

Host and Direct Antitumor Effects and Profound Reduction in Tumor Metastasis with Selective EP4 Receptor Antagonism

Li Yang,¹ Yuhui Huang,² Rut Porta,² Kiyoshi Yanagisawa,¹ Adriana Gonzalez,¹ Eric Segi,⁴ David H. Johnson,^{1,3} Shuh Narumiya,⁴ and David P. Carbone^{1,2,3}

Departments of ¹Medicine and ²Cancer Biology and ³The Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee and ⁴Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto, Japan

Abstract

Prostaglandin E₂ (PGE₂), one of the major metabolites of cyclooxygenase-2, has been implicated in tumorigenesis and tumor progression in several human cancers, including colorectal and lung. Here, we show that one of the PGE₂ receptors, the EP4 receptor, plays an important role in metastasis in both of these tumor types. Using i.v. injected Lewis lung carcinoma (3LL), we found that tumor metastasis to lung was significantly reduced when mice were treated with a specific EP4 antagonist ONO-AE3-208 or when EP4 receptor expression was knocked down in the tumor cells using RNA interference technology. Host EP4 receptors also contributed to tumor metastasis and tumor growth with decreased metastasis and tumor growth observed in EP4 receptor knockout animals. *In vitro* tumor cell adhesion, motility, invasion, colony formation, and Akt phosphorylation were all significantly inhibited when 3LL cells were treated with the EP4 receptor-specific antagonist. When the cells were treated with an EP4-specific agonist (AE1-734), we observed a worsening of these same features *in vitro*. Treatment with ONO-AE3-208 also profoundly decreased liver metastases after intrasplenic injection of MC26 colon cancer cells. Our data show that selective antagonism of EP4 receptor signaling results in a profound reduction in lung and colon cancer metastasis. Selective antagonism of the EP4 receptor may thus represent a novel therapeutic approach for the treatment of cancer and especially its propensity to metastasize. (Cancer Res 2006; 66(19): 9665-72)

Introduction

Several lines of evidence show the functional importance of overproduction of cyclooxygenase (COX)-2 and one of its major metabolite, prostaglandin E₂ (PGE₂), in many human cancers, including colorectal tumors and lung cancer (1–3). Preclinical data indicate that overproduction of COX-2 and PGE₂ is associated with increased tumor angiogenesis (4, 5), metastasis (6–8), apoptosis (9, 10), cell cycle regulation (11), and immune suppression (12, 13). Therefore, COX-2 inhibitors, the nonsteroidal anti-inflammatory drugs, have been tested in the treatment and prevention of cancers of the colon (14), lung (15, 16), breast (17), and prostate (18). However, these have met with limited success and, sometimes, severe side effects (19–21).

Note: Current address for R. Porta: Department of Medical Oncology, Institut Catala Oncologia, Hospital Josep Trueta, Girona, Spain.

Requests for reprints: Li Yang, Vanderbilt University Medical Center, 646 PRB, 2220 Pierce Avenue, Nashville, TN 37232. Phone: 615-936-3320; Fax: 615-936-1790; E-mail: li.yang@vanderbilt.edu.

©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-1271

One of the reasons for this lack of apparent efficacy of the COX inhibitors may be that the COX-2 enzyme produces multiple products with pleiotropic effects in addition to PGE₂, in addition to the fact that there are four PGE₂ receptors with distinct functions. For example, pulmonary overexpression of another COX product, prostacyclin, has been shown to significantly reduce lung tumor incidence in mice (22, 23). In addition, each of the four receptors for PGE₂ signaling has discrete biological effects. For example, although both the EP2 and EP4 receptors couple through G protein α (s) to elevate cyclic AMP production, the EP4 receptor, but not EP2, is known to induce T-cell factor-mediated transcriptional activity through phosphatidylinositol 3-kinase (PI3K) as well as protein kinase A (24, 25). The EP4 receptor can also activate the extracellular signal-regulated kinases 1 and 2 by way of PI3K, leading to the induction of early growth response factor-1, a transcription factor traditionally associated with wound healing (24, 25). These pathways are important for cancer cell survival and the progression of the tumors. EP4 receptor signaling was also found to be central in COX-2-dependent, CD44-mediated, and matrix metalloproteinase (MMP)-2-mediated invasion in non-small cell lung cancer invasion (26), osteolytic bone metastasis of breast cancer *in vivo* (27), and breast cancer cell invasion *in vitro* (28, 29). Furthermore, an enhanced expression of EP4 protein and an enhanced signaling were found in several cancers (30–32), including the cancerous epithelia of gallbladder carcinoma with different depths of invasion (33) and in ovarian carcinoma cell invasion (32). In human glioblastoma samples, EP4 receptor expression was correlated with increasing necrosis grade, an indication for reduced survival of the patients (31).

These reports and others suggest that targeting the EP4 receptor signaling could lead to reduced tumor growth and metastasis (29, 30, 34–36) more effectively than COX-2 inhibitors. In this report, we find support for this hypothesis by showing that selective pharmacologic inhibition of EP4 signaling has profound effects on tumor metastasis *in vitro* and *in vivo* as well as colorectal tumor growth. Our data show that manipulation of PG metabolism downstream from COX-2 produces profound anticancer effects and provide the basis for novel approaches for cancer drug development and improved understanding of the pathogenesis of cancer.

