Chemotherapy Induces the Expression of Cyclooxygenase-2 in Non–Small Cell Lung Cancer

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Abstract

Purpose: To determine the effect of taxane-based chemotherapy on intratumoral levels of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) in patients with non–small cell lung cancer (NSCLC).

Experimental Design: Lung specimens obtained at the time of surgery were used to measure levels of COX-2 and PGE_2 in tumors and adjacent nontumorous tissues in three subsets of NSCLC patients who underwent: (A) surgical resection only ($n = 16$); (B) surgical resection after preoperative taxane-based chemotherapy ($n = 13$); or (C) surgical resection after preoperative chemotherapy coadministered with the selective COX-2 inhibitor, celecoxib 400 mg bid ($n = 17$).

Results: Levels of intratumoral PGE_2 were nearly 3-fold higher among patients who received preoperative chemotherapy compared with those treated by surgery alone ($P < 0.001$). This difference was abrogated by the addition of celecoxib to preoperative chemotherapy ($P < 0.001$). Amounts of intratumoral COX-2 were ~3-fold higher in groups of patients who received preoperative chemotherapy with celecoxib ($P < 0.0001$) or without celecoxib ($P < 0.001$), compared with the group who underwent surgical resection only. Importantly, statistically significant positive correlations between COX-2 and PGE_2 were observed in the surgery only ($r = 0.502$, $P = 0.047$) and preoperative chemotherapy groups ($r = 0.740$, $P = 0.004$); this correlation was abrogated when celecoxib was given with chemotherapy ($r = 0.005$, $P = 0.98$).

Conclusions: Treatment with chemotherapy led to increased amounts of COX-2 and PGE_2 in NSCLC. Cotreatment with celecoxib abrogated the increase in levels of PGE_2 but not COX-2 induced by chemotherapy. Importantly, these results clearly show that levels of a pharmacologic target (i.e., COX-2) can be affected by both the intrinsic molecular properties of a tumor and therapy.

There are two isoforms of cyclooxygenase (COX). The COX enzymes catalyze the first step in the synthesis of prostaglandins from arachidonic acid. COX-1 is constitutively expressed in most normal tissues and seems to mediate various physiologic functions (1). In contrast, COX-2 is absent in most normal tissues but is induced by a variety of mitogenic and proinflammatory stimuli (2–6). Increased levels of COX-2 and prostaglandin E_2 (PGE_2) have been observed in a variety of malignancies, including non–small cell lung cancer (NSCLC; refs. 7–19). Several mechanisms can potentially account for the tumor promoting effects of COX-2–derived prostaglandins. Prostaglandins can enhance tumor growth and metastasis by stimulating cell proliferation, angiogenesis, and invasiveness in addition to inhibiting apoptosis and immune surveillance (7, 20–26). Importantly, the formation and growth of tumors is reduced in mice engineered to be *COX-2* deficient (27–29). Moreover, selective inhibitors of COX-2 suppress the growth of experimental tumors (7, 30–34).

In addition to its potential role in tumor promotion and progression, overexpression of COX-2 may also reduce the response of malignant cells to cytotoxic therapy. For example, we have previously shown that taxanes induce COX-2 and prostaglandin biosynthesis *in vitro* and postulated that this may reduce the efficacy of taxanes (35). Theoretically, coadministration of a selective COX-2 inhibitor with taxane-based chemotherapy would overcome any decrease in efficacy related to taxane-mediated induction of COX-2. In support of this hypothesis, the addition of a selective COX-2 inhibitor augmented taxane-mediated inhibition of tumor growth in an experimental model of NSCLC (36). A phase II clinical trial suggested that the addition of a selective COX-2 inhibitor might enhance the response to preoperative paclitaxel and carboplatin in patients with NSCLC (37).

Despite the above findings, little is known about the effects of cytotoxic chemotherapy on levels of COX-2 and PGE_2 in human malignancies. In the current study, levels of COX-2 and

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PGE_2 were determined in three subsets of NSCLC patients who received different treatments: (a) primary surgical resection without preoperative chemotherapy, (b) surgical resection after preoperative taxane-based chemotherapy, and (c) surgical resection after preoperative taxane-based chemotherapy coadministered with the selective COX-2 inhibitor celecoxib.

To our knowledge, this study provides the first evidence in humans that chemotherapy leads to significant increases in levels of intratumoral COX-2 and PGE_2. Cotreatment with celecoxib abrogated the increase in levels of PGE_2 but not COX-2 induced by chemotherapy. Importantly, these results clearly show that levels of pharmacologic targets (e.g., COX-2) within tumors can be affected by both the intrinsic molecular properties of the tumor and therapy.

