

## Curcumin Sensitizes Human Colorectal Cancer Xenografts in Nude Mice to $\gamma$ -Radiation by Targeting Nuclear Factor- $\kappa$ B – Regulated Gene Products

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**Abstract Purpose:** How colorectal cancer develops resistance to  $\gamma$ -radiation is not fully understood, but the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) and NF- $\kappa$ B – regulated gene products have been proposed as mediators. Because curcumin, a component of turmeric (*Curcuma longa*), has been shown to suppress NF- $\kappa$ B activation, whether it can sensitize the colorectal cancer to  $\gamma$ -radiation was investigated in colorectal cancer xenografts in nude mice.

**Experimental Design:** We established HCT 116 xenograft in nude mice, randomized into four groups, and treated with vehicle (corn oil), curcumin,  $\gamma$ -radiation, and curcumin in combination with  $\gamma$ -radiation. NF- $\kappa$ B modulation was ascertained using electrophoretic mobility shift assay and immunohistochemistry. Markers of proliferation, angiogenesis, and invasion were monitored by immunohistochemistry and Western blot analysis.

**Results:** Curcumin significantly enhanced the efficacy of fractionated radiation therapy by prolonging the time to tumor regrowth ( $P = 0.02$ ) and by reducing the Ki-67 proliferation index ( $P < 0.001$ ). Moreover, curcumin suppressed NF- $\kappa$ B activity and the expression of NF- $\kappa$ B – regulated gene products (cyclin D1, c-myc, Bcl-2, Bcl-xL, cellular inhibitor of apoptosis protein-1, cyclooxygenase-2, matrix metalloproteinase-9, and vascular endothelial growth factor), many of which were induced by radiation therapy and mediate radioresistance. The combination of curcumin and radiation therapy also suppressed angiogenesis, as indicated by a decrease in vascular endothelial growth factor and microvessel density ( $P = 0.002$  versus radiation alone).

**Conclusion:** Collectively, our results suggest that curcumin potentiates the antitumor effects of radiation therapy in colorectal cancer by suppressing NF- $\kappa$ B and NF- $\kappa$ B – regulated gene products, leading to inhibition of proliferation and angiogenesis.

It has been estimated that 41,420 patients will be diagnosed with rectal cancer in 2007 in the United States (1). Most patients with rectal cancer present with locally advanced disease, where preoperative chemoradiation therapy is an integral component of treatment because it reduces the risk of local recurrence and increases the probability of sphincter-preserving surgery (2). Unfortunately, only ~20% of patients achieve complete pathologic responses to preoperative chemoradiation therapy

mainly because of their resistance to radiation therapy (3). Increasing this response rate with novel radiosensitization strategies may permit selective avoidance of radical surgical resections for a subset of patients (4). Why the response to radiation is so limited, is not understood. The role of numerous signaling pathways, including reactive oxygen species, cyclooxygenase 2 (COX-2), phosphoinositide 3-kinase, multidrug resistance proteins, Bcl-2, survivin, growth factors, and transcription factors, such as signal transducers and activators of transcription 3 (STAT3) and nuclear factor- $\kappa$ B (NF- $\kappa$ B), have been implicated in radioresistance (5). Most of these pathways have been shown to be linked with the NF- $\kappa$ B pathway.

Although associated with cell proliferation, invasion, angiogenesis, and metastasis, NF- $\kappa$ B has been closely linked with radioresistance in multiple tumors (6). Numerous studies suggest that the prosurvival signaling mediated by NF- $\kappa$ B is linked to radiation resistance and poorer clinical outcomes with colorectal cancer. First, radiation therapy is known to activate NF- $\kappa$ B (7–9). Second, NF- $\kappa$ B and NF- $\kappa$ B – regulated gene products, including Bcl-xL, cyclin D1, matrix metalloproteinase 9 (MMP-9), vascular endothelial growth factor (VEGF), and COX-2, contribute to the development of radiation resistance within tumor cells (10–16). Third, constitutive activation of NF- $\kappa$ B has been observed in colorectal cancer cells (7) but not in normal colorectal ductal epithelial cells (17, 18).

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Fourth, progressive increases in NF- $\kappa$ B levels correlate with transition of normal colonic epithelial cells to adenomas, dysplasia, and, finally, invasive adenocarcinomas (17, 18). Therefore, inhibition of the NF- $\kappa$ B pathway may inhibit the growth of colorectal tumors and synergize with radiation therapy.

Curcumin (diferuloylmethane), a derivative of the spice turmeric (*Curcuma longa*; Fig. 1A), is known to suppress NF- $\kappa$ B activation (19) and down-regulate the expression of NF- $\kappa$ B-regulated gene products involved in survival (Bcl-2, Bcl-xL, XIAP, and cIAP-1), proliferation (COX-2, cyclin D1, and c-myc), angiogenesis (VEGF and IL-8), invasion (MMP-9), and metastasis (ICAM-1, VCAM-1, and ELAM-1; refs. 20–22) of the tumor. Plummer et al. showed that curcumin inhibits COX-2 expression in colon cells by inhibition of NF- $\kappa$ B activation via the NIK/IKK signaling complex (23). This phytochemical has been shown to modulate various mechanisms linked with radioresistance, such as quenching reactive oxygen species (24), down-regulating COX-2, multidrug resistance protein, Bcl-2, and survivin expression (22, 25, 26), inhibiting phosphoinositide 3-kinase/AKT activation (27), suppressing growth factor signaling pathways (28), and inhibiting signal transducers and activators of transcription 3 activation (29). Moreover, in current clinical trials, curcumin has been found to be pharmacologically quite safe.

Whether curcumin can sensitize colorectal tumors to radiation *in vivo* is not understood. In this study, we tested this hypothesis by measuring the effect of curcumin on the growth of colorectal cancer xenografts in nude mice exposed to radiation. We found that curcumin sensitized colorectal cancers to radiation by down-regulating NF- $\kappa$ B-regulated gene products, leading to inhibition of proliferation and angiogenesis.

