Curcumin Suppresses the Paclitaxel-Induced Nuclear Factor-κB Pathway in Breast Cancer Cells and Inhibits Lung Metastasis of Human Breast Cancer in Nude Mice

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Abstract Currently, there is no effective therapy for metastatic breast cancer after surgery, radiation, and chemotherapy have been used against the primary tumor. Because curcumin suppresses nuclear factor-κB (NF-κB) activation and most chemotherapeutic agents activate NF-κB that mediates cell survival, proliferation, invasion, and metastasis, we hypothesized that curcumin would potentiate the effect of chemotherapy in advanced breast cancer and inhibit lung metastasis. We tested this hypothesis using paclitaxel (Taxol)-resistant breast cancer cells and a human breast cancer xenograft model. As examined by electrophoretic mobility gel shift assay, paclitaxel activated NF-κB in breast cancer cells and curcumin inhibited it; this inhibition was mediated through inhibition of IκBα kinase activation and IκBα phosphorylation and degradation. Curcumin also suppressed the paclitaxel-induced expression of antiapoptotic (XIAP, IAP-1, IAP-2, Bcl-2, and Bcl-xL), proliferative (cyclooxygenase 2, c-Myc, and cyclin D1), and metastatic proteins (vascular endothelial growth factor, matrix metalloproteinase-9, and intercellular adhesion molecule-1). It also enhanced apoptosis. In a human breast cancer xenograft model, dietary administration of curcumin significantly decreased the incidence of breast cancer metastasis to the lung and suppressed the expression of NF-κB, cyclooxygenase 2, and matrix metalloproteinase-9. Overall, our results indicate that curcumin, which is a pharmacologically safe compound, has a therapeutic potential in preventing breast cancer metastasis possibly through suppression of NF-κB and NF-κB–regulated gene products.

Although early-stage breast cancer is highly treatable, no effective treatment is available for metastatic breast cancer that follows surgery, radiation, and chemotherapy for the primary tumor (1). Paclitaxel (Taxol) is currently used as the front-line chemotherapeutic agent in breast cancers (1); however, because the drug frequently induces drug resistance (2–4), probably through the activation of nuclear factor-κB (NF-κB; ref. 5), it is not useful in treating advanced breast cancer.

Curcumin (diferuloylmethane), a polyphenol (see Fig. 1A) derived from turmeric, *Curcuma longa*, is a pharmacologically safe and effective agent that can block NF-κB activation. Curcumin has been shown by us and others to suppress NF-κB activation induced by various inflammatory stimuli (6) through inhibition of the activation of IκBα kinase (IKK) activity needed for NF-κB activation (7, 8). Based on the paclitaxel and curcumin data, we hypothesized that curcumin would improve the therapeutic outcome of paclitaxel treatment for breast cancer. We tested this hypothesis using breast cancer cells and a nude mouse xenograft model. Our goal was to determine whether curcumin can suppress paclitaxel-induced NF-κB activation and NF-κB–regulated gene products and prevent breast cancer metastasis to the lung. We found that curcumin did block paclitaxel-induced NF-κB activation and NF-κB–regulated gene expression in breast cancer cells and inhibited breast cancer metastasis to the lung in nude mice.

Materials and Methods

Materials. Paclitaxel was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). It was dissolved in ethanol as a 10 mmol/L stock solution and stored at 4°C. Penicillin, streptomycin, Iscove's modified Dulbecco's medium, RPMI 1640, and fetal bovine serum were obtained from Life Technologies, Inc. (Grand Island, NY). Tris, glycine, NaCl, SDS, bovine serum albumin, and phorbol 12-myristate 13-acetate were obtained from Sigma Chemical Co. (St. Louis, MO) The following polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-p65, against the epitope corresponding to amino acids mapping within the amino terminal domain of human

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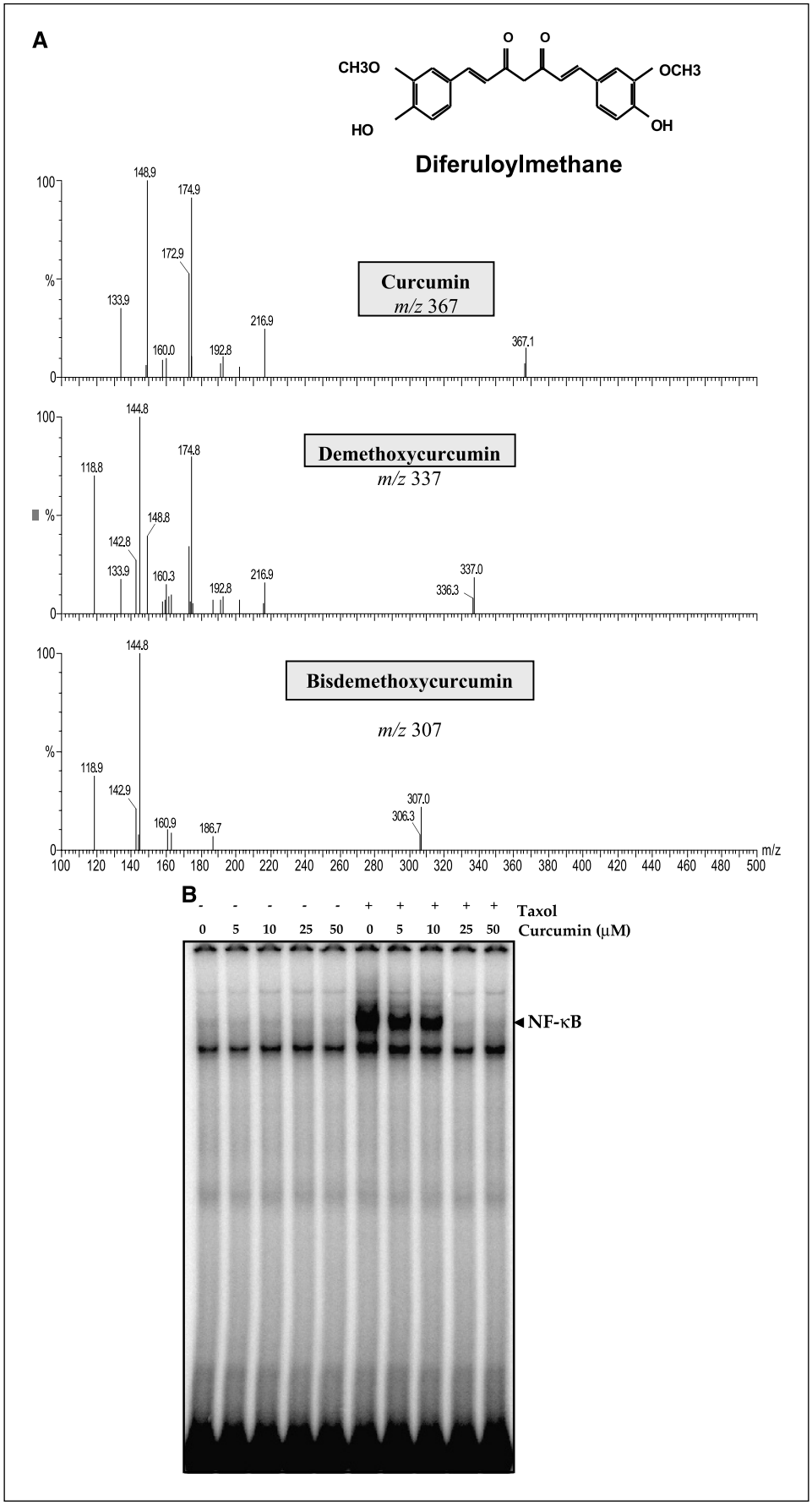

