

Selective Cyclooxygenase-2 Inhibitor Prevents Cisplatin-induced Tumorigenesis in A/J Mice

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Cisplatin is used to treat lung cancer; however, it is also a known carcinogen. Cyclooxygenase-2 (COX-2) inhibitors have been shown to prevent carcinogen-induced experimental tumors. We investigated the effect of a COX-2 inhibitor, celecoxib, on cisplatin-induced lung tumors. One hundred twenty 4-week-old A/J mice were divided into 6 groups: group 1, no treatment; group 2, low-dose celecoxib (150 mg/kg); group 3, high-dose celecoxib (1,500 mg/kg); group 4, cisplatin alone; group 5, cisplatin plus low-dose celecoxib; and group 6, cisplatin plus high-dose celecoxib. Mice in groups 4-6 were administered cisplatin (1.62 mg/kg, i.p.) once a week for 10 weeks between 7 and 16 weeks of age. All mice were sacrificed at week 30. Tumor incidence was 15.8% in group 1, 25% in group 2, 26.3% in group 3, 60% in group 4, 50% in group 5, and 50% in group 6. Tumor multiplicity was 0.2, 0.3, 0.3, 1.3, 1.0, and 0.6 in groups 1-6, respectively. Tumor multiplicity in the cisplatin-treated mice was reduced by celecoxib treatment in a dose-dependent manner ($p < 0.05$, group 4 vs. group 6). Celecoxib significantly reduced COX-2 expression in cisplatin-induced tumors ($p < 0.01$, group 4 vs. group 6).

Key words: cisplatin, non-small cell lung cancer, celecoxib, cyclooxygenase-2, chemoprevention

Lung cancer is a leading cause of cancer mortality in most developed countries, with a five-year survival rate less than 15% [1]. In recent years, improvements in lung cancer therapy (chest radiotherapy and cisplatin-based chemotherapy) have increased the survival rate in lung cancer patients. [2, 3]. Thus, second primary cancers involving small-cell lung cancer have increased [4]. The risk of secondary lung cancer in patients with non-small-cell

lung cancer has been estimated to be 1-2% per patient per year [5], whereas the risk for patients with small-cell lung cancer has increased to > 2-10% per patient per year 10 years after the initial treatment [5]. Cisplatin is widely used in the treatment of malignant diseases; however, it has also been shown to be a carcinogen in experimental animals [6].

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclooxygenase (COX) enzyme family. The COX enzyme has 2 isoforms: COX-1, a housekeeping enzyme that is constitutively expressed in most tissues [7, 8], and COX-2, which is typically undetectable under basal conditions; however, it is expressed in

response to stimulation with growth factors and inflammatory cytokines [9, 10]. COX-2 is commonly overexpressed in malignant tumors in human and animal models [11–15], suggesting a causative association between increased COX-2 expression and tumorigenesis. Several investigations have studied the efficacy of COX-2 as an anticancer therapeutic agent. For example, the COX-2-specific inhibitor NS398 inhibited mouse lung tumorigenesis induced by chronic administration of tobacco-specific nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [16–18]. Celecoxib has been reported to cause colorectal adenoma [19] and colorectal polyps to regress [20] and to prevent the formation of colorectal adenomas and cancers [21–22].

We previously demonstrated that (–)-epigallocatechin gallate prevented cisplatin-induced lung tumorigenesis in A/J mice [23]. We observed COX-2 overexpression in cisplatin-induced lung tumors in the present study, and subsequently investigated the efficacy of celecoxib in preventing cisplatin-induced tumorigenesis in the A/J mouse model.

Materials and Methods

Animals and chemicals. We studied 120 4-week-old female A/J mice (Jackson Laboratories, Bar Harbor, ME, USA) weighing approximately 15 g each. The animals were housed five per plastic cage and were given free access to tap water and standard laboratory chow (MF; Oriental Yeast Co., Ltd, Tokyo, Japan). The mice were kept in an air-conditioned room maintained at $55 \pm 10\%$ humidity with a 12-h light/dark cycle in the Animal Center for Medical Research, Okayama University Medical School. The celecoxib and cisplatin were kindly provided by Pharmacia, Ltd. (Tokyo, Japan) and Nippon Kayaku Co., Ltd. (Tokyo, Japan), respectively.

