Expression and localization of cyclo-oxygenase isoforms in non-small cell lung cancer

D.N. Watkins*, J.C. Lenzo*, A. Segal†, M.J. Garlepp*,‡, P.J. Thompson*

ABSTRACT: The beneficial effects of cyclo-oxygenase (COX) inhibitors in both colon cancer and adenomatous polyps suggest a role for the prostanoid pathway in epithelial malignancy. Although variable prostanoid synthesis in non-small cell lung cancer (NSCLC) has been demonstrated in freshly obtained tissue, COX messenger ribonucleic acid (mRNA) and protein localization in such tumours had not been investigated ex vivo.

Thirty-four cases of primary NSCLC were examined for both constitutive (COX-1) and inducible COX (COX-2) by means of in situ hybridization and immunohistochemistry. COX-1 mRNA expression was absent or below the level of detection via in situ hybridization, COX-1 immunohistochemistry demonstrated uniform faint cytoplasmic staining in tumour cells and stromal inflammatory cells. Semiquantitative analysis of COX-2 expression in NSCLC demonstrated the highest levels of both mRNA and protein in adenocarcinoma cells (n=10, p<0.005 compared with large cell and squamous cell carcinoma), intermediate and variable expression in large cell carcinoma (n=11) and low or absent expression in squamous cell tumours (n=13). Levels of COX-2 expression in infiltrating inflammatory cells was the same in all tumour types.

In conclusion, tumour cell cyclo-oxygenase-2 rather than cyclo-oxygenase-1 expression may account for the variable prostanooid production seen in non-small cell lung cancer, and primary lung adenocarcinoma expresses the highest levels of cyclo-oxygenase-2. Assessment of cyclo-oxygenase-2 expression ex vivo should be performed in studies examining the potential therapeutic effects of cyclo-oxygenase inhibitors in non-small cell lung cancer.

isolated lung cancer tissue [16, 17] suggest that NSCLC, and in particular primary lung adenocarcinoma, is associated with the increased production of PGE2. Moreover, the cellular origins of prostanoid production and the localization of COX isomers has not been studied in human NSCLC. Given the varied prostanoid production associated with NSCLC in vivo, it was hypothesized that expression of COX-2 in NSCLC varied according to histopathological tumour type. The aim of this study was to examine the expression and localization of both COX-1 and COX-2 mRNA and protein in human NSCLC specimens obtained following surgical resection, and to correlate this expression with tumour phenotype.

**Materials and methods**

**Source of materials**

Unless otherwise stated, all were obtained from Sigma (Sydney, Australia).

**Tissue preparation**

Archival formalin-fixed, paraffin-embedded lung cancer specimens from patients undergoing lung resection selected at random (n=34) were obtained from the Dept of Pathology, Queen Elizabeth II Medical Centre (Nedlands, Western Australia).

**In situ hybridization**

Localization of COX-1 and COX-2 mRNAs was performed by means of in situ hybridization, as previously described [18]. All specimens were treated identically. Sections were hybridized with an 35S-recombinant uridine triphosphate (rUTP)-labelled antisense ribonucleic acid (RNA) probe, or a complementary sense probe as a negative control. Probes were transcribed using a plasmid template containing a portion of the COX-1 or COX-2 template, consisting of sections incubated with antibody preadsorbed with diaminobenzidine in tris(hydroxymethyl)aminomethane-buffered saline (pH 7.6) for 5 min, the sections were then recorded as the mean of those of the six areas examined. Subsequently, qualitative review of all sections was performed on all specimens by two investigators (D.N. Watkins and A. Segal).

**Statistical analysis**

Semiquantitative scores of both in situ hybridization and immunohistochemical signals were expressed as mean±SEM. Comparisons between groups were made using one-way analysis of variance and Tukey’s test for multiple comparisons. A p-value <0.05 was considered significant. Correlation between in situ hybridization and immunohistochemical scores was performed using linear regression analysis [21].

**Results**

**Histopathological analysis of tumour specimens**

Thirty-four cases of NSCLC were studied, comprising 10 adenocarcinomas, 11 large cell carcinomas and 13 squamous cell carcinomas. In all cases, review of the sections used for COX localization matched the pre-existing diagnosis. The pathological features of these tumours are summarized in table 1.

**Localization of cyclo-oxygenase-1 in non-small cell lung cancer specimens**

Scattered low-level hybridization signals for COX-1 mRNA were seen throughout the tumours. This was
were absent or faintly positive in the tumour cells. In three cases, diffuse weakly positive in situ hybridization signals and cytoplasmic immunostaining were observed within the major part of the carcinoma, whereas in one case, strong focal positivity for both COX-2 mRNA and protein were seen in carcinoma cells distant from central areas of keratinization. Variable levels of COX-2 expression were detected in infiltrating inflammatory cells in a similar fashion to that observed in both large cell carcinoma and adenocarcinoma. Once again, stromal areas demonstrated no specific hybridization signal or immunoperoxidase staining. Control sections for both in situ hybridization and immunohistochemistry were negative.

Representative sections from both in situ hybridization and immunohistochemical studies are shown in figure 1.

