[CANCER RESEARCH 58, 3761–3764, September 1, 1998]

Advances in Brief

Increased Expression of Cyclooxygenase 2 Occurs Frequently in Human Lung Cancers, Specifically in Adenocarcinomas[1]

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Abstract

Cyclooxygenase (COX)-2 expression was immunohistochemically examined in 59 human lung cancers as well as in normal and premalignant lung specimens. In contrast to scattered weak reactivity seen in normal peripheral airway epithelial cells, markedly up-regulated COX-2 expression was detected in about one-third of atypical adenomatous hyperplasias and carcinoma *in situ* specimens, and a significant increase in COX-2 expression was observed in 70% of invasive adenocarcinoma cases. Interestingly, the proportion of adenocarcinoma cells with marked COX-2 expression was much greater in lymph node metastases than in the corresponding primary tumors. In contrast, small cell carcinomas showed virtually negligible expression, and squamous cell carcinomas showed infrequent and low expression. These findings suggest that an increase in COX-2 expression may be associated with the development of adenocarcinomas and possibly with acquisition of an invasive and metastatic phenotype.

Introduction

Human lung cancers are classified into four major categories based on their histological features, *i.e.*, adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma. Although the incidence of lung cancer is, unfortunately, still rapidly increasing in Japan, adenocarcinoma is, in absolute terms, the most frequently occurring subtype. Adenocarcinoma has also surpassed squamous cell carcinoma as the most common histological type of lung cancer in the United States (1). From the etiological point of view, adenocarcinoma is known to be the least closely related to cigarette smoking, suggesting that chemopreventive efforts minimizing the risk of adenocarcinoma may have a significant impact on the reduction of lung cancer occurrence in individuals without active smoking habits.

COX-1[4] and COX-2 are the rate-limiting enzymes involved in the conversion of arachidonic acid to prostanoids. COX-1 is considered a housekeeping gene responsible for various physiological functions such as cytoprotection of the stomach, vasodilation in the kidney, and production of a proaggregatory prostanoid, thromboxane, by the platelets (2). Recent studies have suggested that COX-2, which was isolated as an inducible immediate-early gene, may have various other functions in addition to its well-known role in inflammatory reactions (2, 3). It is noteworthy that COX-2 has also been linked to carcinogenesis (4–8). Oshima *et al.* (9) recently provided direct genetic evidence that formation of intestinal polyps in $Apc^{\Delta 716}$ knockout mice was dramatically suppressed by crossing with COX-2 knockout mice, indicating that induction of COX-2 represents an early rate-limiting step. Moreover, a number of clinical and epidemiological studies suggest that NSAIDs induce a significant, and often complete, regression of colonic polyps in patients with familial adenomatous polyposis and reduce the risk of colon cancer also in nonfamilial adenomatous polyposis subjects (10–15). Although there have been relatively few studies published on the possible effects of NSAID use on the risk of cancers at sites other than the large bowel, aspirin use was found to be associated with statistically lower risk of cancers of the lung and breast among participants in the National Health and Nutrition Examination Survey (16). To date, however, very little is known about COX-2 expression in normal and neoplastic cells of the lung.

We report here a detailed analysis of COX-2 expression in 59 lung cancer cases representing all major histological subtypes as well as in normal and premalignant lung specimens. The results showed that significantly increased COX-2 expression occurred preferentially in adenocarcinomas. In addition, markedly higher and more homogeneous COX-2 expression was observed in lymph node metastases of adenocarcinomas than in the corresponding primary tumors.

