Overexpression of Cyclooxygenase-2 in Squamous Cell Carcinoma of the Urinary Bladder[1]

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ABSTRACT

Epidemiological studies indicate that the development of squamous cell carcinoma of the urinary bladder is closely associated with chronic inflammation of the urinary tract, but the underlying mechanism is unknown. Cyclooxygenase (COX)-2 is involved in tumorigenesis in many tumors. The purpose of this study was to investigate the role of COX-2 in squamous cell carcinoma of the urinary bladder by immunoblot and immunohistochemical analyses. COX-2 protein was undetectable in normal bladder samples, but was expressed in 29 of 29 (100%) squamous cell carcinomas and in 8 of 8 (100%) squamous metaplasias. The expression of COX-2 showed intense, homogenous cytoplasmic immunostaining in squamous cell carcinomas. In contrast, COX-2 was heterogeneously expressed in 6 of 12 (50%) cases of transitional cell carcinoma of the bladder combined with squamous cell carcinoma, consistent with previous findings. We provide the first evidence that COX-2 is expressed in squamous cell carcinomas of the urinary bladder and in the precursor lesions, indicating its involvement in the development of this type of malignancy.

INTRODUCTION

COX[3] exists in two isoforms, COX-1 and COX-2, and catalyzes the conversion of arachidonic acid to prostaglandin H_2 (1). Whereas COX-1 is expressed constitutively in most normal tissues and is required for their normal physiological function, COX-2 usually is not detectable, but is induced in response to various stimuli such as cytokines, growth factors, oncogenes, and tumor promoters (1).

Several lines of evidence suggest that COX-2 is important in carcinogenesis. For example, epidemiological studies have shown that nonsteroidal anti-inflammatory drugs, which are COX inhibitors, reduce the risk of colorectal cancer (2). Recently, COX-2 was found to be up-regulated in various forms of

Table 1 Immunohistochemical detection of COX-2 in squamous cell carcinoma in the urinary bladder

Patient	Histopathological findings	COX-2 expression	
		% positive cells	Intensity
1	G1, pT3a	>80	Very high
2	G2, vpT3b	>80	Very high
3[a]	G1, pTx	>80	Very high
4	G3, pT3b	>80	Very high
5	G3, pT3b	>60	High
6	G1, pT3b	>80	Very high
7	G3, pT4	>80	Very high
8	G1, pT3b	>80	High
9	G3, pT4	>80	Very high
10	G3, pT3b	>80	Very high
11	G3, pT3a	>80	Very high
12	G3, pT4	>80	Very high
13	G1, pT3b	>80	Very high
14	G1, pT4	>80	Very high
15	G1, pT3b	>80	Very high
16[a]	G1, pTx	>80	Very high
17	G3, pT3b	>80	Very high
18	Normal epithelium	None	
19	Normal epithelium	None	
20	Normal epithelium	None	
21	Normal epithelium	None	
22	Normal epithelium	None	

[a] All patients had invasive tumors and underwent radical resection of the tumor, except patients 3 and 16, who were inoperable and underwent transurethral biopsy of the tumor.

cancer, whereas COX-1 levels were relatively constant (3–7). Furthermore, COX-2 knockout mice reduced the development of intestinal polyps in adenomatous polyposis coli mutant mice, a murine model of familial adenomatous polyposis (8). In addition to the genetic evidence implicating COX-2 in carcinogenesis, newly developed COX-2-selective inhibitors suppressed tumor formation in experimental animals (8, 9).

Chronic inflammation is a recognized risk factor for epithelial carcinogenesis. Both epidemiological (10) and animal studies (11) have indicated that chronic urinary tract infection is implicated in the development of bladder cancer, especially the squamous cell type of bladder cancer. Patients who are paraplegic secondary to spinal cord injury (12) or infected with *Schistosoma haematobium* (13) are particularly vulnerable to the development of squamous cell carcinoma of the bladder because of the high association with chronic bacterial infection in the urinary tract. However, the mechanism of the increased risk of squamous cell carcinoma of the bladder by chronic inflammation is not understood.

In the present study, we demonstrate that COX-2 is markedly expressed in all squamous cell carcinomas examined, suggesting that chronic inflammation stimulates the production of COX-2 and that an increased COX-2 level, in turn, induces the development of squamous cell carcinoma of the bladder.

