Overexpression of cyclooxygenase-2 in non-small cell lung cancer


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Summary Evidence is accumulating to suggest that the inducible isoenzyme of cyclooxygenase (COX)-2 is up-regulated in human cancers and epidemiological studies indicate that COX inhibitors may have a protective effect on the development of lung cancer. We used immunohistochemistry and Western blotting to investigate COX expression in lung tumour specimens and three lung cancer cell lines. Sixty-five archival lung tissue samples, including 46 squamous cell and 6 adenocarcinoma lung resections, and 13 small cell lung cancer (SCLC) biopsies were studied. Dense and intense cytoplasmic COX-2 staining was found in all 52 resections from non-small cell lung cancer (NSCLC). The staining was diffuse and much stronger than adjacent respiratory epithelium. COX-2 staining was relatively weak in the majority of the SCLC samples. The bronchial and bronchiolar epithelium in the surrounding normal lung structures showed uniform COX immunoreactivity with apical concentration of the stain. There was no increase in COX-1 staining in any tumour type. Western blot analysis of the cancer lines revealed significantly higher expression of COX-1 in CORL23 line and COX-2 in two NSCLC cell lines (MOR/P; A549) compared with the expression of COX-1 and COX-2 in cultured normal bronchial epithelial cells. Our findings demonstrated COX-2 overexpression in NSCLC.

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Introduction

The burden of lung cancer to society is enormous. This is one of the leading causes of cancer-related death worldwide and is associated with almost one quarter of all cancer deaths, overtaking breast, prostate, and colon cancer. Lung cancer is highly resistant to conventional cancer therapy and although survival rates have improved slightly in recent years more than 90% of patients diagnosed with lung cancer die from their disease.1,2 Therefore, the identification of the mechanisms preventing or controlling the development and progression of this deadly disease is urgently required and will lead to more effective lung cancer management strategies.

Recent research has shown that both human and experimental tumours contain increased amounts of prostaglandin (PG) E2 which modulates cytokine balance, inhibits host immunity and may play an important role in carcinogenesis.3,4 PG E2 is produced from endogenous arachidonic acid by
cyclooxygenase (COX). Two COX genes have been identified, COX-1 and COX-2. The inducible isoform COX-2 has been found to be up-regulated in a variety of different tumours, including those of the gastrointestinal tract, lung, head and neck, breast. This reflects the increasing interest in the arachidonic acid cascade and particularly COX-2 as potential targets for cancer prevention.

COX non-selective and selective inhibitors have been shown to have anti-neoplastic and prophylactic efficacy against human cancer and in mouse models of this disease. Epidemiological studies indicate that the use of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of morbidity and mortality from gastrointestinal, lung and breast cancers. Sulindac has been shown to decrease the size and number of the colorectal adenomas in patients with familial adenomatous polyposis (FAP). Several experimental studies have also demonstrated that the administration NSAIDs suppresses the tumourigenesis in animal models and cancer cell lines. Tsubouchi et al. used the MTT assay and PGE2 enzyme immunoassay to determine that meloxicam inhibits the growth and PGE2 production of both A549 and PC14 cancer cell lines. Celecoxib, a selective COX-2 inhibitor and a potent anti-angiogenic agent, has been reported to inhibit the growth of lung and colonic tumours implanted into recipient mice.

The majority of the previous studies have concentrated on human colorectal tumours, lung adenocarcinomas and other gastrointestinal cancers. The published so far data regarding the expression of COX isoforms in squamous cell lung cancer remain controversial. Several studies have shown that COX-2 is also up-regulated in squamous cell lung cancer compared with normal lung tissue. Watkins et al. have demonstrated low or absent expression of COX-2 in 9 out of 13 squamous cell lung cancer specimens. This was followed by a larger study conducted by Hasturk revealing that only 1 of the evaluated samples (9 out of 46) expressed increased levels of COX-2. In this study, we examined the expression levels of COX-1 and COX-2 in primary non-small cell lung cancers (NSCLC) and small cell lung cancer (SCLC) and showed a significant increase in COX-2 expression not only in lung adenocarcinomas but also in squamous cell lung tumours. Here we evaluated the COX-1 and COX-2 immunoreactivity in a large number of squamous cell lung cancer patients and report COX-2 overexpression in this type of NSCLC. In addition we assessed the COX-1 and COX-2 levels in three cultured cancer cell lines and compared them with these in normal bronchial epithelial cells. The results demonstrated up-regulated COX-2 in the NSCLC adenocarcinoma but not large cell lung cancer cell lines.

