Cyclooxygenase-2 expression is abundant in alveolar type II cells in lung cancer-sensitive mouse strains and in premalignant lesions

Sarah A. Wardlaw, Thomas H. March and Steven A. Belinsky

Lovelace Respiratory Research Institute, PO Box 5890, Albuquerque, NM 87185, USA

© Oxford University Press 1371

Carcinogenesis vol. 21 no. 7 pp. 1371–1377, 2000

Overexpression of cyclooxygenase-2 (COX-2) is seen in a high percentage of human colon tumors, lung adenocarcinomas and other cancers. Inhibition of this enzyme represses human colon tumorigenesis and decreases lung tumor multiplicity in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-exposed A/J mice. The purpose of this investigation was to characterize the expression of cyclooxygenase-2 (COX-2) during tumor progression in the A/J mouse lung and to compare the results with expression in other cancer-susceptible and several cancer-resistant mouse strains. Analysis of normal A/J mouse lung showed that type II alveolar epithelial cells express high levels of COX-2 protein and mRNA, indicating that COX-2 is present constitutively in this tumor progenitor cell prior to any carcinogen exposure. Examination of lung-cancer-resistant (C3H/HeJ, C57BL/6J, DBA/2J) and other lung-cancer-susceptible (A/WySnJ, SWR/J) strains showed similar levels of COX-2 mRNA expression in the three susceptible strains and lower levels of expression in two of the resistant strains, indicating a possible correlation between COX-2 expression in type II cells and lung cancer susceptibility. COX-2 protein expression was observed in A/J lung tumors at all stages of development. Variation and occasional absence of protein expression were also observed in A/J lung tumors, particularly in adenomas and adenocarcinomas, suggesting that COX-2 is not obligatory for maintenance of the malignant phenotype. In support of this conclusion, treatment of xenografted cell lines derived from malignant murine pulmonary tumors with COX-2 inhibitors produced only a slight repression of growth. However, the frequent expression of COX-2 in early lesions in the A/J mouse lung combined with the known reduction in tumor number in animals treated with COX-2 inhibitors prior to carcinogen exposure indicate that COX-2 could be a promising target for lung cancer chemoprevention. In addition, high levels of COX-2 expression in the normal tumor-progenitor cells of lung-cancer-sensitive mice indicate that COX-2 may play a role in lung cancer susceptibility.

Introduction

Lung cancer is currently the leading cause of death due to cancer in the USA. With 80 million current and former smokers in the USA today, and half of lung cancer diagnoses occurring in former smokers, the population at risk of developing lung cancer remains large. Developments in the treatment of lung cancer have not produced a significant increase in survival rate over the past 25 years. Therefore, one key to reducing lung cancer mortality is the development of methods for the early detection and prevention of lung cancer.

A new class of compounds targeting cyclooxygenase-2 (COX-2) has recently shown promise for the chemoprevention of several solid tumors. Cyclooxygenases catalyze the rate-limiting step in the synthesis of prostaglandins from arachidonic acid. The COX-2 isomerase is generally expressed at low or undetectable levels and induced in response to environmental insults and internal stimuli like cytokines and growth factors. In contrast, COX-1 is constitutively expressed in most tissues and involved in cellular homeostasis. Epidemiological studies have revealed a reduced incidence of colon cancer in people who regularly ingested aspirin, a non-steroidal anti-inflammatory drug (NSAID) (1), and clinical studies have shown the regression of pre-existing colorectal adenomas in patients with familial adenomatous polyposis treated with NSAIDs (2). NSAIDs also significantly reduce tumor multiplicity in animal models of colorectal cancer (3,4).

The anti-inflammatory effects of NSAIDs are mediated in part by inhibition of COX-1 and -2. Approximately half of human colorectal adenomas and 80–90% of adenocarcinomas express high levels of COX-2 relative to normal epithelium (5). Similarly, COX-2 protein and mRNA expression levels are also elevated in most adenocarcinomas and in about one-third of premalignant lesions in human lung (6,7). Squamous cell carcinomas, but not small cell carcinomas of the lung also express COX-2 (6,7). NSAIDs and a specific inhibitor of COX-2 (NS-398) inhibit both in vitro and in vivo growth of murine and human lung-cancer-derived cell lines (8,9), suggesting that this pathway may be a good target for chemoprevention in the lung.

