Use of Cyclooxygenase-2 Inhibition to Enhance the Efficacy of Immunotherapy


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ABSTRACT

Antitumor effects of cyclooxygenase-2 (COX-2) inhibition have been reported in a wide variety of tumor models and in human cancers, both as chemoprevention and therapy. Human mesothelioma tumors have been shown to overexpress COX-2 and high levels of COX-2 protein have been demonstrated to be a prognostic factor, indicating poor outcome in this tumor. In this study, we determined that inhibition of COX-2 by oral administration of Rofecoxib significantly slowed but did not cure the growth of small tumors in mesothelioma-bearing mice. Large tumors were unaffected. This effect was dependent on the presence of CD8+ T cells and was associated with increased tumor-infiltrating lymphocytes. Because these activities are consistent with a mechanism that results in a decrease in the immunosuppressive environment of the tumor, we additionally examined the effect of COX-2 blockade combined with Ad.IFN-β therapy, a treatment that we have previously demonstrated results in expansion of antitumor CD8+ CTLs and cures a high percentage of small mesothelioma tumors in mice. Ad.IFN-β therapy combined with COX-2 inhibition was associated with an increased number of T cells within tumors and resulted in cures of small tumors, significant inhibition of the growth of large established tumors, and inhibition of the growth of metastatic tumor foci after surgical debulking. The additive effects of these modes of treatment suggests that it would be rational to combine COX-2 inhibition with immuno- and immunogene therapy approaches (perhaps in conjunction with surgical debulking) in human clinical trials of treatment of mesothelioma and other tumors.

INTRODUCTION

COX-1 catalyzes the rate-limiting step in the formation of prostaglandins from arachidonic acid. Two forms exist: the constitutive COX-1 and the inducible COX-2. Compared with COX-1, COX-2 leads to increased formation of PGE2 that appears to contribute to the cancer phenotype (1). Parhar and Lala (2) noted as early as 1988 that PGE2 secreted by macrophages facilitated metastases and inhibited NK cells and that COX-2 inhibition combined with IL-2 cured metastases in a mouse model of mammary adenocarcinoma. Antitumor effects of COX-2 inhibition have been reported in a wide variety of tumor models and in human cancers, both as chemoprevention and therapy (3–8). The mechanisms by which COX-2 blockade exerts its antitumor activity appear to vary by tumor type but include direct inhibition of tumor cell growth, blockade of angiogenesis, suppression of apoptosis, and decreased carcinogenic metabolite production (i.e., malondialdehyde; Ref. 8). Another very important activity of COX-2 is its conversion of arachidonic acid into prostaglandins. Two forms exist: the constitutive COX 1 catalyzes the rate-limiting step in the formation of prostaglandins from arachidonic acid. Two forms exist: the constitutive COX-1 and the inducible COX-2. Compared with COX-1, COX-2 leads to increased formation of PGE2 that appears to contribute to the cancer phenotype (1). Parhar and Lala (2) noted as early as 1988 that PGE2 secreted by macrophages facilitated metastases and inhibited NK cells and that COX-2 inhibition combined with IL-2 cured metastases in a mouse model of mammary adenocarcinoma. Antitumor effects of COX-2 inhibition have been reported in a wide variety of tumor models and in human cancers, both as chemoprevention and therapy (3–8). The mechanisms by which COX-2 blockade exerts its antitumor activity appear to vary by tumor type but include direct inhibition of tumor cell growth, blockade of angiogenesis, suppression of apoptosis, and decreased carcinogenic metabolite production (i.e., malondialdehyde; Ref. 8). Another very important activity of COX-2 inhibition may be reversal of suppression of cell-mediated immunity. Decreasing PGE2 levels appears to alter the balance between immunosuppressive and immunostimulatory cytokines such as IL-10 and IL-12 (9), leading to more effective antitumor immune responses (3). In addition, recent studies demonstrate that COX-2 inhibition or decreased expression of the PGE2 EP2 receptor prevent tumor-induced suppression of dendritic cell activity in vivo (10, 11).

The incidence of MM, an asbestos-related malignancy, is increasing worldwide. There is no currently available effective therapy for this tumor and mortality remains disturbingly high with median survival of ~6–12 months. Novel therapies are desperately needed to treat this devastating disease (12). Human mesothelioma tumors have been shown to overexpress COX-2 (13), and high levels of COX-2 protein have been demonstrated to be a prognostic factor indicating poor outcome in this tumor (14), suggesting that COX-2 may contribute to the pathogenesis of MM. Blockade of COX-1 and/or COX-2 has some direct antiproliferative effects on human mesothelioma cell lines and has been reported to restore the depressed lymphokine-activated killer cell activity ex vivo (15). On the basis of these observations, the first goal of the present study was to evaluate the effect of a COX-2 inhibitor on mesothelioma growth in an animal model.

