J Lung Cancer 2007;6(1):15-23

Cyclooxygenase-2 (COX-2) Inhibitors Reduce Immune Tolerance through Indoleamine 2,3-dioxygenase (IDO)

Purpose: Cyclooxygenase-2 (COX-2) and its metabolite, PGE_2 affect multiple tumorigenesis, including angiogenesis, invasion, and tumor-induced immune suppression. Their overexpression is association with impaired immune cell function in many tumors. Indoleamine 2,3-dioxygenase (IDO) is an emerging immuno-regulatory enzyme that can catalyze the initial rate-limiting step in tryptophan catabolism, by causing tryptophan depletion can block T lymphocyte activation, and thus, enable tumor cells to escape from immune system. Although the potential of immunosuppression associated with tumor-produced COX-2 has been suggested, the mechanism of immunosuppression in tumor immunology is not yet well defined. Thus, we hypothesized that the tumor immunity of COX-2 could be partly due to IDO-dependent immune tolerance. To test this hypothesis, we evaluated IDO expression in cancer cells treated with selective COX-2 inhibitor. **Materials and Methods:** The A549 human adenocarcinoma cell line, murine Lewis lung carcinoma (LLC) cell line and C57Bl/6 mice were used for *in vitro* and *in vivo* studies. *In vitro* studies, A549 cells were treated with various concentrations of COX-2 inhibitor (PTPBS) or PGE_2. IDO enzyme activity and protein expression were checked by IDO enzyme activity assay and Western blotting. *In vivo* study, the 20 mice were randomized into normal control, LLC inoculated control, and low and high selective COX-2 inhibitor (celecoxib 25 or 250 mg/kg/day) treated LLL inoculated mice groups (n=5 per group). At one month, mice were sacrificed and tumor mass was isolated for quantification of IDO expression by immunohistochemical stain and western blotting. **Results:** *In vitro* studies, PTPBS treated A549 cells showed a significant decreased in IDO enzyme activity and expression but PGE_2 treated A549 cells showed increased in IDO expression. *In vivo* studies, the tumor mass and lung metastasis were attenuated by celecoxib (respectively, $p<0.05$, $p<0.01$). Compared with the LLC inoculated control group, mice treated with celecoxib had significant reductions in IDO expression of tumor mass (IDO immunohistochemical stain and western blotting). **Conclusion:** The present study reveals that COX-2 inhibitor serves to restore the tumor-induced IDO expression and promotes antitumor reactivity in an immunocompetent murine lung cancer model. These findings further support the suggestion that COX-2 inhibitor is a potential pharmacological immunotherapy in cancer. **(J Lung Cancer 2007;6(1):15－23)**

Key Words: COX-2, IDO, Immune tolerance

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Received: May 10, 2007
Accepted: May 29, 2007

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This work was supported by the Korean Association for the Study of Lung Cancer.

INTRODUCTION

Tumors work out a variety of schemes to create a favorable environment for tumor development and to counteract the effects of host immune effector cells, and these abilities of tumors explain why the immune system often unable to eradicate tumor cells. Recently, cyclooxygenase (COX) has received much attention because of its ability to reduced the anti-tumor capability of the immune system, and to reduce inflammation, carcinogenesis, apoptosis, metastasis and angio-

genesis(1～4). Moreover, because COX-2 can enhance PGE2 production and subsequent cytokine imbalance in vivo, tumor expression of COX-2 may be instrumental in the abrogation of tumor-induced T cell-mediated antitumor responses(3). Although the potential of immunosuppression associated with tumor-produced COX-2 has been suggested, the mechanism of immunosuppression in tumor immunology is not yet well defined.

According to Sayama et al., cyclooxygenase inhibitors almost completely suppress interferon-mediated IDO induction in mouse lung slices(5). IDO is an enzyme that catalyzes the initial and rate-limiting step in the catabolism of trytophan along the kynurenine pathway. Thus, by depleting tryptophan locally, IDO appears to block the proliferation of alloreactive T lymphocytes. These cells are extremely sensitive to tryptophan shortages, which cause their arrest in the G1 phase of the cell cycle(6). IDO over-expression was also observed in cells exposed to interferon-γ, and in certain types of activated macrophages and dendritic cells, thus suggesting a role for IDO in immune response regulation(7～9).

Because COX-2 expression can reduced tumor immunity, we hypothesized that this effect of COX-2 might be due in part to IDO-induced immune tolerance. To test this hypothesis, we evaluated IDO expression in cancer cells treated with COX-2 inhibitors.

