Inhibition of Pancreatic and Lung Adenocarcinoma Cell Survival by Curcumin is Associated with Increased Apoptosis, Down-regulation of COX-2 and EGFR and Inhibition of Erk1/2 Activity

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Abstract. *Background: Several studies suggested that curcumin inhibits growth of malignant cells via inhibition of cyclooxygenase-2 (COX-2) activity. Other studies indicated that epidermal growth factor receptor (EGFR) is also inhibited by curcumin in vitro and in vivo. Moreover, recent investigations revealed an intracellular cross-talk between EGFR signaling and the COX-2 pathway. Our aim was to evaluate whether the curcumin inhibitory effect on the survival of cancer cells is associated with simultaneous down-regulation of COX-2 and EGFR and inhibition of Erk1/2 (extra-cellular signal regulated kinase) signaling pathway. Materials and Methods: Lung and pancreas adenocarcinoma cell lines co-expressing COX-2 and EGFR (PC-14 and p34, respectively) and those expressing EGFR but deficient in COX-2 (H1299 and Panc-1, respectively) were exposed for 72 h to curcumin (0-50 μM). Cell viability was assessed by the XTT assay. Apoptosis was determined by FACS analysis. COX-2, EGFR, ErbB-2 and p-Erk1/2 expressions were measured by Western blot analysis. Results: Curcumin's inhibitory effect on survival and apoptosis of lung and pancreatic adenocarcinoma cell lines was significantly higher in the COX-2-expressing cells than in the COX-2-deficient cells. In the p34 and PC-14 cells, curcumin decreased COX-2, EGFR and p-Erk1/2 expressions in a dose-dependent manner. However, in the Panc-1 and H1299 cell lines, which did not express COX-2, curcumin did not affect EGFR levels. Conclusion: Curcumin co-inhibited COX-2 and EGFR expression and decreased Erk1/2 activity. This inhibition was associated with decreased survival and enhanced induction of apoptosis in lung and pancreatic adenocarcinoma cells.*

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Cyclooxygenase-2 (COX-2) has been shown to be one of the key factors in carcinogenesis. COX-2 mRNA and protein levels are up-regulated in transformed cells (1, 2), as well as in both pre-malignant and malignant tissues, including pancreas and lung tumors (3). The up-regulation of COX-2 was found to be associated with increased proliferation (4), anti-apoptotic effects (5, 6), increased malignancy (7) and promotion of angiogenesis (8). In a landmark study, using a murine model of familial adenomatous polyposis (FAP), Oshima *et al.* (9) showed that in APC^{716} knock-out mice, the number and size of adenomas was reduced in the $COX\text{-}2^{-/-}$ mice compared to the COX-2 wild-type mice.

Several studies suggested that non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors may serve in the prevention and treatment of cancer (10). Their anti-neoplastic properties have been primarily attributed to their ability to block COX-2 activity, which results in inhibition of cell proliferation and induction of apoptosis (11-13). The long-term safety issue associated with the use of COX-2 inhibitors was, however, recently questioned due to the increased cardiovascular and thrombotic toxicity that had been observed with their long-term use (14). The search for new potent COX-2 inhibitors with very low profiles of side-effects has raised particular interest in curcumin, a diferuloylmethane derived from the *Curcuma longa* plant. Curcumin is found in turmeric, curry and mustard and is a potent antioxidant that has been used for centuries as a dietary factor and in herbal therapy in several Eastern countries (15). Moreover, recent clinical trials (16, 17) have shown that curcumin is non-toxic, even at very high doses.

Similar to other COX-2 inhibitors, curcumin possesses both anti-inflammatory (18) and anti-tumor properties (19). Its anti-neoplastic efficacy has been demonstrated in several *in vitro* studies and in animal models (20-22).

The mechanism of action of curcumin appeared not to be limited to COX-2 inhibition [reviewed in (23)]. One of the promising targets that emerged is the epidermal growth factor receptor (EGFR) (24, 25), which is overexpressed in a variety of malignancies and plays an important role in tumor cell growth and metastasis (26, 27), as well as in the resistance of cancer cells to cytotoxic drugs (28, 29). Moreover, several studies have shown an intracellular cross-talk between EGFR signaling and the COX-2 pathway (30, 31). Krysan *et al.* (32) recently showed that the COX-2 product, prostaglandin E_2 (PGE_2), is able to transactivate the EGFR pathway through four G protein-coupled receptors (GPCRs), resulting in the promotion of cancer cell growth and motility.