There are a number of studies suggesting that mesothelioma may be susceptible to immunotherapy (16–18). Our group previously reported success in treating small mesothelioma tumors in mice with liposomes containing immunogenic cDNA (19) and even more impressive responses with a single i.p. dose of Ad.IFN-β, an adenovirus expressing murine IFN-β (16, 20). Both therapies acted through a CD8+ T-cell-dependent immune response associated with increased TILs. As with most immunotherapy approaches, however, these therapies were much less effective in larger tumors. Preliminary data in the Ad.IFN-β system (see below) suggested that this lack of efficacy in large tumors was not attributable to a failure to produce CTLs but to a failure of existing CTLs to adequately enter and kill cells within the tumor. Given that one of the effects of COX-2 inhibition is postulated to be reduction of the immunosuppressive environment within the tumor, the second goal of this study was to test the hypothesis that COX-2 inhibition would complement Ad.IFN-β therapy by allowing the CD8+ T cells greater access to the tumor environment and/or allowing improved cytotoxic functions. We therefore investigated the effect of COX-2 blockade combined with Ad.IFN-β in mice bearing small and large tumors.

In this article, we report three novel findings: (a) a previously unreported antitumor effect of COX-2 inhibition in a mesothelioma model; (b) an observation that the mechanism of this antitumor effect was associated with increased numbers of TILs; and (c) the presence of complementary antitumor effects between COX-2 inhibition and therapy with Ad.IFN-β when treating tumors.

MATERIALS AND METHODS

Animal Models and Tumor Cell Lines. All experiments used either female BALB/c mice (6–8 weeks old; weight, ~20–25 g) or female CB-17 SCID (6–8 weeks of age; weight, ~20–25 g) that were obtained from Taconic Laboratory (Germantown, NY). All animals were housed in the animal facility at the Wistar Institute (Philadelphia, PA) and were maintained in a pathogen-free facility for at least 1 week before each experiment. Each experiment used at least 5 mice/condition and was repeated at least once for confirmation of results. The University of Pennsylvania and the Wistar Institute approved all

Received 3/26/03; revision 8/19/03; accepted 9/5/03.

Grant support: National Cancer Institute Grant PO1 CA 66726.

P.D. and T.T. contributed equally to this manuscript.

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The abbreviations used are: COX, cyclooxygenase; PGE2, prostaglandin E2; IL, interleukin; MM, malignant mesothelioma; Ad.IFN-β, adenovirus-encoding IFN-β; i.i., intratumoral; NK, natural killer; SCID, severe combined immunodeficiency; PBS, fetal bovine serum; ptf, plaque forming unit; TIL, tumor-infiltrating lymphocyte.

CANCER RESEARCH 63, 7845–7852, November 15, 2003

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The mouse mesothelioma cell line AB12 was originally generated by i.p. implantation of asbestos fibers in BALB/c mice and has been characterized previously (16, 19). AB12 cells were cultured and maintained in high-glucose DMEM (Mediatech, Washington, DC) supplemented with 10% FBS (Georgia Biotechnology, Atlanta, GA), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine.

AB12 Flank Tumor Model. We injected 5 × 10^5 AB12 cells in 0.1 ml of serum-free media s.c. Animals were treated 5 days later when ~3-mm tumor nodules were visualized. Mice treated with Ad.IFN-β were injected with a single i.t. dose of 1 × 10^9 or 1 × 10^6 pfu as described. We performed measurements of tumors twice weekly, and animals were sacrificed when they met predetermined criteria established for minimizing pain and suffering. Tumor volumes were estimated using the formula \( \pi \times \text{long axis} \times \text{short axis}/6 \).

Recombinant Adenovirus Vectors. Production and characterization of the adenovirus encoding murine IFN-β has been described in detail (16, 20). Vector preparations were shown to be free of wild-type adenovirus. We determined the particle to pfu ratio of each preparation in 293 cells; this ranged from 17:1 to 70:1.

Transduction Efficiency. It was observed that COX-2 inhibition in combination with Ad.IFN-β therapy had a more significant antitumor effect than either therapy alone (see “Results”). To determine whether COX-2 inhibition affected levels of viral transduction, BALB/c mice fed Rofecoxib-containing chow or regular chow were injected i.p. with Ad.IFN-β. Forty-eight h later, mice were sacrificed and peritoneal fluid harvested by a lavage with 5 ml of saline. IFN-β levels in the peritoneal fluid were assayed by ELISA as described previously (16).

In Vivo Depletion of CD4+ and CD8+ T Cells. To deplete specific effector cell subsets before and during treatment with Rofecoxib chow in the flank model tumor AB12 model, BALB/c mice were injected with 200 μg of purified monoclonal antibodies purified from the anti-CD4+ hybridoma GK1.5 or the anti-CD8+ hybridoma 53–67 (obtained from the American Type Culture Collection [Manassas, VA]). Injections were administered 3 days and 1 day before inoculation with AB12 cells. Thereafter, a maintenance dose of antibody was injected i.p. every 7 days throughout the entire experimental period to ensure depletion of the targeted cell type. CD4+ and CD8+ T-cell depletion was confirmed by flow cytometry of splenic suspensions at the time of tumor injection and at later time points.