MATERIALS AND METHODS

1) Cell culture

The A549 human adenocarcinoma cell line was obtained from the American Type Culture Collection(Manassas, VA, USA) and were grown in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM) at 37°C in a humidified 5% CO_2 water-jacketed incubator. Experiments were performed with A549 cultured in the above-mentioned medium supplemented with 100 uM L-tryptophan. Test compounds of interest were added 30 min before treating cells with IFN-γ (1,000 U/ml or 10,000 U/ml). After culturing for 24 h, cells were lysed and IDO determined.

2) Drugs and chemical compounds

To prepare IFN-γ stimulated A549 cells, cells were incubated with 1,000 U/ml or 10,000 U/ml of IFN-γ (LG Life Science Ltd., Seoul, Korea) in RPMI 1640 medium containing 10% FBS in 5% CO_2 at 37°C.

For *in vitro* studies, 1-methyl tryptophan (MT, Sigma, MO, USA) was formulated in dimethyl sulfoxide (DMSO) containing 0.1N HCL to improve its solubility. 1 μM 1-MT was added into the culture at the same time. The COX-2 inhibitor (PTPBS) was purchased from Sigma-Aldrich (MO, USA). For experiments with COX-2 inhibitor, A549 cells were treated with various concentrations of PTPBS (10 or 50 μM) in RPMI 1640 medium containing 10% FBS in 5% CO_2 at 37°C, whereas control cells were treated with DMSO vehicle. At the end of treatment, the cell lysates were prepared for western blot analysis of IDO.

3) IDO activity assay

IDO activity was measured using the method of Takikawa et al.(8) and modified by Kudo and Boyd(10). Briefly, A549 cells were harvested by trypsin digestion, washed twice, and re-suspended in 1 ml of a buffer containing NaCl (130 mM) and Tris-Mops (50 mM) at pH 7.4. The cells were homogenized by sonification for 30 s on ice at 100 W, and centrifuged at 12,000 g for 5 min at room temperature. Samples of the supernatant were taken to determine the protein content by the Lowry assay using bovine serum albumin as standard. Following this, 0.4 ml of the supernatant was added to an equal volume of a solution containing L-tryptophan (1 mM), methylene blue (20 μM), ascorbic acid (40 mM), catalase (200 U/ml), and potassium phosphate buffer (100 mM), pH 6.5. Both the enzyme suspension and incubation buffer were pre-heated to 37°C before mixing. This mixture was incubated for 30 min at 37°C, and the reaction was stopped by adding 0.2 ml of 30% (w/v) trichloroacetic acid. The mixture was then incubated at 50°C for 30 min to hydrolyze the *N*-formylkynurenine produced by IDO to kynurenine, and centrifuged at 12,000 g at room temperature to remove sediment. The supernatant (0.8 ml) was added to 0.8 ml of 1% (w/v) *p*-dimethylaminobenzaldehyde in acetic acid. The absorbance at 480 nm was determined

4) Western blot analysis

A549 cells (1×10^6 cells/well) were harvested and washed twice with PBS. The cell pellet lysed with MPER lysis buffer

(mammalian protein extraction reagent, Pierce, Rockford, IL, USA) and 1 : 100 dilution of proteinase inhibitors (Pierce, Rockford, IL, USA). To remove insoluble materials, cell lysates were centrifuged at 14,000 rpm for 5 min, and 1 vol of Laemmli's sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 4 mg/100 ml bromophenol blue, and 125 mM Tris-HCL; pH 6.8, Bio-Rad Laboratories) was added to the supernatant. After incubation at 95°C for 5 min, total proteins were separated by SDS-PAGE in 10% acrylamide gels. The running gel was electro-transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories), which was blocked with 5% fat-free skimmed milk in TBS/0.05% Tween 20 for 1 h at room temperature. Membrane incubated with anti-human IDO mAb (1 : 1000, Sigma-Aldrich, MO, USA). The membranes were stripped and reblotted with monoclonal anti-β-actin antibody (Sigma Aldrich, MO, USA) to verify equal loading of protein in each lane. The membrane incubated with HRP-linked secondary antibody (VECTOR, Burlingame, CA, USA) for 1 h at room temperature. Proteins were detected using the Super-signal West picochemiluminescent substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The relative intensity of the IDO band was determined using the SIGMASCAN-PRO program V5.01 (MA, USA). These experiments were performed at least three times using separate sets of cultures.