Because the in vivo target for chemopreventive compounds is premalignant lung disease, studies in animal models could be invaluable for evaluating the efficacy of the compounds and the interactions between the COX-2 pathway and tumor progression. One model that holds promise for these studies is the A/J mouse. The induction of lung tumors in the A/J mouse progresses through several morphologically distinct stages. Tumor initiation by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is characterized first by a proliferation of type II cells along the alveolar septae. These premalignant hyperplastic lesions contain an activated Ki-ras gene, an alteration present in some human adenocarcinomas, and progress to adenomas and ultimately carcinomas (10). Recent studies have shown that treatment of A/J mice with NSAIDs or NS-398, beginning 2 weeks prior to NNK exposure, decreased lung tumor multiplicity and volume (8). The purpose of our investigation was to extend these findings by delineating COX-2 expression patterns...
during murine tumor progression, comparing expression seen in A/J lungs to expression in other mouse strains and human tumors, and by examining the effect of a specific COX-2 inhibitor on gene transcription and enzyme activity in xenografted murine lung tumors.

Materials and methods

Sample preparation and RNA isolation

Murine type II alveolar epithelial cells were isolated by protease digestion of six pooled lungs (per strain) followed by sequential centrifugal elutriation as described previously (11).

Lung tumors were diagnosed in 6-week-old A/J and C57Bl/6J mice by i.p. injection of 50 µg/kg NNK three times per week for 7 weeks. Lung tumors were collected 20–68 weeks after initiation of treatment. Lungs were fixed by inflation with 4% paraformaldehyde, embedded in paraffin and cut into 5 µm sections. Dissected tumors were stored at −80°C.

The cell lines CL13, CL25, CL30 and I033 were derived from A/J lung tumors induced by NNK. The MNNK cell line is an NIH 3T3 transformant generated by transfection of A/J lung tumor DNA containing a mutated Ki-ras gene. The Spon4 cell line was derived from a spontaneously arising A/J lung tumor.

Total RNA was isolated from type II cells, tumors and cell lines by homogenization in TRI Reagent (Molecular Research Center, Cincinnati, OH), phenol/chloroform extraction and isopropanol precipitation. RNA pellets were then resuspended in buffer, incubated with DNase I, extracted with phenol/chloroform, precipitated with isopropanol and resuspended at −80°C.

Immunohistochemistry

Human and murine lung sections were deparaffinized in xylene and rehydrated in a graded series of ethanol/H2O baths. Sections were incubated for 30 min at room temperature in 1% hydrogen peroxide in methanol, rinsed in H2O, then incubated in boiling antigen retrieval citra solution (Biogenex, San Ramon, CA) for 5 min. Sections were cooled to room temperature, rinsed in H2O, then incubated in 1% hydrogen peroxide in methanol, rinsed in H2O, and then overnight at 4°C in a blocking solution composed of 1.5% normal goat serum in AB. A polyclonal rabbit anti-mouse COX-2 antibody (Cayman Chemical Co., Ann Arbor, MI), diluted 1:600 in primary antibody diluting goat serum in AB. A polyclonal rabbit anti-mouse COX-2 antibody (Cayman Chemical Co.) was diluted 15 000 in AB as described in the instructions accompanying the VectaStain Elite H PAP/DAB kit (Vector Laboratories, Burlingame, CA) diluted 1:500 in PAPD. After rinsing with AB the sections were incubated for 30 min at room temperature with Avidin DH: biotinylated horseradish peroxidase complex and then overnight at 4°C in a blocking solution composed of 1.5% normal goat serum in AB. A polyclonal rabbit anti-mouse COX-2 antibody (Cayman Chemical Co., Ann Arbor, MI), diluted 1:600 in primary antibody diluting buffer (PABD; Biomeda), was then applied to the sections. Serial sections were incubated with purified rabbit IgG as immunostaining controls. After 2 h the sections were rinsed with AB and incubated for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:500 in PABD. After rinsing with AB the sections were incubated for 30 min at room temperature with Avidin DH: biotinylated horseradish peroxidase H complex in AB as described in the instructions accompanying the VectaStain Elite ABC kit (Vector Laboratories). The sections were rinsed in H2O, and a diaminobenzidine (DAB) solution was prepared and applied as instructed (DiCaIn, DiCaIn Laboratories). After removal of this solution, the sections were rinsed in H2O, counterstained with hematoxylin, dehydrated and coverslipped with a xylene-based mounting medium. Sections incubated with rabbit IgG rather than primary antibody showed little or no staining.