## Materials and Methods

**Materials.** Curcumin (77.5% curcumin; 4.21% bisdemethoxycurcumin, 18.27% demethoxycurcumin; also called C3 complex) was kindly supplied by Sabinsa. Polyclonal antibodies against p65 (recognizing the epitope within the NH<sub>2</sub> terminal domain of human NF- $\kappa$ B p65), ICAM-1, cyclin D1, MMP-9, survivin, cIAP-1, procaspase-3, and procaspase-9 and monoclonal antibodies against VEGF, COX-2, c-myc, Bcl-2, and Bcl-xL were obtained from Santa Cruz Biotechnology. The liquid 3,3'-diaminobenzidine + substrate chromogen system-horseradish peroxidase used for immunohistochemistry was obtained from DakoCytomation. Penicillin, streptomycin, DMEM/F12 medium, and fetal bovine serum were obtained from Invitrogen. All other chemicals were obtained from Sigma Chemicals unless otherwise stated.

**Cell lines.** Human colon cancer cell line HCT 116 was obtained from the American Type Culture Collection and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin.

**Animals.** Male athymic *nu/nu* mice (4 wk old) were obtained from the breeding colony of the Department of Experimental Radiation Oncology at University of Texas M. D. Anderson Cancer Center. The animals were housed four per cage in standard mouse Plexiglas cages in a room maintained at constant temperature and humidity under 12-h light and dark cycles and fed with regular autoclaved chow diet with water *ad libitum*. None of the mice exhibited any lesions, and all were tested pathogen free. Before initiating the experiment, we acclimatized all mice to a pulverized diet for 3 d. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee.

**Xenograft implantation of HCT 116 cells.** HCT 116 cells were harvested from subconfluent cultures after a brief exposure to 0.25%

trypsin and 0.2% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum. The cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of single cells with >90% viability were used for the injections. Mice were anesthetized with ketamine-xylazine solution, and  $2 \times 10^6$  cells were injected s.c. into the right leg of each mouse using a 27-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Company).

**Experimental protocol.** One week after implantation, mice were randomized into the following treatment groups ( $n = 9$ -10) based on the tumor volume measured by Vernier calipers: (a) untreated control (corn oil, 100  $\mu$ L daily); (b) curcumin alone (1 g/kg), once daily orally; (c) radiation alone (4 Gy, twice weekly); and (d) combination of curcumin (1 g/kg), once daily orally, and radiation (4 Gy, twice weekly; given 1 h after curcumin). For irradiation, animals were anesthetized and immobilized in the treatment position with their right legs extended. Radiation was delivered at a dose rate of 1.25 Gy/min through a single posterior to anterior collimated 3-cm cobalt beam with a 5-mm bolus placed over the tumor. Tumor volume was measured at sequential time intervals, and the final tumor volume was calculated as  $V = 4/3\pi W^2L$ , where  $W$  is half of the shorter axis diameter and  $L$  is half of the longer axis diameter. The time to increase in tumor volume to  $5\times$  baseline values was estimated for each animal. The median times and SEs were calculated for each cohort, and the various groups were compared using unpaired Student's *t* test. Mice were sacrificed, and half of the tumor tissue was formalin-fixed and paraffin-embedded for immunohistochemistry and routine H&E staining. The other half was snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . H&E staining confirmed the presence of tumor(s) in each specimen.

**NF- $\kappa$ B activation in colorectal tumor samples.** To assess NF- $\kappa$ B activation, we isolated nuclei from colorectal tumor samples and carried out electrophoretic mobility shift assays as previously described (30).

**Immunolocalization of NF- $\kappa$ B p65, VEGF, COX-2, and MMP-9 in tumor samples.** The nuclear localization of p65, COX-2, VEGF, and MMP-9 was examined using an immunohistochemical method described previously (30).