Phenotypic Analysis. Flow cytometry was carried out on splenocytes harvested from tumor-bearing mice as described previously (16). Briefly, tumor-bearing mice were treated with regular or Rofecoxib-containing chow. Seven days after initiation of treatment, the mice were sacrificed, and the spleens harvested and lymphocytes isolated. Flow cytometry was performed using fluorescently conjugated monoclonal antibodies for CD4, CD8, DX-5 (to identify NK cells), and CD11c (to identify dendritic cells; Southern Biotech, Birmingham, AL). Samples were analyzed using a Becton-Dickinson FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA).

Morphological Analysis. For immunohistochemistry, we snap-froze tumors in Tissue Tek OCT (Sakura Finitek, Torrance, CA), made 5–7-μm sections on gelatin-coated slides, and fixed them in cold acetone. We performed immunoperoxidase staining by using the avidin-biotin-peroxidase Vector Elite ABC kit (Vector Laboratories, Burlingame, CA). Primary antibodies were antimesothelioma CD4 (GR 1.5) and antimesothelioma CD8 (y-2) purchased from Southern Biotech. We developed sections with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with 1% hematoxylin.

Tumor Neutralization Test (Winn Assay). Winn assays were performed as described previously (21). Spleen cells were isolated and CD8+ T cells were purified using the MACs system (Miltenyi Biotec, Auburn, CA). Briefly, splenocytes were reacted with anti-CD8+ microbeads and run through a magnet-mounted column (Miltenyi Biotec) that allowed the CD8+ cells to pass but bound the head-labeled CD8+ cells. Removal of the column from the magnet released the CD8+ cells, which were then eluted. This cell population contained >90% CD8+ cells by FACS (data not shown).

The CD8+ T-cell-enriched population from normal, tumor-sensitized or tumor-sensitized and treated mice was admixed with viable tumor cells at a ratio of 1 purified CD8+ splenocyte for each tumor cell and the mixture inoculated s.c. into naïve BALB/c mice. Tumor growth was measured after 15 days and expressed as the mean ± SE of 5 mice/group.

**COX-2 Inhibition:** Specific COX inhibition was achieved using the COX-2 inhibitor Rofecoxib (MK-0966) obtained as a gift from Merck Frosst Canada & Co. (Kirkland, Quebec, Canada). This was incorporated into mouse chow by Test Diet (Richmond, IN) at a concentration of 0.0075%. Mice were fed this chow according to experimental design.

**Measurement of Rofecoxib Levels in Serum.** Mice were sacrificed and blood harvested by phlebotomy of the left ventricle. Blood was allowed to clot and serum removed by pipette. Plasma Rofecoxib levels were measured by high-performance liquid chromatography as previously described (21) and detected at a wavelength of 275 nm.

**Debulking and Metastatic Foci Experiments.** To test for antitumor effects of COX-2 inhibition or COX-2 inhibition combined with Ad.IFN-β that were undetectable in mice bearing large tumors, a model using debulking and metastatic foci was devised. In these experiments, 20 BALB/c mice were injected in one flank with 5 × 10^5 AB12 cells. On day 6, 10 mice were injected with 1 × 10^9 pfu of Ad.IFN-β i.t. The other 10 mice were injected i.t. with a similar volume (100 μl) of vehicle. Three days later, all 20 mice had their tumors surgically removed, and 5 × 10^5 AB12 cells were injected in the opposite flank from the debulking. This injection was designed to create a standard residual or metastatic tumor burden. In the group previously treated with Ad.IFN-β, 5 mice were fed Rofecoxib chow and 5 were fed control chow. In the vehicle-pretreated mice, 5 mice were fed Rofecoxib chow and 5 were fed control chow. Tumor volumes were measured twice weekly.

**Statistics.** Tumor volume data at a given time point was analyzed by comparing the tumor volumes of the control group with the treatment groups for each experiment using ANOVA with appropriate posthoc testing (Wilcoxon test) when significant differences (P < 0.05) were found. Calculations were made using StatView (Cary, NC).

SEs for each set of tumor measurements were calculated and represented as y-axis error bars on each graph. In some instances, the error bars cannot be seen because they are smaller than the size of the graphed symbol.

**RESULTS**

**Oral Administration of COX-2 Inhibitor Results in Adequate Serum Levels.** To establish that adequate levels of COX-2 inhibitor were achieved in mouse serum after oral administration of Rofecoxib chow, animals (both BALB/c and SCID mice) were sacrificed at various time points and serum collected for analysis. Serum from mice fed Rofecoxib-impregnated chow (0.0075%) contained a plateau level of 0.077 (SE ± 0.009) μg/ml Rofecoxib, whereas mice fed regular chow had undetectable levels. Serum levels were detectable as soon as 24 h after feeding, and plateau levels were reached after 9 days of administration (data not shown). These levels are very similar to those recently reported by Yao et al. (22).

**Tumor Growth Is Suppressed in Mice Fed the COX-2 Inhibitor Rofecoxib.** To investigate the effect of COX-2 blockade on the ability of mouse mesothelioma cells to form tumors, we injected AB12 mesothelioma cells into the flanks of mice and fed them control or Rofecoxib chow. Tumor size was measured twice weekly. As shown in Fig. 1A, injected tumor cells in the control chow animals grew into large tumors over the course of 40 days. In contrast, animals fed Rofecoxib chow showed marked tumor inhibition. On day 21, the tumor size in the control group was 471 mm^2 compared with 92 mm^2 in the COX-2-inhibited group (P < 0.001). Interestingly, growth inhibition was only maintained 20–25 days after injection when tumor growth resumed at a rate similar to that of control tumors. These data demonstrate that treatment with COX-2 inhibition therapy was able to significantly inhibit the growth of injected mesothelioma tumor cells for ~21 days.