5) Animal experimental procedure

Eight-week old C57Bl/6 mice were purchased from the ORIENT. Co. Ltd. (Gapyeong, Korea). Animals were housed in climate-controlled quarters (24±1°C at 50% humidity) under a 12 h light/12 h dark cycle. The Lewis Lung Carcinoma (LLC) cell line, originally derived from a C57Bl/6 mouse, was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). LLC cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS), L- glutamine, penicillin and streptomycin. Each mouse was inoculated with a subcutaneous injection of LLC cells (3×10^6 in 0.1 ml PBS) into the right forelimb after weighed individually. These 20 mice were then randomized into normal control, LLC inoculated control, and low and high selective COX-2 inhibitor (celecoxib 25 or 250 mg/kg/day; Pharmacia Biotech, Seoul, Korea) groups (n=5 per group). Drugs were administered by oral gavage q.i.d. in a solution of 0.5% methylcellulose (Sigma-Aldrich, MO, USA) and containing 0.025% Tween 20 (Sigma-Aldrich, MO, USA) starting 7 days after LLC inoculation and continued for 21 days. After 4 weeks, mice were sacrificed, and a blinded observer measured total tumor volumes, using a caliper across two perpendicular diameters every 3 days. Tumor volumes were calculated from shortest and longest diameters of xenografts. The tumor volume was deduced according to the formula(11): volume (mm^3)=(shortest diameter)^2×(longest diameter)×0.5. Both body weights and tumor sizes were reme-asured before sacrifice on the 28th day. All tumors were excised and fixed in 4% paraformaldehyde.

6) Analysis of microscopic metastasis

Mice bearing tumors were sacrificed on day 28, lungs were removed, rinsed with saline and fixed in 4% paraformaldehyde. Formalin-fixed whole lungs were sectioned at 2 mm intervals and embedded in paraffin. These embedded tissues were then sectioned at 4 μm thick and placed on glass slides, and stained with hematoxylin and eosin (H&E). Lung sections were then analyzed for tumors microscopically under 20× and 100× magnification. Tumor nodules were identified as densely packed large mitotic cells stained strongly with eosin against the normal lung tissue background.

7) Immunohistochemistry

Paraffin-embedded tumor mass sections were prepared on poly-L-lysine-coated slides at 4 μm. Sections were dewaxed in xylene and rehydrated in graded alcohol baths. Endogenous peroxidase was then blocked by incubating in 3% H_2O_2 in methanol. Nonspecific mouse antigen was blocked with blocking reagent (Zymed, CA, USA). Primary mouse monoclonal anti-IDO Ab (Upstate, NY, USA) was applied at 1 : 150 dilution in primary antibody diluting buffer at 4°C for 1 hour. Detection was through a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Zymed, CA, USA) and a DAB chromagen. Slides were counterstained with hematoxylin. Images were viewed with an Olympus BX60 microscope and captured with a cooled charge-coupled device camera (Magnafire; Olympus, Melville, NY). Images were then imported into Adobe Photoshop (Adobe Systems, CA, USA) as TIFF files. For each section, the extent of IDO staining was graded on a scale of 0 to (2+), with 0 representing no detectable

staining, (1+) positive staining in ≤50% of tumor cells, and (2+) representing positive staining in >50% of tumor cells.

8) Statistics

All data are expressed as means±standard error of mean (SE). One-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups. Scheffé's *F*-test was used to correct for multiple comparisons when statistical significances were identified by ANOVA. A p value of less than 0.05 was considered significant.

RESULTS

1) *In vitro* studies

(1) IDO activity induction is mediated by IFN-γ: In order to evaluate the effect of IFN-γ on IDO activity, NCI-H292 lung cancer cells were treated with IFN-γ, and IDO activities were determined using IDO immunoassays and enzyme activity assays. IFN-γ induced IDO expression in a dose-dependent manner (Fig. 1A), and IFN-γ induced IDO enzyme activity was reduced by IDO inhibitor (1-MT) (Fig. 1B).

(2) Effect of COX-2 inhibitor on IFN-γ induced IDO expression: The role of COX-2 inhibitor on IFN-γ induced IDO expression was assessed. The treatment with COX-2 inhibitor resulted in reduced IDO enzyme activity, dose-dependently (Fig. 2A). Western blot analysis of IDO protein in A549 cells after 72 h of COX-2 inhibitor treatment showed that IDO expression was dose-dependently reduced (Fig. 2B).