To the best of our knowledge, the current study is the first to show that the inhibitory effect of curcumin on lung and pancreas cancer cell line survival is associated with the simultaneous down-regulation of COX-2, EGFR and p-Erk1/2.

Materials and Methods

Cell culture and reagents. Human lung (H1299) and pancreas (Panc-1) carcinoma cell lines were obtained from the American Type Culture Collection (ATCC). The human lung carcinoma PC-14 cell line was obtained from Dr. Isaiah Fiddler (UT M.D. Anderson Cancer Center, Houston, TX, USA). P34 is a human pancreatic cell line that was developed in our lab, as previously described (33). All the cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Israel) supplemented with 10% fetal calf serum (FCS), 1% penicillin and 1% streptomycin (full medium) at 37˚C, in an atmosphere of 95% oxygen and 5% CO_2. Curcumin (97% purity) was purchased from Merck (Whitehouse Station, NJ, USA).

Cell viability assay. The cells (1-2x10^3 cells/well) were seeded in 96-microwell plates for 24 h and were then incubated in full medium containing the test drugs. After 72 h of treatment, cell viability was assessed by the ability of metabolically active cells to reduce tetrazolium salt to colored formazan compounds (XTT assay). The absorbance was measured with an ELISA reader (wavelength 450 nm). The data are expressed in mean values from three similar experiments, each performed in triplicate.

Flow cytometry analysis. The cells were plated at a density of 0.5x10^6 per 10-cm dish for 24 h and were then incubated in full medium with tested drugs at selected concentrations. Following 72 h of treatment, the adherent and non-adherent cells were collected during their exponential growth and counted, after which they were washed in phosphate-buffered saline (PBS) and the pellet was fixed in 3 ml ethanol for 1 hour at 4˚C. The cells were resuspended in 1 ml PBS and incubated for 30 min with 0.15 mg/ml RNAse at 37˚C. The cells were then stained with 5 μg/mL propidium iodide (PI) for 1 h before analysis by flow cytometry using a standard protocol for cell cycle distribution and cell size. Data acquisition was performed on a FACScan and analyzed by CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data for at least 10,000 cells were collected for each data file. Necrotic cells were detected by counting cells following staining with trypan blue before fixation and they were excluded from the calculation of apoptotic cells. All experiments were done three times and gave similar results.

Protein extraction and Western blotting. Exponentially growing intact and treated cells were collected and washed three times in ice-cold PBS, as described earlier. The cell pellets were re-suspended in lysis buffer (20 mM Tris-HCI pH 7.4, 2 mM EDTA, 6 mM 6-mercaptoethanol, 1% NP-40, 0.1% SDS and 10 mM NaF, plus the protease inhibitors, leupeptin 10 μg/ml, aprotinin 10 μg/ml and 0.1 mM phenylmethyl-sulfonylfluoride). The protein concentration of each sample was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). For Western blotting, samples containing 50 μg of total cell lysate were loaded onto a 10% SDS-polyacrylamide gel and were subjected to electrophoresis. Proteins were transferred to "Hybond-C" membranes (Amersham, Arlington Heights, IL, USA) in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol), using a Trans Blot transfer apparatus (Bio-Rad Laboratories, CA, USA) at 70 mA for 12-18 hours at room temperature. The membranes were blocked with blocking buffer (PBS/0.2% Tween-20/0.5% gelatin) for 1 h at room temperature and subsequently washed three times for 5 min in washing buffer (PBS/0.05% Tween-20). They were incubated with polyclonal human anti-COX-2, EGFR, ErbB-2 and p-Erk1/2 for 1 h at room temperature, then washed as described above and incubated with anti-goat secondary antibodies (1:2000) for 1 h at room temperature. Additional washes were carried out as described previously, and immune detection was performed using the ECL Western blotting detection system (Amersham).