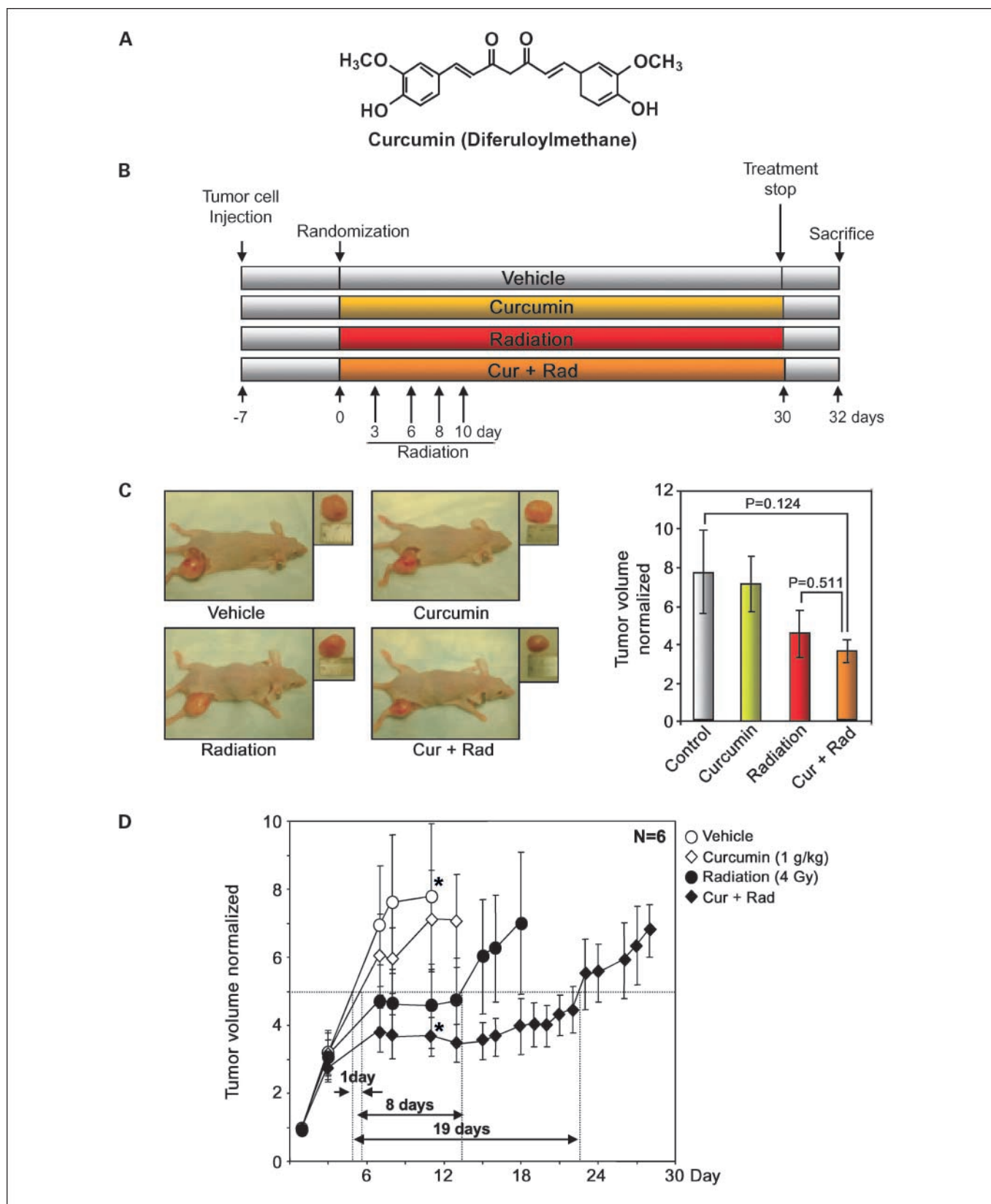
**Ki-67 immunohistochemistry.** Frozen sections (5  $\mu$ m) were stained with anti-Ki-67 (rabbit monoclonal clone SP6, NeoMarkers) antibody as previously described (31). Results were expressed as percentage of Ki-67<sup>+</sup> cells  $\pm$  SE per  $40\times$  magnification. A total of ten  $40\times$  fields were examined and counted from three tumors of each of the treatment groups. The values were initially subjected to one-way ANOVA and then compared using unpaired Student's *t* test.

**Microvessel density.** Frozen sections (5  $\mu$ m) were fixed in cold acetone and stained with rat anti-mouse CD31 monoclonal antibody (PharMingen) as previously described (32). The CD31 stained slides were observed under a Leica DM4000B fluorescence microscope (Leica Microsystems, Inc.) equipped with SPOT-RITKE digital camera (Diagnostic Instruments), and the images were acquired and stored using SPOT advanced software (Diagnostic Instruments). The stored images were processed using NIH ImageJ software. The vessel density in each image was estimated by measuring the pixel intensity in each field of view. The vessel density of each group was represented as intensity per pixel. A total of 20 high power fields were examined from three tumors of each of the treatment groups. The values were initially subjected to one-way ANOVA and then compared using unpaired Student's *t* test.

**Western blot analysis.** The protein expression for bcl-2, cFLIP, survivin, IAP1, procaspase-3, procaspase-9, COX-2, c-myc, cyclin D1, MMP-9, and MMP-9 in colorectal tumor samples were examined by Western blot analysis as previously described (30).

## Results

The aim of the present study was to determine whether curcumin can sensitize colorectal cancers to radiation in a