2) *In vivo* studies

(1) LLC tumor growth was delayed in normal syngeneic mice treated with COX-2 inhibitor: Initially, we hypothesized

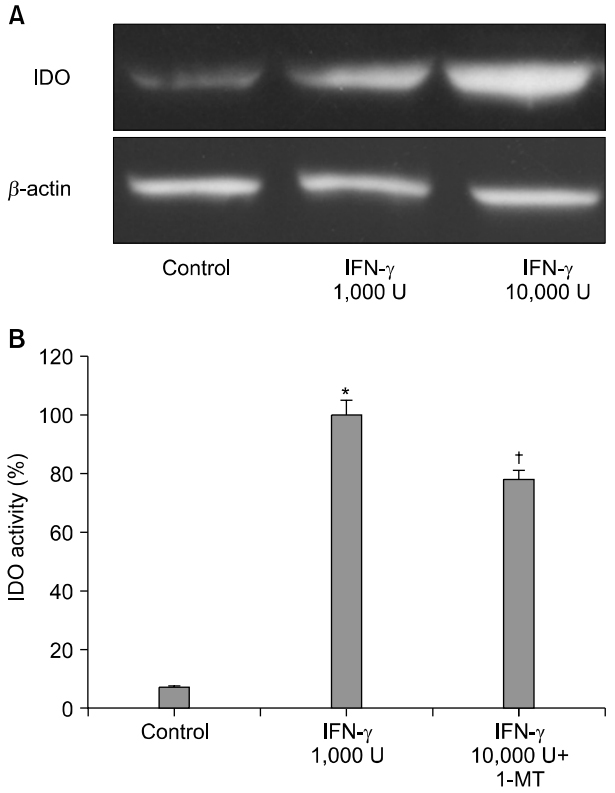

Fig. 1. IDO expression in A549 cells stimulated with IFN-γ. (A) After incubating A549 cells with IFN-γ for 24 h, IDO expression was measured by Western blotting. (B) IDO activities were determined by measuring kynurenine formation and were calculated as percentages with respect to control. Data are means±SEM of three experiments (*p<0.01 vs. control, †p<0.05 vs. IFN-γ treated group).

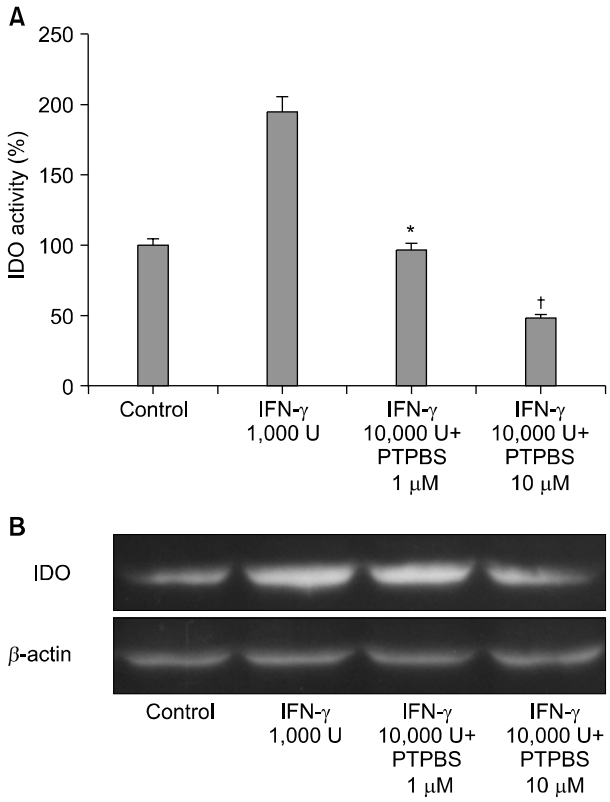

Fig. 2. Effect of COX-2 inhibitor (PTPBS) on the activity of IDO produced by A549 cells in response to IFN-γ. (A) IDO activities were determined by measuring kynurenine formation and were calculated as percentages versus control. Data are means±SEM of three experiments (*p<0.01 vs. IFN-γ 1,000 U/ml, †p<0.05 vs. IFN-γ 1,000 U/ml+PTPBS 1 μM). (B) IDO expressions in A549 cells treated with IFN-γ plus PTPBS.

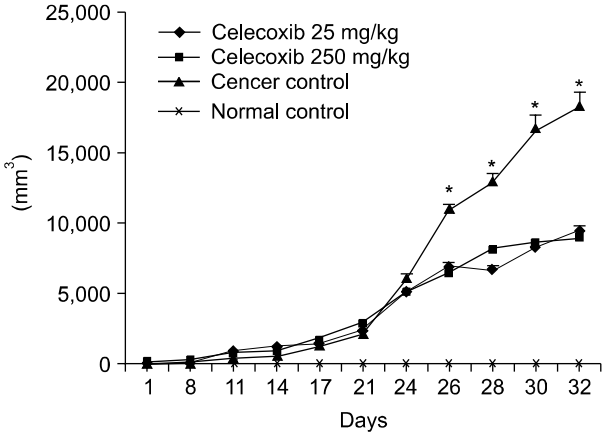

Fig. 3. The growth curve of LLC tumors, *in vivo*. Tumors were measured in mice using a caliper in two perpendicular diameters, including the longest diameter and the shortest perpendicular diameter. A significant differences in tumor size was observed between the untreated LLC control group and celecoxib treated mice (*$p < 0.05$ vs. celecoxib treated mice).