Methods

Study population

Non-small lung cancer patients

Lung resection specimens were obtained retrospectively from 46 patients with squamous cell carcinoma [median age 68, range 47–76 years; 37 males] and 6 patients with adenocarcinoma [median age 69, range 53–71 years; 2 male]. Forty-eight (92.3%) were smokers or ex-smokers. All patients had been initially assessed as operable and had undergone pulmonary resection. No patients had other treatment for their lung cancer including chemo- or radiotherapy prior to the surgical intervention. Three patients were on a regular anti-hypertensive treatment and 6 were on inhaled bronchodilators as required. Samples from tumour nodules were examined and compared with the surrounding histologically normal areas of lung parenchyma.

Small cell lung cancer patients

Bronchial biopsies were obtained retrospectively from 13 patients [median age 66, range 48–81 years, 11 males]. Ten (76.9%) were smokers or ex-smokers. Two patients were taking anti-hypertensive and three were on inhaled bronchodilators intermittently. Three patients had a limited and 10 extensive disease.

No patient included in the study had been on regular medication with aspirin or NSAIDs according to the medical records.

Tissue specimens and immunohistochemistry

Sixty-five archival lung tissue samples, including 46 squamous cell, 6 adenocarcinoma lung resections, and 13 SCLC biopsies were studied. Paraffin embedded tissue blocks were sectioned at 2–3 μm thickness and mounted on APES coated slides. The specimens were deparaffinized in fresh xylene and rehydrated by immersion in graded series of dilute solutions of ethanol. The tissue sections were then microwaved in a large vessel containing 1 l of 10 mM citrate buffer (2.1 g citric acid monohydrate and 1 g NaOH in 1 l de-ionized water, pH 6) for 10 min at full power followed by 10 min at 50% power in a 800W microwave oven. The slides were rapidly cooled, removed from the citrate buffer and then washed in Tris buffered saline prior to immunohistochemistry. Slides were immersed first in 0.3% hydrogen...
peroxide/methanol for 10 min to block endogenous peroxidase activity. Normal swine serum was used as a blocking agent to reduce background staining and to dilute the primary and secondary antibodies. Immunostaining was performed using Streptavidin biotin complex/horse-radish peroxidase immunodetection system at room temperature. All slides were sequentially incubated with monoclonal mouse COX-1 or COX-2 antibodies at the dilution 1:50 for 1 h, biotinylated goat secondary antibody for 30 min and avidin-biotin peroxidase (ABC) complex solution (ABC Quick kit protocol) for 1 h. Immunostaining was visualised with 3,3′-diaminobenzidine (DAB) solution (5 mg DAB and 100 μl 3% hydrogen peroxide in 10 ml pH 7.6 Tris buffer) for 10 min. Sections were then treated with 0.8% copper sulphate in 0.8% sodium chloride for 10 min, counter-stained in haematoxylin, dehydrated, cleared in xylene substitute and mounted in XAM. Negative controls with the primary antibody absent, or replaced with an unrelated isotype-matched mouse IgG were included. These controls always showed no colour development. We have previously demonstrated the specificity of these antibodies for COX-1 and COX-2 using both Western blotting and immunocytochemistry.37–39

**Cell cultures**

**Normal human bronchial epithelial cells (NHBEC)**

Normal human bronchial epithelial cells (NHBEC 4653) obtained from Biowhittaker (Walkersville, MD, USA) were cultured as previously described.37,40

**A549 (lung adenocarcinoma)**

A549 cell line was purchased from the European Culture Collection. The cells were cultured according to a method we have previously described.38,41 Frozen and stored cells were thawed at 37°C, washed twice with PBS, seeded in 24 well tissue culture plates and incubated in humidified 5% CO2/95% air at 37°C. The culture medium containing Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal calf serum (FCS), penicillin G (100 μg ml−1), streptomycin (100 μg ml−1), amphotericin B (2.5 μg ml−1) and L-glutamine (4 mM) was changed every 2 days. Once confluent the cells were growth arrested in serum-free media for 24 h.