Materials and Methods

Patient Samples. A panel of 59 primary lung cancer specimens including 9 small cell carcinomas, 23 adenocarcinomas, 22 squamous cell carcinomas, 3 large cell carcinomas, and 2 adenosquamous cell carcinomas were examined in the present study. Premalignant lesions including three atypical adenomatous hyperplasias, seven adenocarcinomas *in situ*, four squamous metaplasias and dysplasias, and four squamous cell carcinomas *in situ* were also assessed. In addition, four normal lung specimens taken from patients with metastatic colon cancer were used to examine COX-2 expression in individuals without lung cancer. In the present study, the term "adenocarcinoma *in situ*" is used in accordance with the criteria used by Hung *et al.* (17) so that "adenocarcinoma *in situ*" includes the lesions with nuclear features falling within the cytological criteria of malignant cells but without invasive or destructive growth. In addition, lymph node metastases present in 13 adenocarcinoma cases were also examined for COX-2 expression.

Immunohistochemistry. Four-μm-thick sections of formalin-fixed and paraffin-embedded tissue samples were immersed in 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, microwaved in citrate-phosphate buffer (pH 6.0) for antigen retrieval, and incubated with 10% normal goat serum for 30 min to block nonspecific binding. Rabbit polyclonal antibody specific for human COX-2 (IBL, Gunma, Japan) was then applied as the primary antibody at a dilution of 1:25 at 4°C overnight, followed by a standard staining procedure using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Nonimmunized rabbit serum was used for the negative control.

Evaluation of COX-2 Immunostaining. The intensity and extent of positivity of every stained specimen was graded on a scale of 0–3 by two blinded observers (T. H. and Y. Y.) on three separate occasions in a coded manner. In cases of occasional discrepancy in the interpretation, consensus was achieved after discussion and with the aid of a multiheaded microscope.

Staining intensities in normal, premalignant or cancerous cells were evaluated by using reactions in smooth muscles and vascular endothelial cells as internal built-in controls. When positive reactions in the subjects were similar to those in the internal control cells, they were scored as grade 2. Tissue specimens showing significantly more intense staining than the internal control cells were recorded as grade 3, and cells with a readily noticeable reduction in staining intensity as grade 1. Tissue samples showing undetectable or negligible expression were scored as grade 0. The degree of positive reaction showing the grade 2 or 3 intensity was further categorized into four classes: 0,

Received 6/4/98; accepted 7/17/98.

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[1] This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan; by a grant for Biomedical Research from Bristol Myers Squibb; by a grant from the Smoking Research Foundation; and by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, Sports and Culture, Japan.

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[4] The abbreviations used are: COX, cyclooxygenase; NSAID, nonsteroidal antiinflammatory drug; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

