

Results—Curcumin inhibited the proliferation and the post-irradiation clonogenic survival of multiple colorectal cancer cell lines. Radiation stimulated NF-κB activity in a dose- and time-dependent manner while curcumin suppressed this radiation-induced NF-κB activation via inhibition of radiation-induced phosphorylation and degradation of IκBα, inhibition of IKK activity, and inhibition of Akt phosphorylation. Curcumin also suppressed NF-κB regulated gene products (Bcl-2, Bcl-x_L, inhibitor of apoptosis protein-2, cyclooxygenase-2, and cyclin D1).

Conclusions—Our results suggest that transient inducible NF-κB activation provides a pro-survival response to radiation that may account for development of radioresistance. Curcumin blocks this signaling pathway and potentiates the anti-tumor effects of radiation therapy.

Keywords

Curcumin; Radiation; NF-κB; Radiosensitization; Colorectal Cancer

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Introduction

Rectal cancer frequently presents at a locally advanced stage where pre-operative chemoradiation therapy (CRT) is used to downstage the tumor, make it more resectable, and less likely to recur after surgery(1). It is known that response to CRT confers improved disease-free and overall survival rates, reduces the likelihood of local recurrence, and improves the chances of anal sphincter preservation at the time of surgery(2-5). Furthermore, complete pathological response to CRT confers the best clinical outcomes and permits selected patients to undergo less extensive resections, further minimizing toxicity of treatment(2, 6). Unfortunately, only about 20% of patients achieve complete pathological responses to pre-operative CRT(7). Efforts to improve this rate have focused on overcoming the resistance of colorectal cancers to radiation therapy by intensifying the radiation dose or by using radiosensitizing cytotoxic chemotherapy(7-10), neither of which has significantly improved outcomes to date and both of which are associated with increased toxicity.

The transcription factor nuclear factor kappaB (NF-κB) regulates several genes that induce cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis and treatment resistance in a wide range of tumors(11). NF-κB plays a significant role in conferring a survival advantage to colorectal cancers and resistance to radiotherapy. Constitutive activation of NF-κB has been observed in colorectal cancer cells(12) but not in normal colorectal ductal epithelial cells(13, 14). Progressive increases in NF-κB levels correlate with transition of normal colonic epithelial cells to adenomas, dysplasia, and finally, invasive adenocarcinomas(14, 15). NF-κB promotes tumor growth via inhibition of apoptosis and induces proteins, such as cyclin D1 and cycloxygenase-2 (COX-2) that are implicated in colorectal carcinogenesis(16-18). Also, NF-κB plays a pivotal role in promoting radioresistance in colorectal cancer(12, 19-21). Taken together, these evidences suggest that NF-κB serves as a mediator of a pro-survival phenotype in colorectal cancer and its suppression could potentiate the radioresponse in colorectal cancer.

Curcumin (diferuloylmethane), a polyphenol from the rhizomes of *Curcuma longa*, is the major constituent of the yellow spice turmeric, a commonly used flavoring agent in Asian cooking. In a variety of tumor cells, curcumin has been reported to inhibit proliferation and angiogenesis, to induce apoptosis or cell cycle arrest, and to cause regression of tumors in preclinical models(22-29). In colorectal cancer specifically, curcumin has demonstrated potent anti-tumor activity and chemopreventive potential *in vitro* and *in vivo*(30, 31), however, its exact molecular mechanism of action remains elusive.

In the present study, we have investigated the role of curcumin in modulating the radioresponse of colorectal cancer cells *in vitro*. Curcumin enhanced the anti-tumor effects of radiation in a clonogenic assay. While dissecting the mechanism underlying this radiosensitization, we observed that sub-lethal doses of radiation induced NF-κB transiently in a dose-dependent manner in colorectal cancer cells and curcumin inhibited this response. This was mediated by inhibition of radiation-induced Akt phosphorylation, inhibitor of κB kinase (IKK) activation, inhibitor of κB alpha (IκBα) phosphorylation, and IκBα degradation. Curcumin also down-regulated NF-κB–regulated anti-apoptotic and colorectal carcinogenic gene products. Our studies help to elucidate a pro-survival, anti-apoptotic signaling response of surviving colorectal cancer cells to irradiation that is mediated by NF-κB activation and modulation of radioresponse by curcumin via inhibition of this inducible radioresistance pathway.

Methods and Materials

Cells and Reagents

Colorectal cancer cell lines HCT116, HT29, and SW620 were kindly provided by Dr. Ray Meyn (MD Anderson Cancer Center). HCT116 cells were cultured in DMEM:F12 (50:50). HT29 cells were cultured in DMEM and SW620 cells were cultured in L-15. All media were supplemented with 10% FBS and 1% Penicillin-streptomycin (Invitrogen, Grand Island, NY). Curcumin was procured from LKT laboratories (St. Paul, MN). Antibodies to IκBα, inhibitor of apoptosis protein (IAP)-2, cyclin D1, and Bcl-X_L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to COX-2 was from BD Biosciences (San Diego, CA). Anti phospho-IκBα$^{Ser32/36}$ and anti phospho-Akt^{Ser473} were from Cell Signaling (Beverly, MA). Anti-Bcl-2 antibody was from DakoCytomation (Carpinteria, CA) and anti-β-actin was from Sigma (St. Louis, MO). Millimolar (mM) stock solutions of curcumin in dimethyl sulfoxide (DMSO) were stored at -20°C. For every experiment, the appropriate curcumin stock was diluted 1:1000 in culture medium immediately prior to use to obtain the respective micromolar (μM) concentrations. The control groups received DMSO diluted accordingly (final DMSO concentration was 0.1% in all groups).