Table 2 Immunohistochemical detection of COX-2 in transitional cell carcinoma with squamous cell carcinoma in the urinary bladder

| | | COX-2 expression | | | |
| | | Transitional cell carcinoma | | Squamous cell carcinoma | |
Patient	Histopathological findings	% positive cells	Intensity	% positive cells	Intensity
1	TCC > SCC;[a] T1b	20	Moderate	>80	Very high
2[b]	TCC > SCC; pTx	None		>80	Very high
3	TCC > SCC; pT1b	15	Very high	>80	Very high
4	TCC > SCC; pT1a	20	High	>80	Very high
5	TCC > SCC; pT1b	None		>80	Very high
6	TCC = SCC; pT1b	None		>80	Very high
7	TCC > SCC; pT1b	20	Low	>80	Very high
8	TCC > SCC; pT1b	None		>80	Very high
9[b]	TCC > SCC; pTx	None		>80	Very high
10[b]	TCC > SCC; >pT2	40	High	>80	Very high
11[b]	SCC > TCC; >pT2	None		>80	Very high
12	TCC > SCC; >pT3a	>80	Very high	>80	Very high

[a] TCC, transitional cell carcinoma; SCC, squamous cell carcinoma.
[b] All patients underwent radical resection of the tumor, except for patients 2, 9, 10, and 11, who were inoperable and underwent transurethral biopsy of the tumor.

Table 3 Immunohistochemical detection of COX-2 in squamous metaplasia tissue in the urinary bladder

Patient	COX-2 expression[a]
1	High
2	Very high
3	Moderate
4	Very high
5	Very high
6[a]	Very high
7[a]	Very high
8	Very high
9	Very high
10	Very high

[a] Squamous metaplasia at the luminal side was stained.
[b] All patients except patients 6 and 7 had pathologically confirmed transitional cell carcinoma or squamous cell carcinoma of the urinary bladder.

Fig. 1 Immunoblot analysis of COX-2 in squamous carcinoma tissues of the bladder. COX-2 expressions in representative tumor (*T*) and nontumorous (*N*) transitional epithelial tissues are shown. *Samples T1–5* correspond to patients 1, 4, 5, 9, and 12, and *samples N1 and 2* correspond to patients 18 and 19 in Table 1.

MATERIALS AND METHODS

Tumor Samples. Tissue samples were obtained from 37 patients who had undergone radical cystectomy (20 patients), radical cystectomy with low anterior resection (1 patient), total pelvic exenteration (1 patient), or transurethral resection (15 patients) between January 1990 and December 1998. Samples included 17 muscle invasive squamous cell carcinomas with or without squamous metaplasias (pure squamous cell carcinomas; Table 1), 12 transitional cell carcinomas with squamous cell carcinomas (Table 2), and 8 squamous metaplasias with or without transitional cell carcinomas or squamous cell carcinomas (Table 3). Nontumorous epithelial tissues were obtained from five patients who had undergone radical cystectomy for transitional cell carcinoma of the bladder. The median age at surgery was 65.4 years (range, 48–75 years). All experiments were performed after obtaining informed consent according to institutional rules.

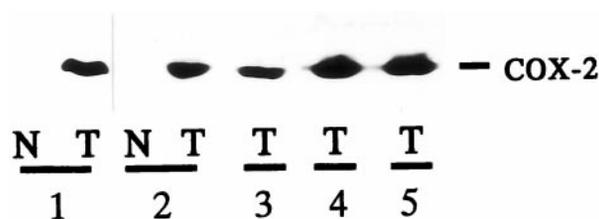The tissue was quickly frozen in liquid nitrogen, and the remaining portion of the specimen was fixed in 10% formalin in PBS and embedded in paraffin. Histopathological findings were assessed according to the criteria of the Japanese Urological Association (14). The degree of tumor differentiation was classified graded a scale from 1 (well differentiated) to 3 (poorly differentiated).

Immunoblot Analyses. Samples were homogenized and lysed in modified radioimmunoprecipitation assay buffer (7). The lysates were centrifuged at $10,000 \times g$ for 20 min at 4°C, and the supernatants were used for immunoblot analysis. Samples containing 50 μg of protein were resolved in 8% SDS-polyacrylamide gels and electrophoretically transferred to a sheet of nitrocellulose. The blots were incubated overnight with a goat polyclonal anti-COX-2 (cat. no. sc-1745; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody (1:200 dilution). The nitrocellulose membrane was incubated with horseradish peroxidase-conjugated antigoat IgG antibody (cat. no. sc-2020; Santa Cruz Biotechnology). Localized horseradish peroxidase activity was detected using the enhanced chemiluminescence Western blotting system (Amersham, Buckinghamshire, United Kingdom). To confirm antibody activity, anti-COX-2 antibody was incubated with the blocking peptide (cat. no. sc-1745p; Santa Cruz Biotechnology) before use.