Statistical analysis. The results were calculated as mean±SE. The difference between the intact and treated cells was evaluated by the one-way Student's *t*-test using an SPSS software package (SPSS Inc., Chicago, IL, USA). Statistical significance ($p<0.05$) was established by the *post hoc* Tukey's pairwise comparison.

Results

Effect of curcumin on cell survival. A dose-dependent inhibitory effect of curcumin on survival was found in all tested human lung (H1299, PC-14) and pancreas (p34, Panc-1) carcinoma cell lines (Figure 1). Notably, the curcumin effect was significantly higher on the PC-14 (IC_{50}=10 μM) and p34 (IC_{50}=15 μM) carcinoma cell lines, both expressing high levels of COX-2, than on the COX-2-deficient H1299 (IC_{50}=20 μM) and Panc-1 (IC_{50}=25 μM) carcinoma cell lines (Table I).

Induction of apoptosis by curcumin. The extent of apoptosis was assessed by flow cytometry analysis following 72 h of exposure of the cells to the different concentrations of curcumin. Curcumin increased the percentage of cells with sub-diploid DNA content, the hallmark of apoptosis, in a dose-dependent manner in the lung (PC-14) and the

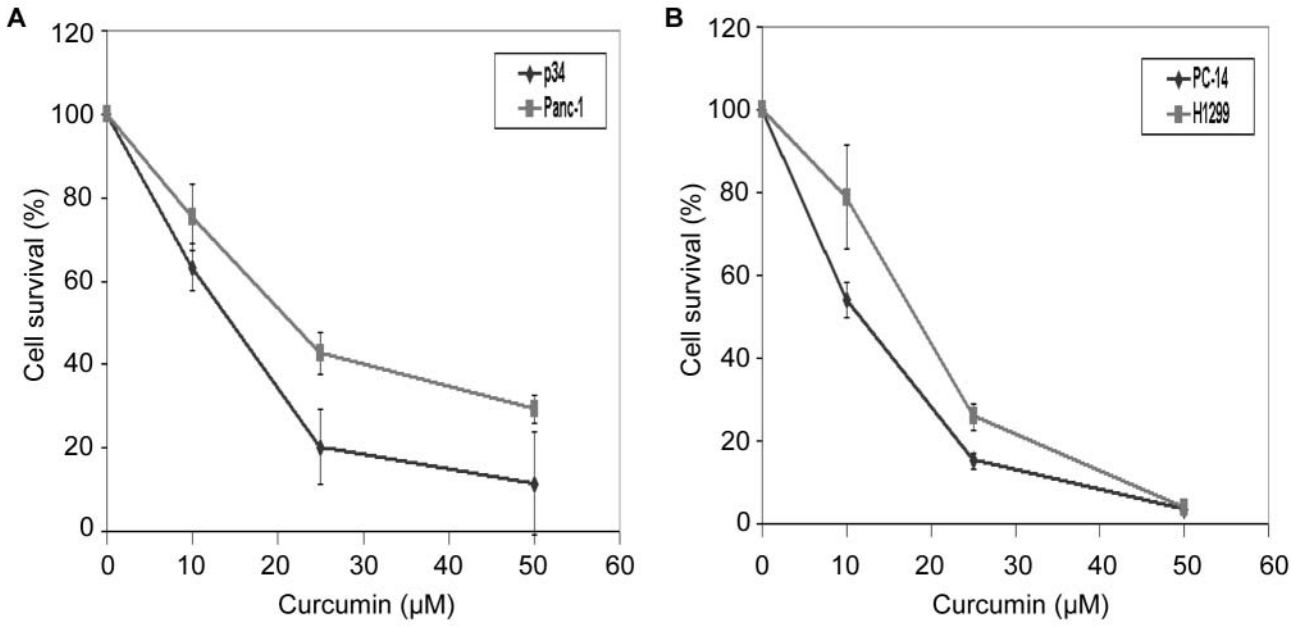

Figure 1. *Effect of curcumin on survival of pancreas (A) and lung (B) adenocarcinoma cell lines expressing COX-2 (PC-14, p34) and COX-2 deficient (H1299, Panc-1). The cells were exposed for 72 h to different concentrations of curcumin. The data are mean±SE values from three individual experiments performed in triplicate.*