**MOR/P (lung adenocarcinoma)**

MOR/P cell line was a generous gift from Dr. P. Twentyman (Cambridge, UK). The P indicates that these are parental cell lines and not drug resistant derivatives of these lines.42 Frozen cells were thawed at 37°C, diluted gradually and cultured in pre-warmed tissue culture media RPMI supplemented with 10% FBS, penicillin G (100 μg ml−1), streptomycin (100 μg ml−1), and L-glutamine (2 mM). Initially the cells were seeded into flasks T175 (50 ml). The media was changed every 1–2 days. When confluent the media was aspirated, the cells washed in PBS and trypsinised in 1–2.5 ml of trypsin/EDTA for 2–5 min at room temperature followed by 5 min at 37°C. The cells were then re-suspended in 10 ml media and seeded into new flasks, or stored frozen in freezing mixture (5%DMSO in BCS) as 1 ml aliquots in cryovials. For the experiments the trysinised cells were re-suspended in PBS, and grown in 24-well culture plates.

**COR-L23/P (large cell lung carcinoma)**

COR-L23, P (parental) cell line was also a generous gift from Dr. P. Twentyman (Cambridge, UK). The cells are derived from a large cell anaplastic tumour. They were grown using identical conditions to MOR/P cell line.

**Western blot**

COX-1 and COX-2 protein levels in normal human bronchial epithelial cells were compared with the levels in the NSCLC lines A549, COR-L23 and MOR/P. To determine the basal COX isoform distribution in these cell lines we carried out Western blotting analysis as previously described. Protein aliquots of 30 μg of the samples were used.38,41 We used the same antibodies as for the immunohistochemistry.

**Scoring of sections**

The whole sections were examined. They were coded and scored semi-quantitatively. The extent and intensity of the expression of COX isoenzymes (brown cytoplasmic staining) were assessed as follows: 0 = none; 1 = weak and patchy; 2 = weak but extensive or strong but patchy, and 3 = strong and extensive.

**Materials**

Monoclonal mouse anti-ovine COX-1 with cross-reactivity to human (Cat N 160110) and monoclonal mouse anti-human COX-2 (Cat N 160112) antibodies were purchased from Alexis Corporation (Bingham, Nottingham, UK, normal swine serum from Sera-lab (Belton, Loughborough, UK), Vectastain, DAB kit from Vector Laboratories (Bretton, Peterborough, UK). Mayer’s hematoxylin, DMEM, penicillin,
streptomycin, L-glutamine, amphotericin-B were all purchased from Sigma (Poole, Dorset, UK), and xylene substitute mountant was purchased from Shandon (Astmoor Runcorn, Cheshire, UK). The A549 cell line was purchased from the European Culture Collection. COR-L23 and MOR/P cell lines were a generous gift from Dr. P. Twentyman (Cambridge, UK). The NHBE 4653 cells, Bronchial Epithelial Cell Growth Medium Bullet kit (BEGM Bullet kit) or Small Airway Epithelial Cell Growth Medium Bullet kit (SAGM Bullet kit), and Reagent Pack were obtained from Bio Whittaker (Walkersville, MD, USA).

Statistical analysis

Statistical analysis was performed using the statistical software SPSS. Differences in staining between NSCLC and SCLC sections, and the adjacent normal lung tissue were compared using the Wilcoxon signed rank and Mann–Whitney U tests. χ² test, Fishers exact test, or χ² test for trend was employed to assess the relationship between the clinicopathological characteristics and the expression of Cox-2 (recategorised as 1 or 2 vs. 3 because of small numbers). A P value lower than 0.05 was regarded as statistically significant. Results are given as the median (inter-quartile range). The results were evaluated twice and the intra-observer variability was assessed using kappa analysis.

Results

Immunohistochemistry

The results are summarised in Table 1 and Figs. 1 and 2. The differences in COX-1 and COX-2 expression among the normal lung structures, NSCLC and SCLC sections were assessed qualitatively and semi-quantitatively. The sections were re-scored twice and the intra-observer variability was assessed as 10 discrepancies out of 90 observations [kappa agreement 0.796 (SEM 0.06)]. The original value was used in the statistical analysis.

Dense and intense granular cytoplasmic COX-2 staining was found in all 52 resections from NSCLC (Fig. 2a). Although COX-2 was most consistently expressed in the adenocarcinoma sections than in squamous cell lung cancers the difference did not reach significance when they were scored separately. The staining was diffuse and much stronger than adjacent respiratory epithelium. Poorly differentiated carcinomas seemed to be more strongly stained for COX-2 when compared with well-differentiated areas. COX-2 staining was significantly lower in the majority of the SCLC samples [COX-2 score 3 (3, 3) vs. 2 (1, 2.5); NSCLC vs. SCLC; P<0.001], although some samples still showed moderate or strong staining (Fig. 2b).

