Celecoxib Decreases Ki-67 Proliferative Index in Active Smokers

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Abstract

Purpose: This study evaluated the feasibility of cyclooxygenase-2 (COX-2) inhibition for lung cancer chemoprevention. We hypothesized that treatment with oral Celecoxib, a selective COX-2 inhibitor, would favorably alter the biomarkers of lung cancer risk as measured by the Ki-67 proliferative labeling index (Ki-67 LI).

Experimental Design: Twenty active heavy smokers were enrolled into a pilot study and treated with Celecoxib for 6 months. Bronchoscopies with bronchial biopsies were done before and after 6 months of Celecoxib treatment. H&E stain for histologic grading and immunohistochemical examination for Ki-67 LI, COX-2, and survivin were carried out on serially matched biopsy samples to determine responses to treatment.

Results: Treatment with Celecoxib significantly reduced Ki-67 LI in smokers by 35% (P = 0.016), and increased the expression of nuclear survivin by 23% (P = 0.036) without significantly changing that of cytoplasmic survivin.

Conclusions: Our findings suggest that oral Celecoxib may be capable of modulating the proliferation indices and apoptotic balance in bronchial tissue of active smokers.

Lung cancer is the leading cause of cancer death in the U.S. among both men and women, with a 5-year survival rate of only 14%. More Americans continue to die from lung cancer than from colon, breast, and prostate cancer combined. Whereas smoking prevention and cessation remain essential in the overall strategy for lung cancer prevention, these approaches have limited effectiveness. Even when smoking cessation is successful, ex-smokers continue to be at significant risk (1–3). This realization provides the impetus to explore chemopreventive strategies using pharmacologic or natural agents that are capable of impeding carcinogenesis to inhibit the development of cancer (4, 5).

Ample preclinical data suggests that the cyclooxygenase-2 (COX-2)/prostaglandin-E2 (PGE2) signaling pathway plays a pivotal role in conferring the malignant phenotype (6–13). Produced primarily by the action of cyclooxygenases on the free arachidonic acid liberated from membrane phospholipids, overproduction of PGE2, which is predominantly generated by up-regulation of COX-2, is associated with a variety of carcinogenic mechanisms. These mechanisms include abnormal expression of epithelial growth factors, epithelial and microvascular proliferation, resistance to apoptosis, and suppression of antitumor immunity. COX-2 expression has also been shown to be a poor prognostic indicator in non–small cell lung cancer (14). Previous studies have shown elevated PGE2 levels in the bronchoalveolar lavage fluid of patients with bronchogenic carcinoma (15, 16). Furthermore, treatment with chemotherapy leads to increased amounts of COX-2 and PGE2 in non–small cell lung cancer and cotreatment with Celecoxib abrogates the increase in levels of PGE2 (17). In animal models, inhibition of COX-2 and PGE2 synthesis suppresses lung tumorigenesis (7, 18). These data suggesting the antineoplastic effect of COX-2 inhibitors provide the rationale for evaluating their potential in the chemoprevention of bronchogenic carcinoma.

According to the field cancerization concept, key molecular and biochemical events are thought to occur before altered cellular morphology is apparent. In fact, emerging data suggests that histologic response to chemoprevention may not be enough to determine their efficacy (19). In addition to modulation of histopathology, many chemopreventive trials have used various markers known to be causally linked to lung cancer as surrogate end point biomarkers (SEBM), including the assessment of cell proliferation with Ki-67. Ki-67 is a proliferation marker expressed in all phases of the cell cycle except in resting cells (20). Because abnormal epithelial proliferation is a hallmark of tumorigenesis, the measurement of Ki-67 labeling indices (Ki-67 LI) in bronchial tissues as a SEBM for lung cancer chemoprevention trials has attracted significant interest. Elevated levels can be detected in areas where squamous metaplasia is lacking (20). As such, high Ki-67
L1 may also be a useful marker for lung cancer risk. Indeed, elevated Ki-67 LI has been reported to be an unfavorable prognostic factor in non–small cell lung cancers (21).