In vitro cytotoxicity

The effect of curcumin on tumor cells was assessed using the XTT cell proliferation kit (Roche Applied Science, Indianapolis, IN). Briefly, cells were seeded in 96-well plates (3,000 cells per well) and grown overnight. Next day, the medium was aspirated and cells were exposed to different concentrations of curcumin. Following 4 h, curcumin was aspirated; the wells were rinsed and replenished with fresh medium. After further 48 h of culture, cell viability was determined by XTT assay. Results are expressed as percent cell viability for each concentration of curcumin with respect to DMSO controls.

Clonogenic survival

Cells were treated with vehicle control (DMSO) or 25 μM curcumin for 4 h and then irradiated with a ^{137}Cs unit (cylindrical source 4.5 cm diameter × 5.3 cm length) at room temperature (dose rate 3.1 Gy/min). Following 3 h, curcumin was washed, cells were trypsinized and specific cell densities were re-plated in 60-mm petridishes and were incubated for colony formation for 10-14 days. Colonies were counted after staining with 0.5% alcoholic crystal violet. The fraction surviving a given treatment was calculated with respect to the survival of unirradiated controls (cells treated with DMSO or curcumin alone).

Electrophoretic mobility shift assays (EMSA)

NF-κB activation was assessed by EMSA as described previously(32). Briefly, nuclear extracts of treated cells were incubated with ^{32}P-end-labeled double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAGGCGTGG-3′ (boldface indicates NF-κB binding sites) for 30 min at 37°C, and the DNA-protein complex was resolved on 6.6% native PAGE. The radioactive bands on dried gels were quantified by a PhosphorImager using ImageQuant software (v 5.1; Molecular Dynamics, Sunnyvale, CA).

Immunobloting

Whole cell lysates were fractionated by SDS-PAGE, the proteins were electrotransferred to nitrocellulose membranes and probed with appropriate antibody. The blots were next probed with appropriate horseradish peroxidase conjugated secondary antibodies (Santa Cruz, CA)

and developed by enhanced chemiluminescence using ECL™ (GE healthcare, Piscataway, NJ).

Kinase Assay

The effect of curcumin on radiation-induced IKK activation was assessed by an immunocomplex kinase assay(33). Briefly, the IKK complex from whole-cell extracts was precipitated with IKK-α antibody followed by treatment with protein A/G-Sepharose beads (Pierce Chemical, Rockford, IL) for 2 h. The beads were then washed with lysis buffer and assayed in a kinase assay mixture (50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 mCi [γ-^{32}P]ATP, 10 mM unlabeled ATP) with the substrate glutathione *S*-transferase-IκBα (GST-IκBα ; amino acids 1-54) at 37°C. The reaction was terminated after 30 min by boiling the assay mixture with SDS sample buffer. The protein was resolved on 10% SDS-PAGE, the radioactive bands were visualized on dried gels with a PhosphorImager.

Statistics

All the experiments were done at least in triplicates unless otherwise mentioned. Results are expressed as mean ± standard error (SE). Comparisons between groups were performed by two-tailed Student's t tests. Representative data of one of the three independent experiments is shown.

Results

Curcumin inhibited proliferation of colorectal cancer cells

The cytotoxic effects of curcumin alone on colon cancer cell lines were determined using XTT assay. Curcumin inhibited the proliferation of all the cell lines in a dose-dependent manner (Fig. 1a). HCT116 and SW620 cells were equally sensitive to curcumin (IC_{50} 27.7 ± 0.6 μM and 23.4 ± 1.3 μM, respectively; $p = 0.144$) whereas HT29 was least sensitive (IC_{50} 37.4 ± 1.06 μM). p value

Curcumin sensitized colon cancer cells to radiation

Treatment with curcumin (25 μM) significantly enhanced the intrinsic tumor radiosensitivity of both HCT116 and HT29 as assessed by clonogenic survival assays (Fig. 1b). The dose enhancement ratio (DER) calculated at 10% surviving fraction was 1.28 (HCT116) and 1.18 (HT29) respectively. This dose (25 μM) was chosen for further experiments as higher doses were independently toxic to cells and lower doses had less appreciable radiosensitization effects (data not shown).