Materials and Methods

Materials. Rabbit polyclonal anti–COX-2 antiserum (PG-27) was from Oxford Biomedical Research (Oxford, MI) and COX-2 blocking peptide was from Cayman Chemical Co. (Ann Arbor, MI). Antisera to COX-1 and β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Indomethacin and 3,3′-diaminobenzidine were from Sigma Chemical Co. (St. Louis, MO). Secondary anti-rabbit biotinylated antibody was from Vector Laboratories, Inc. (Burlingame, CA). Streptavidin-horseradish peroxidase was from DAKO Corp. (Carpinteria, CA). Superfrost Plus slides were from Fisher Scientific (Pittsburgh, PA).

Patients and tissue specimens. Tissue specimens were obtained from a total of 46 NSCLC patients (16 surgery only, 13 surgery after chemotherapy, and 17 surgery after chemotherapy and celecoxib) who underwent surgery at the New York Presbyterian Hospital. There were no major differences among the three groups with respect to tumor cell type and stage distribution (Table 1). The 17 patients who underwent surgery after chemotherapy and celecoxib were part of a phase II clinical trial that has been previously described (37). Tumor tissue was not available from seven additional patients in this study who had significant pathologic responses. Briefly, patients in this trial received two cycles of paclitaxel and carboplatin 21 days apart. Paclitaxel at 225 mg/m^2 was given as a 3-hour infusion followed by a 1-hour i.v. infusion of carboplatin dosed to an area under the curve of 6 by the Calvert formula. Celecoxib was given orally at a dose of 400 mg twice daily, starting on the first day of chemotherapy until the morning of the surgical procedure. This protocol was reviewed and approved by the institutional review board at the Weill Medical College of Cornell University, and informed consent was obtained from all patients. Separate institutional review board approval was obtained to carry out COX enzyme and PGE_2 analyses on surgically resected lung tissues from patients who underwent surgery only or patients who received preoperative paclitaxel and carboplatin before undergoing surgery. For all patients who received preoperative chemotherapy, thoracotomy was done 3 to 6 weeks after the second cycle of chemotherapy. Samples of primary tumor and adjacent nontumorous lung at least 5 cm away from the tumor were immediately snap frozen and stored at −80°C until analysis.

Table 1. Clinical and pathologic characteristics

	Surgery ($n = 16$)	Preoperative chemotherapy ($n = 13$)	Preoperative chemotherapy + celecoxib ($n = 17$)
Median age (range, y)	67.5 (42-85)	63.0 (43-77)	64.0 (40-80)
Gender			
Male, *n* (%)	8 (50)	4 (31)	10 (59)
Female, *n* (%)	8 (50)	9 (69)	7 (41)
Histology			
Adenocarcinoma	11 (69)	10 (77)	10 (59)
Squamous cell carcinoma	4 (25)	3 (23)	5 (29)
Large cell	1 (6)	0 (0)	2 (12)
Stage			
I	8 (50)	3 (23)	9 (53)
II/III	8 (50)	10 (77)	8 (47)

Prostaglandin E_2 measurements. Levels of PGE_2 were determined as in previous studies (37). Briefly, frozen tissue was thawed in ice-cold buffer containing 150 mmol/L NaCl, 100 mmol/L TBS (pH 8), 1 mmol/L EDTA, and 10 μmol/L indomethacin. Indomethacin, a dual inhibitor of COX-1 and COX-2, was added to prevent *ex vivo* synthesis of PGE_2. Following homogenization, tissues were sonicated twice for 20 seconds on ice and centrifuged at 10,000 × *g* for 10 minutes at 4°C to remove particulate material. The protein concentration of the supernatant was measured using the Lowry protein assay kit (Sigma Chemical). Amounts of PGE_2 in the supernatant were determined by enzyme immunoassay according to the manufacturer's instructions (Cayman Chemical). Levels of PGE_2 were normalized to protein concentrations in the supernatant.