Pre-incubation of primary antibody with increasing concentrations of purified peptide antigen (Cayman Chemical Co.) resulted in decreased and, ultimately, a complete absence of staining.

Staining intensity in hyperplastic lesions and neoplasms was subjectively graded in two parts consisting of intensity and distribution. Intensity was graded on a scale of 0–4+, with 4+ being the most intense (bronchiolar epithelium as a positive control). Distribution of the staining among the cells in a lesion was subjectively graded on a percentage basis (i.e. 1, <25% of the lesion affected; 2, 25–50%; 3, 50–75%; 4, 75–100%). The product of the intensity and distribution gave a subset of the score (e.g. 25–50% of cells in a lesion stained with a 4+ intensity; product = 8; 25–50% of cells stained with a 3+ intensity; product = 6). For the score of an entire lesion, the subsets were summed.

Ribonuclease protection assay

A COX-2-specific cDNA probe was prepared by PCR amplification of a 300 bp region of a commercially available 1.2 kb COX-2-specific cDNA probe (Cayman Chemical Co.). The resulting cDNA was cloned into a PCR II vector using a TA Cloning Kit (Invitrogen, Carlsbad, CA). Ligation was confirmed by restriction enzyme digestion, as well as sequencing. The insert was then transferred to a pTFRamp18 vector (Ambion, Austin, TX). Linearized plasmid was gel purified using a QIAquick Gel Extraction Kit (Qiagen, Santa Clarita, CA), then 32P-labeled using a MAXiScript In vitro Transcription Kit (Ambion). Radiolabeled transcript was gel purified and combined with sample RNA (3–10 µg) as described in the RPA III Kit protocol (Ambion). Following incubation, unhybridized RNA was digested with ribonuclease, then hybridized RNAs were separated on a 5% acrylamide/8 M urea Tris–borate–EDTA gel. The gel was dried and exposed to film. Densitometry was performed using an Alpha Innotech scanning densitometer and accompanying software. A 103 bp radiolabeled cyclophilin probe prepared from a commercially available template (pTRI-cyclophilin, Ambion) was included in all hybridization reactions as a positive/gel-loading control. Yeast RNA was used as a negative control. Radiolabeled marker transcripts were prepared using a Century Marker Template Set (Ambion). All samples were analyzed at least twice.

Lung tumor xenografts

Nude mice were injected s.c. with ~2.5 million CL30 or Spon4 cells in a volume of 0.1 ml. When tumors were palpable, inhibitors were injected i.p. three times per week for 2–3 weeks. Tumor area was measured twice per week. For each cell line, four mice were treated with vehicle only (0.5% methylcellulose, 0.025% Tween-20), pyroline dithiocarbamate (PDTC, 200 mg/kg; Sigma, St Louis, MO), SC-236 (2 mg/kg; generously provided by Searle-Monsanto, Skokie, IL), or a combination of both PDTC and SC-236. Following treatment, the mice were killed and the xenografts were excised. Portions of each xenograft were quick-frozen and stored at −80°C, and the remainder fixed in formalin.

DNA fragmentation analysis

Apoptotic cells were identified in paraffin-embedded tissue sections using the Klenow-FragEL DNA Fragmentation Detection Kit (Calbiochem, San Diego, CA). 3'-OH groups generated by apoptotic endonucleases were labeled with horseradish peroxidase and visualized with DAB as described in the protocol accompanying the kit. Three sham- and three SC-236/PDTC-treated Spon4 xenografts were analyzed. Apoptotic cells were identified based on positive DAB staining as well as characteristic cellular morphology. Apoptotic cells were counted in 400× microscopic fields. Twenty serial fields were examined per tumor.