Table I. *Effect of curcumin on cell survival and induction of apoptosis in the pancreatic and lung human carcinoma cell lines.*

Origin	Cell line	COX-2 expression*	EGFR expression*	Curcumin IC_{50} (μM)#	Apoptosis (%) (25 μM curcumin)
Pancreatic cancer	p34	+	+	15	23.4
	Panc-1	–	+	25	5.1
Non-small cell lung cancer	PC-14	+	+	10	21.0
	H1299	–	+	20	13.6

*COX-2 and EGFR expression: (+) high level, (–) no expression.
#IC_{50}: concentration of compound that inhibits the growth of cells by 50%.

pancreatic (p34) carcinoma cell lines that expressed COX-2 (Figure 2). In contrast, it had only a minor effect on the induction of apoptosis in the lung (H1299) and the pancreatic (Panc-1) carcinoma cell lines characterized by low COX-2 expression (Table I).

Effect of curcumin on COX-2, EGFR and ErbB-2 expression. Western blot analysis revealed detectable levels of EGFR in all four cell lines (Figure 3), whereas ErbB-2 was not detectable in these cell lines (data not shown). High COX-2 levels were found in the pancreatic p34 and the lung PC-14 cancer cell lines (Figure 3 A and B), while in the pancreatic Panc-1 and the lung H1299 cells COX-2 was not expressed (Figure 3 C and D). Curcumin decreased both COX-2 and EGFR expression in a dose-dependent manner in the p34 and the PC-14 cancer cell lines (Figure 3, A and B). In the Panc-1 and the H1299 cells curcumin did not alter EGFR expression (Figure 3 C and D).

Erk1/2 activity in p34 and PC-14 cells treated with curcumin. Since the Erk1/2 signaling pathway is common to both the EGFR and COX-2-signaling pathways, the next step was to test whether curcumin altered Erk1/2 activity. Curcumin treatment resulted in inhibition of p-Erk1/2 in a dose-dependent manner in both the p34 and PC-14 cell lines (Figure 4).