Western blotting. Frozen tissue was thawed in ice-cold lysis buffer [150 mmol/L NaCl, 100 mmol/L Tris (pH 8.0), 1% Tween 20, 50 mmol/L diethyldithiocarbamate, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL trypsin inhibitor, and 10 μg/mL leupeptin]. Tissues were sonicated for 20 seconds on ice and centrifuged at 10,000 × *g* for 10 minutes at 4°C to remove the particulate material. The protein concentration of the supernatant was measured using the method of Lowry et al. (38). Immunoblot analyses for COX enzymes and β-actin were done using methods described in previous studies (35). A computer densitometer (Gel Doc 2000, Bio-Rad, Hercules, CA) was used to quantify the density of the bands. Amounts of COX-2 are expressed in arbitrary units.

Immunohistochemistry. This was carried out using methods that have been reported previously (39). Neutral buffered formalin-fixed tissue was embedded in paraffin. Tissue sections (4 μm) were prepared using a microtome and mounted on Superfrost/Plus slides. Immunohistochemical analysis was done within 24 hours of the sections being cut. Sections were deparaffinized in xylene, rehydrated in graded alcohols, and washed in distilled water. Antigen retrieval was done by steaming the sections in 10 mmol/L citric acid (pH 6.0) for 30 minutes. Subsequently, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Slides were washed thrice in PBS and blocked for 20 minutes with 2% bovine serum albumin. Tissue sections were incubated with antiserum to COX-2 at a 1:1,000 dilution (2% bovine serum albumin in PBS) for 18 hours at 4°C. Control sections were incubated with COX-2 antiserum preabsorbed with a 100-fold excess of blocking peptide. After being washed thrice with PBS, the sections were incubated with biotinylated anti-rabbit antibody at a 1:500 dilution for 1 hour at room temperature. The slides were washed thrice in PBS and labeled using 1:500 streptavidin-horseradish peroxidase for 1 hour at room temperature. The reaction was visualized using 3,3′-diaminobenzidine. Subsequently, the slides were rinsed in tap water and counterstained with hematoxylin. The slides were then dehydrated with ethanol, rinsed with xylene, and mounted.

Statistical analysis. The data for PGE_2 levels (pg/μg protein) in normal and in tumor tissue and for COX-2 levels (arbitrary unit) were positive and had distributions that were skewed towards high values in each of the three groups. Because the data were skewed, means and SDs are not appropriate summaries of typical values of measurements within patient groups. We therefore present medians with nonparametric 95% confidence intervals (95% CI). Generalized γ distributions,

which allow for a great diversity in possible distribution shapes, were fit parametrically to the data by maximum likelihood using SAS Software Proc Lifereg (version 9.0, SAS Institute, Inc., Cary, NC). When generalized γ distributions were fit to data for each patient group, the scale and shape variables for each measurement (PGE_2 in tumor and adjacent normal tissues and intratumoral COX-2 expression) did not differ significantly between groups. This facilitated assessment of statistical significance of comparisons based on the location variables, which correspond to differences in the central values of log-transformed measurements. These differences are equivalent to the ratios of the central values on the original, raw data scale.

Results

Patients included in this investigation underwent surgery between September 1997 and May 2002. Patient characteristics are listed in Table 1. Among the surgery-only cases, males and females were equally represented; however, most patients undergoing preoperative chemotherapy were female (69%), whereas the majority of patients who received celecoxib in addition to preoperative chemotherapy were male (59%). Adenocarcinoma was the most prevalent histology in all groups. In general, there were no major differences among the three groups with respect to cell type and stage distribution.

Chemotherapy increases amounts of intratumoral prostaglandin E_2. The concentrations of PGE_2 were measured in both tumor tissues and adjacent normal tissues in the three defined groups of patients. As displayed in Fig. 1, PGE_2 levels in tumors were 2.78 times as high based on the fitted distributions among patients who received preoperative chemotherapy with median 384 pg/μg protein, and 95% CI (279-1,818) compared with tumors treated by surgical resection only with median 150 pg/μg protein (95% CI, 89-265; $P < 0.001$). Intratumoral PGE_2 levels were 3.77 times as high among chemotherapy patients compared with patients who received preoperative chemotherapy + celecoxib with median 112 (95% CI, 69-193; $P < 0.0001$). Thus, as previously reported (37), the addition of celecoxib to chemotherapy led to a normalization of levels of intratumoral PGE_2.