Experiment design. Mice were treated in accordance with the guidelines of the Okayama University's Institutional Committee on the Treatment of Experimental Animals. The animals were divided into 6 groups of 20: group 1, no treatment control; group 2, low-dose celecoxib (150 mg/kg, diet); group 3, high-dose celecoxib (1,500 mg/kg, diet); group 4, cisplatin treatment; group 5, cisplatin plus low-dose celecoxib (150 mg/kg, diet); and group 6, cisplatin plus high-dose celecoxib (1,500 mg/kg, diet). Mice in

groups 4–6 were treated with cisplatin (1.62 mg/kg, i.p.) once a week for 10 weeks between 7 and 16 weeks of age. Between 5 and 30 weeks of age, mice in groups 2, 3, 5, and 6 were exclusively fed the experimental diet prepared by mixing celecoxib into the standard chow. All mice were sacrificed at week 30, and the number of tumors on the lung surface of each mouse was determined.

Immunohistochemistry. The lungs were inflated with 10% formalin, embedded in paraffin, and cut into several 4- μ m-thick sagittal sections around the greatest dimension of the lung. Tissue sections were deparaffinized in xylene and washed in ethanol. Endogenous peroxidase activity was then inhibited using 0.3% H₂O₂ followed by antigen retrieval. Slides were blocked with rabbit serum antibody (Vector Laboratories, Burlingame, CA, USA). A polyclonal rabbit anti-murine COX-2 primary antibody (Cayman Chemical, Ann Arbor, MI, USA) was added at a 1 : 200 dilution. Biotin-conjugated secondary antibody (Vector) was applied and incubated with avidin biotin enzyme reagent (Vector). Then, diaminobenzidine (DAB, Vector) as a peroxidase substrate was applied. The sections were rinsed in H₂O, counterstained with methyl green, dehydrated, and covered with a xylene-based mounting medium.

COX-2 expression was evaluated in 100 cells from each tumor. The cells expressing COX-2 per high-power microscope field ($\times 400$) were counted 3 times.

Statistical analysis. The Student's *t*-test and chi-square test were used to assess the relationship among categorical variables. The results are expressed as means \pm standard errors, and *p*-values < 0.05 were deemed to be statistically significant.

Results

Fig. 1A shows hematoxylin and eosin staining of a cisplatin-induced tumor from group 4. Table 1 shows the tumor incidence and multiplicity in each group. The tumor incidence was 60% (12/20), 50% (10/20), and 50% (10/20) in groups 4 (cisplatin alone), 5 (cisplatin plus low-dose celecoxib), and 6 (cisplatin plus high-dose celecoxib), respectively. Thus, celecoxib treatment did not significantly reduce tumor incidence. However, mice treated with cisplatin alone developed 1.3 ± 0.3 tumors per mouse, whereas mice treated with cisplatin plus high-dose celecoxib developed 0.6