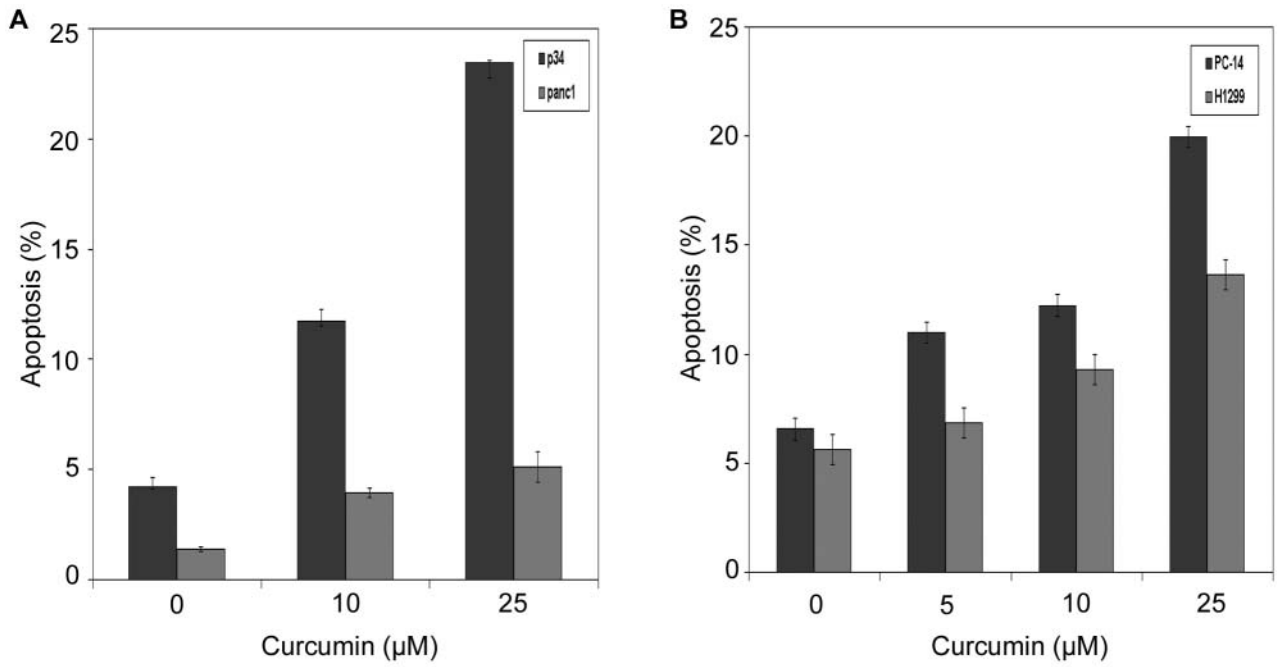

Figure 2. *Effect of curcumin on the induction of apoptosis in pancreas (A) and lung (B) adenocarcinoma cell lines expressing COX-2 (PC-14, p34) and COX-2 deficient (H1299, Panc-1). The cells were treated with different concentrations of curcumin and were harvested for an estimation of apoptotic cells by flow cytometry as described in the Materials and Methods section. The extent of apoptosis was assessed by the sub-G1 population. The values are means±SE from three individual experiments performed in triplicate. Significant differences ($p<0.05$) in the percentage of apoptotic cells after exposure to different concentrations of curcumin (Student's t-test) are observed.*

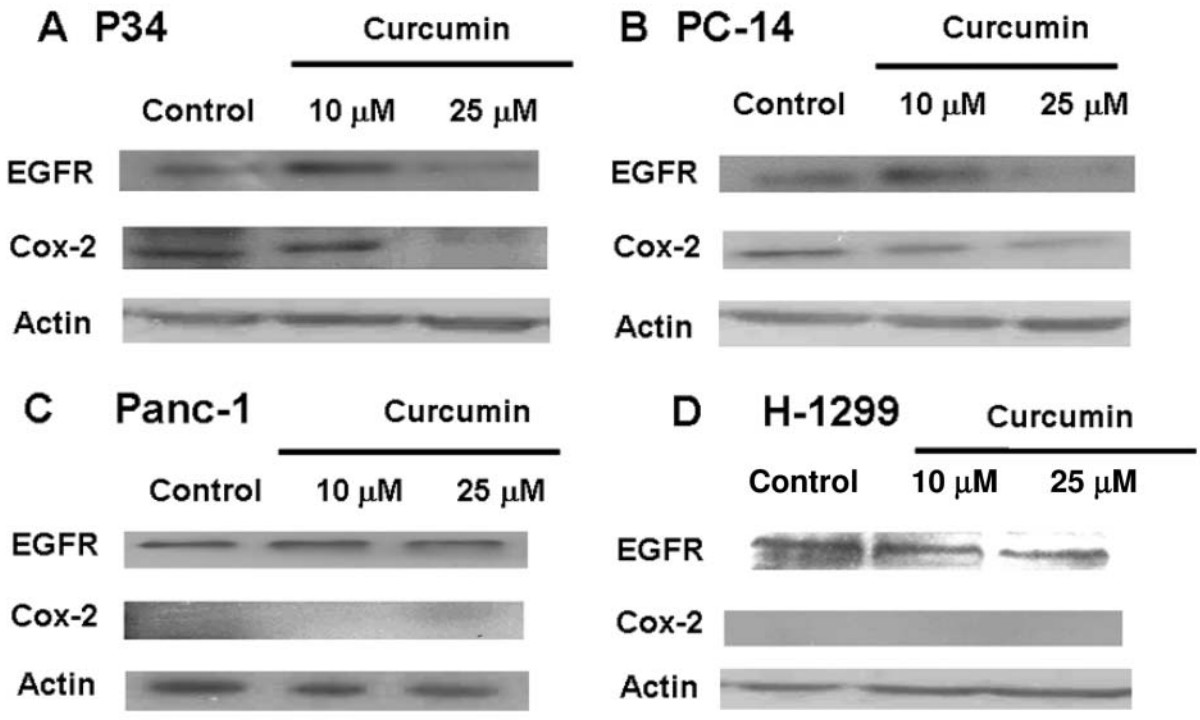

Figure 3. *Effect of curcumin on COX-2 and EGFR expression in p34 (A), PC-14 (B), Panc-1 (C) and H1299 (D) cell lines. The cells were treated with different concentrations of curcumin (0-25 μM) for 72 h and were then analyzed by Western blot as described in the Materials and Methods section. Lower panels: actin expression.*