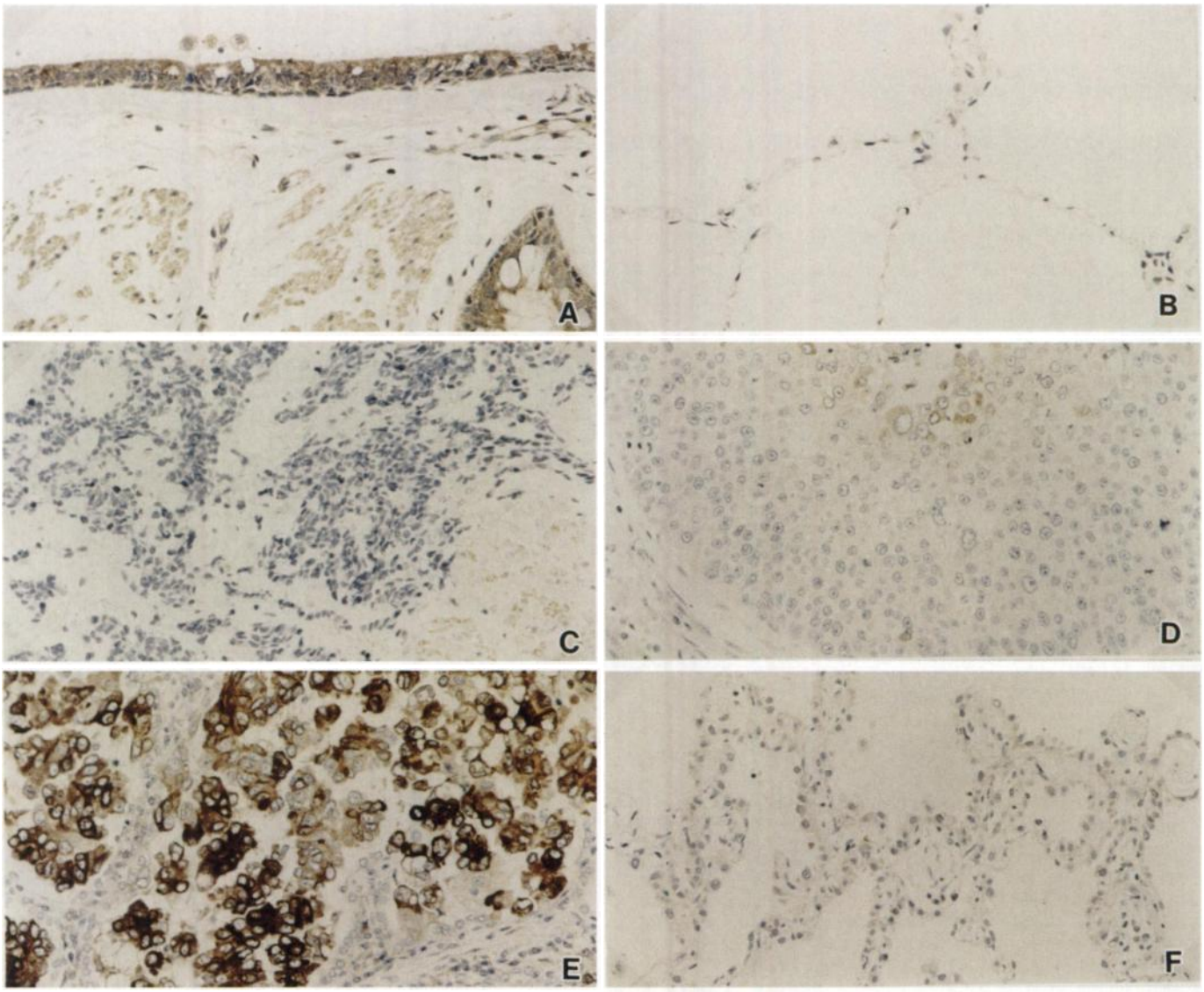

Fig. 1. Representative results of COX-2 immunostaining in human normal, preneoplastic, and carcinomatous lung tissues. COX-2 expression is seen in bronchial epithelium (*A*) as well as bronchial smooth muscles at grade 2 intensity and >60% positivity, whereas pneumocytes (*B*) show scattered positivity of grade 1 intensity. A SCLC case exhibits virtually no immunoreactivity (*C*), whereas weak and occasional staining is detectable in a squamous cell carcinoma specimen (*D*). In contrast, intense and diffuse immunoreactivity is observed in a case of adenocarcinoma (*E*). Atypical adenomatous hyperplasia (*F*) shows occasional staining of weak to moderate intensity.

<30%, 30–60%, >60%. When positive reactions were weak (grade 1 intensity), the degree was not further classified.

Statistical Analysis. The χ^2 test and Fisher's Exact probability test were used to examine the association between COX-2 expression status and clinicopathological features. Logistic regression analysis of clinicopathological parameters potentially related to COX-2 expression was performed to identify which independent factors might jointly have a significant influence. $P < 0.05$ was considered to have statistical significance.

Results and Discussion

COX-2 Expression in Normal Lung Tissues. We initiated the present study by confirming the specificity of the anti-COX-2 antibody with the aid of four lung cancer cell lines, which expressed COX-2 mRNAs at negligible (ACC-LC-176), low (ACC-LC-319), modest (ACC-LC-323), and abundant (ACC-LC-91) levels in Northern blot analysis.[5] Good concordance was observed between immunohistochemical reactivity and the amounts of COX-2 transcripts in each of the cell lines. We then proceeded to examine COX-2 expression in normal lung tissues. COX-2 expression was observed in bronchial epithelial cells, type I and II pneumocytes, smooth muscle cells, vascular endothelial cells, and inflammatory mononuclear cells at various intensities and to various degrees (Fig. 1). Bronchial epithelial cells and stromal cells showed rather ubiquitous and modest expression, most of which corresponded to a grade 2 intensity and >60% positivity (Table 1). In contrast, only scattered weak reactivity was observed in pneumocytes in peripheral airways, most of which were of grade 1 intensity.