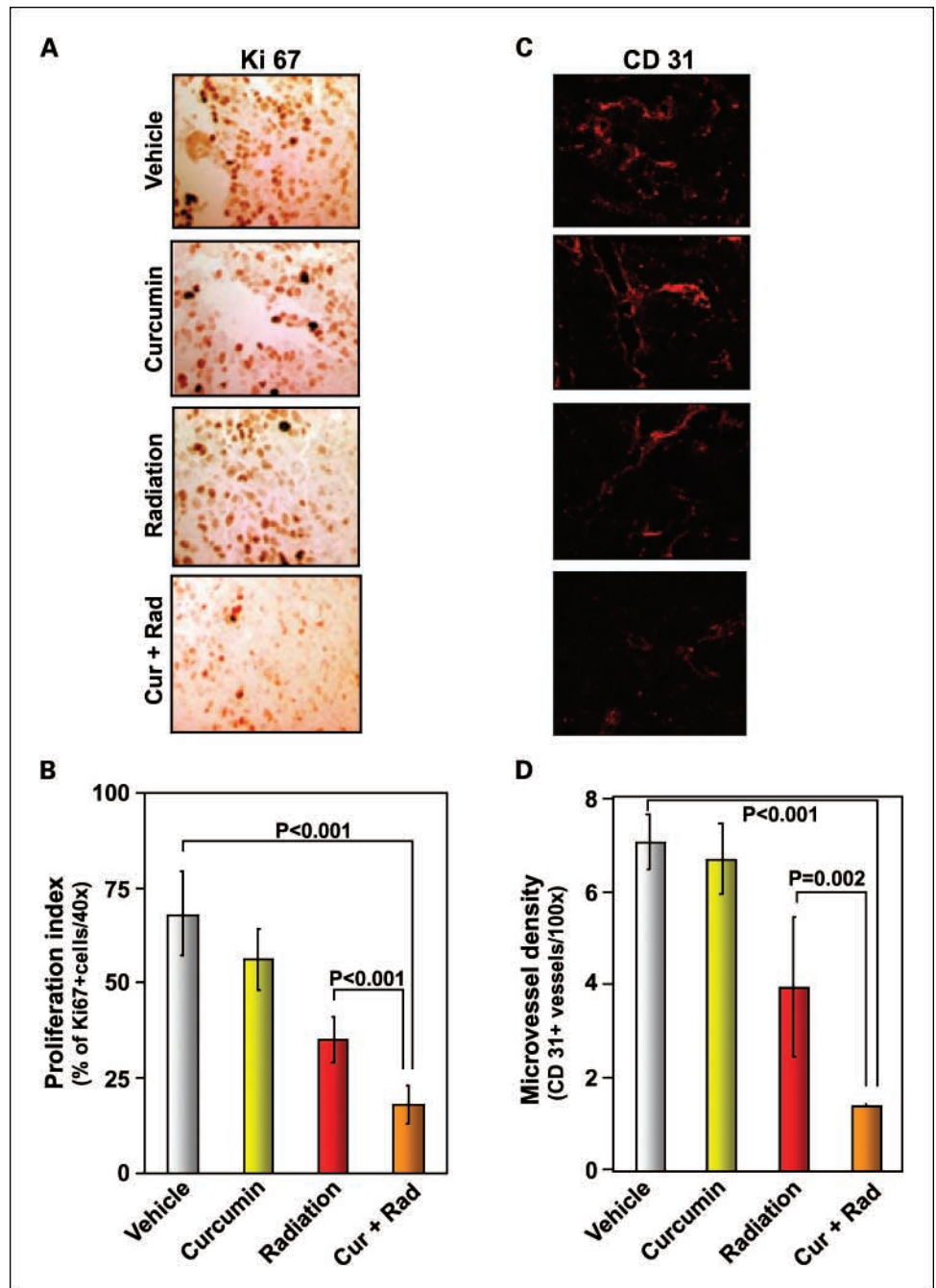
**Fig. 1.** Curcumin sensitizes colorectal tumors to radiation in nude mice. *A*, structure of curcumin. *B*, schematic representation of experimental protocol described in Materials and Methods. Group I was given corn oil (100  $\mu$ L orally daily), group II with curcumin (1 gm/kg orally daily), group III with  $\gamma$ -radiation 3, 6, 8, and 10 d after randomization, and group IV with curcumin (1 gm/kg orally daily) and with  $\gamma$ -radiation 3, 6, 8, and 10 d after randomization ( $n = 6$ ). *C*, necropsy photographs of mice bearing HCT 116 induced colorectal tumors on 10th day (*left*) and the tumor volume in mice (*right*). Columns, mean; bars, SE. *D*, tumor volume measured in different time intervals using Vernier calipers and calculated as described in Materials and Methods ( $n = 6$ ; \*,  $P < 0.001$ ).

mouse xenograft model and to investigate the mechanism by which curcumin radiosensitizes this tumor.

**Curcumin sensitizes colorectal cancers to radiation in vivo.** To determine whether curcumin can sensitize colorectal cancer to radiation, we examined the effect of radiation alone, curcumin alone, or in combination on the growth of subcutaneous HCT 116 xenograft tumors (Fig. 1B). Based on tumor volume measurements on the seventh day after tumor cell implantation, we randomized animals into four groups as described in Materials and Methods. Curcumin treatment was initiated after randomization and continued up to 30 days. Mice were irradiated twice weekly (4 Gy). All mice from the vehicle-treated group and three mice from each of the other groups were

sacrificed on the 10th day due to excessive tumor burden and analyzed for NF- $\kappa$ B and other biomarkers. Representative images of the tumor volume at this time are illustrated in Fig. 1C. At this early time point, the tumor volume in the combined group was significantly lower than that in the curcumin and control groups. Animals from the curcumin, radiation, and combined treatment groups were sacrificed on the 13th, 18th, and 32nd days, respectively. Growth delay after the combined treatment was more than the sum of growth delays caused by either alone (Fig. 1D). Normalized tumor volume reached five times the original volume in ~5 days when mice were treated with vehicle, ~6 days when treated with curcumin, ~14 days when treated with fractionated local

**Fig. 2.** Curcumin potentiates the effect of radiation against tumor cell proliferation and angiogenesis in colorectal cancer xenograft. *A*, immunohistochemical analysis of proliferation marker Ki-67 indicates the inhibition of colorectal tumor cell proliferation in curcumin alone or in combination with radiation-treated groups of animals. *B*, quantification of Ki-67<sup>+</sup> cells as described in Materials and Methods. Columns, mean of triplicate; bars, SE. *C*, immunohistochemical analysis of CD31 for microvessel density in colorectal tumors indicates the inhibition of angiogenesis by curcumin alone and curcumin alone and in combination with radiation. *D*, quantification of CD31<sup>+</sup> microvessel density as described in Materials and Methods. Columns, mean of triplicate; bars, SE.



tumor irradiation, and ~23 days when treated with both curcumin and radiation therapy ( $P = 0.02$ , compared with radiation-only group). The enhancement factor was 2.0, as determined by dividing the normalized tumor growth delay of the combined groups (18 days) by the absolute tumor growth delay of the radiation-only group (9 days).