that LLC tumors grow in C57BL/6 mice due to IDO- induced immune tolerance and that this immune tolerance is attenuated by cyclooxygenase-2. To test this hypothesis, we treated mice with COX-2 inhibitor (celecoxib), to determine this treatment would delay tumor growth. Seven days after LLC cell inoculation, celecoxib was administered by oral gavage for 21 days. A significant delay in tumor growth was observed in the groups of mice treated with celecoxib as compared with the untreated LLC inoculated control group. However, no significant differences were between groups treated with 25mg/kg and 250 mg/kg of celecoxib (Fig. 3).

(2) Effect of Celecoxib on LLC inoculated mouse lung microscopic metastasis: Microscopic metastases were observed in the lungs of most mice with a large tumor (Fig. 4). Metastatic lung tumors were present on lung surfaces and in lung parenchyma (Fig. 4A). Quantitative analysis of metastases

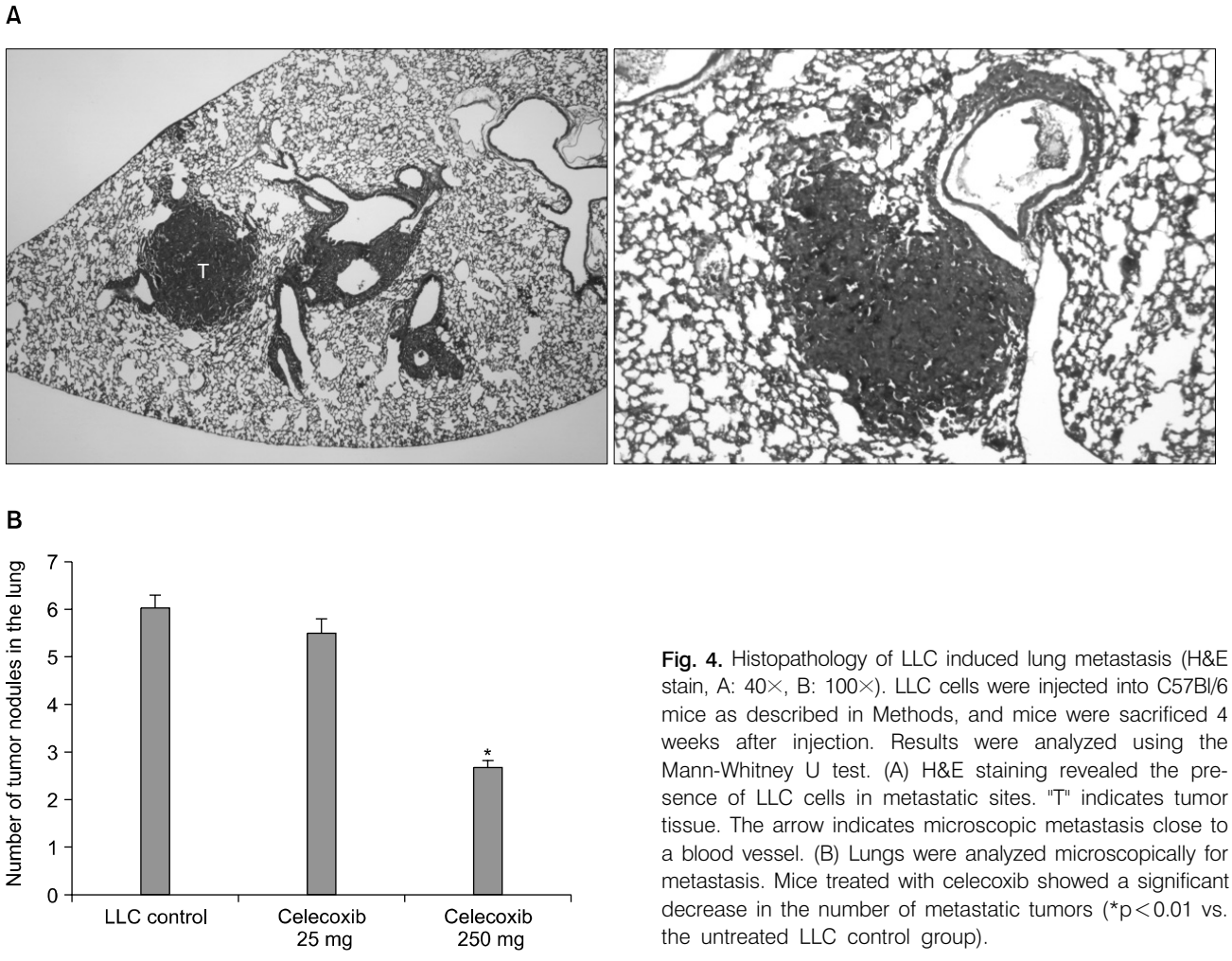

Fig. 4. Histopathology of LLC induced lung metastasis (H&E stain, A: 40×, B: 100×). LLC cells were injected into C57Bl/6 mice as described in Methods, and mice were sacrificed 4 weeks after injection. Results were analyzed using the Mann-Whitney U test. (A) H&E staining revealed the presence of LLC cells in metastatic sites. "T" indicates tumor tissue. The arrow indicates microscopic metastasis close to a blood vessel. (B) Lungs were analyzed microscopically for metastasis. Mice treated with celecoxib showed a significant decrease in the number of metastatic tumors (*$p < 0.01$ vs. the untreated LLC control group).