**Rofecoxib-Dependent Tumor Suppression Is Immunological.** To determine whether the mechanism of this antitumor effect of COX-2 inhibition was immunological, studies were repeated in immunodeficient SCID mice that lack B and T cells. In contrast to the
In contrast, tumors from animals fed a COX-2 inhibitor (COX) were significantly smaller than the size of the symbol. Tumors on control animals showed typical growth.

Effects of COX-2 inhibition in these models, we used antibodies to inhibit the suppressive effects of Rofecoxib chow. Mice fed Rofecoxib-containing chow and depleted of CD4+ T cells had a mean tumor volume of 56 mm3 compared with 276 mm3 in the group depleted of CD4+ T cells and fed normal chow alone, a reduction in tumor volume of ~80% (P < 0.05). These data show that COX-2 inhibition does not act exclusively through CD4+ or CD8+ T cells but rather is dependent on both cell types.  

To determine the effect of COX-2 inhibition on the number of splenic lymphocytes, tumor-bearing mice fed either Rofecoxib or regular chow were sacrificed, the spleens harvested, and the total lymphocyte cell count determined. There were no significant differences (data not shown). To assess whether COX-inhibition altered the relative proportions of lymphocyte subsets, flow cytometric analysis were performed. Some of its antitumor effects through CD8+ T-cell-dependent mechanisms.

The effect of CD4+ T cells on the efficacy of COX-2 inhibition was also examined using antibody depletion (Fig. 2B). Interestingly, depletion of CD4+ T cells in mice fed control chow resulted in suppression of tumor growth. At day 27, mice fed regular chow and depleted of CD4+ T cells (AB12 anti-CD4) had a mean tumor volume of 462 versus 698 mm3 for controls (P < 0.05). This effect has been observed in other tumors and suggests that there may be suppressor (regulatory) CD4+ T cells operative in this model. However, depletion of CD4+ T cells did not inhibit the suppressive effects of Rofecoxib chow. Mice fed Rofecoxib-containing chow and depleted of CD4+ T cells had a mean tumor volume of 56 mm3 compared with 276 mm3 in the group depleted of CD4+ T cells and fed normal chow alone, a reduction in tumor volume of ~80% (P < 0.05). These data show that COX-2 inhibition does not act exclusively through CD4+ or CD8+ T cells but rather is dependent on both cell types.

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The effect of COX-2 inhibition was diminished after CD8 depletion resulting in approximately a 40% reduction in size (P < 0.05). These data show that COX-2 inhibition exerts only some of its antitumor effects through CD8+ T-cell-dependent mechanisms.

The effect of CD4+ T cells on the efficacy of COX-2 inhibition was also examined using antibody depletion (Fig. 2B). Interestingly, depletion of CD4+ T cells in mice fed control chow resulted in suppression of tumor growth. At day 27, mice fed regular chow and depleted of CD4+ T cells (AB12 anti-CD4) had a mean tumor volume of 462 versus 698 mm3 for controls (P < 0.05). This effect has been observed in other tumors and suggests that there may be suppressor (regulatory) CD4+ T cells operative in this model. However, depletion of CD4+ T cells did not inhibit the suppressive effects of Rofecoxib chow. Mice fed Rofecoxib-containing chow and depleted of CD4+ T cells had a mean tumor volume of 56 mm3 compared with 276 mm3 in the group depleted of CD4+ T cells and fed normal chow alone, a reduction in tumor volume of ~80% (P < 0.05). These data show that COX-2 inhibition does not act exclusively through CD4+ or CD8+ T cells but rather is dependent on both cell types.

COX-2 inhibition augments immunotherapy.

Fig. 1. Treatment with Rofecoxib chow (COX-2 blockade) inhibits tumor growth by an immunological mechanism. A, groups of mice (n = 5) were fed normal chow (control) or Rofecoxib-containing (Cox) chow starting at the same time as injection of 5 × 10^5 AB12 (mouse mesotheloma) cells in the flank. Tumor volume was measured over time. Values are mean volume (mm^3). Error bars represent SE (no error bars appear when SE was smaller than the size of the symbol). Tumors on control animals showed typical growth. In contrast, tumors from animals fed a COX-2 inhibitor (COX) were significantly inhibited (P < 0.001) at 21 days. After 25–30 days (despite continued feeding of Rofecoxib chow), the tumors began to grow at a rate similar to control tumors. B, SCID immunodeficient mice were fed control chow (n = 10) or Rofecoxib-containing chow (n = 10) for 7 days before injection of 5 × 10^5 AB12 tumor cells. Tumor volume was measured over time. Values are mean volume (mm^3). Error bars represent SE (no error bars appear when SE was smaller than the size of the symbol). Tumors on control animals showed typical growth. Tumors from mice fed a COX-2 inhibitor (COX) were not significantly suppressed and grew at a similar rate to controls (P > 0.05 at all time points).