Prostaglandin E2 (PGE2) quantitation

Portions of frozen excised xenografts were homogenized on ice in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1 µg/ml leupeptin. The homogenate was then centrifuged at 10 000 g for 20 min at 4°C. The supernatant was re-centrifuged at 10 000 g for 10 min. The resulting supernatant was aliquotted, quick-frozen and stored at −80°C. Protein concentration was determined using a Bradford-based protein assay (Bio-Rad, Hercules, CA). PGE2 concentration was measured using a competitive enzyme immunoassay kit (Cayman Chemicals). The xenograft supernatant was diluted 15 000× prior to analysis by immunoassay. Samples were assayed twice in duplicate. PGE2 concentration was linear with volume of sample assayed, indicating no interfering substances in the xenograft supernatants at this dilution.

Statistical analysis

Statistical analyses comparing data derived from two groups (treated and untreated) were conducted using the Student’s t-test (two-tailed, unpaired, equal variance). Statistical analysis of xenograft growth over time, with and without inhibitor treatment, was conducted by means of a repeated measures analysis of variance. The single, independent variable used was treatment group. Multivariate significance was assessed using the Hotelling–Lawley Trace. A multivariate contrast was used to evaluate trend.

Results

COX-2 protein in A/J type II cells and lung tumors

A/J mouse normal lung tissue, hyperplastic lesions and neoplasms were stained for COX-2 immunoreactivity using a monoclonal antibody and ABC methods. In normal lung tissue, bronchiolar epithelial cells stained most intensely with dark, evenly stained cytoplasmic compartments (Figure 1A and E), whereas type II epithelial cells generally stained less intensely with faint to moderately stained cytoplasm (Figure 1A). Staining of individual cells in hyperplastic and neoplastic lesions generally resembled that in normal type II cells, but intensity of staining across lesions often varied with unevenly distributed faintly and/or intensely staining cells (Figure 1B–F). The distribution of positively stained cells in hyperplastic lesions was often more widespread than in the neoplasms (Figure 1B). Staining intensity was often greater on the peripheries of neoplasms (Figure 1C), where
type II epithelial hyperplasia was often intermingled with neoplastic cells. In addition, the papillary and solid carcinomas had qualitatively different staining patterns. Papillary tumors had limited, often peripheral regions where cells stained diffusely (Figure 1E), whereas mixed tumors with solid areas displayed scattered individual and small groups of cells that stained intensely (Figure 1F). Solid tumors also had peripheral areas of papillary differentiation that stained similarly to the peripheral regions of pure papillary carcinomas. The degree of immunoreactivity, based on the intensity and distribution of the stained cells, was graded semiquantitatively (Materials and methods) in 60 hyperplastic lesions, 45 adenomas and 37 carcinomas from the lungs of 12 A/J mice. Although differences in staining patterns were apparent by subjective evaluation, there was no significant difference in the overall grading scores for COX-2 immunoreactivity between hyperplastic lesions, adenomas or carcinomas (average scores ± SE: hyperplasias = 9.3 ± 0.8, adenomas = 7.6 ± 0.8, carcinomas = 8.6 ± 0.7). However, 22% of hyperplasias had scores ≥15, compared with 7% of adenomas and 8% of carcinomas, indicative of intense, homogeneous immunoreactivity (Figure 2).

NNK-induced pulmonary tumors from C3H/HeJ mice, a strain with low incidence of spontaneous pulmonary neoplasia, were also evaluated for COX-2 immunoreactivity. Staining intensities and distribution in tumors from these mice were similar to those in neoplasms in the A/J strain. However, type II epithelial cells in non-neoplastic C3H pulmonary tissue stained with much less intensity than type II cells in sections from A/J mouse lungs (data not shown).

In addition to the COX-2 immunostaining of mouse lung tumors, specimens from several human pulmonary neoplasms were evaluated by immunohistochemistry for COX-2 expression. Like the mouse tumors, human tumors displayed variation in staining patterns and intensity (data not shown), with papillary tumors showing locally extensive regions of rather diffuse staining and more solid tumors (such as squamous cell carcinomas) displaying multifocal individual cell staining.
Human type II and bronchiolar epithelial cells were routinely negative for COX-2 immunoreactivity.