Fig. 1. *A,* structure and mass spectrophotometric analysis of curcumin. *B,* curcumin inhibited paclitaxel-dependent NF-κB activation. MDA-MB-435 cells (2×10^6/mL) were preincubated with different concentrations of curcumin for 2 hours at 37°C and then treated with 50 μmol/L paclitaxel for 16 hours. Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods.

NF-κB p65; anti-p50, against a 15-amino-acid-long peptide mapping at the nuclear localization sequence region of NF-κB p50; anti-IκBα, against amino acids 297 to 317 mapping at the carboxy terminus of IκBα/MAD-3; and anti–c-Rel and anti–cyclin D1, against amino acids 1 to 295, which represents full-length cyclin D1 of human origin. Phospho-IκBα (Ser^{32}) antibody was purchased from New England BioLabs (Beverly, MA). Anti-IKKα and anti-IKKβ antibodies were kindly provided by Imgenex (San Diego, CA). Anti–cyclooxygenase 2 (COX-2) antibody was purchased from Transduction Labs (now Invitrogen, Carlsbad, CA) and anti–matrix metalloproteinase (MMP-9) antibody was purchased from Cell Sciences, Inc. (Norwood, MA).

Curcumin. Curcumin with a purity of >98% was obtained from either LKT Laboratories (St. Paul, MN) or Sabinsa Corp. (Piscataway, NJ). To ensure identity and quality of the curcumin used in this study, curcumin was dissolved in 50:50 methanol/0.2% formic acid and maintained at 5°C in a refrigerated autosampler before analysis by electrospray ionization liquid chromatography tandem mass spectrometry. Curcuminoids (curcumin, demethoxycurcumin, and bisdesmethoxycurcumin) were separated on a Phenomenex Gemini 5 μm C18 2 × 100 mm analytic column using a linear acetonitrile/0.1% formic acid gradient. Detection was accomplished using a Waters Quattro Micro tandem mass spectrometer equipped with electrospray positive ionization capability. Each of these compounds was quantified by using a standard calibration curve prepared from a curcumin reference standard obtained from Sigma-Aldrich Chemical. The calibration curve was prepared by making a 1 mg/mL stock solution of the authentic material in methanol and then serially diluting the stock solution to 1,000, 500, 250, 100, 50, 10, 5, and 1 ng/mL with methanol. A calibration curve was then prepared using the mass spectrometry quantification software. The ratios of the three curcuminoids in the curcumin used in this study were as follows: curcumin, 87.2% (detected at *m/z* 367); demethoxycurcumin, 10.5% (detected at *m/z* 337); and bisdemethoxycurcumin, 2.3% (detected at *m/z* 307; see Fig. 1A). Percentages were calculated based on the peak areas for each of the curcuminoids detected.

Cell lines. We used the human breast cancer cell line MDA-MB-435. We have previously shown that this cell line is tumorigenic and metastatic in nude mice (9).

Nuclear factor-κB activation. To determine NF-κB activation, we carried out electrophoretic mobility gel shift assay on nuclear extracts of paclitaxel-treated cells essentially as previously described (6).

IκBα degradation. To determine the effect of curcumin on paclitaxel-dependent IκBα degradation, cytoplasmic extracts were prepared as previously described (6) from MDA-MB-435 cells (2×10^6/mL) pretreated with curcumin for 2 hours and then exposed to 50 μmol/L paclitaxel for various times. The extracts were then resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against IκBα, and detected by chemiluminescence (ECL, Amersham, Piscataway, NJ). The bands obtained were quantitated using Personal Densitometer Scan v1.30 using Imagequant software version 3.3 (Amersham).

IκBα phosphorylation. To determine the effect of curcumin on paclitaxel-dependent IκBα phosphorylation, cytoplasmic extracts were prepared from MDA-MB-435 cells (2×10^6/mL) treated with 50 μmol/L curcumin for 2 hours and then treated with 50 μmol/L paclitaxel for various times. The extracts were then resolved on 10% SDS polyacrylamide gels and analyzed by Western blotting using antibody against phosphorylated IκBα (Amersham).

IκBα kinase assay. To determine the effect of curcumin on paclitaxel-induced IKK activation, we did IKK by a method described previously (10).

RNA analysis and reverse transcription-PCR. MDA-MB-435 cells were cultured at a density of 1×10^6 cells/mL and kept overnight in serum-containing medium. Cells were washed and then treated with either tumor necrosis factor (TNF; 1 nmol/L), curcumin (50 μmol/L), paclitaxel (50 μmol/L), or their combination as indicated. The growth medium was removed, cells were suspended in Trizol reagent, and total RNA was extracted according to the instructions of the manufacturer (Invitrogen). Two micrograms of total RNA were converted to cDNA by Superscript reverse transcriptase and then amplified by platinum Taq polymerase using Superscript One Step reverse transcription-PCR (RT-PCR) kit (Invitrogen). The relative expression of COX-2 was analyzed using quantitative RT-PCR with β-actin as an internal control. The RT-PCR reaction mixture contained 25 μL of 2× reaction buffer, 2 μL each of RNA and forward and reverse COX-2 or β-actin primers, and 1 μL of RT/platinum Taq in a final volume of 50 μL. The primer sequences for COX-2 were as follows: sense 5′-TTCAAATGAGATTGTGGGAAAA-TTGCT-3′ and antisense 5′-AGATCATCTCTGCCTGAGTATCTT-3′. For β-actin, the primer sequences were as follows: sense 5′-GGGTCAGAAG-GATTCCTATG-3′ and antisense 5′-GGTCTCAAACAT GATCTGGG-3′. The reaction was done at 50°C for 30 minutes, 94°C for 2 minutes, 35 cycles at 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute with extension at 72°C for 10 minutes. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and were photographed.