COX-2 Expression in Lung Cancers and Their Premalignant Lesions. COX-2 expression in neoplastic cells of the lung was then examined immunohistochemically. Based on the results obtained with nonneoplastic epithelial cells, we classified tumors showing grade 3 intensity and >30% positivity for grade 2 or 3 as high expressers of COX-2. Diffuse and intense expression of COX-2 was frequently detected in adenocarcinomas, whereas 16 (70%) of 23 adenocarcinomas could be considered high expressers of COX-2 (Fig. 1 and Table 1). In contrast, eight (89%) of nine SCLCs did not express COX-2 at detectable levels, whereas modest COX-2 expression was observed in the remaining single case. Positive reactions were observed in a fraction of the squamous cell carcinomas, but even in cases with high COX-2 expression, positivity was often limited to cells in the superficial layer of the stratified carcinoma foci with keratinization (Fig. 1).

[5] Unpublished observation.

Table 1 *COX-2 expression in human lung cancers as well as in normal and premalignant lung tissues*

Intensity (highest) / Percentage with grade 2 or 3	*n*	0	1	2			3			Cases with high expression[a]
		—	–							
		0	0	<30%	30–60%	>60%	<30%	30–60%	>60%	
Normal and premalignant/preinvasive lesions										
Central airway										
Bronchial epithelium	4	0	0	0	0	4	0	0	0	0
Squamous metaplasia	4	0	2	0	1	1	0	0	0	0
Squamous dysplasia	4	0	2	0	0	2	0	0	0	0
Carcinoma *in situ*	4	0	3	0	0	1	0	0	0	0
Peripheral airway										
Types I and II pneumocytes	4	0	4	0	0	0	0	0	0	0
Atypical adenomatous hyperplasia	3	0	0	2	0	0	0	1	0	1 (33%)
Carcinoma *in situ*[b]	7	0	0	3	2	0	0	1	1	2 (29%)
Stromal cells										
Smooth muscles	12	0	0	0	0	12	0	0	0	0
Vascular endothelial cells	12	0	0	0	1	11	0	0	0	0
Lung cancers										
SCLC	9	8	0	0	0	1	0	0	0	0
NSCLC										
Adenocarcinoma	23	0	1	2	0	1	3	2	14	16 (70%)
Squamous cell carcinoma	22	11	1	1	1	0	5[c]	3	0	3 (14%)
Large cell carcinoma	3	1	0	0	0	1	1	0	0	0
Adenosquamous carcinoma	2	0	0	0	0	0	1	1	0	1 (50%)

[a] The presence of cells expressing COX-2 at grade 3 intensity and positive reactions is seen in >30% of tumor cells with grade 2 or 3 of intensity.
[b] Lesions with nuclear features falling within the cytological criteria of malignant cells but without apparent stromal invasion.
[c] Only a few carcinoma cells with grade 3 intensity were seen and mostly at the foci with keratinization.

We next examined COX-2 expression in premalignant lesions to gain an insight into its possible connection with malignant progression of lung epithelial cells. In the peripheral airway, markedly up-regulated expression of COX-2 was detected in one (33%) of three atypical adenomatous hyperplasias that are generally considered to be premalignant lesions of adenocarcinoma, as well as in two (29%) of seven carcinoma *in situ* cases. These results indicate that COX-2 expression is up-regulated in only a fraction of premalignant lesions of peripheral airways and that further induction appears to take place during progression. In contrast, none of the squamous metaplasias, dysplasias, and carcinomas *in situ* exhibited a high expression of COX-2, as could be expected from the rare occurrence of an increase in COX-2 expression in squamous cell carcinomas.