**Semiquantitative analysis of cyclo-oxygenase-2 expression**

Expression of COX-2 mRNA and protein in both tumour cells and infiltrating inflammatory cells was analysed semiquantitatively. The results of this analysis are summarized in table 2 and figure 2. There was a strong positive correlation ($r^2=0.895$, $p<0.0001$) between the two blinded observers scores for both mRNA and protein expression. A positive correlation ($r^2=0.730$, $p<0.001$) between tumour cell in situ hybridization and immunohistochemical scores was observed. Levels of COX-2 mRNA in adenocarcinoma cells were significantly increased compared with squamous cell carcinoma ($p<0.05$). Although there was a trend towards lower levels of COX-2 mRNA expression in large cell tumours compared with adenocarcinoma, this difference did not reach statistical significance. COX-2 protein expression was also greatest in adenocarcinoma cells, and was significantly greater than that observed in both large cell carcinoma ($p<0.001$) and squamous cell carcinoma ($p<0.001$). Levels of COX-2 expression in infiltrating inflammatory cells did not vary significantly with tumour type, and nor was there a significant correlation between COX-2 expression in tumour cells and adjacent inflammatory cells.

**Discussion**

This study investigated the differential expression of COX isoforms in NSCLC specimens obtained at thoracotomy. Although levels of COX-1 mRNA expression appeared to be below the level of detection by means of in situ hybridization, COX-1 immunohistochemistry suggested widespread low-level cytoplasmic protein expression in lung cancer cells, as well as in stromal and inflammatory cells. By contrast, COX-2 mRNA and protein expression was variable between tumour specimens, the most intense signals being seen in adenocarcinoma cells, with intermediate levels of expression in large cell tumours and the lowest levels in squamous cell carcinoma. These data suggest that COX-2, but not COX-1, is differentially regulated and expressed in NSCLC, and is most consistently expressed in primary lung adenocarcinoma. The possibility that tumour stage rather than phenotype may have influenced COX-2 expression cannot be excluded. However, this seems unlikely based on the following facts: 1) all tumours were deemed surgically resectable and

<table>
<thead>
<tr>
<th>Tumour Pathology</th>
<th>Number</th>
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<td>Adenocarcinoma</td>
<td>10</td>
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<tr>
<td>Well differentiated</td>
<td>2</td>
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<td>Moderately differentiated</td>
<td>6</td>
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<tr>
<td>Poorly differentiated</td>
<td>2</td>
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<tr>
<td>Large cell carcinoma</td>
<td>11</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>13</td>
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<td>Well differentiated</td>
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<td>Moderately differentiated</td>
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<tr>
<td>Poorly differentiated</td>
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Fig. 1. – Cyclooxygenase (COX) localization in three primary lung carcinomas. Representative sections from each tumour type are shown: a, d, g, j, m) adenocarcinoma; b, e, h, k, n) large cell carcinoma; and c, f, i, l, o) squamous cell carcinoma. Haematoxylin and eosin stained section; d–f, j–l) in situ hybridization study using antisense probe (d–f) COX-1; and j–l) COX-2); and g–i, m–o) immunohistochemistry study using COX-specific polyclonal antibody (g–i) COX-1; and m–o) COX-2). (Internal scale bar=15 μm (o) and applies to all sections of the figure.)
therefore fall within a relatively narrow range of staging, and 2) in the only comparable study, Hida et al. [22] showed no relationship between COX-2 immunostaining and tumour stage.

Overexpression of COX-2, rather than COX-1, has been described in malignant colonic tumours [8, 9]. These findings are supported by immunoblot analysis of COX-1 and COX-2 protein expression in malignant and normal colonic tissue [10]. Furthermore, biochemical evidence of enhanced PG production has been reported in both colon cancer [23] and primary human lung cancers ex vivo [24]. The findings in the present study suggest that, in NSCLC, expression of COX-2 by tumour cells is the mechanism responsible for the variable upregulation of prostanooid biosynthesis in primary lung cancers observed ex vivo [24], and that tumours of the adenocarcinoma phenotype are more likely to express COX-2. In addition, the expression of COX-2 in large cell carcinoma may reflect the presence of glandular differentiation not readily detectable on routine histological examination.

In a recent immunohistochemical study, Hida et al. [22] demonstrated COX-2 expression in NSCLC, but not in small cell lung cancer, and suggested, via semiquantitative analysis of immunostaining, that COX-2 expression in such tumours was upregulated compared to noncancerous airway epithelium. COX-2 expression, by means of both in situ hybridization and immunohistochemistry, has recently been investigated in nonmalignant airway epithelial cells from patients undergoing surgery for primary lung cancer [4]. Focal COX-2 mRNA expression was observed in small numbers of columnar epithelial cells, and corresponding COX-2 protein expression localized predominantly to the apical surface of the respiratory epithelial cells [4]. These findings are qualitatively different from the COX-2 expression seen in adenocarcinoma in the present study, both in the intensity of mRNA expression and the diffuse cytoplasmic localization of COX-2. However, semiquantitative comparison, given the methodology used in both studies [19], is not appropriate when comparing gene expression in solid tumours to that in lumenal structures such as airways.