Although angiogenesis inhibition may function when T cells are present because of the immune mediated genesis of antiangiogenic factors, this experiment indicates that the effects of COX-2 inhibition in this model are not because of direct antiproliferative effects or angiogenesis inhibition but are dependent on B or T lymphocytes.

To further evaluate the apparent immune-dependent antitumor effects of COX-2 inhibition in these models, we used antibodies to selectively deplete CD8+ T cells from tumor-bearing mice fed Rofecoxib-containing chow (Fig. 2A). As above, mice fed Rofecoxib chow have significantly smaller tumors than those fed normal chow. The mean tumor volume at day 24 was 561 mm^3 in the control group versus 176 mm^3 in the Rofecoxib chow-fed group. COX-2 inhibition resulting in a 70% reduction of size (P < 0.05). CD8+ T-cell depletion in tumor-bearing mice fed normal chow led to markedly accelerated tumor growth. This finding is consistent with our detection of CTL activity in untreated animals (see below). Tumors in CD8+ T-cell-depleted mice fed Rofecoxib also grew faster than control tumors; however, growth was significantly less than in the CD8+ T-cell-depleted mice fed normal chow. The mean tumor volume at day 24 was 1820 mm^3 in the CD8+ T-cell-depleted mice fed normal chow group versus 1074 mm^3 in the CD8+ T-cell-depleted mice fed Rofecoxib chow group. The effect of COX-2 inhibition was diminished after CD8 depletion resulting in approximately a 40% reduction in size (P < 0.05). These data show that COX-2 inhibition exerts only some of its antitumor effects through CD8+ T-cell-dependent mechanisms.
was performed on the splenic lymphocytes from tumor-bearing animals fed either Rofecoxib or regular chow using monoclonal antibodies for CD4, CD8, DX-5 (NK cells), and CD11c (dendritic cells). There were no significant changes in the percentages or absolute numbers of these cells in response to COX-2 inhibition (data not shown).

Recent in vitro work has suggested that PGE2 may inhibit dendritic cell function (10, 11). Thus, one potential mechanism for our observation was that COX-2 inhibition resulted in an increase in the number or activity of CTLs. To test this hypothesis, we assayed for CTLs using an in vivo tumor neutralization assay (Winn assay). CD8+ lymphocytes were purified from the spleens of AB12 tumor-bearing mice that had been fed either regular chow Rofecoxib-containing chow. These isolated CD8+ lymphocytes were then mixed ex vivo with AB12 tumor cells at a ratio of 1:1 and injected into the flanks of naïve Balb/C mice. Tumor growth was assessed after 15 days. At this time, tumor cells that had not been mixed with lymphocytes grew to a size of 312 mm3 (Fig. 3, control/bar 1). Addition of CD8+ T cells from tumor-bearing mice fed with normal chow to injected AB12 cells (Fig. 3, bar 2) inhibited tumor growth by ∼50% (Fig. 3, bar 1 versus bar 2; P = 0.01), indicating the presence of some CTL activity against the tumor. Importantly, tumor-bearing mice fed COX-2 inhibitor had virtually the same level of CTL activity (Fig. 3, bar 2 versus bar 3).

These data show that efficacy of COX-2 inhibition was not attributable to increased numbers or activity of circulating CTLs. As an alternative mechanism, we tested whether COX-2 inhibition increased the ability of existing CTLs to enter or persist within the tumor. We therefore took tumor-bearing mice that had been fed either normal or Rofecoxib-containing chow and sacrificed them 6 days after the s.c. injection of 5 × 105 AB12 cells. Tumors were sectioned and stained with anti-CD4 and anti-CD8 antibodies to confirm the presence of TILs. As shown in Fig. 4 (top panel), mice fed normal chow showed minimal infiltration of T cells into the tumor. In contrast, tumors from mice fed Rofecoxib-containing chow (Fig. 4, second panels) showed clear increases in CD4+ and CD8+ T-cell infiltration. These results support the hypothesis that one effect of COX-2 inhibition is enhanced accumulation of lymphocytes within tumors.

**COX-2 Blockade Inhibits the Growth of Small but Not Large Established Tumors.** To test the effect of COX-2 blockade in established tumors, mice were fed normal chow and then injected with 5 × 105 AB12 cells in the flank. Tumor growth was monitored until the size was ∼100 mm3 (4–6 days after injection) at which time animals were continued on normal chow or switched the chow containing COX-2 inhibitor. As shown in Fig. 5, mice fed Rofecoxib-containing chow showed significant tumor growth inhibition compared with controls fed normal chow. The mean tumor volume at day 25 was 1135 mm3 in the control group versus 390 mm3 in the Rofecoxib chow-fed group (P < 0.05). As we observed previously (Fig. 1A), after 21–25 days, the rate of growth of the tumor approached that of the control group despite continued ingestion of COX-2 inhibitor.