Chemotherapy increases cyclooxygenase-2 expression. In an attempt to elucidate the mechanism underlying the observed differences in intratumoral PGE_2 in the three groups of patients, Western blots for COX-2 were done. Levels of COX-2 were 3.33 times as high based on the fitted distributions in tumors after preoperative chemotherapy with median 8.2 and 95% CI of 0.6 to 14.0 compared with tumors from patients treated with surgery only with median 2.2 (95% CI, 1.0-4.1; $P < 0.001$) and were 2.74 times as high in tumors among chemotherapy patients treated with celecoxib compared with tumors from patients who underwent surgery only with median 5.0 (95% CI, 3.6-7.0; $P < 0.0001$; Fig. 2). Thus, the increased levels of intratumoral PGE_2 observed after preoperative chemotherapy were associated with a significant increase in amounts of intratumoral COX-2. Remarkably, the addition of celecoxib at 800 mg/d to chemotherapy reduced the levels of intratumoral PGE_2 to those found in adjacent normal lung (Fig. 1) despite persistent overexpression of COX-2 in the tumors (Fig. 2). These findings are consistent with celecoxib inhibiting COX-2 enzyme activity not protein expression. In Fig. 3, representative results are shown from tumors in each of the three patient groups to illustrate the main findings of the study. Preoperative chemotherapy led to increased amounts of intratumoral COX-2

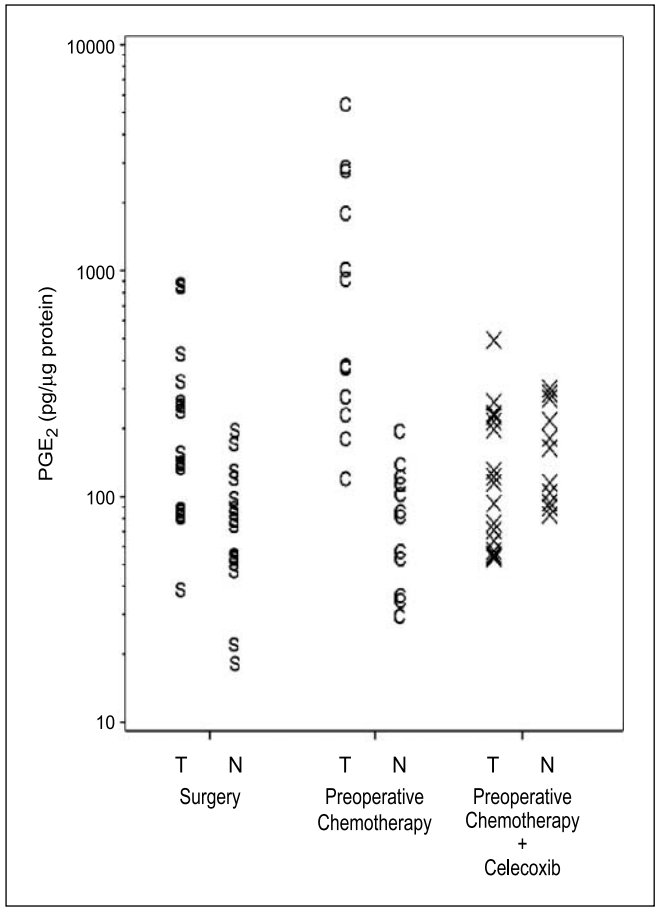

Fig. 1. Concentrations of intratumoral PGE_2 are elevated in tumors from patients treated with preoperative chemotherapy compared with those treated by surgical resection alone and those treated with preoperative chemotherapy + celecoxib. At the time of surgery, tumor (*T*) and adjacent nontumorous (*N*) tissue were obtained from NSCLC patients who received surgery alone ($n = 16$), preoperative chemotherapy ($n = 13$), or preoperative chemotherapy and celecoxib ($n = 17$). Log-transformed data for each individual patient are displayed within study groups: S, surgery; C, preoperative chemotherapy; and X, preoperative chemotherapy + celecoxib. Levels of intratumoral PGE_2 were increased in tumors from patients treated with preoperative chemotherapy compared with those treated by surgery alone ($P < 0.001$) and those treated with preoperative chemotherapy + celecoxib ($P < 0.0001$).

and PGE_2 compared with tumors from patients who underwent surgery only. The addition of celecoxib to preoperative chemotherapy led to a significant reduction in intratumoral PGE_2 although COX-2 levels were elevated compared with tumors from patients who underwent surgery only. In contrast to COX-2, levels of COX-1 were similar among the three groups. In untreated NSCLC, COX-2 is overexpressed in neoplastic epithelial cells (14, 17). Given the discovery that preoperative chemotherapy led to increased amounts of COX-2, immunohistochemistry was carried out to localize COX-2. Theoretically, chemotherapy might have altered expression in neoplastic cells, stroma, or both. As shown in Fig. 4, the expression of COX-2 was exclusively limited to neoplastic cells with no stomal staining. Immunoreactivity was lost when the antiserum to COX-2 was preincubated with a COX-2 blocking peptide.