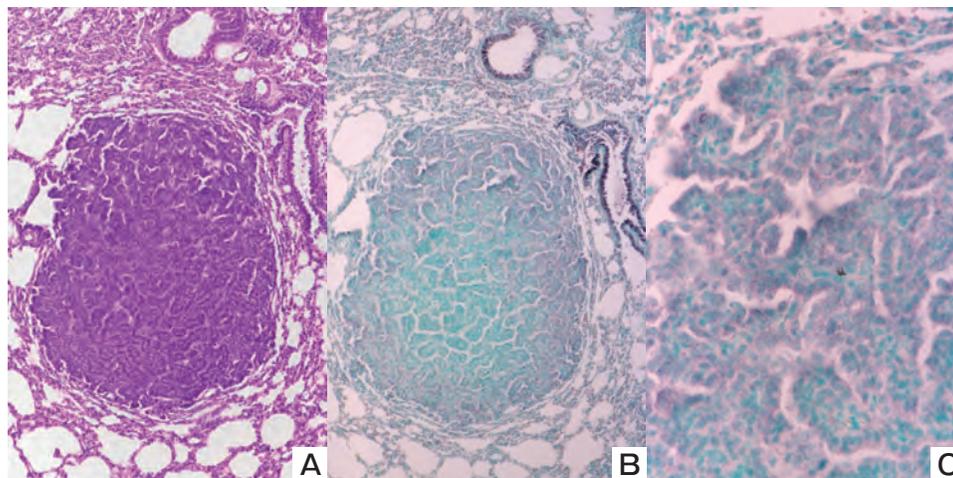


Fig. 1 Hematoxylin and eosin and immunohistochemical staining in cisplatin-induced tumors. (A) Lung tumor stained with hematoxylin and eosin ($\times 250$). (B) Lung tumor stained with COX-2 antibody ($\times 250$). The blue-stained tumor cells indicate overexpression of COX-2. (C) A higher magnification of panel B ($\times 400$).

Table 1 The inhibitory effects of celecoxib on gross tumor incidence and multiplicity in A/J mice

Group no.	Tumor incidence ^a	Tumor multiplicity ^b
1 (tap water only)	3/19 (15.8%)	0.2 \pm 0.1
2 (celecoxib 150 mg/kg)	5/20 (25.0%)	0.3 \pm 0.1
3 (celecoxib 1,500 mg/kg)	5/19 (26.3%)	0.3 \pm 0.1
4 (cisplatin 1.62 mg/kg)	12/20 (60.0%)	1.3 \pm 0.3
5 (cisplatin 1.62 mg/kg + celecoxib 150 mg/kg)	10/20 (50.0%)	1.0 \pm 0.3
6 (cisplatin 1.62 mg/kg + celecoxib 1,500 mg/kg)	10/20 (50.0%)	0.6 \pm 0.1

^aDifference between cisplatin and cisplatin plus celecoxib was not statistically significant by chi-square test.

^bNumber of tumors per mouse: group 4 vs. group 6, $p < 0.05$ (Student's *t*-test).

± 0.1 tumors per mouse, indicating that high-dose celecoxib significantly reduced the number of cisplatin-induced lung tumors ($p < 0.05$). No differences in tumor incidence or multiplicity were found among groups 1 (control), 2 (low-dose celecoxib), and 3 (high-dose celecoxib) mice.

The analysis of COX-2 expression in the tumors revealed intense COX-2 staining in the cisplatin-induced tumor cells (group 4; Fig. 1B, 1C). In contrast, COX-2 expression was weak in the cisplatin-induced tumors treated with high-dose celecoxib (group 6; Fig. 2). Fig. 3 shows that the high dose of celecoxib significantly reduced the percentage of cells overexpressing COX-2 ($p < 0.01$).

After the initiation of cisplatin treatment at week 7, the body weight of the mice in groups 4, 5, and 6 was lower than that of the mice in the non-cisplatin

groups (1, 2, and 3; Fig. 4). However, the mice in group 5 (cisplatin plus low-dose celecoxib) did not lose weight after the administration of cisplatin. Thus, low-dose celecoxib prevented cisplatin-induced weight loss, and the mice in group 5 weighed significantly more than did the mice that received cisplatin alone (group 4) at week 30 ($p < 0.05$).

Discussion

Pulmonary tumors have been reported to occur in A/J mice 18 weeks after cisplatin treatment [14]. In the present study, we demonstrated that COX-2 was overexpressed in cisplatin-induced lung tumors. Furthermore, we found that administration of high-dose celecoxib significantly reduced tumor multiplicity and reduced the number of COX-2-expressing tumor