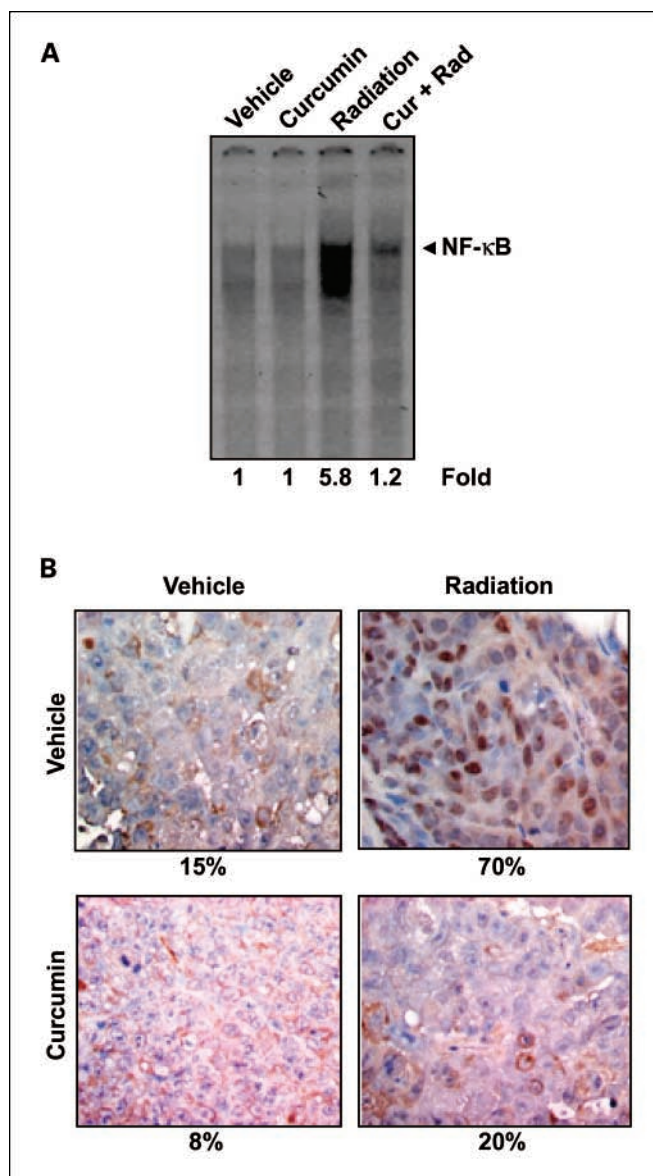
**Curcumin potentiates the effect of radiation on biomarker of tumor cell proliferation.** Proliferation of tumor cell clonogens between radiation doses can significantly affect the overall efficacy of therapy (33, 34). To explain the greater enhancement factor for the *in vivo* tumor regrowth delay study than for the *in vitro* clonogenic assay, we hypothesized that curcumin mediates its antitumor effects via inhibition of proliferation. Therefore, we examined the expression of the cell proliferation marker Ki-67 in tumor tissues from the four groups. The results in Fig. 2A and B showed that the combination of curcumin with radiation significantly down-regulated the expression of Ki-67 in tumor tissues when compared with radiation alone ( $P < 0.001$ ).

**Curcumin potentiates the effect of radiation on a biomarker of angiogenesis.** The higher radiosensitization in xenografts than in monolayer cultures when radiation therapy is combined with other cytostatic agents has been attributed to the capacity of these agents to inhibit angiogenesis (35, 36). We thus analyzed the effect of curcumin on angiogenesis by examining the expression of CD31, a marker of microvessel density. The results in Fig. 2C and D showed that the combination of radiation and curcumin significantly suppressed the expression of CD31 in tumor tissues when compared with radiation alone ( $P = 0.002$ ).

**Curcumin suppresses radiation-induced NF- $\kappa$ B activation in the tumor.** Because both proliferation and angiogenesis are regulated by NF- $\kappa$ B activation (6), whether curcumin mediates its effects through modulation of NF- $\kappa$ B was investigated. We determined the nuclear levels of p65 NF- $\kappa$ B expression by electrophoretic mobility shift assay in HCT 116 xenograft tissue after treatment in all four cohorts of animals. Radiation therapy significantly increased the activity of NF- $\kappa$ B, whereas concurrent treatment with curcumin decreased this inducible NF- $\kappa$ B activity to nearly baseline levels (Fig. 3A). We also determined the nuclear levels of p65 NF- $\kappa$ B expression by immunohistochemistry. The analysis for p65 NF- $\kappa$ B translocation to the nucleus showed a smaller percentage of cells positive for nuclear staining in the combined treatment group than the radiation-alone group (Fig. 3B). Thus, these results show that curcumin down-regulates radiation-induced NF- $\kappa$ B activation in the tumor.

**Curcumin down-regulates the expression of COX-2, VEGF, and MMP-9.** NF- $\kappa$ B is known to regulate the expression of gene products associated with angiogenesis, invasion, metastasis, and proliferation. Whether curcumin mediates its effects through modulation of these gene products was investigated. Consistent with NF- $\kappa$ B data, immunohistochemistry results indicate that radiation alone induced the expression of COX-2 (Fig. 4), and curcumin suppressed both constitutive and radiation-induced expression of COX-2 in the tumor xenografts. Our results also showed that MMP-9, a critical factor in invasion, was induced by radiation, and curcumin suppressed the expression quite effectively. The same was true for VEGF, a critical factor in angiogenesis.

**Curcumin down-regulates the expression of NF- $\kappa$ B-regulated gene products.** NF- $\kappa$ B regulates tumor survival through regulation of expression of survivin, Bcl-2, cFLIP, and IAP-1 (37–40);



**Fig. 3.** Curcumin inhibited radiation induced NF- $\kappa$ B activity in colorectal tumors. *A*, detection of NF- $\kappa$ B by DNA binding in colorectal tumor tissue samples showed the inhibition of NF- $\kappa$ B by curcumin. The numbers indicated are fold activation in relation to human myeloid KBM-5 cells as one. *B*, immunohistochemical analysis of nuclear p65 showed the inhibition of NF- $\kappa$ B by curcumin alone or in combination with radiation. Percentage indicates p65 nuclear positive cells. Samples from three animals in each group were analyzed, and a representative data is shown.