Transient transfection and luciferase assay. MDA-MB-435 cells were seeded at a concentration of 1.5×10^5 per well in six-well plates. After overnight culture, the cells in each well were transfected with 2 μg DNA consisting of *COX-2* promoter luciferase reporter plasmid along with 6 μL of LipofectAMINE 2000 (Life Technologies) by following the protocol of the manufacturer. The COX-2 promoter (−375 to +59) was amplified from human genomic DNA by using the primers 5′-GAGTCTCTTATTTATTTTT-3′ (sense) and 5′-GCTGCTGAG-GAGTTCCTGGACGTGC-3′ (antisense; kindly provided by Dr. Xiao-Chun Xu, M. D. Anderson Cancer Center, Houston, TX). After a 6-hour exposure to the transfection mixture, the cells were incubated in medium containing curcumin (25 μmol/L) for 12 hours. The cells were then exposed to TNF (1 nmol/L) or paclitaxel (50 μmol/L) for 24 hours and then harvested. Luciferase activity was measured by using the Promega luciferase assay system according to the protocol of the manufacturer and detected by using Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA). All experiments were done in triplicate and repeated at least twice to prove their reproducibility.

Metastasis therapy experiments. Female athymic nude mice were injected with 2×10^6 MDA-MB-435LVB human breast cancer cells (a variant of MDA-MB-435) selected for high incidence of spontaneous metastases into the mammary fatpad as described previously (9). When the mean tumor diameter reached 10 mm (58-60 days after cell injection), mice were anesthetized, the tumors were removed, and the incisions were closed. The animals were randomly assigned to treatment groups (15 were included in each group) and fed powdered diet (5053 Pico Lab Rodent Chow; Charles Rivers Laboratories, Raleigh, NC) or diet mixed with 2% w/w curcumin from day 5 after tumor removal until the end of the study. Administration of curcumin in the diet did not alter the weight of mice and consumption of food with the supplement was not noticeably different from consumption of the standard diet. On days 10, 17, and 24 after tumor removal, mice were injected i.p. with 10 mg/kg paclitaxel prepared in Cremophor vehicle (Cremophor EL/ethanol 1:1, diluted 1:4 with PBS after the addition of paclitaxel) or with the Cremophor vehicle alone (reagents were from Sigma Chemical). Five weeks after tumor removal, mice were killed and autopsied, and the incidence and numbers of visible lung metastases were recorded. The lungs were removed and fixed in 10% buffered formalin, and paraffin-embedded sections were stained with H&E. The animal experiments were done with approval from the Institutional Animal Care and Use Committee.

Statistical analyses. Data were analyzed using the unpaired two-tailed Student's *t* test (tumor weight), Fisher's exact test (incidence of metastasis), and Mann-Whitney test (numbers of lung metastases). $P < 0.05$ was considered significant.