Radiation induced dose- and time-dependent NF-κB activation

Tumor cells were treated with graded doses of radiation (0-10 Gy) and incubated for 3 h before harvesting and preparation of nuclear extracts for assessing NF-κB activation. Radiation activated NF-κB in a dose-dependent manner in all three cell lines (Fig. 2a, 2c and 2e). Radiation dose inducing maximum NF-κB activation (10 Gy), was chosen for further studies. To discern the time course of NF-κB induction post-irradiation, cells were exposed to 10 Gy and harvested after different time intervals post irradiation. Marginal induction of NF-κB was seen in cells harvested immediately after irradiation (Fig. 2b) which increased significantly within 1 h. These NF-κB levels peaked at 3 h and started declining gradually after 6 h (Fig. 2b, 2d, and 2f).

Curcumin suppressed radiation-induced NF-κB activation

Cells treated with DMSO (control) or curcumin (25 μM) for 4 h were exposed to single dose of radiation (10 Gy) and harvested at different time points post-radiation for assessing NF-κB activation. Radiation induced NF-κB activation within 30 min, and the NF-κB levels increased with increasing incubation time, peaking at 3 h post-irradiation (Fig. 3a). Curcumin effectively inhibited NF-κB at all these time points (Fig. 3a). Based on this time course study, we examined effect of curcumin on NF-κB activation in these cell lines. Cells treated with DMSO or curcumin (10 and 25 μM) for 4 h were exposed to single dose of radiation (10 Gy) and harvested 3 h post-radiation. In all these cells, radiation induced significant levels of NF-κB in the untreated cells (Fig. 3 b-d). Curcumin treatment significantly inhibited this NF-κB activation in all these cells at both 10 and 25 μM.

Curcumin inhibited radiation-induced phosphorylation and degradation of IκBα, activation of IKK, and activation of Akt

To investigate the mechanism of radiation-induced NF-κB activation and curcumin's action along this pathway, we interrogated the canonical pathway leading up to degradation of IκBα and release of cytoplasmic NF-κB for nuclear translocation and DNA-binding. HCT116 was chosen for further studies as the degree of induction of NF-κB following radiation (6-fold change) was neither as low as HT-29 (3.5-fold) nor as high as SW-620 (10.5-fold), curcumin was moderately effective as an inhibitor of NF-κB and it is a commonly used colorectal cell line with intact wild-type p53 that senses and regulates downstream effects of radiation-induced DNA damage. Cells were exposed to 6 and 10 Gy of radiation and harvested at different times post-irradiation. Radiation induced IκBα degradation in a dose dependent manner (extent of degradation being higher in 10 Gy treated cells than in 6 Gy) (Fig. 4a). In cells pre-treated with curcumin (25 μM), this radiation induced IκBα degradation was significantly inhibited (Fig. 4b).

IκBα is phosphorylated at Ser 32/36 or Tyr 42 residues before degradation by polyubiquitination. To determine whether radiation signals phosphorylation of IκBα, cells were incubated with a proteasome inhibitor (ALLN) for 30 min before exposure to 10 Gy radiation. Phosphorylation of IκBα was monitored at different time intervals post irradiation. Corresponding to the previously noted temporal pattern of IκBα degradation, increasing levels of phosphorylated IκBα were noted at a similar time frame (Fig. 4c) confirming that radiation phosphorylates Ser 32/36 residues of IκBα.

Since the most common effector molecule for phosphorylation and subsequent degradation of IκBα is IKK(34), we investigated the role of radiation and curcumin on IKK activation. As seen in Fig. 4d, IKK activation occurred within 2 h following radiation and this activation was almost completely suppressed by curcumin.

One of the known activators of IKK is Akt/PKB, which also mediates tumor cell resistance via induction of pro-survival signaling. Hence, we investigated the effects of curcumin on Akt activation. Radiation induced phosphorylation of Akt^{Ser473} in HCT116 cells and curcumin significantly inhibited this phosphorylation (Fig. 4e). Taken together, these results suggest that curcumin blocks proliferative signals by targeting both IKK activation and AKT phosphorylation.

Curcumin suppressed NF-κB regulated gene expression

Because NF-κB regulates the expression of the anti-apoptotic and proliferative proteins such as IAPs(35, 36), Bcl-x_L(37), Bcl-2(38), cyclin D1(39) and COX-2(40), we examined whether curcumin can modulate the expression of these anti-apoptotic gene products in HCT116 cells. Immunoblot analysis showed that radiation induced these anti-apoptotic

proteins and that curcumin suppressed them (Fig. 5). It is of particular note here that although radiation did not induce COX-2 levels in these cells, the constitutive levels of this protein were completely suppressed by curcumin.

Discussion

The primary objective of this study was to determine whether curcumin can sensitize colorectal cancer cells to radiation and to investigate the mechanism of action of this radiosensitization. We used colorectal cancer cells HCT116 (wild type p53, mutant K-ras), HT29 cells (mutant p53, wild type K-ras), and SW620 (mutant p53 and K-ras). Curcumin inhibited proliferation and the post-irradiation clonogenic survival of these cell lines. Transient dose-dependent radiation-induced NF-κB was suppressed by curcumin via inhibition of radiation-induced phosphorylation and degradation of IκBα, inhibition of IKK activity, and inhibition of Akt phosphorylation. Curcumin also suppressed NF-κB regulated gene products (Bcl-2, Bcl-x_L, IAP-2, COX-2, cyclin D1 and VEGF). Our results suggest that transient radiation-inducible NF-κB activation provides a pro-survival response to radiation that may account for development of radioresistance. Curcumin blocks this signaling pathway and potentiates the anti-tumor effects of radiotherapy.