### COX-2 mRNA in murine type II cells, lung tumors and lung tumor-derived cell lines

COX-2 mRNA levels were measured in A/J mouse alveolar type II cells, lung tumors and lung-tumor-derived cell lines to determine whether the heterogeneity seen for COX-2 protein expression was due to differences in transcript levels. In addition to A/J cells, type II cells from two other lung-cancer-sensitive mouse strains (A/WySn and SWR/J) and three lung cancer-resistant mouse strains (C3H/HeJ, C57BL/6J and DBA/2J) were analyzed to determine whether COX-2 levels in normal type II cells correlate with lung cancer susceptibility. Similarly high levels of COX-2 mRNA were found in the type II cells from all three lung-cancer-sensitive mouse strains (Figure 3A and B) [A/J level set at 100%, A/WySnJ = 114 ± 8% (average of two measurements ± range), SWR/J = 116 ± 16%]. COX-2 mRNA levels in the lung-cancer-resistant strains were 19 ± 8% and 57 ± 11% of A/J levels in the C3H/HeJ and C57BL/6J strains, respectively. In contrast, COX-2 mRNA levels in the resistant DBA/2J strain were higher than A/J levels (168 ± 3%).

COX-2 mRNA levels in tumors isolated from NNK-treated A/J mice varied (Figure 3D), reflecting the heterogeneity seen for COX-2 protein in A/J lung tumors. Transcript levels were measured in 17 adenocarcinomas. The eight tumors shown in Figure 3D display a five-fold variation in mRNA levels (not including Tumor 1 with a transcript level below detection limits). Further examination of nine tumors revealed a seven-fold variation in COX-2 message levels in six of those tumors and transcript levels below detection limits in three tumors (data not shown). There did not appear to be a correlation between tumor size and mRNA levels.

COX-2 mRNA levels also varied among the six lung-tumor-derived cell lines examined (Figure 3C). Expression varied by an average of 35-fold, with the highest level seen in the Spon4 cell line that was derived from a spontaneously arising A/J mouse lung tumor.

### Effect of COX-2 inhibitors on the growth of lung tumor-derived xenografts

A specific inhibitor of COX-2 activity (SC-236) and an inhibitor of COX-2 transcription (PDTC) were tested alone and in combination for their effect on the growth of lung-tumor-derived xenografts implanted s.c. in nude mice. Treatment was initiated ~1 week after implantation, when palpable tumors were clearly evident. Mice were injected with vehicle and inhibitors three times per week. The doses of SC-236 and PDTC were chosen based on doses shown by Chinery et al. (12) to inhibit the growth of colon tumor-derived xenografts. Chinery et al. used the structurally similar COX-2 inhibitor SC-58125, rather than SC-236 (12). SC-236 has a 10-fold lower IC50 than SC-58125, so a 5-fold smaller dose of SC-236 was used. The most dramatic effect observed was the repression of the growth of Spon4 xenografts as a result of combined SC-236/PDTC treatment (Figure 4). After 2 weeks of treatment, the SC-236/PDTC-treated xenografts had increased in size by ~5-fold, whereas untreated xenografts increased in size by ~7-fold. Statistical analysis (Materials and methods) indicated no significant (P > 0.05) differences between the four treatment groups. The effect of these inhibitors on the growth of CL30 xenografts was also examined, with no growth repression observed following 2 weeks of treatment (data not shown). The rapid growth of both cell lines in sham-treated mice precluded extending the treatment period.

The effect of SC-236/PDTC treatment on COX-2 transcription and enzymatic activity was measured in Spon4 xenografts. COX-2 protein levels determined by immunohistochemistry were qualitatively similar between sham- and inhibitor-treated tumors (data not shown). COX-2 mRNA levels were also similar between sham- and inhibitor-treated tumors (data not shown). PGE2 levels, indicative of COX-2 enzymatic activity, were measured in supernatants of homogenized xenografts by means of a competitive enzyme immunoassay. Overall, levels of PGE2 were lower in inhibitor-treated xenografts (180 ± 50 pg PGE2/mg protein) compared with sham-treated xenografts (260 ± 55 pg PGE2/mg protein). However, this difference was not statistically significant (P = 0.32).

One mechanism proposed for the reduction in tumor growth seen in other studies is increased apoptosis following exposure to COX-2 inhibitors. The average (±SE) number of apoptotic cells in a 400× microscope field in sham- and inhibitor-treated xenografts was 3.5 ± 0.5 and 4.7 ± 0.4, respectively. This increase in apoptotic cells following inhibitor treatment was not statistically significant (P = 0.16).