Chemotherapy-based induction of cyclooxygenase-2 and prostaglandin E_2 occurs in a subset of patients with non–small cell lung cancer. As shown in the scatter plots in Fig. 5,

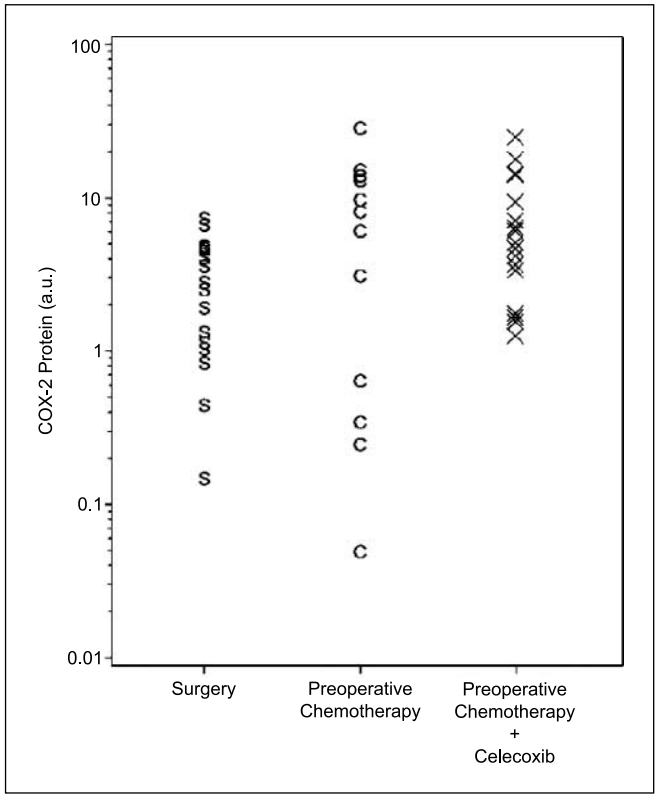

Fig. 2. Amounts of intratumoral COX-2 were significantly higher in both patient groups treated with preoperative chemotherapy, with or without celecoxib treatment, compared with those treated by surgical resection alone. Levels of COX-2 were determined by immunoblot analysis and quantified in arbitrary units (*a.u.*). Log-transformed data for each individual patient are displayed within study groups: S, surgery; C, preoperative chemotherapy; and X, preoperative chemotherapy + celecoxib. Levels of COX-2 were increased in tumors after preoperative chemotherapy with ($P < 0.0001$) or without celecoxib ($P < 0.001$) compared with tumors from patients treated with surgery only.

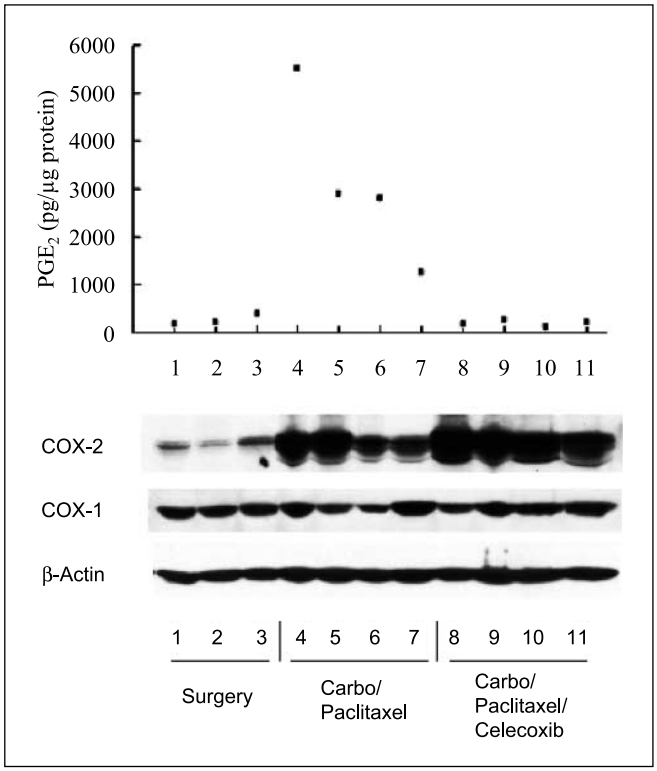

Fig. 3. Preoperative chemotherapy, with or without celecoxib treatment, induced COX-2. Lanes 1 to 3, representative tumor samples from patients treated by surgical resection only. Lanes 4 to 7, representative tumor samples from patients treated by preoperative chemotherapy followed by surgical resection. Lanes 8 to 11, representative tumor samples from patients treated by preoperative chemotherapy + celecoxib followed by surgery. Levels of COX-2 and COX-1 were determined by immunoblot analysis. Intratumoral levels of PGE_2 from the same cases (cases 1-11) were determined by enzyme immunoassay.