The identification of inducible radioresistance in colorectal cancer mediated via the NF-κB pathway is of considerable interest, because this pathway could then serve as a target for developing molecular-based radiosensitization protocols for radioresistant tumors. Activation of NF-κB in cancer cells has been shown to attenuate apoptosis induced by radiation(21). Inhibition of NF-κB, therefore, has the potential to improve radiotherapeutic efficacy by enhancing radiation-induced cell kill. NF-κB can be inhibited by multiple strategies including the use of a modified form of IκBα(19, 20, 41, 42), decoy of NF-κB(43), or proteosome inhibitors(20), all of which have shown promising activity in preclinical studies. One concern with highly specific NF-κB targeting strategies is the likelihood that this could lead to immunosuppression and greater susceptibility to infections and/or lymphoproliferative disorders as has been documented with the use of tumor necrosis factor α (TNF-α) antagonists in autoimmune diseases (44). In contrast, curcumin has a broader spectrum of activity, that possibly cause radiosensitization via multiple mechanisms including inhibition of TNF-α-mediated NF-κB activation(45) (Supplementary Fig. 1) and Akt-mediated inhibition of the MDM2 oncogene(46). Furthermore, the more potent inhibition of inducible NF-κB than constitutive NF-κB and the lack of any toxicity in clinical studies so far suggest that curcumin may have a wide therapeutic window.

The identification of curcumin as a radiosensitizer in colorectal cancer is also of considerable interest, because of its affordability, ease of oral administration, and lack of toxicity in clinical use. In human trials of its safety, doses as large as 12 gm/day have been tolerated with minimal clinical toxicity(47). It has shown clinical activity in a small colorectal polyp prevention study in patients with familial adenomatous polyposis syndrome who had not undergone a prophylactic colectomy and had failed other chemopreventive treatments(48). Lastly, an oral dose of 3.6 gm of curcumin achieves pharmacologically efficacious levels of curcumin in colorectal cancers with negligible distribution of the parent drug outside the gut(49).

An improved understanding of the complex multi-step colorectal carcinogenesis process at the molecular level and the aberrant homeostatic pathways defining resistance to therapies has led to the increasing use of novel targeted agents inhibiting pathways mediating tumor proliferation/ survival and/or tumor angiogenesis. While some of these agents have shown promising activities in the treatment of metastatic colorectal cancer, similar improvements in outcomes following their integration into preoperative CRT have not been observed so far.