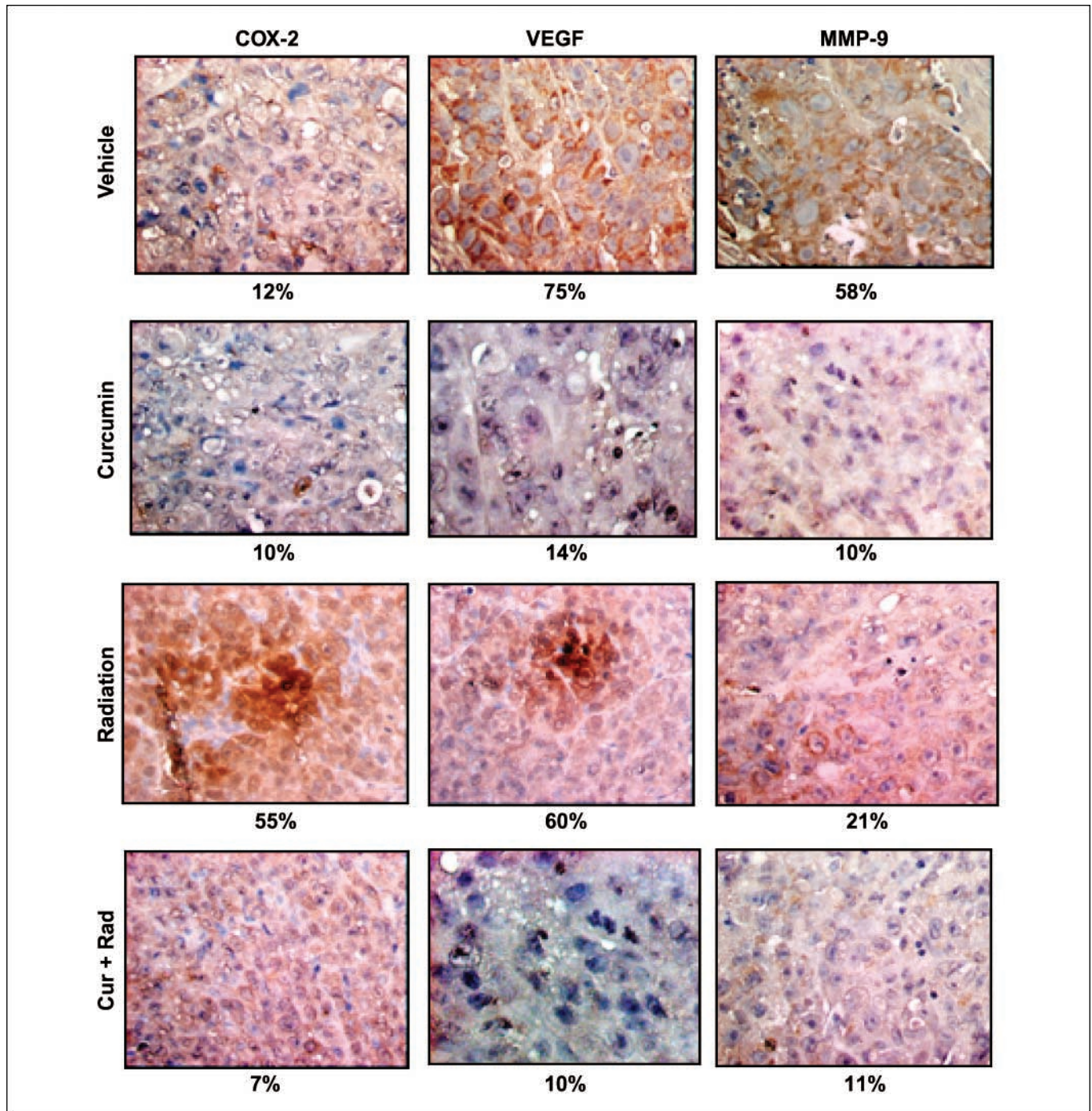
tumor cell proliferation through expression of cyclin D1 and c-myc (41); and angiogenesis and invasion through regulation of COX-2, VEGF, and MMP-9 (42, 43). The overexpression of several of these products has been linked with radioresistance. The effect of radiation and curcumin on the expression of these gene products in the xenografts was also examined by Western blot analysis (Fig. 5). Figure 5A clearly shows that curcumin down-regulated constitutive expression of Bcl-2, cFLIP, survivin, and IAP-1 in the tumor and down-regulated the radiation-induced expression of these gene products and survivin. Results in Fig. 5B show that curcumin induced the activation of caspase-9 and caspase-3, two critical apoptosis-inducing caspases. Consistent with immunohistochemistry data, results

in Fig. 5C indicate that the addition of curcumin suppressed radiation-induced expression of COX-2 and curcumin. Radiation-induced cyclin D1 expression, needed for the G<sub>1</sub>-S cell cycle transition to occur, was also down-regulated by curcumin. In addition, radiation-induced MMP-9 and VEGF were down-regulated by curcumin (Fig. 5D).

Thus, these results suggest that curcumin may sensitize the colorectal cancer to radiation through modulation of NF- $\kappa$ B-regulated gene products in the xenograft mouse model.

## Discussion

The aim of the present study was to determine whether curcumin could sensitize colorectal tumors to radiation therapy. Our results suggest that curcumin enhances the antitumor effects of radiation therapy *in vivo* by suppressing the NF- $\kappa$ B pathway, which regulates tumor survival, proliferation, invasion, and angiogenesis (see Fig. 6). Our results indicate that curcumin inhibits the activation of NF- $\kappa$ B and the



**Fig. 4.** Curcumin down-regulated the expression of NF- $\kappa$ B-regulated gene products in colorectal tumor samples. Immunohistochemical analysis of COX-2, VEGF, and MMP-9 showed the inhibition of COX-2, VEGF, and MMP-9 by curcumin alone or in combination with radiation. Percentage indicates positive staining for the given biomarker. Samples from three animals in each group were analyzed, and a representative data is shown.

expression of NF- $\kappa$ B-regulated gene products in colorectal xenografts.