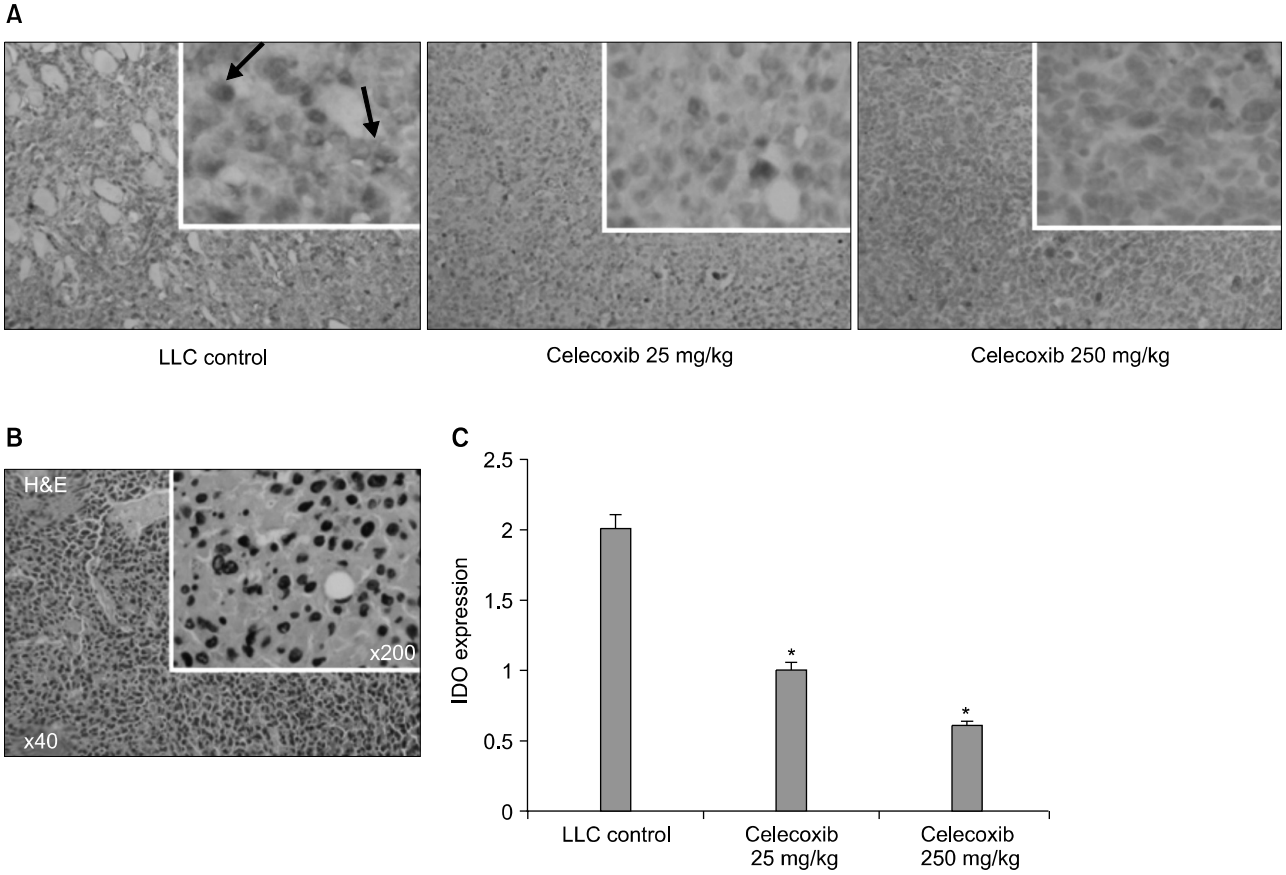

Fig. 5. Immunohistochemical staining for IDO. Celecoxib treated mice showed significantly lower levels of IDO expression in tumor cells and tumor-infiltrating inflammatory cells. (A) Paraffin sections of isolated primary tumors were stained with anti-IDO antibody. Slides were counterstained with hematoxylin and visualized by light microscopy (at ×40 and ×200 magnification). Positively-stained cells are arrowed. (B) Photomicrograph of a tumor (H&E staining), showing original LLC cells in a tumor mass at its injection site. (C) IDO expression was determined by calculating the IDO stained area per microscopic field. IDO expression was significantly lower in celecoxib treatment group (*$p<0.01$ vs. the untreated LLC control group). However, no significant difference was observed between the celecoxib 25 mg and 250 mg groups.

in LLC cells inoculated control mice and celecoxib treated mice was performed by counting the numbers of H&E stained positive tumors on lung surfaces and within lung parenchyma (Fig. 4B). Tumor numbers were found to be significantly reduced in celecoxib treated mice versus LLC inoculated untreated controls.

(3) IDO is expressed by mononuclear cells within LLC tumors and COX-2 inhibitors inhibited this IDO expression: Tumors from LLC bearing mice were immunostained to determine whether IDO is inhibited within tumors by celecoxib. The cytoplasm of tumor cells from controls stained positively for IDO antibody (Fig. 5), but tumor masses obtained from celecoxib treated mice, showed lower levels of IDO immunostaining. IDO expression grades for LLC inoculated control mice and celecoxib (25 or 250 mg/kg) treated mice were 2±0, 1±0.7, and 0.6±0.5, respectively (Fig. 5), and the differences between the LLC control and the celecoxib treated groups were significant ($p<0.05$). The celecoxib modestly decreased the expression of COX-2. In addition, there was a corresponding decrease in IDO expression in the tumor lysates by Western blot analysis (Fig. 6).

DISCUSSION