In general, these approaches have addressed pre-existing or *de novo* chemo- and radio-resistance pathways using highly targeted agents that are prone to being circumvented by downstream signaling via redundant pathways. In contrast to these constitutively active pathways, the relative contribution of acquired and/or inducible resistance pathways to the overall problem of chemo- or radio-resistance has not been actively investigated. In this study, we report a novel strategy for improving rectal cancer therapy, based on combining radiation therapy with curcumin, which inhibits radiation-induced NF-κB in cancer cells. Our studies help to elucidate a pro-survival, anti-apoptotic signaling response of colorectal cancer cells to irradiation mediated by signaling via the NF-κB pathway and one of the mechanisms by which curcumin modulates radioresponse by targeting this inducible radioresistance pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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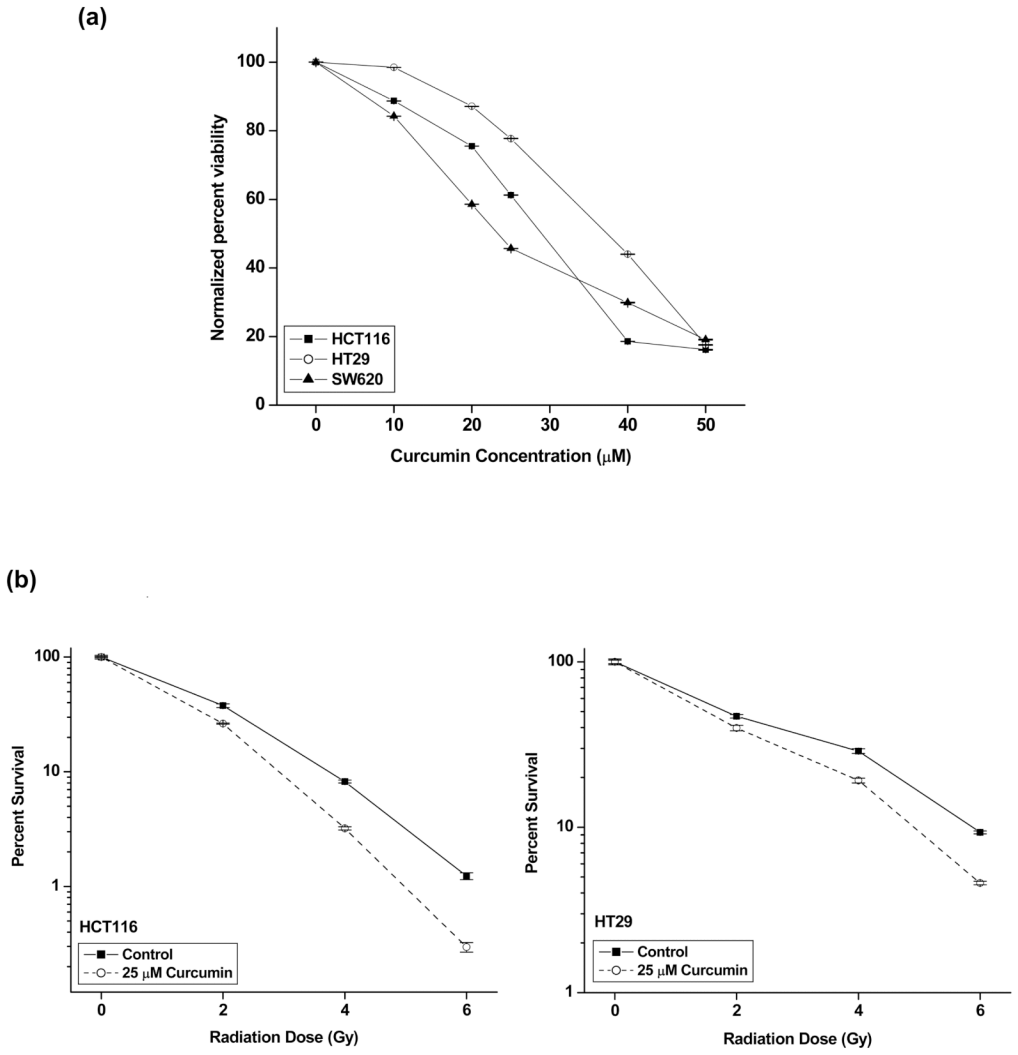

Figure 1. Curcumin augments radiation-induced cytotoxicity in colon cancer cells

(a) Cells (3×10^4/mL) were exposed to different doses of curcumin in 96-well plate for 4 h after which the curcumin was washed and fresh medium was added to cells. Viability was assessed by XTT assay (Roche) after 48 h. The percent viability was calculated with respect to DMSO-treated controls. *Points*, mean of quadruplicates for each concentration; *bars*, SE. **(b)** HCT116 and HT29 cells were exposed to 25 μM curcumin for 4 h, and irradiated at indicated doses. Cells were re-plated for clonogenic assay 3 h post-radiation. The enhancement in radiosensitivity by curcumin was assessed on the basis of percent cell survival in comparison with the controls (cells irradiated with DMSO). *Points*, mean of the sextuplicates, *bars*, SE.