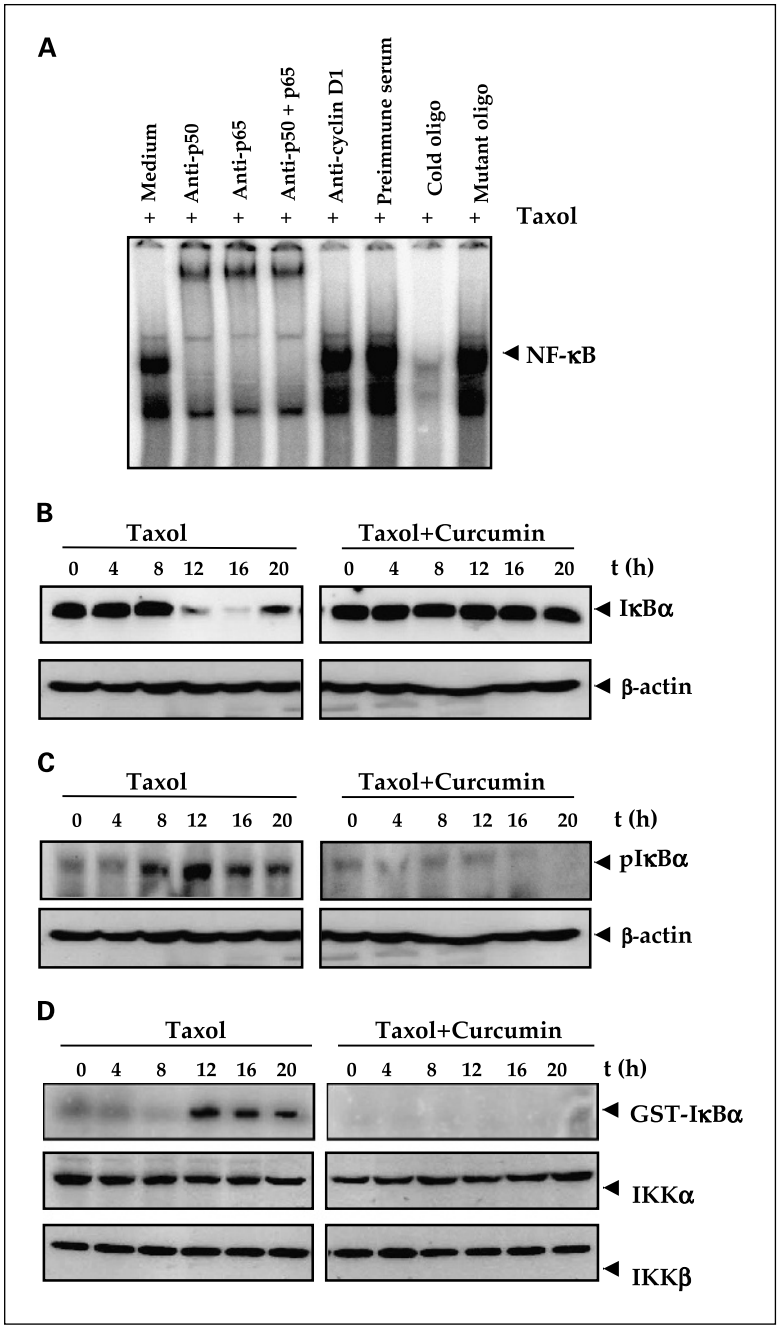

Fig. 2. Curcumin inhibits IκBα phosphorylation, IKK activation, and gene expression. *A,* supershift analysis of paclitaxel-induced NF-κB. MDA-MB-435 cells (2×10^6/mL) were incubated with 50 μmol/L paclitaxel at 37°C, then incubated with different antibodies or unlabeled oligo probes as indicated and examined for NF-κB by DNA binding. *B,* curcumin inhibits paclitaxel-induced IκBα degradation. MDA-MB-435 cells (2×10^6/mL) were incubated with 50 μmol/L curcumin for 2 hours at 37°C, treated with 50 μmol/L paclitaxel for indicated times at 37°C, and tested for IκBα in the cytosolic fractions by Western blot analysis using antibodies against IκBα. Equal protein loading was evaluated by β-actin. *C,* curcumin inhibits paclitaxel-induced IκBα phosphorylation. MDA-MB-435 cells (2×10^6/mL) were incubated with 50 μmol/L curcumin for 2 hours at 37°C, treated with 50 μmol/L paclitaxel for indicated times at 37°C, and tested for phosphorylated IκBα in the cytosolic fractions by Western blot analysis using antibodies against phosphorylated IκBα (*pIκBα*). Equal protein loading was evaluated by β-actin. *D,* curcumin inhibits paclitaxel-induced IKK activity. MDA-MB-435 cells (2×10^6/mL) were treated with 50 μmol/L curcumin for 2 hours and then treated with 50 μmol/L paclitaxel for the indicated time intervals. Whole-cell extracts were prepared and 200 μg of extract was immunoprecipitated with antibodies against IKKα and IKKβ. Thereafter, immune complex kinase assay was done as described in Materials and Methods. To examine the effect of curcumin on the level of expression of IKK proteins, 30 μg of cytoplasmic extracts was run on 7.5% SDS-PAGE, electrotransferred, and immunoblotted with indicated antibodies as described in Materials and Methods.

Histologic sections. Formalin-fixed tissue was paraffin-embedded, sectioned (3-5 μm), and stained with H&E. Sections were evaluated for tumor cell cytology, mitotic rate, growth pattern, necrosis, and associated inflammatory cellular response.

Immunohistochemistry. Immunohistochemical studies were done using paraffin-embedded material, heat-induced antigen retrieval (Tris buffer, pH 8.0 for MMP-9; citrate buffer, pH 6.0 for COX-2 and p65, and pH 7.2 for Ki-67), and antibodies specific for MMP-9 (1:500; R&D Systems, Minneapolis, MN), COX-2 (1:500; Cayman Chemical Co., Ann Arbor, MI), p65 (1:75; Abcam, Cambridge, United Kingdom), and Ki-67 (1:100; DAKO, Carpinteria, CA). The detection system used was the LSAB2 detection kit (DAKO). The slides were counterstained with hematoxylin. Negative and positive controls were also run. Stained slides were analyzed under a bright-field microscope (Labophot-2; Nikon, Tokyo, Japan). Images were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA). At least 500 tumor cells were evaluated for staining positivity in each case.

Results

The aim of this study was to determine if curcumin suppresses paclitaxel-induced NF-κB activation and NF-κB–regulated gene products and whether the combination of paclitaxel and curcumin can be exploited to suppress the incidence and extent of breast cancer metastasis in a mouse xenograft model. For most studies, the human breast cancer MDA-MB-435 cell line was used, which we have previously shown to be tumorigenic and metastatic in nude mice (9).

Curcumin inhibits paclitaxel-induced activation of nuclear factor-κB and IκBα kinase. Electrophoretic mobility gel shift assay (Fig. 1B) indicated that treatment with paclitaxel activated NF-κB in MDA-MB-435 cells. The results in Fig. 1B also show that treatment of cells with curcumin abolished the paclitaxel-induced NF-κB activation; maximum suppression was observed at a 25 μmol/L concentration. Supershift analysis indicated that paclitaxel-activated NF-κB consisted of the p50 and p65 subunits of NF-κB (Fig. 2A).

NF-κB activation by most agents is known to require the phosphorylation and degradation of IκBα, an inhibitor of NF-κB. As expected, curcumin completely suppressed the paclitaxel-mediated degradation of IκBα (Fig. 2B). Similarly, the data also indicate that paclitaxel induced phosphorylation of IκBα and curcumin prevented it (Fig. 2C). The phosphorylation of IκBα is mediated through activation of IKK (5). As shown in Fig. 2D, paclitaxel activated IKK in a time-dependent manner and curcumin suppressed this activation. No effect on the levels of either IKKα or IKKβ was noted.

Curcumin represses paclitaxel-induced nuclear factor-κB–dependent antiapoptotic gene products. NF-κB regulates the expression of the antiapoptotic proteins IAP1/2, XIAP, Bcl-2, and Bcl-xL (11). We investigated whether curcumin can modulate the expression of these antiapoptotic gene products induced by paclitaxel. Paclitaxel induced the activity of these antiapoptotic proteins in a time-dependent manner whereas curcumin suppressed it (Fig. 3A).

Curcumin represses the paclitaxel-induced nuclear factor-κB–dependent gene products involved in the proliferation and metastasis of tumor cells. We also investigated whether curcumin can modulate NF-κB–regulated gene products involved in the proliferation and metastasis of tumor cells. NF-κB activation has been shown to regulate the expression of cyclin D1, c-Myc, COX-2, MMP-9, and intercellular adhesion molecule-1 (12–16).

We thus determined whether paclitaxel also induces all these gene products and whether curcumin inhibits this activation. Western blot analysis using specific antibodies showed that paclitaxel induced the expression of COX-2, c-Myc, and cyclin D1 (Fig. 3B) and of vascular endothelial growth factor, MMP-9, and intercellular adhesion molecule-1 (Fig. 3C) in a time-dependent manner, whereas curcumin suppressed