Association between COX-2 Expression and Clinicopathological Characteristics. In the present study, we observed differential expression patterns of COX-2 in SCLC and NSCLC. Furthermore, increased expression of COX-2 was also shown to be apparently associated with a particular histological subtype within the NSCLC histologies, *i.e.*, adenocarcinoma. We therefore examined the relationship between an increase in COX-2 expression and various clinicopathological features of NSCLC (Table 2). Such an increase was found to be significantly associated with histology ($P = 0.001$ by Fisher's Exact probability test) and a past history of cigarette smoking ($P = 0.01$). However, multivariate analysis using the stepwise method revealed that an increase in COX-2 expression was significantly associated only with histology ($P = 0.001$).

COX-2 Expression in Metastatic Lymph Nodes. Adenocarcinoma is often referred to as one of the most morphologically heterogeneous carcinomas, and it frequently shows an admixture of well- to poorly differentiated lesions in a single given tumor. During the course of this study, we sometimes observed more intense expression of COX-2 in poorly differentiated lesions than in well- or moderately differentiated lesions (Fig. 2*A*). These findings led us to compare COX-2 expression in primary tumors and in their corresponding metastatic lymph nodes to assess a possible association between COX-2 expression and tumor progression. Thirteen of 23 cases with adenocarcinoma histology had metastases to the regional lymph nodes and thus were considered relevant. Although 10 of the 13 cases were classified as high expressers of COX-2 at both primary and metastatic sites, two of the remaining three cases that expressed COX-2 at low levels in primary tumors exhibited high expression only in lymph node metastases. More striking was the homogeneity of COX-2 expression in metastatic cells (Fig. 2*B*). In fact, the vast majority of metastatic tumor cells showed very intense COX-2 staining (grade 3) in 8 of the 13 cases examined, which represented a marked increase in the proportion of highly expressing cells in metastases when compared with that in the corresponding primary tumor sites.

The present study has shown that a significant increase in COX-2 expression was preferentially observed in adenocarcinomas, in contrast to virtually negligible expression in small cell carcinomas and significantly less frequent and lower expression in squamous cell carcinomas. The present findings of up-regulated COX-2 expression

Table 2 *Relationship between clinical characteristics and high expression of COX-2 in NSCLC*

	COX-2		
	Low	High	*P*
Number of cases	30	20	
Sex			0.417
Male	24	14	
Female	6	6	
Age			0.918
Median	58	58	
Range	32–77	43–74	
Histology			0.001[a]
Adenocarcinoma	7	16	
Squamous cell carcinoma	19	3	
Large cell carcinoma	3	0	
Adenosquamous carcinoma	1	1	
Tumor size (pT)			0.133
1	7	9	
2	13	9	
3	9	1	
4	1	1	
Nodal involvement (pN)			0.969
0	14	9	
1	7	4	
2	7	5	
3	2	2	
Disease stage			0.789
1	12	9	
2	2	2	
3A	13	6	
3B	3	3	
Smoking history			0.012[a]
Smoker	26	11	
Nonsmoker	4	9	

[a] Multivariate analysis using the stepwise method revealed that an increase in COX-2 expression was significantly associated only with histology ($P = 0.001$), whereas smoking did not show independent relationship with COX-2 expression status.