Although the role of COX-2 expression in NSCLC biology has not been extensively investigated, experimental studies using both in vitro and animal models support the potential importance of COX-2 upregulation in epithelial malignancy [14, 25]. Lewis lung carcinoma cells synthesize large amounts of PGE2 [26], and nonspecific COX inhibitors reduce their growth and metastatic potential in vivo [14, 27], suggesting that PGE2 production may provide a survival advantage for clones of proliferating lung cancer cells [14, 24]. Interestingly, primary rat tracheal cells cultured in vitro undergo mucociliary differentiation in the presence of retinoic acid which is associated with COX-2 expression and PGE2 formation [28], suggesting a specific link between the mucociliary phenotype and COX-2 induction. Although it is tempting to speculate that such a link exists in primary lung adenocarcinoma, specific studies on the expression and inhibition of COX-2 in NSCLC are required to test this hypothesis.

In colonic carcinogenesis models, early induction of COX-2 has been observed in azoxymethane-induced colonic tumours in rats [29], and in colonic epithelium exposed to a high level of dietary fat intake [30]. In human colon cancer cell lines, inhibition of the COX pathway is associated with reduced tumour growth [31] and the induction of apoptosis [12]. Moreover, specific COX-2 inhibitors reduce the growth of colonic tumour cell lines in vivo and of solid tumours in nude mice [32]. Similar studies in NSCLC systems are needed to confirm the importance of COX-2 in this context. Based on the findings in the present study, it can now be hypothesized
that COX-2 expression may be significant in the pathogenesis and propagation of lung cancer, particularly adenocarcinoma.

In the present study, evidence consistent with upregulation of COX-2 mRNA in adenocarcinoma cells, by means of in situ hybridization, and a strong correlation between mRNA and protein expression in the NSCLC cases studied was demonstrated. These data suggest that transcriptional events may be important in COX-2 induction and expression in NSCLC. This contention is supported by extensive evidence of transcriptional regulation of COX-2 in vitro [7, 33], and by the recent demonstration of activation of the COX-2 transcriptional apparatus in both colon and breast cancer cell lines [34]. Studies in breast cancer [34] and in several human NSCLC cell lines [35] have suggested that mutations in the ras oncogene family are directly implicated in the transcriptional activation of COX-2. Interestingly, ras mutations have been identified in 30–50% of primary lung adenocarcinomas, but are less frequent in large cell carcinoma (21%) and squamous cell carcinoma (5%) [36]. The present findings of marked COX-2 expression in primary lung adenocarcinoma is consistent with the hypothesis that transcriptional activation by ras may be important in the upregulation of COX-2 and the production of prostanoids in NSCLC.

Studies in NSCLC have demonstrated that PGE2 is the dominant prostanoid synthesized in both cell lines [16] and fresh human lung cancer tissue [24]. Many authors have speculated that tumour-derived prostanoids, particularly PGE2, may be important in tumour invasion, the formation of metastases, and the inhibition of macrophage and T cell responses (reviewed in [14]). More recently, tumour COX-2 expression and prostanoid production have been directly implicated in colon cancer angiogenesis [15]. Given the present findings of marked COX-2 overexpression in adenocarcinoma, and the clinically aggressive behaviour of this tumour when associated with oncogenic ras mutations [1], it seems likely that COX-2 expression is important for tumour growth, tissue invasion and metastasis. The potential mechanisms by which tumour-derived prostanoids may provide a survival advantage in NSCLC require further investigation. In particular, studies in colon carcinoma in vitro have shown that COX-2 inhibitors may induce apoptosis by a mechanism independent of PGE2 [32], suggesting that additional mechanisms may also exist in lung adenocarcinoma.

Recent interest in the effects of cyclooxygenase inhibitors on early carcinogenesis and tumour growth in colon cancer suggests that these agents may be of value in high-risk patients with adenomatous polyposis, and as an adjunctive therapy in established colon cancer (reviewed in [8]). In addition, emerging evidence linking the expression of cyclooxygenase-2 with early events in colon carcinogenesis as well as the promotion of tumour growth and metastasis suggests that cyclooxygenase-2 specific inhibitors may be of value in this regard [8]. If in vitro studies of non-small cell lung cancer suggest that cyclooxygenase-2 inhibition is associated with growth inhibition, reduction in metastatic potential and the induction of apoptosis, clinical trials of cyclooxygenase-2 inhibitors in non-small cell lung cancer would seem warranted. Given that all patients in the present study were deemed surgically resectable, it is possible that a selection bias towards less aggressive tumours may have occurred. Despite this, the findings in this study suggest that adenocarcinoma and some large cell malignancies are the tumours most likely to respond to cyclooxygenase-2 inhibition. Given the variable expression of cyclooxygenase-2 observed within each non-small cell lung cancer phenotype, the authors propose that immunohistochemical analysis of tumour cyclooxygenase-2 expression be a prerequisite for any clinical trial of cyclooxygenase inhibitors in this form of malignancy.

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References