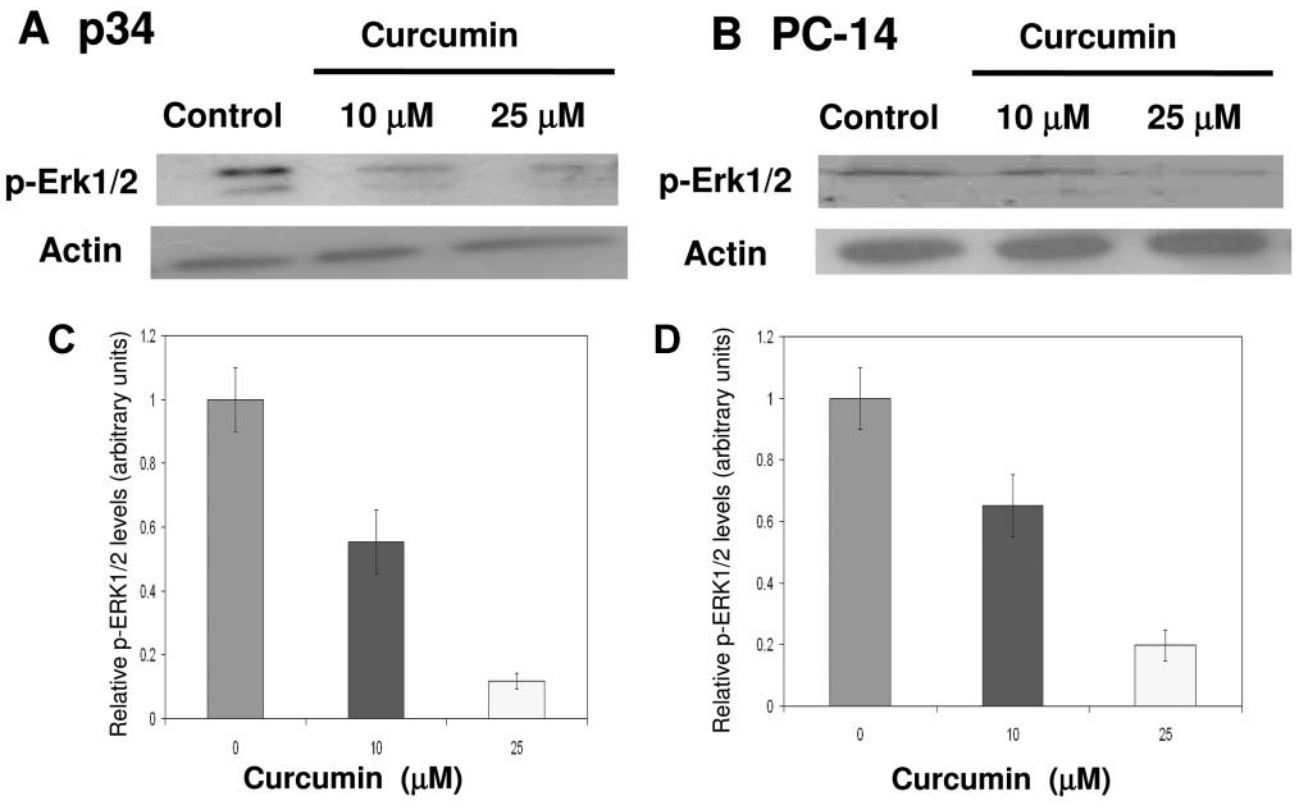

Figure 4. *Effect of curcumin on p-Erk1/2 expression in p34 (A) and PC-14 (B) cell lines. The cells were treated with different concentrations of curcumin (0-25 μM) for 72 h and were then analyzed by Western blot as described in the Materials and Methods section. Quantification of Western blot experiments in (C) p34 and (D) PC-14 cells. The values are expressed in relation to the respective control, which was given an arbitrary value of 1.*