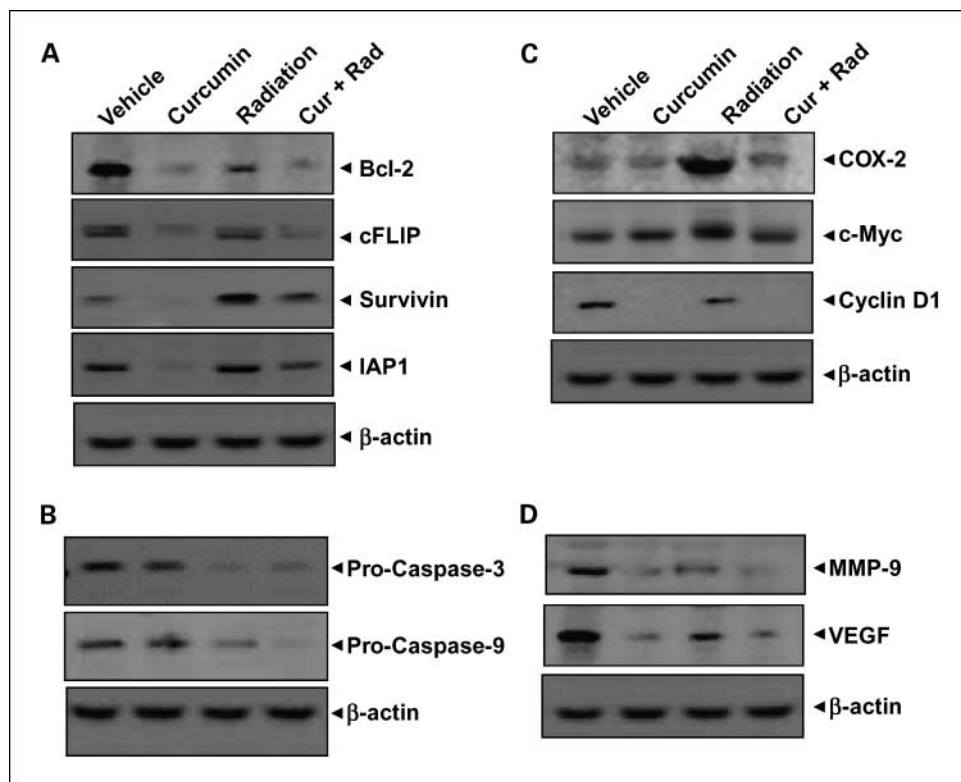
The biological response of tumors to ionizing radiation is mediated through multiple interrelated mechanisms. One such mechanism, the continued proliferation of tumor clonogens that survive a dose of ionizing radiation, has been implicated in the adverse local tumor control after treatment prolongation and/or treatment delays during a course of fractionated radiation therapy (33, 34, 44, 45). Using a spectrum of pharmacologic agents, inhibition of this proliferation between treatment fractions, otherwise known as repopulation, has been shown to sensitize tumors to radiation therapy in preclinical models (46–50). The magnitude of radiosensitization achieved with an agent that influences this repopulation is expected to be greater when multiple fractions of radiation are used. Indeed, in the current study, the enhancement of efficacy of radiation therapy with the addition of curcumin was more amplified in the multifraction *in vivo* radiation therapy as evidenced by the reduced proliferation index (Ki-67 staining), *c-myc* levels, and cyclin D1 levels in the combined treatment group compared with the radiation-alone group. In addition to the proliferation index of a tumor, the composite tumor response to radiation therapy is also influenced by the rate of tumor cell apoptosis, a counterbalance to proliferation that reduces clonogenic burden. Our results suggest that the addition of curcumin amplifies this apoptotic response beyond that observed with radiation alone (Fig. 5C and D).

To explain the potent radiosensitization noted in the tumor regrowth delay assay, we also evaluated the possibility that curcumin might inhibit mechanisms beyond merely proliferative growth inhibition and apoptosis induction. One such mechanism is tumor angiogenesis, a host-mediated biological

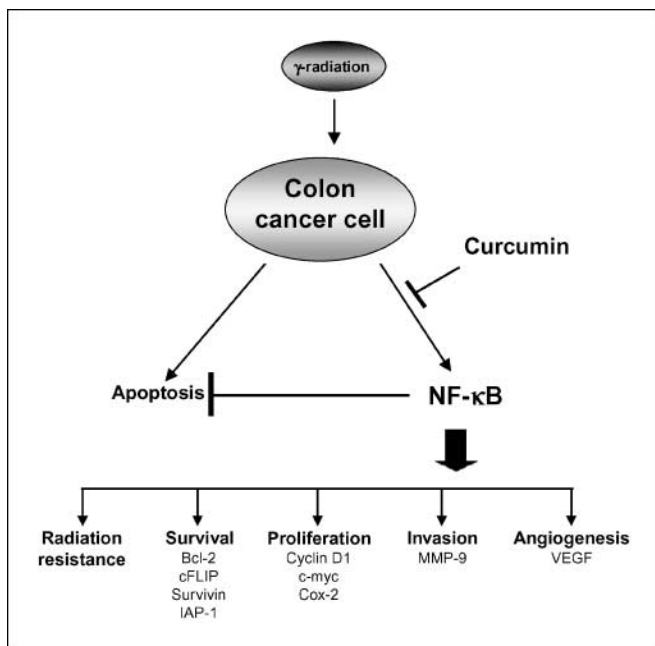
process that contributes to tumor cell survival in a xenograft microenvironment: when tumors outgrow their supply of oxygen and nutrients, neovasculature is recruited. A spectrum of pharmacologic agents, including antibodies, kinase inhibitors, and soluble VEGF receptors, in antiangiogenic therapy have been shown to promote tumor radiosensitization (51). In the current study, the microvessel density within xenograft tumors in the combined treatment group was lower than that in the radiation-alone group (Fig. 3C and D) and was associated with a decrease in COX-2, MMP-9, and VEGF expression (Fig. 5A and B).