Tissue Staining and Evaluation. Tissue samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, and
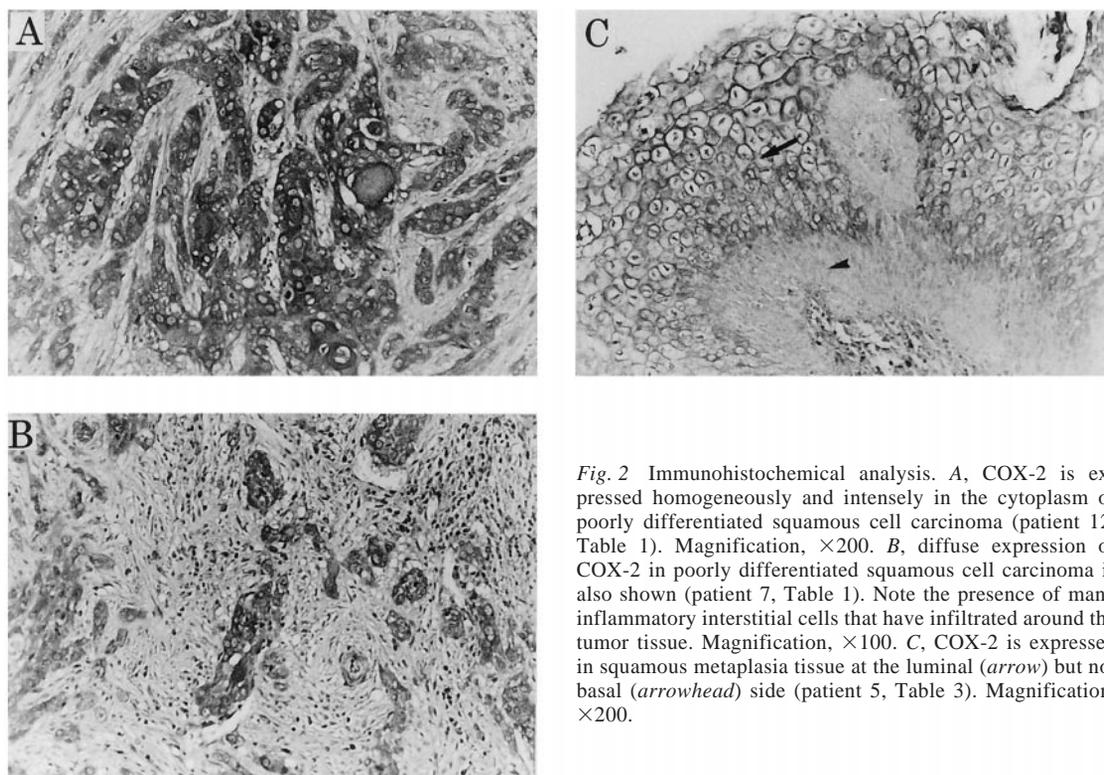
Fig. 2 Immunohistochemical analysis. *A*, COX-2 is expressed homogeneously and intensely in the cytoplasm of poorly differentiated squamous cell carcinoma (patient 12, Table 1). Magnification, ×200. *B*, diffuse expression of COX-2 in poorly differentiated squamous cell carcinoma is also shown (patient 7, Table 1). Note the presence of many inflammatory interstitial cells that have infiltrated around the tumor tissue. Magnification, ×100. *C*, COX-2 is expressed in squamous metaplasia tissue at the luminal (*arrow*) but not basal (*arrowhead*) side (patient 5, Table 3). Magnification, ×200.

cut into 3-μm thick sections. After initial examination of the H&E-stained slides, serial sections from one representative paraffin block were immunostained. Sections were deparaffinized with xylene, dehydrated with 98% ethanol, and treated in a microwave for 10 min at 65°C. Endogenous peroxidase was inactivated by immersing the slides in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature. Sections were incubated in 5% skim milk for 30 min at room temperature and then incubated with a goat anti-COX-2 polyclonal antibody, diluted 1:200 with 5% skim milk in PBS for 1 h at room temperature. Sections were then washed with PBS and incubated for 30 min with biotinylated goat antirabbit IgG at room temperature. After washing, sections were incubated for 30 min with avidin-biotin-peroxidase complex (Vectastain kit; Vector Laboratories, Inc., Burlingame, CA). Color was developed by 0.005% (v/v) diaminobenzidine (Nakarai Chemicals, Kyoto, Japan) and 0.008% (v/v) hydrogen peroxide in PBS for 20 min. Sections were counterstained with hematoxylin and mounted on slides. For negative controls, the primary antibody was omitted from samples or preincubated with the blocking peptides as described previously (7).