As part of a pilot phase Ila study to evaluate the feasibility of Celecoxib as a chemopreventive agent for lung cancer, 20 heavy current smokers were recruited and treated with a 6-month course of oral Celecoxib. To determine the effects of Celecoxib on altering SEBM of carcinogenesis in the lung, serial bronchoscopies with bronchoalveolar lavage and biopsy were done. Previously, we reported that treatment with oral Celecoxib in this cohort inhibited the production of PGE2 and interleukin-10 in the lung microenvironment (22). In the present report, we evaluated the effect of oral Celecoxib on modulating Ki-67 LI, histopathology, COX-2, and survivin expression in bronchial biopsies. Our findings support the hypothesis that oral administration of Celecoxib is capable of modulating Ki-67 LI in the bronchial tissue of active smokers.

Materials and Methods

**Celecoxib clinical study design.** Active heavy smokers 45 years of age or older, with a smoking history of at least 20 pack-years, were recruited and treated with oral Celecoxib (Celebrex, Pfizer, New York, NY) at a dose of 400 mg, twice daily for 6 months. Written informed consent was obtained in accordance with the University of California at Los Angeles Institutional Review Board. Patients were screened with chest X-ray and fluorescence bronchoscopy (Xillix, Vancouver, Canada) to rule out the presence of lung cancer as previously described (22). Bronchial biopsies were obtained under fluorescence examination from areas with abnormal fluorescence, as well as at predetermined sites (main carina, carina between right upper lobe and bronchus intermedius, right middle lobe and right lower lobe, right lower lobe anterior and medial basal segment, lingua and upper division bronchus, and left upper lobe and left lower lobe). Nineteen subjects underwent repeat fluorescence bronchoscopy at 6 months with serial bronchial mucosal biopsies at each intensity was determined. For immunohistochemical staining, batch processing and analyses were carried out on paired sections from matched biopsies obtained pre- and posttreatment from each subject in order to eliminate interassay variability. Negative controls using nonimmune sera showed no staining.

**Statistical analysis.** The effects of 6 months of Celecoxib treatment on histopathology, Ki-67, COX-2, and survivin immunostaining were determined by comparing baseline values with those obtained at 6 months of treatment using paired \( t \) tests and ANOVA. Changes in response to treatment were analyzed in two ways: \( (a) \) per subject, in which composite scores among biopsies were generated for each subject before and after treatment, by averaging the scores of all biopsies obtained from each subject at the same time point; \( (b) \) per biopsy, in which histology grading or composite scores for immunohistochemical stain for each biopsy site was compared before and after treatment.

**Results**

**Subject characteristics.** A total of 20 subjects were enrolled, but one withdrew from the study for personal reasons unrelated to the study. The 19 subjects who completed the trial consisted of 9 males and 10 females with a mean age of 55 and a mean smoking history of 43 pack-years (Table 1). All subjects maintained their smoking habits and mean pack-years increased slightly to 43.8 at the time of the second bronchoscopy. Ten subjects had evidence of airflow obstruction defined as a \( \text{FEV}_1 < 80\% \) predicted (mean of 66%). Six subjects had at least one family member with a history of lung cancer. The Celecoxib dose of 400 mg twice daily was well tolerated with no reports of serious adverse events related to the study drug.

**Effects of 6 months of oral Celecoxib treatment on bronchial histopathology.** A total of 200 paired biopsies were available for evaluation. At baseline, 47 were grade 1 (normal), 18 were grade 2 (hyperplasia), 32 were grade 3 (squamous metaplasia),

Table 1. Baseline subject characteristics

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean (range)</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>55 (47-78)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>9/10</td>
</tr>
<tr>
<td>Smoking history (pack-years)</td>
<td>43 (20-80)</td>
</tr>
<tr>
<td>Ethnicity (A/B/C/H)*</td>
<td>1/2/14/2</td>
</tr>
<tr>
<td>COPD (n)</td>
<td>10</td>
</tr>
<tr>
<td>Family history of lung cancer (n)</td>
<td>6</td>
</tr>
</tbody>
</table>