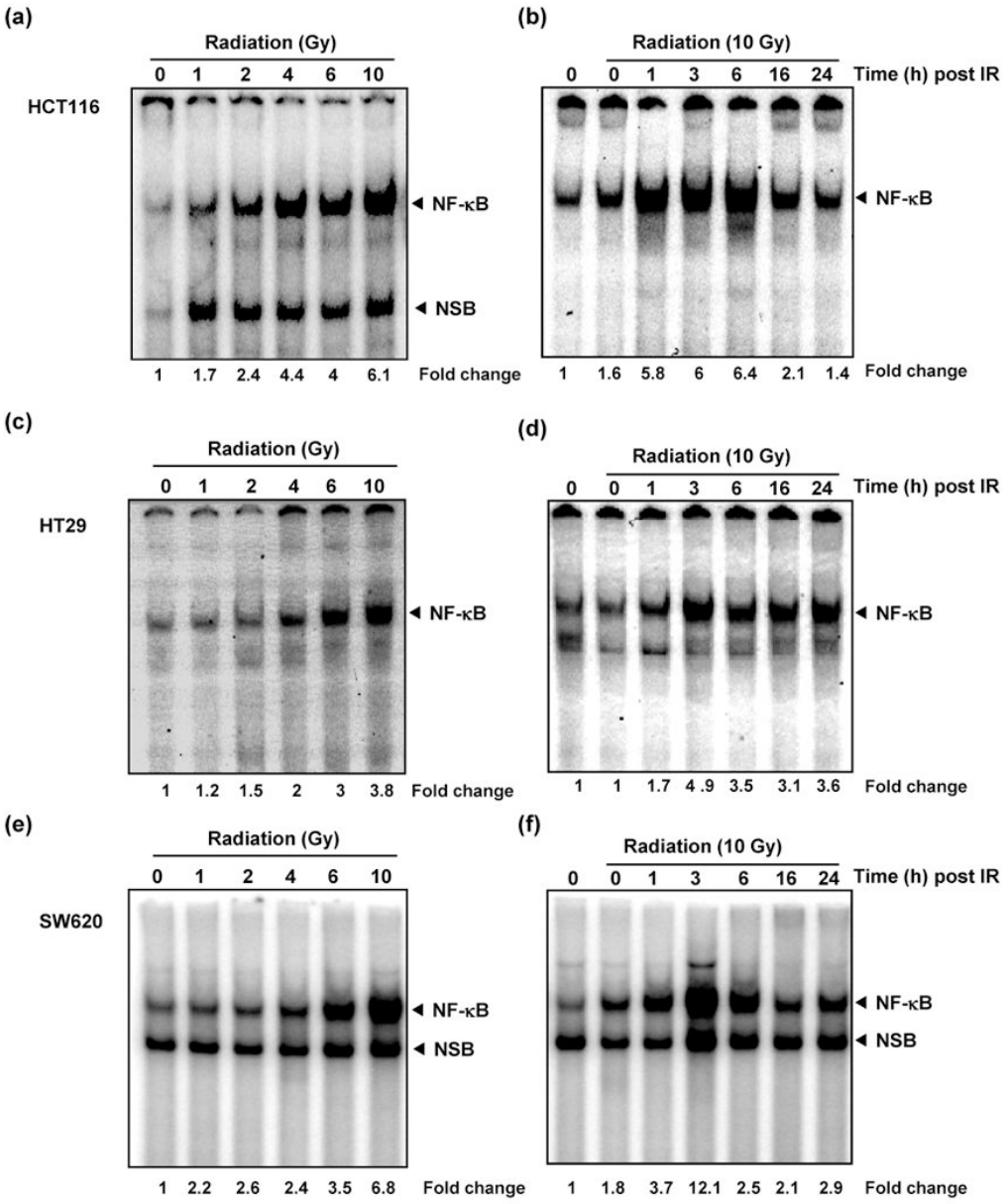

Figure 2. Radiation induced NF-κB in colorectal cancer cells

Dose dependent increase in NF-κB activity was observed in HCT116 **(a)**, HT29 **(c)**, and SW620 **(e)** cells. Colon cancer cells (2×10^5/mL) were exposed to graded doses of radiation and harvested 3 h after irradiation. Nuclear extracts were probed for NF-κB activation by EMSA. The time course of radiation-induced NF-κB activation after 10 Gy irradiation in HCT116 **(b)**, HT29 **(d)**, and SW620 **(f)**. Cells (2×10^5 /mL) were exposed to radiation, harvested post-radiation at indicated time points, and probed for NF-κB. Activation of NF-κB was expressed as fold increase of DNA-bound protein over untreated controls (basal levels). The band labeled NF-κB represents the p65:p50 heterodimer, the predominant functionally active NF-κB dimer whereas the lower band is a non-specific band formed by the inactive NF-κB homodimer (p50:p50)