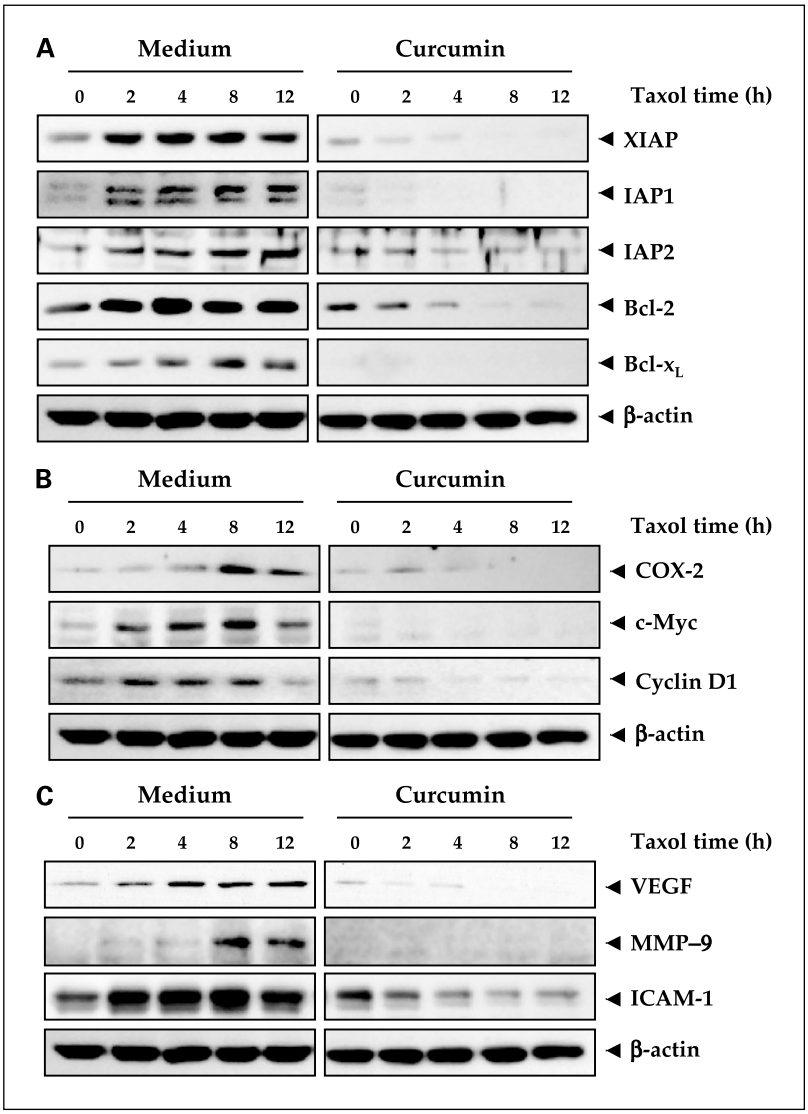

Fig. 3. Curcumin inhibits induction of paclitaxel-induced NF-κB – regulated gene products. *A,* curcumin inhibits induction of XIAP, IAP1, IAP2, Bcl-2, and Bcl-xL induced by paclitaxel. MDA-MB-435 cells (2×10^6/mL) were left untreated or incubated with 50 μmol/L curcumin for 2 hours and then treated with 50 μmol/L paclitaxel for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against specific gene products. Equal protein loading was evaluated by β-actin. *B,* curcumin inhibits induction of COX-2, c-Myc, and cyclin D1 by paclitaxel. MDA-MB-435 cells (2×10^6/mL) were left untreated or incubated with 50 μmol/L curcumin for 2 hours and then treated with 50 μmol/L paclitaxel for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against specific gene products. Equal protein loading was evaluated by β-actin. *C,* curcumin inhibits induction of vascular endothelial growth factor (*VEGF*), MMP-9, and intercellular adhesion molecule-1 (*ICAM-1*) by paclitaxel. MDA-MB-435 cells (2×10^6/mL) were left untreated or incubated with 50 μmol/L curcumin for 2 hours and then treated with 50 μmol/L paclitaxel for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against specific gene products. Equal protein loading was evaluated by β-actin.

it (Fig 3B and C). These results support our postulate that curcumin blocks paclitaxel-induced NF-κB–regulated gene products.

Curcumin inhibits paclitaxel-induced activation of cyclooxygenase-2 messenger RNA and promoter activity. Whether curcumin affects the transcriptional regulation of paclitaxel-induced gene products was also examined. As shown in Fig. 4A, both TNF (our control) and paclitaxel induced COX-2 mRNA and curcumin completely suppressed the induction. Furthermore, curcumin inhibited TNF- and paclitaxel-induced COX-2 promoter activity (Fig. 4B).

Curcumin potentiates the cytotoxicity of paclitaxel toward breast cancer cells. Both paclitaxel and curcumin can induce apoptosis of breast cancer cells in culture (2, 3, 17). In agreement with these reports, we found that both curcumin and paclitaxel alone suppressed the growth of breast cancer cells but the combination of the two compounds together was more effective than either one alone (Fig. 5A).

Curcumin inhibits human breast cancer metastasis to the lung in a mouse xenograft model. We next investigated the ability of curcumin to modulate human breast cancer metastasis to the lung in a nude mouse xenograft model. We found that curcumin plus paclitaxel significantly suppressed the incidence of breast cancer metastasis in lung tissue (Fig. 5B). Macroscopic lung metastases were seen in 96% of mice in the control group. Treatment with 10 mg/kg paclitaxel only modestly reduced the incidence of metastasis. However, both curcumin alone and curcumin plus paclitaxel treatments significantly reduced both the incidence and numbers of visible lung metastases. Because the size of the tumor in the mammary fatpad can influence the resulting metastatic burden (9), mice were assigned randomly to the different treatment groups. There was no statistically significant difference in primary tumor weight in the different