### Discussion

The results of this study indicate that COX-2 is a promising target for chemopreventive compounds due to its presence during the earliest stages of lung carcinogenesis and that COX-2 expression in normal tumor-progenitor cells may be linked to lung cancer susceptibility. COX-2 expression in alveolar type II cells correlated with susceptibility to the development of carcinogen-induced lung cancer in five of the six mouse strains examined. This is a high correlation rate given the complexity of the factors involved in determining susceptibility to lung cancer development. Multiple genetic loci are postulated to modulate murine lung cancer susceptibility. For example, pulmonary adenoma resistance (PAR) loci have recently been identified in the mouse genome. The presence of two of these PAR loci in an SMXA recombinant inbred mouse strain produced an adenoma-resistant phenotype even though this strain carries susceptible alleles of all four known lung cancer susceptibility genes (13). Murine susceptibility has been correlated with a polymorphism in the Ki-Ras gene (14).
Cyclooxygenase-2 in A/J mouse lung

Fig. 3. COX-2 mRNA in lung cells, tumors and tumor-derived cell lines as determined by ribonuclease protection assay. (A) COX-2 mRNA in alveolar type II cells isolated from the lungs of A/J (lane 1), A/WySnJ (lane 2), SWR/J (lane 3), C3H/HeJ (lane 4), C57BL/6J (lane 5) and DBA/2J mice (lane 6). (B) Quantitation of COX-2 mRNA in alveolar type II cells isolated from the lungs of the mouse strains shown in (A), normalized to the amount of cyclophilin (cyclo.) mRNA and expressed as a percentage of the level measured in A/J mice. Each bar represents the average of two measurements ± range. (C) COX-2 mRNA levels in MNNK (lane 1), Spon4 (lane 2), IO33 (lane 3), CL13 (lane 4), CL25 (lane 5) and CL30 (lane 6) cell lines. (D) COX-2 mRNA levels in eight tumors from eight A/J mice treated with NNK. For all ribonuclease assays, 10 µg of total RNA was loaded per lane.

more, in the C3A mouse, generated by crossing A/J and C3H mice, the Ki-Ras gene was preferentially mutated on the A/J allele in tumors induced by NNK (15).

The Ras/ERK signaling pathway appears to play a role in the regulation of COX-2 expression. Human non-small-cell lung cancer cell lines with mutations in Ki-Ras have high expression levels of COX-2, and inhibition of Ras activity in these cell lines decreases COX-2 expression (16). Rat intestinal epithelial cells and fibroblasts transfected with Ha-Ras overexpress COX-2, whereas inhibitors of ERK ameliorate this response (17). However, differences in COX-2 levels between sensitive and resistant mouse strains may also be due to differences in regulatory factors unrelated to the ras pathway. Hida et al. (6) observed increased COX-2 expression in 70% of adenocarcinomas, whereas, historically, mutation of the Ki-Ras gene has been detected in only 30% of this histologic tumor type. Kim and Fischer (18) have identified several cis- and trans-acting factors required for COX-2 expression in mouse skin carcinoma cells, pointing out the complexity of COX-2 regulation. This multi-factorial nature of murine lung cancer susceptibility and COX-2 regulation most likely underlies the lack of full concordance between lung cancer susceptibility and endogenous COX-2 expression levels among the mouse strains examined in our studies.

COX-2 overexpression in normal alveolar type II cells may be directly involved in increasing the sensitivity of these cells to the effects of carcinogens and enhancing tumor development after initiation. Clear evidence for a role for COX-2 in tumorigenesis has been substantiated by a multitude of studies. For example, overexpression of COX-2 in rat intestinal epithelial cells increases their tumorigenic potential by increasing cellular adhesiveness and resistance to apoptosis (19). Overexpression of COX-2 in tumors, and the consequent increase in prostaglandin secretion, may alter the release of cytokines such as IL-10 and IL-12 from lymphocytes, macrophages and the tumors’ own cells, resulting in the repression of the host immune response (20). Recent work also indicates that cells overexpressing COX-2 secrete pro-angiogenic factors that may contribute to blood vessel formation in tumors (21).