* A/B/C/H, Asian/Black/Caucasian/Hispanic.
Effects of Celecoxib on Ki-67 LI in bronchial mucosa. To determine the effects of oral Celecoxib on cell proliferation, Ki-67 expression at matched biopsy sites were compared before and after treatment. On average, 6 months of Celecoxib decreased Ki-67 LI by 35% (9 ± 1.22 at baseline versus 5.8 ± 0.9 at 6 months; Fig. 3A). When Ki-67 LI was analyzed using patient-specific composite scores, 12 subjects’ composite scores had decreased, whereas nine subjects’ scores had increased. Although in subjects with increased Ki-67 composite scores, all increases were relatively small (Fig. 3C). There was a significant correlation between the pathology grades of the bronchial biopsies and Ki-67 expression both before and after treatment (baseline, r = 0.248, P = 0.011; final, r = 0.426, P < 0.001). Higher levels of Ki-67 LI also correlated with higher pack-years of smoking at (baseline, r = 0.493, P = 0.032; Fig. 3D; at 6 months, r = 0.204, P = 0.025). A micrograph of Ki-67 staining before and after treatment is shown in Fig. 5A and B.

Effects of Celecoxib treatment on COX-2 expression in bronchial mucosa. In general, the overall frequency and intensity of COX-2 expression in biopsied bronchial epithelial cells was low. No staining was detected in 48 paired biopsies. Four subjects (18 paired biopsies) had no detectable COX-2 staining throughout the bronchial tree. In these samples, COX-2 staining did not correlate with either histopathology or Ki-67 expression at baseline or following treatment. Celecoxib treatment also did not consistently or significantly alter COX-2 expression in bronchial biopsies.

Effects of Celecoxib on survivin expression in bronchial mucosa. Survivin is an important inhibitor of apoptosis. Previous results have shown that the levels of survivin are regulated by COX-2 expression levels (23). As such, we examined the expression of survivin in biopsied bronchial epithelium before and after treatment. In our cohort, we found an appreciable amount of survivin staining in both the cytoplasm and the nucleus. There was consistently more staining in the basal cells, and nuclear survivin staining was noted predominantly in the upper layers of the epithelium. Oral Celecoxib modestly increased the expression of nuclear survivin without significantly altering that of cytoplasmic survivin (Fig. 4). Survivin expression did not correlate with histopathology. A micrograph of survivin staining before and after treatment is shown in Fig. 5C and D. The presence of airflow obstruction did not correlate with changes in histopathology, Ki-67, COX-2, or survivin expression.

Discussion

This pilot study focused on the feasibility of using an oral COX-2 inhibitor, Celecoxib, as a chemopreventive agent to protect against the development of lung cancer in active smokers. Previously, we reported that oral Celecoxib, at a dose of 400 mg twice daily, is capable of inhibiting the synthesis of the biochemically relevant marker, PGE₂, within the lung microenvironment of active smokers. We now report that oral Celecoxib reduced Ki-67 LI with a concomitant trend of a shift of histopathology toward lower grade. Celecoxib also modestly increased nuclear survivin expression without affecting cytoplasmic survivin expression. Collectively, these findings suggest that in addition to modulating PGE₂ production, Celecoxib may be capable of altering additional SEBM of carcinogenesis in the lung microenvironment.

Table 2. Histopathology grading of bronchial biopsy samples at baseline and after 6 months of Celecoxib

<table>
<thead>
<tr>
<th>Histopathologic grading</th>
<th>Normal (1)</th>
<th>Hyperplasia (2)</th>
<th>Squamous metaplasia (3)</th>
<th>Mild dysplasia (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before (n)</td>
<td>47</td>
<td>18</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>After (n)</td>
<td>54</td>
<td>16</td>
<td>30</td>
<td>0</td>
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Fig. 1. Histologic grade at baseline and 6 months.
The identification of appropriate SEBM that are capable of reliably predicting therapeutic efficacy is an essential goal in the field of lung cancer chemoprevention (24). An appropriate SEBM must be integrally involved in the process of carcinogenesis, such that modulation of expression correlates highly with disease course. The expression of the marker should differ in normal versus premalignant or at-risk epithelium, and should be easily and reproducibly measurable from biological specimens obtained in clinical trials. Furthermore, the expression of such a marker should respond to chemopreventive treatment and not fluctuate spontaneously or have a high spontaneous regression rate (25). Although many SEBM have been proposed and evaluated based on their correlation with tumor biology, none of these biomarkers have been validated in prospective studies (26).