Table 1  COX immunoreactivity.

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<th>NSCLC</th>
<th>SCLC</th>
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<tr>
<td></td>
<td>n = 52</td>
<td>n = 13</td>
<td></td>
</tr>
<tr>
<td>COX-1</td>
<td>2 (0, 2)</td>
<td>1 (0, 1)</td>
<td>P = 0.482</td>
</tr>
<tr>
<td>COX-2</td>
<td>3 (3, 3)</td>
<td>1 (1, 1)</td>
<td>P &lt; 0.001</td>
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P values for different comparisons are shown. The values are given as the median (inter-quartile range). NSCLC—non-small cell lung cancer; SCLC—small cell lung cancer; N1—normal lung tissue adjacent to NSCLC; N2—normal lung tissue adjacent to SCLC; COX—cyclooxygenase.

Figure 1  COX-2 expression in NSCLC vs. SCLC. NSCLC—non-small cell lung cancer; SCLC—small cell lung cancer; COX—cyclooxygenase; (1) weak and patchy staining; (2) weak but extensive or strong but patchy staining and (3) strong and extensive staining.
There was no increase in COX-1 staining in any tumour type compared with the normal surrounding lung tissue, but moderate cytoplasmic staining was commonly seen (Fig. 2c). Some very large bizarre tumour cells were stained strongly with both COX-1 and COX-2.

Relationship between COX-2 expression and clinicopathological characteristics

No significant association was found between COX-2 expression and various clinicopathological parameters including age ($P = 0.52; P = 0.13$ for NSCLC & SCLC, respectively), sex, smoking and pack-year smoking history, and the stage of the disease in both NSCLC and SCLC specimens (Table 2). There was a significant correlation between the expression of COX-2 and tumour differentiation in NSCLC. We determined no significant relationship between the survival of patients with NSCLC and SCLC and the level of COX-2 immunoreactivity ($P = 0.317; P = 0.488$, respectively).

A similar relationship between the COX-2 levels and the clinical characteristics of the patients with squamous cell and adenocarcinomas to those calculated for NSCLC patients as a whole was found when the data were reanalysed separately.

COX-2 expression in cancer cell lines

Western blot analysis of the three cancer cell lines revealed significantly higher COX-1 expression in COR-L23 line and COX-2 expression in two NSCLC cell lines (MOR/P and A549) in comparison with COX-1 and COX-2 expression in cultured normal bronchial epithelial cells, respectively (Fig. 3).

Discussion

Our study demonstrated that COX-2 is overexpressed in NSCLC and two cancer cell lines. All 52 NSCLC resections including 46 squamous cell and 6 adenocarcinomas showed intense cytoplasmic
COX-2 staining, significantly higher compared to normal lung tissue. This is one of the first studies to include a large number of squamous cell lung cancer specimens. By contrast, there was no significant difference in COX-2 immunoreactivity in the SCLC sections compared with the normal surrounding lung tissue. COX-1 showed weak and patchy cytoplasmic expression in the majority of the examined lung cancer specimens, as well as in the normal adjacent lung tissue. Cultured human NSCLC adenocarcinoma cell lines showed elevated levels of COX-2 but not COX-1 when compared to NHBEC.

Our findings are similar to previously published observations which have used immunohistochemical analysis and molecular biological methods, and reported overexpression of COX-2 protein in non-small cell lung cancer, particularly adenocarcinomas. Subsequent research has looked at the COX immunoreactivity in squamous cell lung cancer, particularly in small cell lung cancer.
cancer and suggested that COX-2 protein has similar pattern in both types of NSCLC specimens, which was also the case in our study.\(^4\) Watkins et al.,\(^5\) however, found that the most intense expression of COX-2 mRNA was in lung adenocarcinoma cells, followed by that in large cell tumours and the lowest levels were observed in squamous cell carcinomas. A recently published immunohistochemical study including 101 NSCLC resections (51 adenocarcinoma and 46 squamous cell lung cancers) showed increased COX-2 levels in a small proportion 9 of 46 (20%) of the examined squamous cell lung carcinomas. Also a relatively small number of the examined adenocarcinomas 21 of 51 (41%) were assessed as COX-2 positive.\(^3\) Wolff et al.\(^1\) using two methods Northern blot analysis and immunohistochemistry demonstrated elevated COX-2 not only in adenocarcinoma but also in all examined (11 out of 11) squamous cell lung carcinoma specimens. Up-regulation of COX-2 has been observed in squamous cell carcinomas of head and neck, skin, oesophagus, cervical and urinary bladder cancers as well.\(^8,9,45,46\) The discrepancies in the reported findings may reflect the different methods used in these studies and particularly the different antibodies and their concentration. The studies, however, in which two different methods for evaluating COX expression were utilised, have detected COX-2 overexpression in different squamous cell carcinomas.\(^9,12,45–48\)