We next tested the effect of instituting COX-2 blockade in mice bearing larger established tumors (400 or 1000 mm3). In contrast to the effects in small tumor, no significant tumor growth suppressing
effect was observed compared with controls fed normal chow for 400 mm$^3$ tumors (data not shown) or for tumors that were 1000 mm$^3$ in size (Fig. 7).

Rofecoxib Chow (COX-2 Blockade) Augments Efficacy of Immunogene Therapy. Although Rofecoxib alone was not sufficient to eradicate tumors, we hypothesized that its ability to augment the ability of existing CTLs to enter and/or kill tumor cells would make it an excellent agent to combine with approaches that could increase and expand tumor-specific T cells. Our previous work with models of i.p. mesotheliomas had shown that an Ad.IFN-β was highly effective at curing early-stage tumors (16) and that the mechanism of this effect was dependent on CD8$^+$ T cells (16, 20). We conducted similar experiments with flank tumors. We observed that administration of Ad.IFN-β to established flank tumors resulted in marked antitumor activity (see below). Treatment of flank tumors with Ad.IFN-β also led to marked increases in CD8$^+$-mediated CTL activity as measured by the Winn assay (Fig. 3, bar 4).

We therefore tested the effect of combined therapy in established AB12 flank tumors. When tumors were ~100 mm$^3$, four groups were established. Group 1 received normal chow and no additional therapy. Group 2 were fed Rofecoxib-containing chow and no additional therapy. Group 3 received normal chow and was injected with $1 \times 10^8$ pfu Ad.IFN-β, a dose that would inhibit but not eradicate the tumors. Group 4 received combined therapy with Rofecoxib-containing chow and the same dose of Ad.IFN-β. As shown in Fig. 6, up until day 24 all three treatments markedly inhibited tumor growth ($P < 0.001$ versus control for all groups). After 25–30 days, tumors in mice treated with Rofecoxib-containing chow began to grow rapidly. Tumors treated with Ad.IFN-β alone also began to grow. In contrast, all tumors were completely eradicated in the group 4 mice receiving the combination of Rofecoxib-containing chow and the subtherapeutic dose of Ad.IFN-β. None of these cured animals demonstrated tumor growth when subsequently injected with $5 \times 10^5$ AB12 cells on day 40 after initial treatment with COX-2 inhibition and Ad.IFN-β (data not shown).

As with most immunotherapeutic approaches, the efficacy of Ad.IFN-β therapy was reduced in moderate-sized tumors and had little or no effect on large i.p. tumors (16). It was therefore of interest to determine whether addition of COX-2 inhibition to Ad.IFN-β therapy might allow treatment of animals with larger tumors. Mice were thus injected with AB12 cells, and tumors were allowed to grow to very large sizes, volumes of ~1000 mm$^3$. At this time, the animals were started on Rofecoxib-containing chow, injected with $10^7$ pfu Ad.IFN-β, or received both therapies. As shown in Fig. 7, COX-2 inhibitor therapy alone had no effect on tumor growth. Injection of Ad.IFN-β alone also had minimal effects. In contrast, the combination of COX-2 inhibition and Ad.IFN-β therapy significantly ($P < 0.01$ versus all three other groups at day 45) inhibited the growth of the tumors for >25 days. A similar growth inhibitory effect was noted for moderate-sized tumor with volumes of ~500 mm$^3$ (data not shown).

Rofecoxib Chow (COX-2 Blockade) Can Be Effectively Combined With Neoadjuvant Immunotherapy to Inhibit the Growth of Metastatic Foci after Debulking. The finding that COX-2 blockade augmented the ability of i.t. Ad.IFN-β therapy to eradicate small tumors suggested that a potentially effective clinical use of this combination would be to use this treatment in a setting where an i.t. injection could be followed by debulking leaving only minimal disease behind. The use of debulking in mesothelioma is well accepted as a way to improve symptoms, although it does not increase overall survival (23, 24). It is possible that antitumor immune therapies administered to patients bearing large tumors might stimulate responses that would eradicate small tumors but would be inhibited or ineffective against the large tumors. Thus, the presence of a large tumor at the time of therapy might alter the response to the therapy. We modeled this clinical scenario by treating mice bearing large tumors (500 mm$^3$), waiting 3 days, then surgically debulking the
The combination of COX-2 blockade and Ad.IFN-β therapy inhibits the growth of metastatic tumor foci. Mice were injected with AB12 cells, and when tumors reached a size of ~500 mm³, groups 1 and 2 were injected i.t. with saline and groups 3 and 4 injected i.t. with 10⁹ pfu of Ad.IFN-β. Three days later, all groups had their tumors surgically removed followed by injection of 5 × 10⁹ AB12 cells into the contralateral flank. Group 1 (None) and Group 3 (β) received no additional treatment. Group 2 (Cox) and Group 4 (β and Cox) were fed Rofecoxib chow. The growth of the metastatic focus was followed by measuring tumor size twice weekly. Both COX-2 blockade and Ad.IFN-β therapy, before debulking, significantly slowed the growth of the contralateral tumor versus debulking alone (P < 0.05 and P < 0.0001, respectively). However, the combination of both therapies resulted in virtually complete suppression of tumor growth on the contralateral flank (P < 0.01 versus all three other groups).