chemotherapy-mediated induction of COX-2 and PGE_2 was only observed in a subset of patients. For example, COX-2 levels were similar to those found in the surgery alone group (Fig. 5A) in ~7 of 13 patients in the chemotherapy only patients (Fig. 5B) and 12 of 17 patients treated with chemotherapy + celecoxib (Fig. 5C). However, a total of 11 of 30 patients who received preoperative chemotherapy with or without celecoxib had COX-2 levels that exceeded COX-2 levels in any patient in the surgery alone group.

***Cyclooxygenase-2 is the rate-limiting step in prostaglandin E_2 synthesis* in vivo.** To determine whether COX-2 is a rate-limiting enzyme in PGE_2 biosynthesis in NSCLC, the levels of intratumoral PGE_2 were plotted against the amounts of COX-2 as determined by Western blotting for each of the three groups of patients. As seen in Fig. 5A and B, there was a positive correlation between levels of PGE_2 and COX-2 levels in the surgery only group ($r = 0.502$, $P = 0.047$) as well as in patients treated by preoperative chemotherapy only ($r = 0.740$, $P = 0.004$). This positive correlation was abrogated when celecoxib was given with chemotherapy ($r = 0.005$, $P = 0.98$; Fig. 5C).

Discussion

To our knowledge, this study provides the first evidence in humans that treatment with chemotherapy can enhance intratumoral levels of COX-2 and PGE_2. This finding has both practical and theoretical implications. Previous studies have shown that levels of COX-2 and PGE_2 are commonly increased in different tumor types (7–19, 40–43). Generally, the magnitude of these increases is quite variable both within and between tumor types (8, 11, 13, 39). The current findings imply that intratumoral levels of COX-2 and prostanoids need to be interpreted in the context of whether cytotoxic therapy was given before tissue acquisition.

Numerous *in vitro* studies have shown that either oncogene activation or tumor suppressor gene inactivation result in enhanced expression of COX-2 and increased synthesis of PGE_2 (7, 40, 44, 45). An effort has been made to translate these *in vitro* findings to human tumors. For example, both overexpression of HER-2/*neu* and mutation of *TP53* have been associated with elevated levels of COX-2 in human malignancies (44, 46, 47). Based on the findings in this study, it is clear that the ability to identify molecular determinants of COX-2 expression will be compromised if samples are obtained from subjects with varying treatment histories. Therefore, future correlative studies need to be done with a detailed knowledge of any treatment that was given before the tumor sample was obtained.

We observed a positive correlation between intratumoral COX-2 expression and PGE_2 levels in patients who were treated with surgery alone (Fig. 5A) or preoperative chemotherapy