In addition to being expressed in normal A/J type II cells, expression of the COX-2 protein was frequently seen in hyperplasias in this animal model. This observation, combined with the significant decrease in the number of NNK-induced lung tumors following pre-/early treatment of the A/J mouse with a specific inhibitor of COX-2 activity (8), supports a role for COX-2 in the earliest stages of tumor progression and makes COX-2 a promising target for chemoprevention studies. COX-2 expression has also been reported in early hyperplastic lesions in the human lung (6,7), linking an elevation of this protein to the earliest stages of human lung adenocarcinoma development. Conflicting data have been published regarding COX-2 expression in normal human alveolar epithelial cells (6,7,20), indicating a need for additional investigations.

In our studies of NNK-induced A/J mouse lung tumors, COX-2 expression was more variable and focal in location within adenocarcinomas than observed within hyperplasias. This variation in COX-2 expression has been observed in other types of cancers, including human pancreatic adenocarcinoma,
murine skin carcinoma and human breast carcinoma (22–24). Most significantly, such variability has also been observed in human lung adenocarcinoma (6,7), further supporting the use of the A/J mouse model for the study of human lung cancer. In cell culture COX-2 expression has proved to be highly responsive, changing rapidly in the presence of many growth factors, cytokines and other inflammatory mediators. For this reason it is not surprising that in some tumors and tumor-regions COX-2 expression becomes down-regulated. Loss of COX-2 expression in some tumors as a result of tumor progression does not alter the value of COX-2 inhibition for lung cancer chemoprevention since, by definition, the target of chemopreventive compounds are early lesions. In fact, examination of the mechanism(s) of such down-regulation could lead to the design of new chemopreventive strategies.

In preliminary chemopreventive studies, two A/J lung tumor-derived cell lines that expressed moderate to high levels of COX-2 were grown s.c. in nude mice and were then treated with COX-2 inhibitors. The repression in growth may have been due, in part, to an increase in apoptosis. Although PDTC, an inhibitor of transcription, was essential for maximum growth repression by SC-236, decreased transcription of COX-2 was not detected. The repression of xenograft growth by PDTC/SC-236 was not as dramatic as seen for xenografts derived from mouse intestinal epithelial cells and human colon cancer-derived cell lines (12,25,26). It is possible that the COX-2 inhibitor used in those studies (SC-58125) was more effective, or the appropriate dose of SC-236 for inhibiting this pathway in lung tumor-derived xenografts was not obtained. Another possibility for the lack of significant growth repression of the two lung adenocarcinoma-derived cell lines is that later-stage lung tumors are not dependent on COX-2, a hypothesis supported by the previously discussed variability of COX-2 expression in A/J lung adenocarcinomas. Thus, future chemopreventive studies targeting the lung will be aimed at inhibiting COX-2 during the earliest stages of tumor development. This approach is supported by one study to date in which pre-/early treatment with NS-398, a specific inhibitor of COX-2, was shown to be effective in reducing tumor number in A/J mice initiated with NNK (8).

In a study of six different colon cancer cell lines, only the two cell lines with the highest levels of COX-2 expression showed any repression of proliferation in response to COX-2 inhibitor treatment (27). Similarly, in our studies the lung-tumor-derived cell line that expresses high levels of COX-2 protein (Spon4) showed a partial response, whereas the CL30 cell line that expresses much lower protein levels was refractory. This may provide insight into predicting the response of premalignant lesions to these inhibitors. If efficacy is directly correlated to protein levels, this would further support preneoplasia as a target, where high levels of COX-2 protein are more often seen. The A/J mouse model and derived tumor cell lines should be invaluable for understanding the biology associated with inhibition of COX-2 and for validating this enzyme as a target for chemoprevention of human adenocarcinomas.

Acknowledgements
We would like to acknowledge the technical contributions of Raymond N.DuBois (Department of Medicine/GI Division, Vanderbilt University Medical Center) and Rebecca Shattuck-Brandt, a member of his laboratory. We would like to thank Margaret Ménéchre and Justin Kubatko for their statistical analysis of the data describing the effects of COX-2 inhibitors on the growth of lung tumor-derived xenografts in nude mice. This investigation was supported by National Institutes of Health, National Research Service Award ES05823-02 from the National Institute of Environmental Health Sciences, by Cooperative Agreement DE-FC04-96AL64066 with the United States Department of Energy, and by National Institutes of Health Grant ES08801. Research was conducted in facilities fully accredited by the American Association of Laboratory Animal Care.

References


Received November 3, 1999; revised March 22, 2000; accepted March 29, 2000