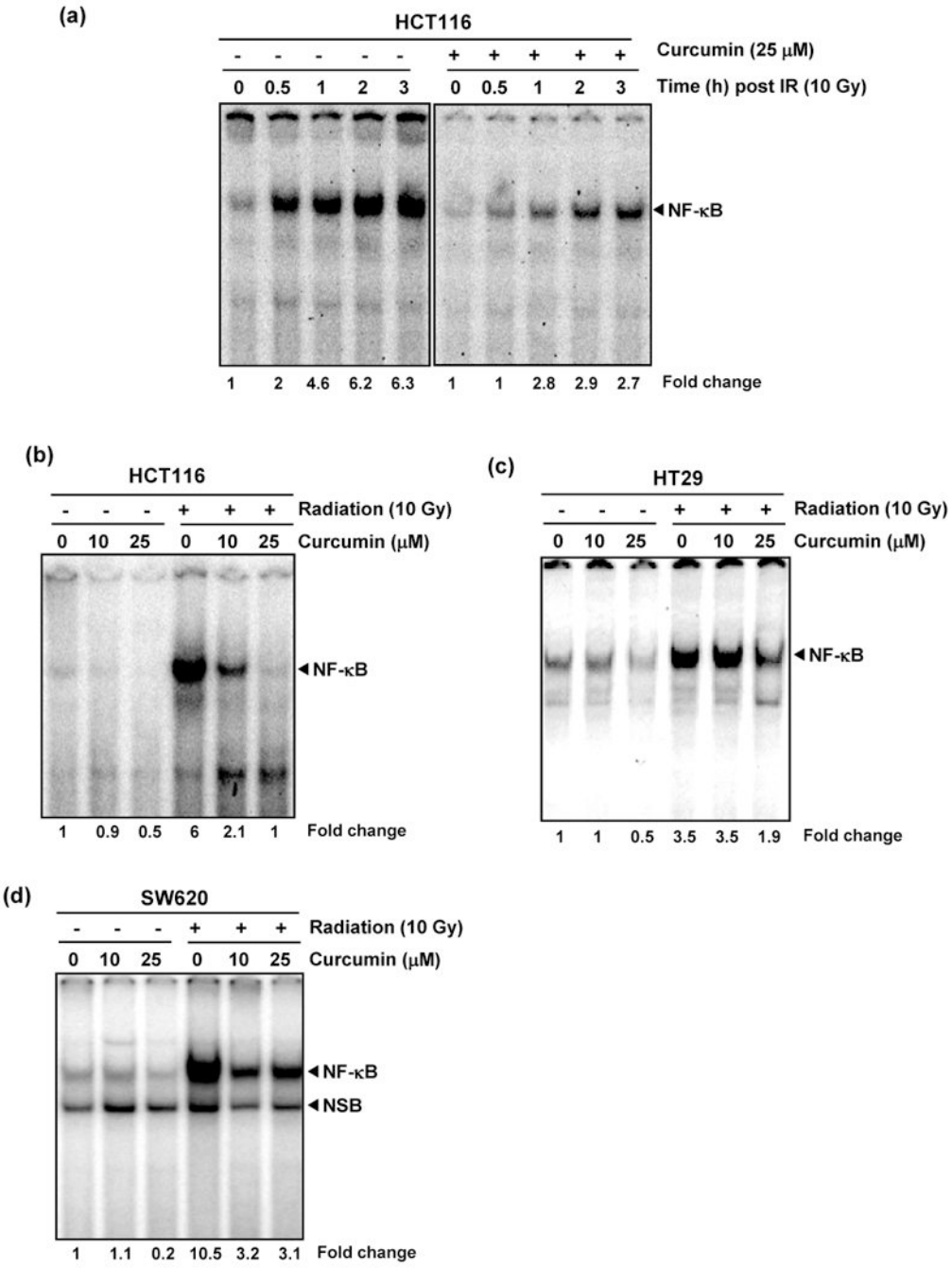

Figure 3. Inhibition of radiation-induced NF-κB activation by curcumin

Curcumin inhibited radiation-induced activation of NF-κB. **(a)** HCT116 cells were incubated with curcumin (25 μM) for 4 h, exposed to radiation (10 Gy) and nuclear extracts prepared at indicated time points post IR were probed for NF-κB. Consistent suppression of radiation-induced NF-κB activity was noted at all time points **(b-d)**. Suppression of radiation-induced NF-κB was also observed in HCT116 **(b)**, HT29 **(c)** and SW620 **(d)** cells at both 10 and 25 μM concentrations, with a greater response observed for higher doses curcumin.