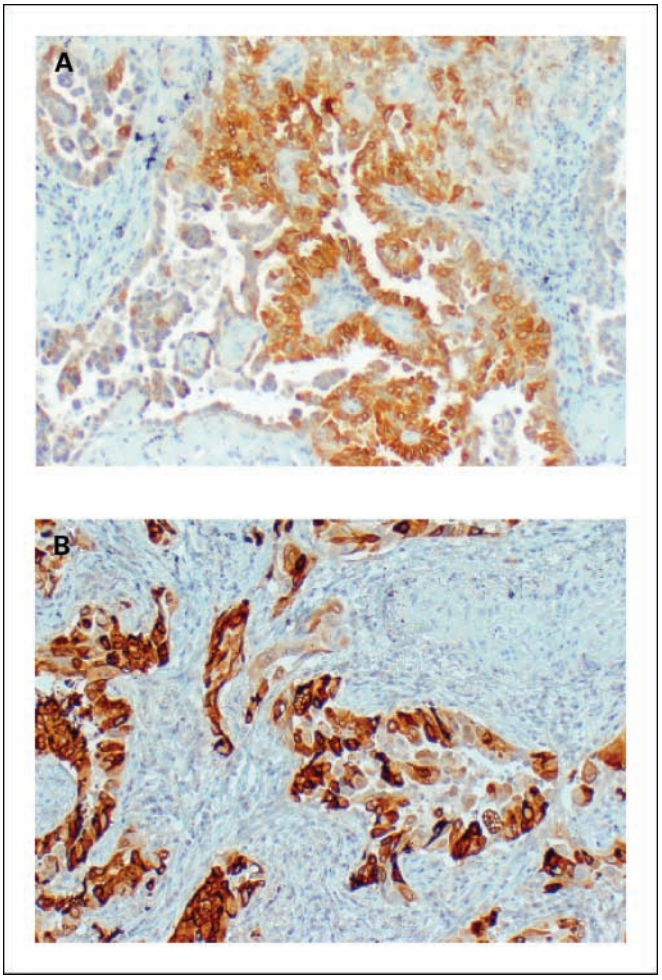

Fig. 4. COX-2 is expressed in neoplastic cells in NSCLC. Immunohistochemistry was used to evaluate COX-2 expression in tumor samples from patients treated with surgery alone or preoperative chemotherapy followed by surgery. Representative sections. *A,* adenocarcinoma treated by primary surgical resection. *B,* poorly differentiated squamous cell carcinoma after preoperative chemotherapy. Both show localization of COX-2 expression to neoplastic cells.

followed by surgery (Fig. 5B). Notably, the addition of celecoxib 400 mg bid to chemotherapy abrogated this positive correlation (Fig. 5C). This dose was chosen because it was previously reported to cause a reduction in the number of colorectal adenomas in a clinical trial (48). These findings in human tissues are consistent with existing *in vitro* evidence that COX-2 is rate limiting for PGE_2 synthesis in tumor cells. It is intriguing to note that the inductive effect of chemotherapy on levels of COX-2 and PGE_2 was only observed in a subset of tumors. Tumor tissue was unavailable from patients who had significant pathologic responses to preoperative chemotherapy. This limited our ability to determine whether intratumoral levels of COX-2 correlated with clinical response. Differences in amounts of COX-2 following chemotherapy could simply reflect the biological heterogeneity of tumors. Alternatively, chemotherapy may have induced COX-2 and PGE_2 in virtually all cases with normalization of levels in some tumors during the interval of time between the last dose of chemotherapy and surgery. Recently, levels of urinary PGE-M, a stable end metabolite of PGE_2, were reported to reflect systemic production of PGE_2 (49). Future studies can be done to determine if