At the present time, no consensus exists on how best to identify individuals at high-risk for lung cancer chemoprevention trials and to monitor for therapeutic efficacy. Even histopathology (preneoplasia grading), which is generally considered the gold standard, may not be sufficiently predictive. For instance, Mao et al. reported the persistence of loss of heterozygosity despite complete histologic response in the premalignant lesions of the head and neck in patients treated with 13-cis-retinoic acid, IFN-α, and α-tocopherol (19). The persistence of genetic alteration may explain the high recurrence of premalignant lesions shortly after stopping chemoprevention therapy. In addition, resumed pathologic progression to cancer development, after a delay in treated patients, may also be due to persisting clonal genetic abnormalities that are unaffected by the treatment.

Measurement of proliferative indices such as Ki-67 expression has attracted considerable interest in recent years. Lee et al. reported that Ki-67 staining is markedly elevated in current smokers but decreases substantially after smoking cessation, however, remains measurable many years after smoking cessation (20). The Ki-67-LI also correlates with histologic abnormalities. Moreover, Ki-67 expression has been reported in the absence of metaplasia (20, 27), and may more accurately reflect the level of tobacco-induced damage throughout the lung. These characteristics suggest that Ki-67 may have added value, beyond that of histopathology, in predicting lung cancer risk and as a SEBM for chemopreventive efficacy.

Recently, Celecoxib has been reported to decrease Ki-67 expression in cervical cancer (28). Suppression of cell proliferation as measured by Ki-67 LI has also been reported to accompany polyp regression in a chemoprevention trial in patients with familial adenomatous polyposis (29). Whether or not Celecoxib is capable of modulating Ki-67 LI in the lung is unclear. Our findings, for the first time, provide evidence to support that Celecoxib may be capable of decreasing Ki-67 expression and proliferative activity in bronchial mucosa in continuing smokers.

Dysregulation of apoptosis is an essential feature of carcinogenesis, and apoptosis induction has been widely investigated and consistently supported in studies that seek to define the
potential antineoplastic mechanisms of COX-2 inhibition. We recently reported that non–small cell lung cancer cells that overexpress COX-2 were found to have a significantly increased resistance to radiation and drug-induced apoptosis. Levels of survivin strongly correlated with COX-2 expression (23).

Survivin is differentially expressed in normal tissues versus cancer (30–32). It is critical for normal embryonic development, and it is virtually absent in most normal differentiated cells, except in certain highly proliferative areas within normal tissues (33–35). Recently both constitutive and PGE$_2$-inducible survivin expression have been shown in human monocyte-derived dendritic cells (36). By contrast, survivin is highly expressed in most human tumors. In addition to apoptosis, survivin has been implicated to play pivotal roles in regulating cell cycle and mitosis. Caldas et al. (37) reported that the diverse functional role of survivin may be in part, due to splice variants leading to the production of various isoforms that regulate the balance between proliferation and cell death. When expression of all survivin forms is eliminated by small interfering RNA transfections, cells undergo both apoptosis and defective cell division (37). The various isoforms may account for the complex biological roles of survivin and the controversies surrounding its utility as a prognostic factor. In addition, the pattern of survivin expression may influence the prognostic implication of survivin. For example, when nuclear or cytoplasmic staining of survivin was scored separately, nuclear staining was linked with favorable prognosis in gastric cancer (38), breast cancer (39), and osteosarcoma (40), whereas cytoplasmic survivin was not found to be prognostic. In contrast, a recent study in esophageal cancers showed that nuclear survivin was associated with poor survival (41).