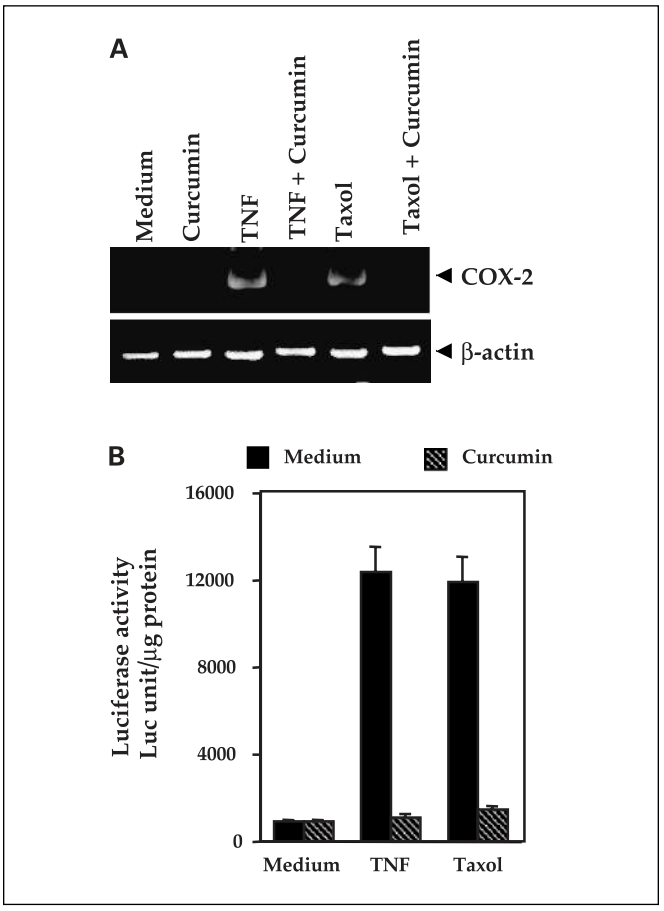

Fig. 4. Curcumin inhibits paclitaxel induction of COX-2 mRNA and COX-2 promoter activity. *A,* curcumin inhibits induction of COX-2 mRNA by paclitaxel. MDA-MB-435 cells (5×10^6/mL) were left untreated or incubated with 50 μmol/L curcumin for 3 hours and then treated with either paclitaxel (50 μmol/L) or TNF (1 nmol/L) for 3 hours. Total mRNA was isolated and probed for COX-2 as indicated in Materials and Methods. Equal loading was evaluated by β-actin. *B,* curcumin inhibits induction of COX-2 promoter–dependent luciferase activity. MDA-MB-435 cells (1×10^6 per well) were left untreated or incubated with 50 μmol/L curcumin for 3 hours and then treated with either paclitaxel (50 μmol/L) or TNF (1 nmol/L) for 36 hours. Whole-cell extracts were prepared and analyzed for luciferase activity as described in Materials and Methods.

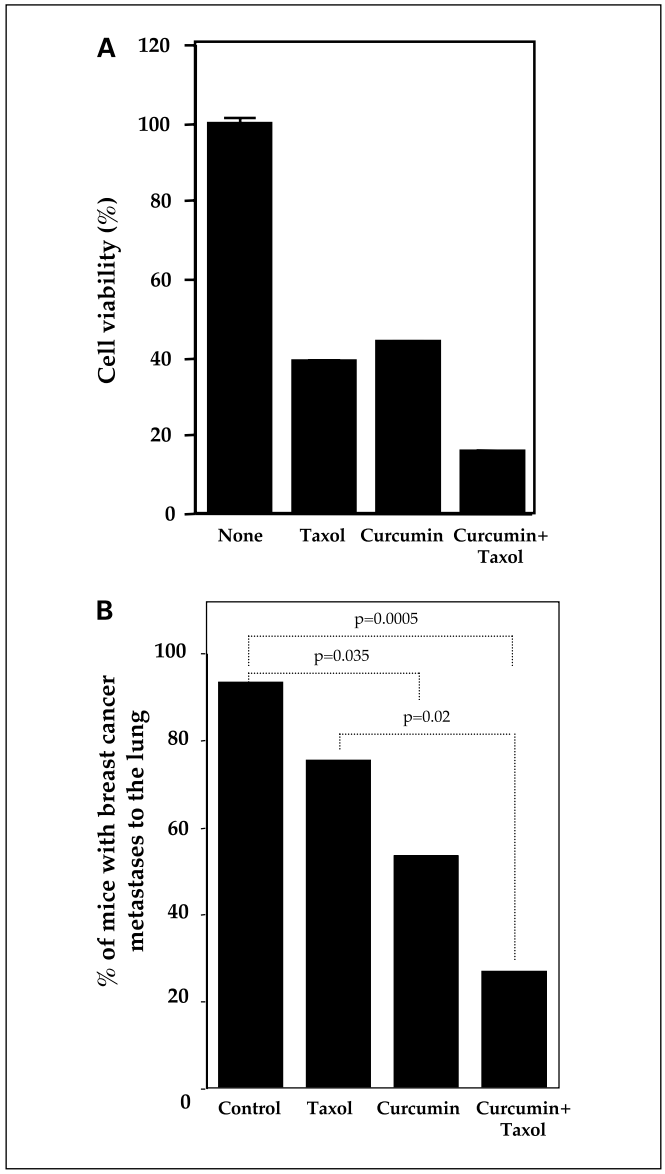

Fig. 5. *A,* curcumin potentiates the cytotoxic effects of paclitaxel against human breast cancer MDA-MB-435 cells. Cells (50,000/mL) were incubated in triplicate with either 50 μmol/L curcumin or 10 μmol/L paclitaxel or in combination for 72 hours, and then cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. *B,* curcumin, paclitaxel, and their combination inhibit breast cancer metastasis to the lung. Incidence of lung tumor metastasis was examined in mice after primary tumors were removed surgically. *P* values were determined using Fisher's exact test.

groups, suggesting that differences in the incidence and number of metastases were the result of treatments and not the initial tumor size (data not shown).

Examination of histologic sections of the lungs confirmed the presence of metastatic disease. Microscopic metastases were found in lungs of 28% of mice treated with curcumin plus paclitaxel although there was no macroscopic disease evident. Most of the micrometastases were solitary foci consisting of only a few cells, suggesting that the combination of curcumin and paclitaxel inhibited the growth of breast cancer tumor cells seeded in the lung before removal of primary tumors.

Curcumin down-regulates nuclear factor-κB, cyclooxygenase-2, and matrix metalloproteinase-9 in breast tumor metastasis to the lungs. The status of NF-κB in tumor tissues derived from control (*A*), curcumin-fed (*B*), paclitaxel-treated (*C*), and paclitaxel together with curcumin–fed (*D*) animals was examined by immunohistochemistry (Fig. 6). We found that paclitaxel increased the expression of NF-κB in tumor tissues (control, 45%; paclitaxel, 60%). Curcumin inhibited the expression of NF-κB in tissue derived from both curcumin-fed (30%) and curcumin plus paclitaxel–treated (35%) animals.

Immunohistochemistry also revealed the absence of MMP-9 protein expression in normal lung epithelium and high expression (1 of 8), low expression (5 of 8), or lack of expression (2 of 8) in lung tumors harvested from control and paclitaxel-treated animals (Fig. 7). In contrast, tumors harvested from curcumin- and curcumin-paclitaxel groups showed low expression (6 of 8) or lack of expression (2 of 8). COX-2 expression was consistently absent in normal lung epithelium (Fig. 7, *middle*) but metastatic lung tumors from the control and paclitaxel-only treatment groups were positive (10 of 10) for the presence of COX-2. The metastatic carcinomas from curcumin- and curcumin plus paclitaxel–treated mice stained weakly positive (4 of 8) or not at all (4 of 8). The overall findings support our *in vitro* evidence that curcumin down-regulates paclitaxel-induced COX-2 and MMP-9 expression in metastatic breast carcinoma to the lung.