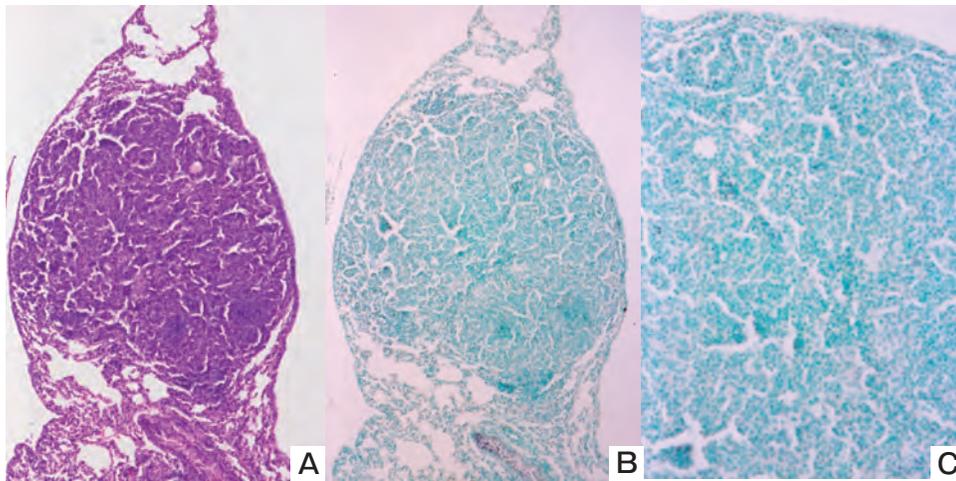


Fig. 2 Hematoxylin and eosin and immunohistochemical staining in cisplatin-induced tumors treated with celecoxib (1,500 mg/kg). **(A)** Lung tumor stained with hematoxylin and eosin ($\times 100$). **(B)** Lung tumor stained with COX-2 antibody ($\times 100$). The majority of tumor cells did not overexpress COX-2. **(C)** A higher magnification of panel B ($\times 400$).

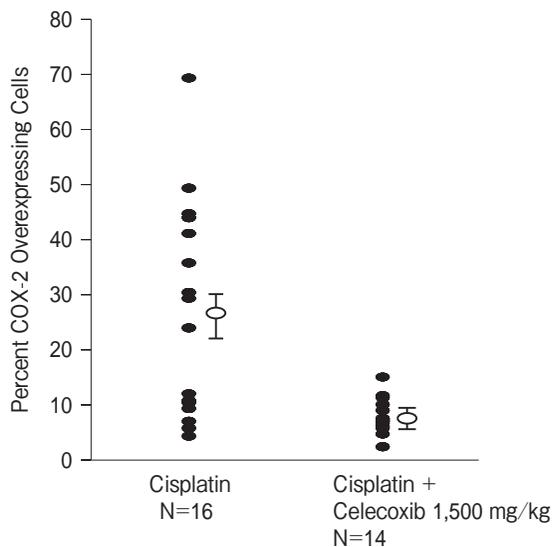


Fig. 3 Effect of celecoxib on COX-2 expression in cisplatin-induced tumor cells. The number of COX-2-expressing cells in a sample of 100 cells from each cisplatin-induced tumor was counted 3 times. Percent COX-2 positive rates (mean \pm SE) of group 4 (cisplatin) and group 6 (cisplatin + celecoxib 1,500 mg/kg) were $26.7 \pm 5.0\%$ and $7.9 \pm 0.9\%$, respectively ($p < 0.01$). Statistical significance was determined using Student's *t*-test.

cells. To our knowledge, this is the first report showing that a COX-2 inhibitor prevented cisplatin-induced tumorigenesis in a mouse model.

NSAIDs have been shown to prevent the development of various human tumors, including colon [24–

26], breast [27], esophageal [28], and lung [29] neoplasms by inhibiting COX-2 activity. COX-2 expression is induced by inflammatory disease and in response to cytokines, growth factors, and other tumor promoters. Additionally, COX-2 expression plays several distinct roles during oncogenesis [24–29]. The concentration of COX-2 protein has been shown to be significantly higher in urethane-induced tumors and carcinogenesis in A/J mouse lung tissue compared with control lung tissue [14]. Furthermore, NNK was shown to cause increasingly higher levels of COX-2 expression with progressive stages of lung carcinogenesis in rats on a high-fat diet [30]. Moreover, we reported that COX-2 may play a role in the progression from atypical adenomatous hyperplasia to adenocarcinoma in human lung tissue [31]. The nature of the association between increased COX-2 expression and tumorigenesis and the mechanisms underlying the COX-2 inhibition of tumorigenesis in terms of angiogenesis [32], apoptosis [33], and inflammation [34] remain to be elucidated in our cisplatin-induced lung tumor model.