Additional studies are being designed to clarify the mechanism of inhibition of angiogenesis by curcumin and the mechanism of radiosensitization by inhibition of angiogenesis in this model. One possibility is that curcumin exerts a direct effect on vascular endothelial cells to modulate their radio-response. Alternatively, down-regulation of tumor cell proliferative signaling via inhibition of Akt (mediated by growth factor receptor pathways among others) may lead to decrease in VEGF expression. Decreased VEGF expression may lead to more efficient oxygenation of tumors via normalization of aberrant and leaky vascular channels within tumors and reduction in interstitial fluid pressure (52). This reoxygenation of tumors enhances radiosensitivity because normoxic cells are substantially more sensitive to radiation-induced cell killing than hypoxic cells (53).

Numerous cell signaling mechanisms have been implicated in radioresistance, including reactive oxygen species, COX-2, phosphoinositide 3-kinase, multidrug resistance proteins, Bcl-2, survivin, growth factors, and transcription factors, such as signal transducers and activators of transcription 3 and NF- $\kappa$ B (5). Perhaps central to all these pathways is the transcription



**Fig. 5.** Curcumin down-regulated the expression of NF- $\kappa$ B-regulated gene products in colorectal tumor samples. **A**, Western blot showing that curcumin and radiation together inhibit the expression of NF- $\kappa$ B-dependent antiapoptotic genes, such as bcl-2, cFLIP, survivin, and IAP1 in colorectal tumor tissues. **B**, Western blot showing that curcumin and radiation together inhibit the expression of procaspase-3 and procaspase-9 in colorectal tumor tissues. **C**, Western blot showing that curcumin and radiation together inhibit the expression of NF- $\kappa$ B-dependent proliferative genes, such as COX-2, cyclin D1, and c-myc. **D**, Western blot showing that curcumin and radiation together inhibit the expression of NF- $\kappa$ B-dependent invasive and angiogenic gene products, such as MMP-9 and VEGF in colorectal tumor tissues. Samples from three animals in each group were analyzed, and a representative data is shown.



**Fig. 6.** Schematic representation showing how curcumin sensitizes colon cancer to radiation and inhibits tumor growth.

factor NF- $\kappa$ B. A common mechanism of up-regulation of the mediators of angiogenesis and proliferation described above occurs via activation of NF- $\kappa$ B, which in turn binds to the promoters for these mediators. As described in the introduction, the biological effects of curcumin are at least partly mediated via inhibition of NF- $\kappa$ B. In the current study, inhibition of NF- $\kappa$ B activity and inhibition of NF- $\kappa$ B-regulated gene products by curcumin were documented *in vivo*. Notably, in the radiation-alone group, activation of NF- $\kappa$ B was documented (Fig. 4). Inducible activation of NF- $\kappa$ B inhibits the apoptotic response to ionizing radiation and provides a mechanism for tumors to evade the cytotoxicity of therapy (54, 55). Inhibition of this pathway by curcumin could, therefore, overcome this mechanism of inducible radioresistance and enhance the efficacy of radiation therapy. Another interesting finding was that the radiation therapy increased the levels of COX-2 and c-myc in tumor samples. This is particularly relevant because the expression of COX-2 is related directly to cell proliferation, survival, metastasis, and angiogenesis in colorectal cancers (56–58).

Our results in colorectal cancer are in accordance with reports in other malignancies showing an increased sensitivity of

prostate and breast cancer cells *in vitro* to irradiation after treatment with curcumin. The reported mechanisms of radiosensitization included inhibition of radiation-induced tumor necrosis factor  $\alpha$  activation and induction of apoptosis (59) and inhibition of Akt-mediated inhibition of the MDM-2 oncogene (60). Similar radiosensitization has been shown preclinically by combination radiation therapy and multiple strategies to inhibit NF- $\kappa$ B, including the use of a modified form of I $\kappa$ B $\alpha$  (54, 61–63), a decoy of NF- $\kappa$ B (63), or proteasome inhibitors (62).

In summary, our results suggest that curcumin enhances the efficacy of radiation therapy for colorectal cancer. The underlying mechanisms by which curcumin improves radiation response seem to be multifaceted and involve suppression of proliferation and angiogenesis. Furthermore, the potent *in vivo* radiosensitization effects of curcumin are associated with inhibition of the activity of NF- $\kappa$ B and the expression of NF- $\kappa$ B-regulated gene products that regulate proliferation, resistance to apoptosis, angiogenesis, invasion, and metastasis. Placed in a clinical context, several characteristics of these findings support the investigation of the combination of radiation therapy with curcumin in the treatment of rectal cancer: (a) the clinically relevant dose of curcumin (1 g/kg) used in the current study and its excellent tolerability in human subjects, even at very high doses (64); (b) the clinical evidence that curcumin accumulates specifically in colorectal cancers, possibly via a combination of local absorption and systemic accumulation (65, 66); (c) the clinical evidence that curcumin decreases the size and number of polyps in patients with a genetic predisposition to the development of multiple polyps at a young age (67); and (d) the increasing recognition that transient up-regulation of NF- $\kappa$ B after radiation may mediate an inducible form of radioresistance (54). Radiation therapy alone has been used as the preoperative treatment regimen involving short course of high-dose radiotherapy (5Gy  $\times$  5) followed by total mesorectal excision of the primary rectal cancer shortly thereafter (usually within 2 weeks; ref. 68). However, because conventional treatment of rectal cancer involves chemoradiation therapy more often than radiation therapy alone, the implication of these preclinical findings on traditional rectal cancer treatment remains to be established. Nevertheless, our results support further research on curcumin in anticipation of future radiation therapy trials in patients with colorectal cancer.

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# Clinical Cancer Research

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