The immune system of tumor-bearing hosts fails to respond effectively to tumor antigens. The fact is that although cancers often elicit a vigorous immune response during the early stage, that this immune response is soon down-regulated, which per-

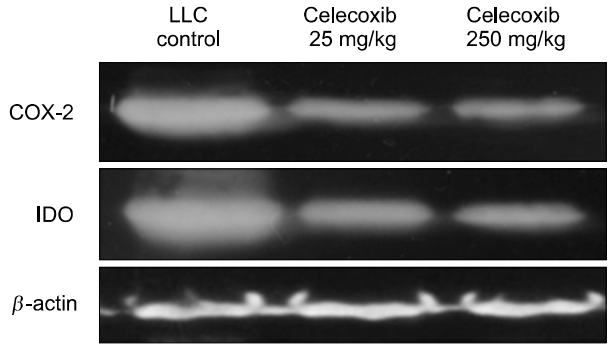

Fig. 6. Inhibition of COX-2 and IDO expression in the mice treated with celecoxib. Representative western blot analysis shows COX-2 and IDO expression in tumor lysates from each group.

mits unabated tumor growth. Tumor-induced immune tolerance also presents a problem for clinical immunotherapy, because the immune system fails to respond to tumor antigens. Unfortunately, the molecular mechanisms by which tolerance is created are poorly understood. In some instances, the products of tumor cells may suppress immune responses. One example of an immunosuppressive tumor product is transforming growth factor-β, which is secreted in large quantities by many tumors, and which inhibits the proliferative and effector functions of lymphocytes and macrophages. More specifically, it was recently demonstrated that IDO can be immunosuppressive(6). IDO is a rate-limiting enzyme in tryptophan catabolism and is emerging as an important immunoregulatory enzyme(12). Conceptually, the suppressive effects of IDO fall into two categories: those mediated by tryptophan depletion, which include its antimicrobial and antiviral effects, and its inhibition of T cell proliferation in some models(6,13,14); and the effects mediated by the toxic downstream metabolites of tryptophan, which include CD4+ T cell apoptosis and the inhibition of T cell proliferation in other models(15,16). By depleting tryptophan from local microenvironments, IDO can block T lymphocyte activation, which are particularly sensitive to the loss of this essential amino acid(17), and undergo G1 phase of the cell cycle arrest at reduced levels(6). This role of nutrient depletion has been extended recently to the regulation of IDO induced immunosuppression. However, although constitutively high IDO expression in tumor cells would presumably have some inhibitory effect on tumor growth, any antiproliferative effect of IDO is likely to be outweighed by its immunosuppressive effect.