Specimens were regarded as COX-2 negative when <5% of the cancer cells were stained and as COX-2 positive when >5% of the cancer cells were stained. Staining intensity was graded into four categories: low, moderate, high, and very high.

RESULTS

Immunoblot Analyses of COX-2 in Squamous Cell Carcinoma and Nontumorous Epithelial Tissues. COX-2 antibodies, pretreated with the blocking peptide, did not react with COX-2 protein from tumor samples, as reported previously (7), demonstrating the specificity of the antibodies used (data not shown). Tissue samples from seven patients (patients 1, 4, 6, and 9–12) with squamous cell carcinoma were analyzed for expression of COX-2 activity. COX-2 was expressed only in the tumor, but not in nontumorous transitional epithelial tissues. Results of immunoblot analysis in representative cases are shown in Fig. 1.

Immunohistochemical Analyses of COX-2 Expression. COX-2 was expressed homogeneously and intensely in the cytoplasm of all pure squamous cell carcinomas (Table 1). The percentage of positively stained cells was >80% in 16 squamous cell carcinomas and > 60% in 1 squamous cell carcinoma. Similarly, COX-2 was markedly expressed in all squamous cell carcinomas combined with transitional cell carcinomas. COX-2 was expressed homogeneously in one case, heterogeneously in five cases, and in six cases no expression was noted (Table 2). The heterogeneous expression pattern of COX-2 in transitional cell carcinoma was consistent with the previous findings reported by our group (7). Squamous metaplastic epithelium at the luminal side also expressed COX-2 (Table 3). Inflammatory interstitial cells frequently infiltrated around squamous cell carcinoma and metaplasia lesions, suggesting the involvement of chronic inflammation in the development of those lesions.

Representative COX-2 immunostaining patterns in squamous cell carcinomas and squamous metaplasias are shown in Fig. 2, *A* and *B* (patients 12 and 7 in Table 1), and Fig. 2*C* (patient 5 in Table 3), respectively.

DISCUSSION

The major findings of this study were that COX-2 was markedly expressed in the cytoplasm of all squamous cell carcinomas examined and that it did not show heterogeneous expression among cases; this expression pattern has never been reported in tumors of the colon (3), lung (4), pancreas (5), and bladder (6, 7). These findings strongly suggest that COX-2 is involved in the development of squamous cell carcinoma of the bladder, especially the early step of carcinogenesis, because squamous cell metaplasia, which is a precursor of some squamous cell carcinomas, also expressed COX-2. In contrast to squamous cell carcinoma, transitional cell carcinoma of the urinary bladder expressed COX-2 less heterogeneously, as reported previously (6, 7). These different expression patterns in two malignancies appear to be logical because they have different etiologies. The development of squamous cell carcinoma closely correlates with chronic urinary tract infection (10–13); therefore, COX-2 plays an important role in inflammation-induced carcinogenesis. Increased COX-2 protein thus may induce tumorigenesis by affecting many biological features of such tissues, including apoptosis (15), cell adhesion (15), angiogenesis (16), and invasiveness (17).

COX-2 can activate various types of procarcinogens to carcinogens by peroxidase activity, but not COX activity, which may activate oncogenes or inactivate tumor suppresser genes (1). For example, benzo[*a*]pyrene, a carcinogen found in cigarette smoke, can be activated by COX-2, and the active form of the product binds to exons of the *p53* gene, which correspond to the p53 mutational hot spots in human lung cancer (18). Notably, significant levels of nitrosoamine compounds, such as volatile nitrosoamines and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, which is a bladder carcinogen for rodents and dogs, have been demonstrated in the urine of individuals with *S. haematobium* infection (19) or even in the urine of patients with chronic urinary tract bacterial infection (20). We suggest that inflammation stimulates production of COX-2 via bacterial lipopolysaccharides or inflammatory cytokines, and the increased level of COX-2 metabolically activates nitrosamines, which are produced in patients with chronic urinary tract infection, resulting in the development of squamous cell carcinoma.

In conclusion, we demonstrate for the first time that COX-2 protein is overexpressed in squamous cell carcinomas and suggest that COX-2 is involved in the development of squamous cell carcinoma of the urinary bladder. Whereas nonsteroidal anti-inflammatory drugs inhibit the production of prostaglandins, they do not inhibit the peroxidase activity of COX-2 (1). To reduce the incidence of squamous cell carcinomas, it is therefore important to inhibit COX-2 at the level of the enzyme as well as at the level of gene expression to inhibit peroxidase activity of COX-2.