Experiments in animal lung tumourigenesis demonstrating increased COX-2 protein levels and PGE synthesis also support our results. By RT-PCR, COX-2 PCR products were detected in mouse lung adenomas.\(^15,16\) Bauer showed not only elevated COX-2 but also COX-1 in mouse lung carcinomas.\(^49\) Lewis lung carcinoma cells produce increased amounts of PGE\(_2\), which are blocked by non-selective COX inhibitors. This leads to reduction of the growth and metastatic potential of the tumour cells and indicates that PGE\(_2\) may play a role in the survival and proliferation rate of lung cancer cells.\(^50\)

Although our results suggested that COX-2 was most consistently expressed in the adenocarcinoma than in squamous cell lung cancer sections, the difference did not reach significance. The strongest staining in our study, however, was observed in poorly differentiated carcinomas. Similar results were observed by other researchers who reported greater expression of COX-2 in poorly differentiated lesions than in well or moderately differentiated areas.\(^34,43\) Poorly differentiated adenocarcinomas have been associated with increased metastatic potential and more adverse prognosis compared to well or moderately differentated adenocarcinomas.\(^43,44\) Wolff’s study,\(^12\) however, has described particularly elevated COX-2 levels in well differentiated adenocarcinomas. The difference among studies suggests that further research including a larger number of tumours is required to clarify this area, which may have a crucial role for the progression of the tumour.

We found no significant relationship between the level of COX-2 expression and the majority of the clinicopathological characteristics of the patients. No other studies have previously reported a significant relationship between COX-2 expression and most of the clinicopathological characteristics of the patients. Khuri et al. have demonstrated a significant correlation between the high level of COX-2 expression and a worse overall survival rate \(P = 0.001\) and a worse disease-free survival rate \(P = 0.022\).\(^4\)

All in vivo studies including ours have shown no difference between COX-1 expression in NSCLC sections and the surrounding normal lung tissue. Similar data have been reported for the COX-1 and COX-2 immunoreactivity in SCLC.\(^12,36\)

The findings of our immunohistochemical study are supported by additional in vitro experiments. We used Western blot analysis to compare the COX-1 and COX-2 expression in NHBEC with this in cultured cancer cell lines. The levels of COX-2 in both adenocarcinoma cell lines A549 and MOR/P were higher than these in NHBEC. Tsubouchi also detected increased COX-2 in the NSCLC cell lines A549 and PC14 whose growth and PGE\(_2\) production was completely blocked by Meloxicam.\(^31\) In contrast, COR-L23 cell line showed overexpression of COX-1 when compared with the NHBEC. Cornetta et al. studied the COX levels in thyroid neoplasms and observed no COX-2 expression in anaplastic carcinomas.\(^51\) In a study of mouse lung carcinogenesis, both COX-1 and COX-2 were found to be up-regulated, and different expression patterns were observed in tumours with different growth patterns.\(^48\) In addition, treatment with a tobacco carcinogen led to induced nuclear factor kappa B (NF\(\kappa B\)) and COX-1 expression in a human macrophage model.\(^51\) We could speculate that COX-1 expression may also be induced in some types of lung cancer, perhaps by specific carcinogens. Our study included no clinical large cell anaplastic lung cancers and only one study to date has included this tumour type and found lower COX-2 levels in large cell than adenocarcinoma.\(^35\) This finding may warrant further investigation, but is beyond the scope of the current paper.

In conclusion, our data indicate that NSCLC, but not SCLC, have the ability to express increased
levels of the inducible COX-2. Further investigations are required to determine whether modulating the expression of COX-2 would improve the prognosis of this deadly disease.

References