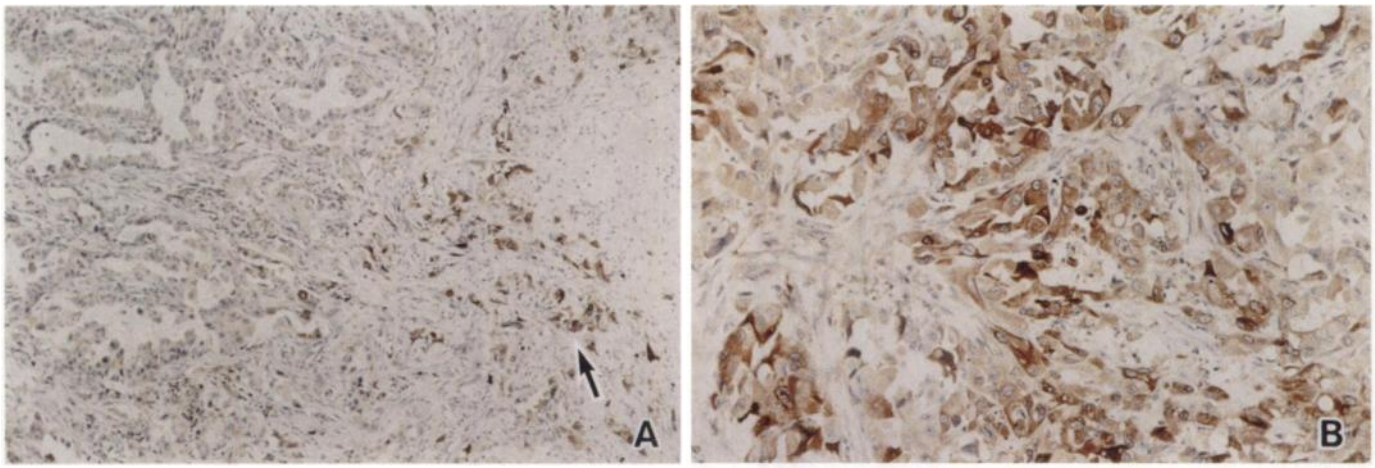

Fig. 2. COX-2 expression in the primary tumor (*A*) and the corresponding lymph node metastasis (*B*) of a representative adenocarcinoma case. A major part of the primary lesion showed a moderately differentiated, papillotubular growth pattern with a limited area of poorly differentiated, infiltrating growth (*arrow*). The primary lesion demonstrated moderate expression of COX-2, whereas the poorly differentiated infiltrating lesion exhibited significantly more intense staining. The metastatic lesion in the lymph node consisted of less differentiated cancer cells with a highly increased COX-2 expression.

in one-third of premalignant lesions and in over two-thirds of adenocarcinomas appear to be compatible with those reported for adenomas and cancers of the colon, *i.e.*, 80–90% of colorectal adenocarcinomas and in 40–50% of premalignant adenomas (3). It should be of considerable interest to examine other types of human cancers to determine whether increased COX-2 expression might be a characteristic feature of human cancers derived from glandular epithelium. Recent studies have demonstrated that COX-2 overexpression may alter the biological behavior of tumor cells in a number of ways (18–20). Tsujii *et al.* (19) reported that constitutive expression of COX-2 could lead to phenotypic changes that alter the metastatic potential of colorectal cancer cells, resulting in 6-fold enhancement of invasiveness. In our study, a marked increase in COX-2 immunoreactivity was often observed in tumor-invasive lesions and in lymph node metastases. The present findings consequently suggest that an increase in COX-2 expression may be associated with tumor progression of adenocarcinoma of the lung and that COX-2 might play a part in the acquisition of an invasive and metastatic phenotype.

It is worth noting that the chemopreventive effect of aspirin has recently been demonstrated in an A/J mice model (21), and that an association between aspirin consumption and reduced lung cancer incidence in men was also reported as the result of an epidemiological study (16). On the one hand, detection of small non-invasive adenocarcinomas is increasingly feasible because of recent advances in diagnostic imaging technologies such as helical computed tomography. On the other hand, accumulating evidence suggests that the presence of multiple atypical adenomatous hyperplasias is more probable in adenocarcinoma cases. Further investigation will therefore be warranted to determine the putative role of COX-2 in the development and progression of lung cancers, and whether the enzyme encoded by this gene represents a potential target for chemopreventive strategies specifically aimed at adenocarcinomas of the lung.

Acknowledgments

We thank H. Ishida for technical assistance.

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Cancer Res 1998;58:3761-3764.