As a corollary to assessing changes in proliferative activity in bronchial mucosa, we evaluated the effects of Celecoxib on the expression of survivin. To our knowledge, the pattern of survivin expression in the bronchial epithelium of active smokers without lung cancer has not been documented. In our cohort, we found an appreciable amount of survivin staining in both the cytoplasm and the nucleus. There was consistently more staining in the basal cells and nuclear survivin staining in the upper layers of the epithelium. Oral Celecoxib modestly increased the expression of nuclear survivin

![Fig. 4. A and B, to determine the effect of oral Celecoxib on survivin expression on bronchial epithelium, cytoplasmic, and nuclear survivin expressions were scored separately. There was consistently more staining in the basal cells and nuclear survivin staining in the upper layers of the epithelium. Nuclear survivin expression increased modestly by 23% (70 ± 8.2 at baseline versus 90 ± 9.8 at 6 months; *, P = 0.036) after treatment without significantly changing cytoplasmic survivin levels. Neither cytoplasmic nor nuclear expression of survivin correlated to bronchial histopathology grading.](image)

![Fig. 5. Representative micrographs of Ki-67 staining of bronchial biopsies from matched bronchial sites before (A) and after (B) 6 months of Celecoxib treatment. Note marked decrease in nuclear staining. Representative micrographs of survivin staining of bronchial biopsies from matched bronchial sites before (C) and after (D) 6 months of Celecoxib treatment. Note increase in nuclear staining (all avidin-biotin complex peroxidase stain; original magnification, ×200).](image)
Celecoxib Decreases Ki-67 Expression in Smokers

without significantly altering that of cytoplasmic survivin. Such a pattern of change in survivin expression may have important clinical implications, as nuclear localization of survivin has been reported to be a positive prognostic factor for survival in advanced non–small cell lung cancer (42). On the contrary, others have reported that nuclear survivin staining is associated with poor prognosis (43). The true clinical significance and the precise molecular mechanisms that might account for such a response are unclear and remain to be elucidated.

Consistent with previous reports, we did not find high levels of bronchial COX-2 expression in our heavy smoker cohort. Furthermore, COX-2 expression did not correlate with histopathology, Ki-67, survivin expression, or response to treatment. These findings suggest that the presence of COX-2 in bronchial epithelium, to the extent detectable by conventional immunohistochemistry, is not essential in predicting the response to Celecoxib. One plausible explanation is that Celecoxib may exert its effect primarily through modulation of other cell types that are present in the lung microenvironment but are not found in bronchial biopsies, such as alveolar macrophages. To this end, we previously reported that Celecoxib decreased alveolar macrophage PGE2 production in a subset of randomly selected subjects (n = 6) following 1 month of treatment during interim analysis. Five out of the six subjects completed the study with follow-up bronchial biopsy. The reduction of alveolar macrophage PGE2 production correlated with the reduction of Ki-67 expression in four of the five subjects, whereas the remaining subject had low levels of Ki-67 expression both at baseline and second bronchoscopy. Alternatively, Celecoxib could be exerting its antineoplastic effects via COX-2-independent mechanisms (44).

We acknowledge that our pilot study is limited by its non-comparative design and small sample size. It is possible that the observed improvement could be a consequence of mechanical removal of the abnormal area or preneoplasia during biopsy. The changes could also be due to spontaneous modulation. For example, changes in smoking habits have been well-documented to be associated with alteration of a variety of biomarkers, including histopathology and Ki-67 LI. It is noteworthy, however, that all of our participants maintained their smoking habits during the trial, thus making spontaneous alteration from smoking cessation less likely an issue. In addition, one would expect to see histopathologic progression or enhancement of proliferative activity in the setting of continuous carcinogen exposure. Nevertheless, the effects of Celecoxib require validation in larger, randomized placebo-controlled trials.

In summary, we found that 6 months of oral Celecoxib treatment is associated with a significant decrease in Ki-67 LI in active smokers and a modest increase in nuclear survivin expression. Our results suggest that oral Celecoxib is capable of affecting the lung microenvironment in active smokers. These findings support the continued investigation of Celecoxib as a chemopreventive agent for lung cancer.

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References
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