IDO-expressing cells are found at several sites of immune tolerance, including thymus, mucosa of the gut, epididymis, and placenta, and have also been shown to be expressed by human monocyte-derived macrophages and dendritic cells(6,18～20). Moreover, with the exception of the epididymis where IDO is constitutively expressed, IDO is inducible by inflammatory mediators, including interferons.

According to Sayama et al., cyclooxygenase plays an important role in the induction of IDO by interferon(5). In the current study, we hypothesized that COX-2 inhibition leads to an antitumor response via the down-regulation of IDO. To support this hypothesis, we examined whether the COX-2 selective inhibitor regulate the induction of IDO in IFN-γ activated tumor cells. *In vitro* studies revealed that COX-2 inhibitor (PTPBS) decreased IDO expression and activity. Thus, based on these in vitro results, we performed in vivo assays in a mouse model to define the impact of tumor COX-2 expression and IDO expression on immune tolerance. And, in vivo study, tumor mass size and metastasis were found to be markedly reduced by COX-2 inhibitor (celecoxib). IDO immunohistochemical staining showed that tumors in mice treated with celecoxib showed lower IDO expressions than untreated controls, and that these expressional reductions correlated with decreased tumor masses and metastasis. The occurrence of non-neoplastic cells expressing IDO within tumor was recently described by Ishio et al., in human hepatocellular carcinoma(21). However, they were unable to identify the cells responsible for IDO expression and referred to them as tumor-infiltrating cells, and thus, they were likely to be macrophages or dendritic cells. Moreover, Uyttenhove et al. found IDO expression in tumor cells in a number of human solid tumors(22). However, the proportion of IDO-positive tumor cells was very low and weak. In addition, they found non-neoplastic, and IDO-positive cells at the peripheries of many tumors. In the present study, IDO was expressed by tumor cells and by tumor-infiltrating inflammatory cells. After considering all the factors, it appears that IDO expression can confer a growth advantage to tumors, at least for those tumors where an immune-mediated rejection could take place.

COX-2 is inducible by a number of inflammatory mediators including IFN-γ and LPS, which also induce IDO. Cyclooxygenase catalyse the conversion of arachidonic acid to prostaglandins. Two isoforms are known, COX-1 is constitutively

expressed in most tissues and produces prostaglandins that are involved in the maintenance of the gastric mucosa, the regulation of renal blood flow, and platelet aggregation(23). On the other hand, the inducible form, COX-2, is expressed in inflamed and neoplastic tissues, and is induced by proinflammatory and mitogenic stimuli, such as, growth factors (epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor) and cytokines (tumor necrosis factor α, interleukins 1α and 1β)(24). COX-2 is expressed in macrophages, synovinocytes, fibroblasts, osteoblasts, tumor cells, and activated endothelial cells(25). Recent research suggests its role in neoplasia, including hyperproliferation, transformation, tumor growth, invasion, and metastasis(26). Because COX-2 can enhance PGE2 production and subsequent cytokine imbalance in vivo, the expression of COX-2 in tumors may be instrumental in the generation of the tumor-induced abrogation of T cell-mediated antitumor responses(3). Inhibition of COX-2 leads to marked lymphocytic infiltration of the tumor and reduced tumor growth. Treatment of mice with COX-2 inhibitor replicated the growth reduction seen in tumor-bearing mice(27). In our studies, the celecoxib doses chosen for the in vivo study were based on the literatures. The low dose (25 mg/kg body weight per day) was based on previously reported work that yielded celecoxib plasma levels of 0.25 μg/ml (0.6 μmol/L)(28). The review by Davies and coworkers on celecoxib pharmacokinetics states that adults taking 800 mg daily have plasma levels of 2.8 μg/ml(29). The plasma levels of celecoxib in the low dose group were thus well below those in human subjects on standard celecoxib dosages. In addition, Williams et al. found a serum concentration of 2.3 μmol/L in mice consuming 250 mg/kg/day without toxicity(30). Thus, we chose a high dose of 250 mg/kg to minimize toxicity for the present study. In the relation of celecoxib dosage and metastatic lung nodules, low dose of celecoxib group (25 mg/kg/day) did not reduced the count of metastatic nodules. This finding suggests that more than standard doses are needed.

We conclude that COX-2 inhibition promote tumor immune response by inhibiting IDO activity. Our findings represent a first demonstration of the tumor COX-2 dependent upregulation of IDO, and thus, immune tolerance. Moreover, these effects were found to be reversed when tumor growth was pharmacologically inhibited by COX-2 inhibitors. These findings lend further support to the suggestion that tumor COX-2 maybe an important pharmacologic or genetic therapeutic target in cancer.

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