Mechanism Of Rofecoxib Augmentation of Ad.IFN-β Therapy.

One potential explanation for why Rofecoxib augmented the effect of Ad.IFN-β therapy was that COX-2 inhibition increased the level of vector transduction or production of IFN-β. To test this possibility, mice fed Rofecoxib-containing chow or regular chow and were injected with Ad.IFN-β in the peritoneum. After 48 h, the mice were sacrificed and peritoneal fluid assayed for IFN-β levels. COX-2 inhibition had no effect on the level of IFN-β in the peritoneal fluid compared with fluid from mice that were not treated with COX-2 inhibition (data not shown).

We also determined whether the combination of COX-2 inhibitor plus Ad.IFN-β resulted in increased activity of CTLs using the Winn assay. As shown in Fig. 2, bar 5, addition of Rofecoxib did not increase the amount of CTL activity over that seen in Ad.IFN-β-treated animals treated with regular chow (Fig. 3, bar 4).

On the basis of our previous experiments, we examined the hypothesis that the efficacy of the combination of COX-2 inhibition and Ad.IFN-β was because of an augmented ability of CTLs to accumulate in the tumors. Mice were injected with 5 × 10⁹ AB12 cells and tumors allowed to grow to 500 mm³. At this time, the tumors were injected with saline or Ad.IFN-β (10⁹ pfu). Rofecoxib-containing chow was then initiated in one group. All animals were sacrificed 6 days later, and tumors were examined by histology for TILs. As shown in Fig. 4 (third set of panels), mice treated with Ad.IFN-β showed a moderate increase in CD4⁺ and CD8⁺ lymphocytes compared with controls. In contrast, mice treated with both Rofecoxib chow and Ad.IFN-β showed even larger numbers of CD4⁺ and CD8⁺ lymphocytes within the tumors.

DISCUSSION

Although COX-2 is overexpressed in MM, its role in the pathogenesis of this tumor is unknown. The first goal of this study was to examine the effect of COX-2 inhibition on the growth of mesothelioma tumors in mice. We therefore studied flank tumor growth in response to treatment with chow containing the specific COX-2 inhibitor Rofecoxib. Oral administration was chosen to mimic the route that a COX-2 inhibitor might be given to patients and measurements of serum levels of Rofecoxib were close to those reported in humans taking 25 mg once daily.

The antitumor effects of COX-2 inhibition appear attributable to immunological effects because inhibition was completely lost in immunodeficient SCID mice (Fig. 1B). It still remains possible that angiogenesis inhibition could be operative if mediated through CD4⁺ and CD8⁺ T cells (i.e., through alterations in chemokine production). To further define the immunological mechanisms responsible for COX-2 inhibitor-induced tumor suppression, we conducted additional studies using antibody depletion of CD4⁺ or CD8⁺ cells. The results are interesting but interpretation is complicated by the effects of antibody depletion in mice fed normal chow. In this model, CD4⁺ T-cell depletion (in mice fed normal chow) inhibited tumor growth (Fig. 2B), suggesting the presence of a population of suppressor (regulatory) CD4⁺ cells. However, when Rofecoxib was fed to the CD4⁺ T-cell-depleted animals, tumor growth was inhibited by 80%. Given SCID mouse results, these data indicate that much of the COX-2 effect is CD8⁺ T-cell dependent. However, not all of the effect appears to be attributable to CD8⁺ T cells. CD8⁺ T-cell depletion led to more rapid growth of the tumors (Fig. 2A). Depletion of CD8⁺ T cells did not completely eliminate the effect of Rofecoxib; COX-2 inhibition blocked tumor growth by ~30%. These depletion data show that COX-2 inhibition does not act exclusively through CD4⁺ or CD8⁺ T cells but rather is dependent on both cell types with CD8⁺ T cells appearing to be more important. These results are quite consistent with the immunohistology showing enhanced infiltration of both CD4⁺ and CD8⁺ T cells (Fig. 4).

There are a number of mechanisms by which COX-2 may have potentially important effects on the immune system. Recent studies have shown that COX-2 inhibition or decreased expression of the PGE₂ EP2 receptor can prevent tumor-induced PGE₂-dependent suppression of dendritic cell activity in vitro (10, 11). However, in our animal model (using a semiquantitative Winn assay), we did not observe increased activity of CTLs in animals receiving COX-2 inhibition. Another mechanism proposed (in lung cancer models) is that COX-2 inhibition may exert its antitumor effect by altering the cytokine ratios of IL-10 and IL-12 within the tumor, thus restoring...
cell-mediated immunity (3). It has also recently been demonstrated that lung cancer cells do not express IL-10 receptors and thus are not able to down-regulate COX-2 expression in response to increased IL-10 levels (25). Furthermore, the COX-2 product, PGE$_2$, can down-regulate IL-2, IL-6, and IFN-$\gamma$, thus preventing activation and trafficking of CTLs necessary for tumor regression (26). Our data showing that COX-2 inhibition did not increase the number or activity of splenic CTLs (Fig. 3) but did increase the number of CD4$^+$ and CD8$^+$ T cells that were visible within the tumors (Fig. 4) is more consistent with the hypothesis that COX-2 inhibition alters the immunosuppressive environment found within the tumor; an environment that both inhibits T-cell migration and inactivates or induces apoptosis in those T cells that do gain entry into the tumor. This hypothesis is being evaluated by additional studies using real-time PCR to profile cytokines such as IL-6, IL-10, transforming growth factor $\beta$, IFN-$\gamma$, and chemokines such as monokine induced by interferon gamma (MIG) and gamma interferon inducible protein 10 (IP-10) in treated versus nontreated tumors are under way. In addition, phenotypic studies of dendritic cells in tumor-bearing mice undergoing COX-2 blockade are ongoing to determine the activation level of dendritic cells in vivo in response to this therapy.