We also measured the proliferation rate using Ki-67 and apoptosis using terminal deoxynucleotidyl transferase–mediated nick end labeling assay in the tissue samples. These results showed 24 ± 5% Ki-67–positive cells in control group, 8 ± 4% in curcumin-fed, 15 ± 5% in paclitaxel-treated, and 16 ± 8% in curcumin together with paclitaxel-treated groups. These results

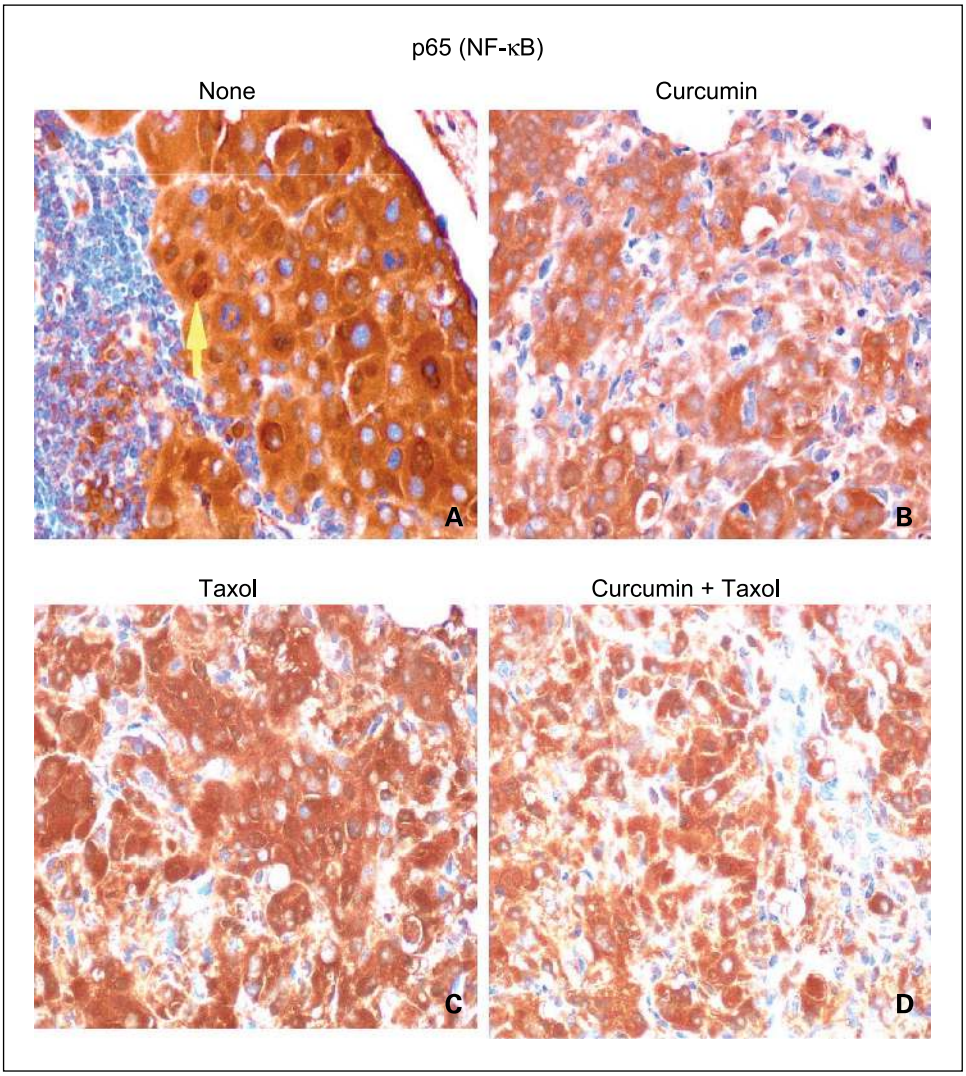

Fig. 6. Curcumin suppresses paclitaxel-induced p65 expression in breast carcinoma metastatic to the lung. Tissue sections were immunostained for p65 subunit of NF-κB metastatic breast carcinomas. Lung metastases from untreated control mice [*A,* tumor cells express strong nuclear (*arrow*) and cytoplasmic p65 immunoreactivity], from curcumin-fed mice (*B,* tumor shows decreased nuclear and cytoplasmic p65 immunopositivity), from paclitaxel-treated mice (*C,* tumor with strong nuclear and cytoplasmic p65 expression), and from curcumin plus paclitaxel – treated mice (*D,* tumor with decreased nuclear and cytoplasmic p65 immunoreactivity) are shown. Magnification, ×200.

clearly show that the curcumin-fed group had the lowest proliferation rate. However, terminal deoxynucleotidyl transferase–mediated nick end labeling assay showed that the apoptosis rates were not significantly different from each other (data not shown).

Discussion

The goal of this study was to investigate the effect of curcumin on paclitaxel-induced NF-κB–regulated gene products and on breast cancer metastasis. We found that paclitaxel activated NF-κB in breast cancer cells and curcumin inhibited this activation through inhibition of IKK activation, IκBα phosphorylation, and IκBα degradation. Curcumin also suppressed paclitaxel-induced expression of antiapoptotic, proliferative, and metastatic proteins and enhanced apoptosis. In a human breast cancer xenograft model, dietary administration of curcumin significantly decreased the incidence of breast cancer metastasis to the lung and this correlated with the suppression of the expression of NF-κB and NF-κB–regulated gene products in the tumor tissue.

Our study indicates that paclitaxel activates NF-κB in human breast cancer cells through a classic NF-κB activation pathway consisting of IKK activation, IκBα phosphorylation and degradation, and NF-κB–regulated gene expression, including cyclin D1, MMP-9, and COX-2. Treatment of breast cancer cells with curcumin completely suppressed the paclitaxel-induced IKK activation, leading to suppression of NF-κB activation. Curcumin also suppressed paclitaxel-induced cyclin D1, MMP-9, and COX-2 in breast cancer cells. Paclitaxel also induced various antiapoptotic gene products in these cells and again their expression was down-regulated by curcumin. Our results are in agreement with previous reports that showed that curcumin inhibits COX-2 (8, 18), cyclin D1 (19), and MMP-9 (20) expression, all of which are regulated by NF-κB. We found that curcumin also down-regulated the expression of paclitaxel-induced COX-2 mRNA and COX-2 promoter activity, indicating regulation at the transcriptional level.