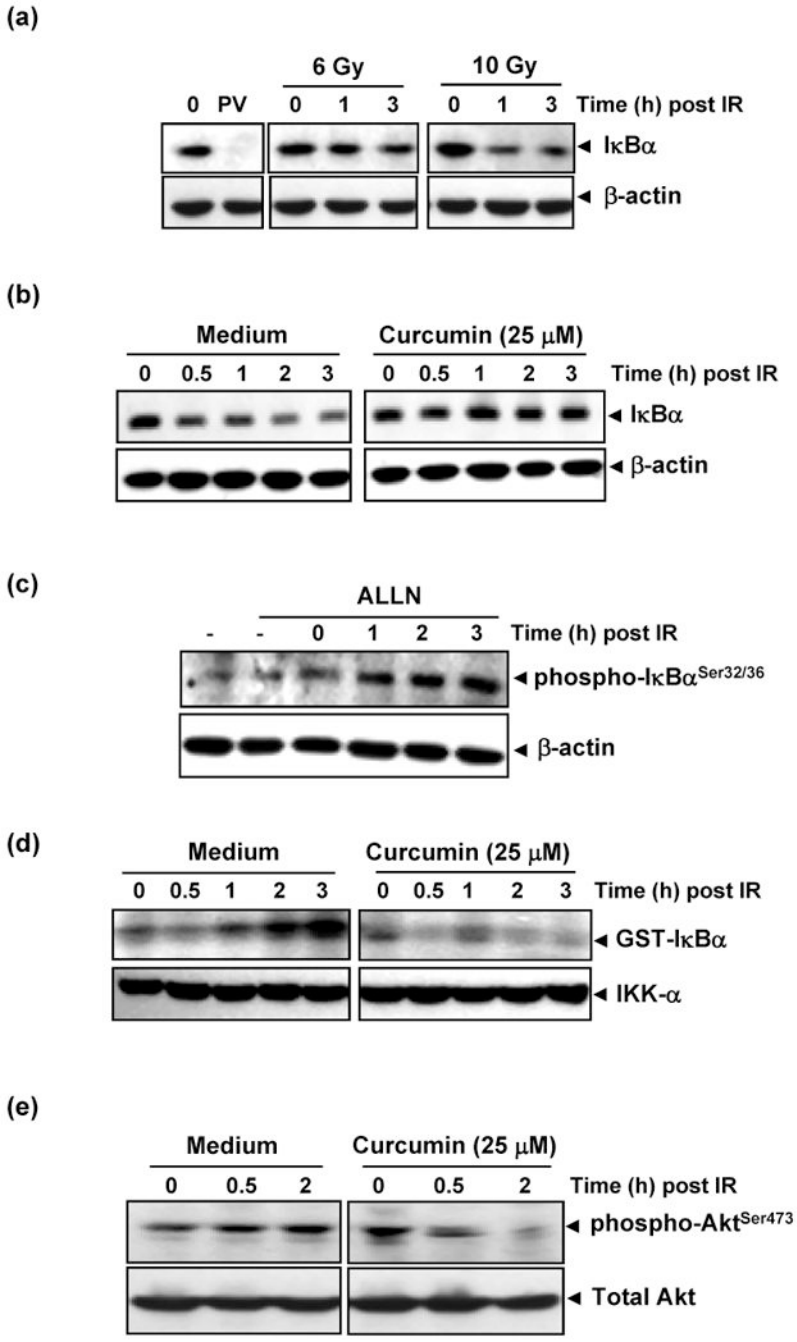

Figure 4. Curcumin inhibits radiation-induced activation of proteins upstream of NF-κB

(a) Radiation induced IκBα degradation. HCT116 cells were exposed to 6 or 10 Gy of radiation and incubated for the indicated times. Pervanadate (PV; 50μM), a protein tyrosine phosphatase inhibitor, which induces IκBα degradation via tyrosine phosphorylation was positive control. Cytoplasmic extracts were prepared and subjected to immunoblotting using anti-IκBα antibody. **(b)** Pre-treatment with curcumin abrogated radiation-induced degradation of IκBα. HCT116 cells were incubated with 25 μM curcumin for 4 h prior to radiation (10 Gy) and cytoplasmic extracts were immunoblotted as described above. **(c)** Radiation induced phosphorylation of IκBα. HCT116 cells were incubated with 50 μg/ml ALLN for 30 min, exposed to radiation (10 Gy), and cytoplasmic extracts obtained at

different time points were subjected to immunoblot analysis using phospho-specific anti-IκBα antibody (Ser32/36) **(d)** Curcumin inhibited radiation-induced IKK activation. HCT116 cells were pre-treated with or without 25 μM curcumin for 4 h and the IKK complex from whole-cell extracts was precipitated with antibody against IKK-α. IKK activity was analyzed by an immune complex kinase assay. The fusion protein GST-IκBα (glutathione *S*-transferase-IκBα) that was subsequently immunoblotted serves as a substrate for IKK. **(e)** Pre-treatment with curcumin inhibited radiation-induced activation of Akt. In experiments similar to those above, cytoplasmic extracts of HCT116 cells treated with and without curcumin prior to radiation were immunoblotted with anti phospho-Akt ^{Ser473} antibody.

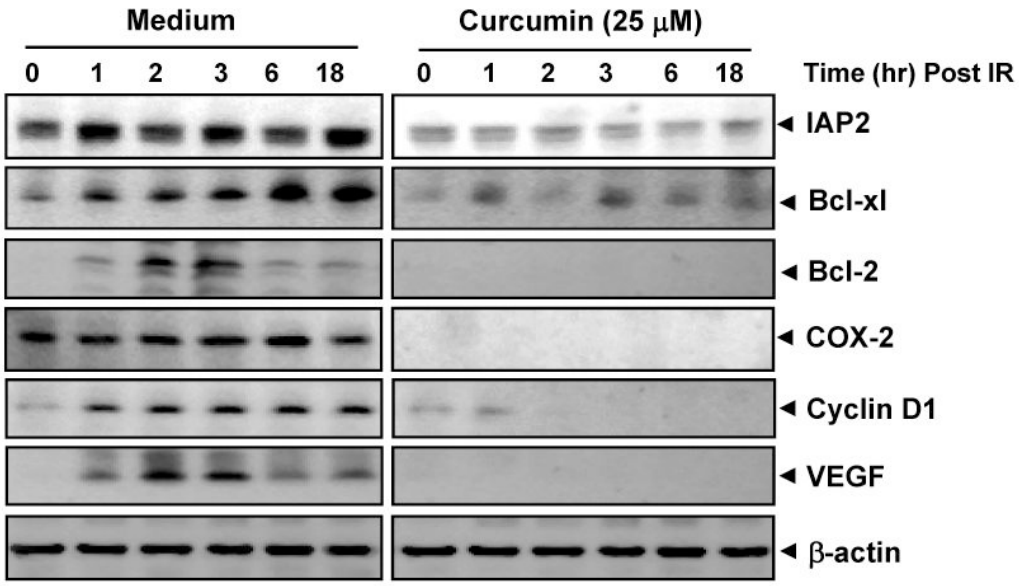

Figure 5. Curcumin inhibits NF-κB regulated gene products

Curcumin suppresses radiation-induced expression of NF-κB-dependent anti-apoptotic (IAP2, Bcl-2, Bcl-$X_{L,}$), inflammatory (COX-2), proliferative (cyclin D1) and angiogenic (VEGF) gene products. Cells were pre-incubated with 25 μM curcumin (4 h), exposed to radiation (10 Gy), and harvested post-radiation at indicated time points for immunoblot analysis.