An important limitation of this Rofecoxib therapy was the observation that only small tumors could be effectively treated. As with most immunotherapies described in animal models or in patients (27), therapeutic efficacy was almost completely lost when mice with moderate and large tumors (Fig. 7) were treated. These results suggest that COX-2 inhibition in patients with MM would thus only be effective in those patients with minimal disease. Because most patients present with extensive tumors, the most likely therapeutic scenario where administration of COX-2 inhibitor would be helpful would be administration as adjuvant therapy after surgical debulking has been performed. This hypothesis is supported by data from our model of residual disease after debulking (Fig. 8) where residual tumor growth was significantly slower in debulked animals fed Rofecoxib. Although somewhat controversial, surgical debulking for MM has a number of advocates and is not uncommonly performed (23, 24). Addition of COX-2 inhibitors may useful in this setting. Preliminary data from our laboratory also suggests that COX-2 inhibition could be useful in lung cancer. Thus, it may be reasonable to suggest a trial of COX-2 inhibition in patients surgically cured of lung cancer but at high risk of recurrence (i.e., stage Iib patients).

Given that COX-2 inhibition had the ability to enhance lymphocyte accumulation in tumors, we postulated that an important new use for COX-2 inhibition therapy could be in combination with immunotherapy. The second goal of this study was to test the hypothesis that COX-2 inhibition would complement Ad.IFN-$\beta$ therapy by allowing the CD8$^+$ T cells greater access to the tumor environment. We have previously shown that Ad.IFN-$\beta$ increased the number of CTLs in an i.p. model of mesothelioma (16). Using the Wnn assay, we confirmed that the activity of CTLs present after viral injection into flank tumors was also markedly increased (Fig. 3). Although these CTLs were able to markedly slow (Fig. 6) or eradicate small tumors (data not shown at the higher dose of $10^9$ plu), they had only minimal effects on growth of larger tumors (Fig. 7). The limited number of stainable CD4$^+$ and CD8$^+$ cells within tumors with Ad.IFN-$\beta$-treated animals (Fig. 3, third row panels) suggests that this failure is likely attributable to an either an inability of the CTLs to effectively enter to the tumor or the rapid apoptosis of these cells within the tumor. The augmented efficacy of COX-2 plus Ad.IFN-$\beta$ therapy in both small (Fig. 6) and very large (Fig. 7) tumors plus the demonstration of increased numbers of CD4$^+$ and CD8$^+$ T cells within the tumors of animals given combined therapy (Fig. 4, bottom panels) supports our hypothesis. We believe that COX-2 inhibition is effective in combination therapy because it targets the immune response at a different stage (CTL access and/or function in the tumor microenvironment) than Ad.IFN-$\beta$ (generation and/or expansion of CTLs).

Given that combination therapy is also much more effective in smaller tumors, our experiments suggest that COX-2 inhibitors could be especially useful in combination with immunogene therapy and surgical debulking in a clinical setting. Using a model of minimal residual disease after surgery, we found that the combination of COX-2 inhibitor plus Ad.IFN-$\beta$ therapy was more effective in preventing recurrences than either agent alone (Fig. 8). We are currently conducting a Phase I trial of Ad.IFN-$\beta$ in patients with mesothelioma. If this therapy proves safe, we anticipate that design of the Phase II study will be administration of Ad.IFN-$\beta$ followed by debulking followed by COX-2 inhibition. A similar approach might be useful for other tumors such as lung cancer.

In summary, we have shown that COX-2 inhibition does block the growth of small mesothelioma tumors through an immunological mechanism that appears to allow more effective accumulation of CTLs in the tumors. This therapeutic effect can be greatly magnified by combining COX-2 inhibition with an immunotherapy (such as Ad.IFN-$\beta$) that works via a different mechanism (increasing the number of CTLs). The use of COX-2 inhibition thus presents a nontoxic, easily available approach that can be potentially be used in conjunction with wide variety of other immunotherapeutic approaches.

ACKNOWLEDGMENTS

We thank Ian Rodger and Pauline LuK of Merck and Merck-Frost-Canada for the gift of Rofecoxib chow and the performance of Rofecoxib serum measurement and Kathleen Proport of the Epidemiology and Biostatistics Department at The University of Pennsylvania for help with statistical analysis of data.

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Use of Cyclooxygenase-2 Inhibition to Enhance the Efficacy of Immunotherapy

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