Constitutive activation of NF-κB and overexpression of COX-2 and cyclin D1 have been detected in human breast cancer (21–25). Agents to suppress NF-κB activation, COX-2, and

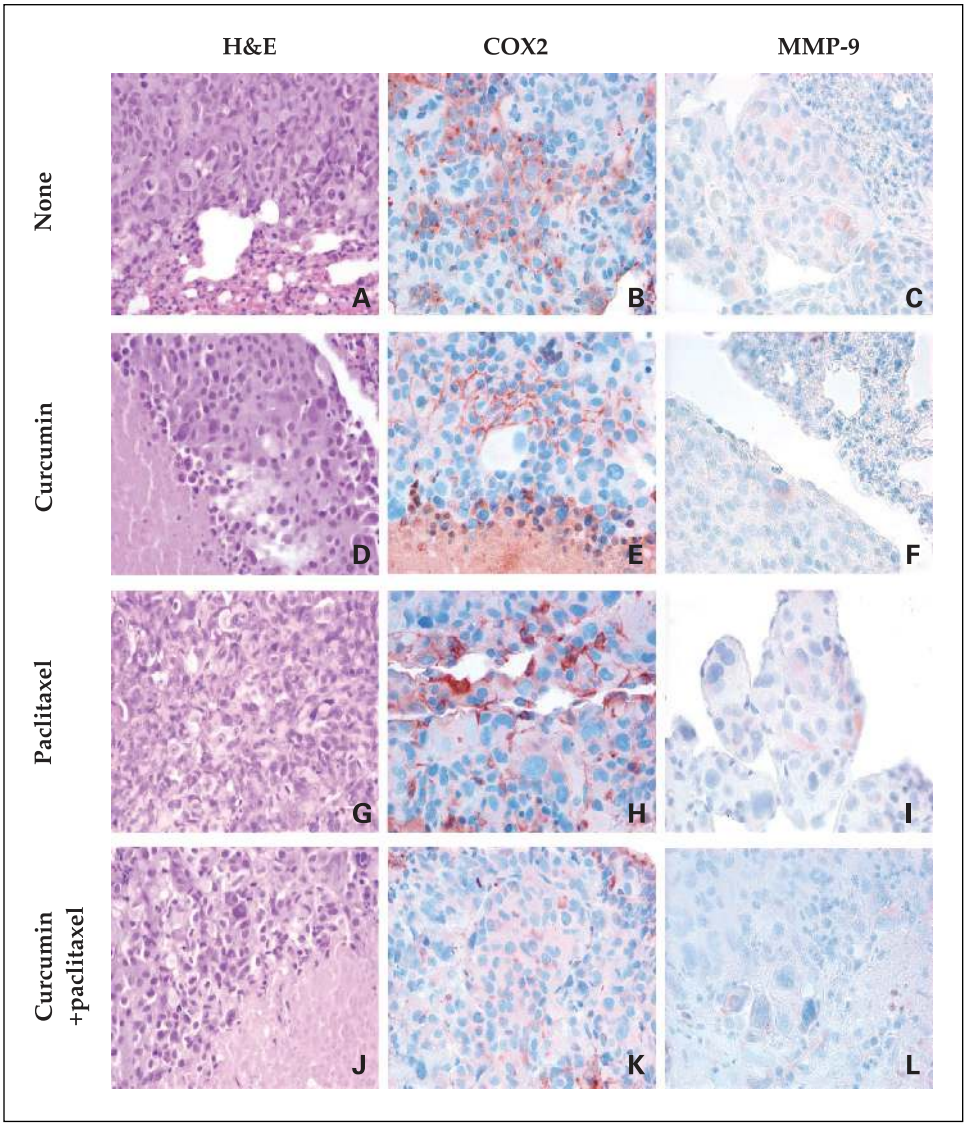

Fig. 7. Effect of curcumin and paclitaxel on the pathology of metastatic breast carcinoma to the lung and on COX-2 and MMP-9 expression. Representative H&E-stained sections (*left column*) and immunohistochemical staining for COX-2 (*middle column*) and MMP-9 (*right column*) of metastatic breast carcinomas are shown. Lung metastasis from untreated control (*A,* carcinoma without necrosis), from curcumin-treated animals (*D,* carcinoma with necrosis), from Taxol-treated animals (*G,* carcinoma with multiple mitosis and without necrosis), and from curcumin plus Taxol – treated mice (*J,* carcinoma with extensive necrosis) are shown. Immunohistochemical staining for COX-2 protein shows strong COX-2 positivity in lung metastasis from untreated mice (*B*), focal positivity in the metastatic carcinoma from curcumin-treated (*E*) and paclitaxel-treated (*H*) mice, and faint positivity in the tumor tissue from curcumin plus paclitaxel – treated mice. Immunohistochemical staining for MMP-9 protein shows many positive tumor cells in the untreated tissue (*C*) and paclitaxel-treated tissue (*I*) and focal faint cytoplasmic positivity in the tumor from curcumin-treated mice (*F*) and curcumin plus paclitaxel – treated tumor (*L*). Scattered immunoreactions are localized to the inflammatory cells (positive internal control).

cyclin D1, as potential therapeutic approaches for breast cancer, are being exploited (26, 27). Furthermore, the role of NF-κB in chemoresistance is well established (28–30). We found that curcumin enhanced the paclitaxel-induced apoptosis of breast cancer cells.

When tested in animals, curcumin suppressed the growth of human breast cancer metastasis in the lung. We found a significant reduction of lung metastasis in mice treated with curcumin plus paclitaxel. Our immunohistochemistry results showed that in tumor tissue derived from animals, paclitaxel induced NF-κB, COX-2, and MMP-9 expression, and treatment of animals with curcumin suppressed the expression of all the gene products.

Paclitaxel induced NF-κB both *in vitro* and *in vivo*, whereas curcumin suppressed it. In tissue sections, NF-κB was evident in both the nucleus and cytoplasm in control as well as paclitaxel-treated groups. Therefore, we cannot rule out the possibility that suppression of breast cancer metastasis by curcumin occurs through mechanisms other than the suppression of NF-κB and NF-κB–regulated gene expression.

How curcumin affects the growth of micrometastatic cells and how this activity may differ from its action in combination with paclitaxel is yet to be determined. Either agent alone or in combination was more effective in reducing lung metastases in mice. It should be noted that the 10 mg/kg dose of paclitaxel used was lower than doses previously shown to be effective against this tumor (31). Using this relatively less effective dose, we showed that the addition of curcumin resulted in antimetastatic therapy that was as effective as higher, potentially toxic doses of the chemotherapeutic drug.

The protocol for the treatment used in our studies can be conveniently used in humans, possibly after surgery. Because of the lack of any dose-limiting toxicity and its potential to suppress metastatic breast cancer, the efficacy of curcumin should be tested in human breast cancer.

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