We found that low-dose celecoxib (group 5) prevented cisplatin-induced weight loss, and that the mice in group 5 weighed significantly more than mice treated with cisplatin alone (group 4) at week 30. The COX-2 inhibitor JTE-522 has been reported to prevent body weight loss in rats with N-nitrosomethylbenzylamine-induced tumors [30], choline-deficient

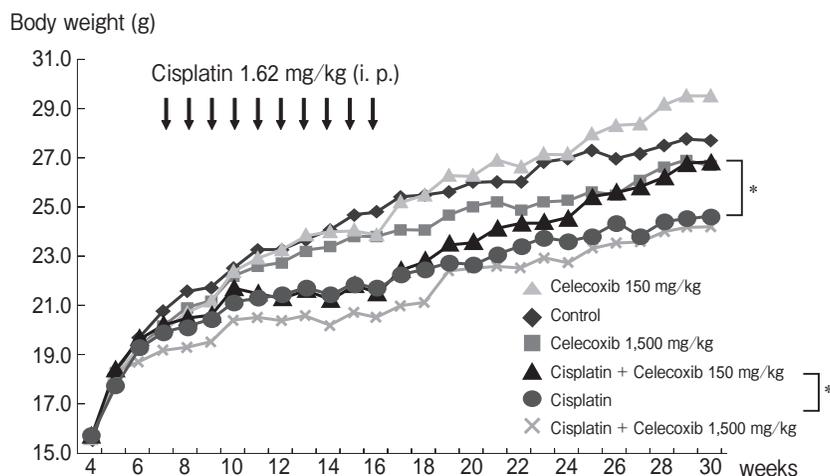


Fig. 4 Change in body weight of the 6 groups over the treatment period. Statistical significance (determined using Student's *t*-test): * $p < 0.05$. Arrows indicate intraperitoneal administration of cisplatin (1.62 mg/kg body weight).

rats, and rats receiving L-amino acid [35]. Carcinogens administered to experimental animals are likely to cause cachexia, resulting in weight loss. A placebo-controlled study carried out in cachectic patients with head and neck or gastrointestinal cancer showed a statistically significant increase in weight in patients receiving celecoxib (200 mg twice daily) compared with those in the placebo arm [36]. Furthermore, a phase II clinical trial showed that celecoxib (300 mg/day for 4 months) significantly increased lean body mass [37]. In that study, celecoxib produced a significant decrease in tumor necrosis factor- α ; however, the mechanism by which low-dose celecoxib prevented the loss of body weight in our mouse model is not clear.

The COX-2 inhibitor rofecoxib is associated with an increased cardiovascular risk in patients with a history of colorectal adenomas [38]. A pooled analysis of 6 randomized trials comparing celecoxib with a placebo showed an increase in cardiovascular risk and provided evidence of an interaction between baseline cardiovascular risk and the effect of celecoxib [39]. The authors suggested that the adverse effect of the celecoxib was most pronounced in the high-risk patients. In contrast, other studies found no clear association between cardiovascular risk and COX-2 inhibitors [40]; however, high-risk patients were at increased risk for COX-2 inhibitor-related adverse cardiovascular events. Celecoxib has been found to be an effective chemopreventive agent for non-melanoma skin cancer in patients at high risk for the disease

[41]. In that study, no statistically significant difference was found in the number of adverse cardiovascular events between participants who received celecoxib and those who received the placebo; however, the drug was administered for only 9 months. In our study, 1,500 mg/kg of celecoxib was found to be a safe dose for long-term treatment in A/J mice. However, it would be necessary to balance the beneficial effects and adverse events associated with celecoxib in a chemopreventative trial in patients who will receive cisplatin treatment in an adjuvant or a neoadjuvant setting.

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