Discussion

There is considerable evidence to suggest that both COX-2 and EGFR play a central role in the development and growth of various cancers, including tumors of the lung and pancreas and, as such, are rational targets for cancer treatment and prevention. In the current study, it was shown for the first time that curcumin inhibited cell survival in lung and pancreatic adenocarcinoma cell lines, an effect that was associated with the down-regulation of both COX-2 and EGFR and inhibition of Erk1/2 activity.

Our data demonstrated that the effect of curcumin on the survival of pancreatic and lung cancer cells and on the induction of apoptosis depended on the status of COX-2. The most significant effect was observed in cell lines that expressed high levels of COX-2 (PC-14 and p34), whereas only a weaker effect was seen in the H1299 and Panc-1 cells, which had a low expression of COX-2. This finding is of therapeutic significance since COX-2 is up-regulated at most stages of tumor progression in lung cancer (34-36) and in the majority of pancreatic carcinomas (37-39).

Recent studies have shown an intracellular cross-talk between the EGFR and COX-2 pathways (40, 41). Activation of EGFR signaling have been reported to lead to AP-1-induced COX-2 transcription and PGE_2 production (42). On the other hand, increased COX-2 transcription also resulted in enhanced production of PGE_2, which synergistically potentiated EGFR expression and activity by multiple pathways (43-45). Herein, we showed that curcumin co-inhibited COX-2 and EGFR expression in COX-2-positive cells (PC-14, p34), while EGFR expression was not affected by curcumin in COX-2-negative cells (H1299, Panc-1). Inhibition of COX-2-derived PGE_2 may have resulted in subsequent down-regulation of EGFR and modulation of EGFR downstream-signaling molecules, such as Erk1/2.

Several experimental and clinical studies were recently initiated to explore the role of co-targeting EGFR and COX-2 in cancer therapy (46-48). Torrance *et al.* (46) showed that combining COX-2 and EGFR inhibitors was more effective than using either agent alone for the suppression of the development of colorectal carcinoma *in vivo*. Similarly, Zhang *et al.* (47) demonstrated a cooperative

effect of the combined treatment on tumor progression *via* blocking both EGFR- and COX-2-related pathways in squamous cell carcinoma of the head and neck.

The three best-characterized signaling pathways induced through EGFR are the Ras-mitogen-activated protein kinase (Ras-MAPK), phosphatidylinositol 3' kinase-protein kinase B (PI3K-PKB/Akt), and phospholipase C-protein kinase C (PLC-PKC) pathways (49-54). Interestingly, several studies have shown that MAPK (Erk1/2) also played a role in the COX-2-signaling pathway (55, 56). On the one hand, activation of EGFR signaling was found to lead to increased MAPK activity, resulting in enhanced COX-2 expression (57, 58). On the other hand, COX-2 overexpression was recently shown to stimulate Erk phosphorylation in non-small cell lung cancer cells (32). Our data are in concordance with these studies. We demonstrated that the inhibitory effect of curcumin on COX-2 and EGFR expression in pancreatic and lung adenocarcinoma cell lines was associated with a decrease in Erk1/2 activity.

A number of studies have shown that NSAIDs and COX-2 inhibitors induced apoptosis in cancer cells *in vitro* and *in vivo* (59-61). Shiff *et al.* (59) have found that the growth inhibition of colon adenocarcinoma cells by NSAIDs was associated with apoptosis. Other studies have found that COX-2 inhibition was associated with the modulation of various pro- and anti-apoptotic factors, such as Bcl-2 (60) and caspase-3 (61). We observed a similar effect: the down-regulation of COX-2 in PC-14 and p34 cells by curcumin resulted in increased apoptotic cell death.

In conclusion, curcumin down-regulated both COX-2 and EGFR and decreased Erk1/2 activity. This inhibition was associated with decreased survival and enhanced induction of apoptosis in lung and pancreatic adenocarcinoma cells.

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