REFERENCES

1. Subbaramaiah, K., Zakim, D., Weksler, B. B., and Dannenberg, A. J. Inhibition of cyclooxygenase: a novel approach to cancer prevention. Exp. Bio. Med., *216:* 201–210, 1997.

2. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celand, P., Booker, S. V., Robinson, R., and Offerhaus, G. J. A. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N. Engl. J. Med., *328:* 1313–1316, 1993.

3. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res., *55:* 3785–3789, 1995.

4. Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H., and Ristimaki, A. Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res., *58:* 4997–5001, 1998.

5. Tucker, O. N., Dannenberg, A. J., Yang, E. K., Zhang, F., Teng, L., Daly, J. M., Soslow, R. A., Masferrer, J. L., Woerner, B. M., Koki, A. T., and Fahey, T. J., III. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res., *59:* 987–990, 1999.

6. Mohammed, S. I., Knapp, D. W., Bostwick, D. G., Foster, R. S., Khan, K. N. M., Masferrer, J. L., Woerner, B. M., Snyder, P. W., and Koki, A. T. Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. Cancer Res., *59:* 5647–5650, 1999.

7. Shirahama, T. Cyclooxygenase-2 expression is up-regulated in transitional cell carcinoma and its preneoplastic lesions in the human urinary bladder. Clin. Cancer Res., *6:* 2424–2430, 2000.

8. Oshima, M., Dinchuk, J. E., Kargman, S., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in APC$^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, *87:* 803–809, 1996.

9. Okajima, E., Denda, A., Ozono, S., Takahama, M., Akai, H., Sasaki, Y., Kitayama, W., Wakabayashi, K., and Konishi, Y. Chemopreventive effects of nimesulide, a selective cyclooxygenase-2 inhibitor, on the development of rat urinary bladder carcinomas initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. Cancer Res., *58:* 3028–3031, 1998.

10. La Vecchia, C., Negri, E., D'Avanzo, B., Savoldelli, R., and Francesci, S. Genital and urinary tract disease and bladder cancer. Cancer Res., *51:* 629–631, 1991.

11. Johansson, S. L., Anderstrom, C., von Schultz, L., and Larsson, P. Enhancement of *N*-[4-(5-nitro-2-furyl)-2-thiazoly]formamide-induced carcinogenesis by urinary tract infection in rats. Cancer Res., *47:* 559–562, 1987.

12. Kaufman, J. M., Fam, B., Jacobs, S. C., Gabilondo, F., Yalla, S., Kane, J. P., and Rossier, A. B. Bladder cancer and squamous metaplasia in spinal cord injury patients. J. Urol., *118:* 967–971, 1977.

13. Bedwani, R., Renganathan, E., Kwhsky, F. E., Braga, C., Seif, H. A., Azm, T. A., Zaki, A., Francesci, S., Boffetta, P., and Vacchia, C. L. Schistosomiasis and the risk of bladder cancer in Alexandria, Egypt. Br. J. Cancer, *77:* 1186–1189, 1998.

14. The Japanese Urological Association and the Japanese Society of Pathology. General Rule for Clinical and Pathological Studies on Bladder Cancer No. 2. Tokyo: Kanehara Publishers B. V., 1993.

15. Tsujii, M., and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. Cell, *83:* 493–501, 1995.

16. Tsuji, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93:* 705–716, 1998.

17. Tsujii, M., Kawano, S., and Dubois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc. Natl. Acad. Sci. USA, *94:* 3336–3340, 1997.

18. Denissenko, M. F., Pao, A., Tang, M., and Pfeifer, G. P. Preferential formation of benzo[*a*]pyrene adducts at lung cancer mutational hot spots in *p53*. Science (Washington DC), *274:* 430–432, 1996.

19. Tricker, A. R., Mostafa, M. H., Spiegelhandler, B., and Preussmann, R. Urinary excretion of nitrate, nitrite, and N-nitroso compounds in schistosomiasis and bilharzia bladder cancer patients. Carcinogenesis (Lond.), *1991:* 547–552, 1989.

20. Tricker, A. R., Stickler, D. J., Chawla, J. C., and Preussmann, R. Increased urinary nitrosamine excretion in paraplegic patients. Carcinogenesis (Lond.), *12:* 943–946, 1991.

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