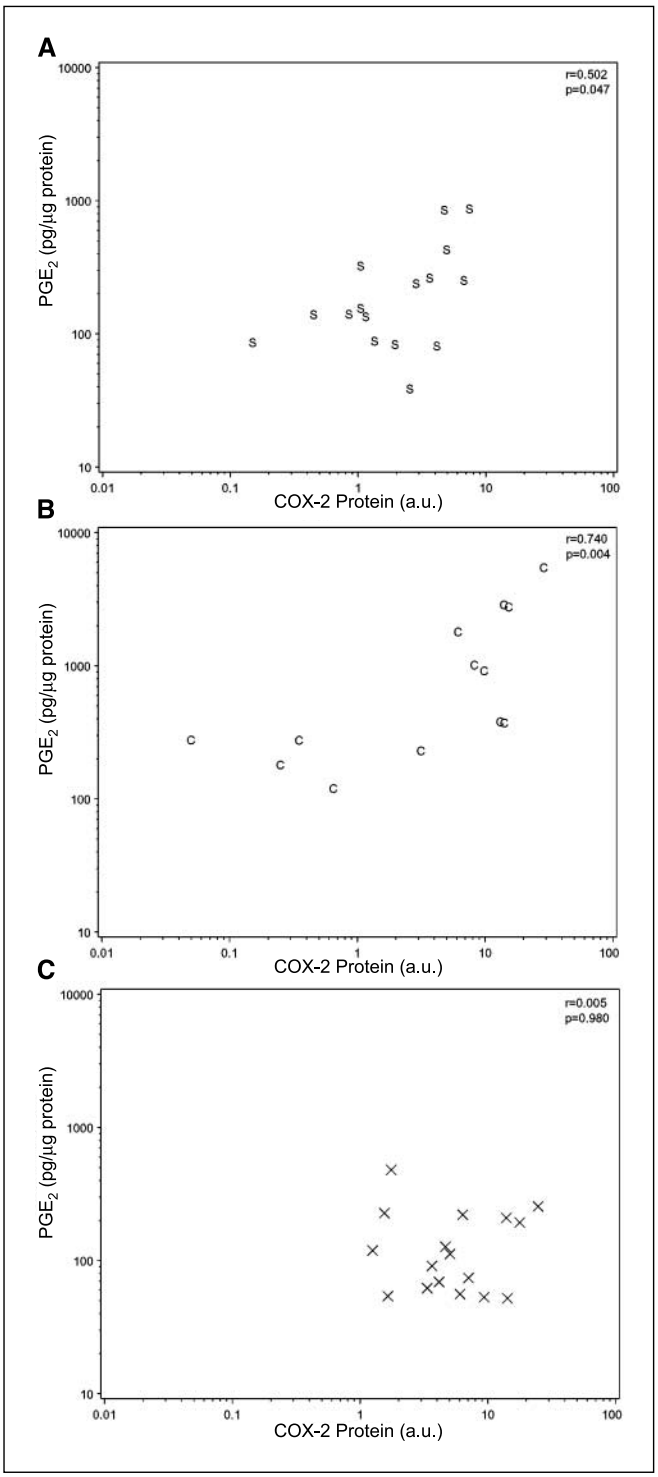

Fig. 5. Scatter plots show the correlation between COX-2 and PGE_2 levels in patients treated by (*A*) surgery only, (*B*) preoperative chemotherapy, or (*C*) preoperative chemotherapy + celecoxib. The positive correlation between COX-2 and PGE_2 in the surgery and preoperative chemotherapy groups was abrogated when celecoxib was given with chemotherapy. Data for each individual patient are displayed within study groups: S, surgery; C, preoperative chemotherapy; and X, preoperative chemotherapy + celecoxib.

cytotoxic chemotherapy induces levels of urinary PGE-M. If so, serial measurements should help to further elucidate the relationship between chemotherapy and deregulated PGE_2 production.

Another interesting question concerns the mechanism(s) underlying the observed increase in amounts of COX-2 in some tumors following chemotherapy. Both deregulated transcriptional and post-transcriptional mechanisms can affect levels of COX-2 (40, 50, 51). In fact, taxanes can both enhance *COX-2* transcription and stabilize COX-2 message *in vitro* (35, 52). Previous studies have suggested that taxanes activate COX-2 transcription by an activator protein-1-dependent mechanism (35). Increased binding of HuR to the COX-2 3′-untranslated region was found to contribute to taxane-induced stabilization of COX-2 mRNA (52). Whether either of these mechanisms is operative *in vivo* should be evaluated. It also is important to acknowledge that the mechanism by which chemotherapy induces COX-2 in tumors *in vivo* may be quite different than the mechanism that was identified during short-term *in vitro* studies. For example, the increase in COX-2 following chemotherapy could be a nonspecific consequence of tissue injury. Interestingly, when compared with surgery alone, treatment with chemotherapy led to increased amounts of COX-2–derived PGE_2 in tumorous but not in normal tissue. This finding is consistent with the ability of cytotoxic chemotherapy to cause greater injury of tumor versus normal cells.

The primary observation of this study, that taxane-based chemotherapy induces COX-2 and PGE_2 in patients with NSCLC, may have important clinical implications. Although cytotoxic therapy may result in cell death in a population of cancer cells, it might induce or repress a number of factors that influence therapeutic efficacy or even further accelerate the emergence of a metastatic phenotype. For example, COX-2 expression may not only inhibit chemotherapy-mediated apoptosis but also promote metastatic clones by enhancing the expression of CD_{44} and matrix metalloproteinases (53, 54). Importantly, in an era of heightened interest in targeted therapy, our data strongly suggest that levels of molecular targets may not be static. This study suggests that molecular changes induced by cytotoxic therapy may affect either the optimal dose or schedule of administration of a targeted therapeutic.

Recently, prolonged use of selective COX-2 inhibitors has been associated with an increased risk of cardiovascular complications including myocardial infarction and stroke. In every clinical situation, the potential risk/benefit ratio of a medication needs to be considered. This is certainly true for NSCLC patients who frequently have a very poor prognosis. Although the current study was not designed to evaluate clinical efficacy, it did show the ability of celecoxib to effectively suppress intratumoral COX-2 activity. Clinical trials are needed to determine whether the promising anticancer activity of celecoxib shown in preclinical studies (7, 40) translates into clinical benefit for cancer patients.

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