Materials and Methods

Materials. Cancer cell lines 3LL and MC26 were obtained from the American Type Culture Collection (Manassas, VA) and maintained per standard cell culture techniques. The EP4 receptor-specific antagonist ONO-AE3-208 [4-(4-cyano-2-[2-(4-fluoronaphthalen-1-yl) propionylamino] phenyl) butyric acid] was kindly provided by Ono Pharmaceutical Co. (Osaka, Japan). Its structures, binding affinities and selectivities, and pharmacokinetic properties have been characterized in Ono Pharmaceutical and Narumiya's laboratory (37, 38). The EP4 receptor-selective agonist (AE1-734) was a kind gift from Dr. S. Narumiya and Ono Pharmaceutical.

Six- to eight-week-old female mice BALB/c and C57BL/6 were purchased from Harlan, Inc. (Indianapolis, IN). F2 progeny of female survivor $EP4^{-/-}$ mice was used in the study with $EP4^{+/+}$ mice as controls due to the fact that most $EP4^{-/-}$ mice die as a result of patent ductus arteriosus (39, 40). Animals were housed in pathogen-free units at Vanderbilt University School of Medicine (Nashville, TN) in compliance with Institutional Animal Care and Use Committee regulations or in Kyoto University (Kyoto, Japan) in compliance with the Committee on Animal Research of Kyoto University.

Construct of EP4 RNA interference. EP4 RNA interference (RNAi) sequence was determined by using the design software of Oligoengine Station (Seattle, WA) and blasted against National Center for Biotechnology Information Web site to make sure it was EP4 specific. The EP4 RNAi sequence is GGAGCAGAAAGAGACGACCTctcttgaacCTCGTCTTTGTGTGCTGG. It was then cloned into pSUPER retroviral vector with Hi-RNA promoter.

EP4 RNAi transfection of 3LL cells. The EP4 RNAi and control vector constructs were transfected into 3LL cells, and the positive transfectants were selected by puromycin, which then were further subcloned, and each clone was identified for the deletion of the EP4 receptor expression.

Isograft models of tumor biology. Single-cell suspensions containing 5×10^5 of 3LL or MC26 cells in 100 μ L PBS were injected s.c. into the right flank of C57BL/6 or BALB/c mice. The size of the tumor was determined by direct measurement of the tumor dimensions in 3 to 4 weeks using calipers. To quantitate tumor volume, the following equation was used: volume = [length \times (width)²] \times 0.5. For Lewis lung carcinoma metastasis model, 5×10^5 of 3LL cells in 100 μ L PBS were injected into the tail vein of C57BL/6. The lung weight and number of tumor nodules were obtained after \sim 3 weeks when the control animals became sick. For the colon cancer metastasis model, 5×10^4 of MC26 cells in 100 μ L PBS were injected into the spleen of BALB/c mice. The spleen was removed 10 minutes after injection. The weight of the liver and tumor nodules in liver were evaluated after \sim 4 weeks. The mice in both colorectal and lung cancer models were pretreated with ONO-AE3-208 3 days before and during the experiment. AE3-208 was given (10 mg/kg/d) orally in the drinking water, which has been shown to result in specific inhibition of EP4 receptor activity *in vivo* (37, 38).

Adhesion assays. Fibronectin [100 μ L of 10 μ g/mL stock in 0.1% bovine serum albumin (BSA)] was coated on each well of 48-well plate overnight at 4°C. BSA (3%) was added for 7 hours at 4°C to block specific bindings. Wells coated with BSA only were served as negative control. 3LL cells, with or without treatment of PGE₂, EP4 agonist and antagonist, as well as EP4 RNAi clones with the vector controls were suspended in RPMI 1640 plus 0.1% BSA at 250,000/mL. Cell suspension (100 μ L) was incubated for 60 minutes at 37°C with triplicates for each treatment. The wells were then washed with PBS twice, fixed with 4% paraformaldehyde for 30 minutes, and stained with 0.2% crystal violet in 2% ethanol for 10 minutes at room temperature. The wells were further washed with tap water by immersion for 2 \times . SDS (1%) was then added to solubilize the adherent cells, and the plate was shaken until the color is uniform. The absorbance at 570 nm was obtained.

Migration and invasion assays. 3LL cells and EP4 RNAi clones as well as the vector controls (1×10^5) were put on the upper Boyden chamber of a Transwell (8- μ m pore size; BD PharMingen, Franklin Lakes, NJ) in 500 μ L serum-free RPMI 1640. RPMI 1640 (2 mL) plus 10% FCS were put in the lower chamber. The cells were cultured at 37°C for 4 hours. The inserts were then removed. Cells in the upper side of the chamber were wiped using a cotton swab, and the inserts were fixed with 100% methanol and stained with crystal violet. The membranes were removed and then put on slides, and migrated cells were counted under a microscope in a blinded fashion. For the invasion assays, the Boyden chamber is modified using a basement membrane matrix preparation, Matrigel, as the matrix barrier. The cells with 1 μ mol/L of PGE₂, EP4-specific agonist or antagonist, EP4 RNAi clones, and vector controls were cultured for 24 hours.

Colony formation assay. Soft agar colony formation assays were carried out in six-well culture plates. 3LL cells, EP4 RNAi clones, and vector controls (7.5×10^3) were suspended in 2 mL of 0.4% Sea Kemp low-melting agarose (Metaphor Agarose, Cambrex, East Rutherford, NJ) with growth medium

(RPMI 1640 supplemented with 10% fetal bovine serum) added on a base layer of 0.8% the agar. The plates were incubated at 37°C in a 5% CO₂ incubator for 2 to 3 weeks. Triplicates were set up for each experimental treatment. The number of colonies was counted under low magnification (\times 100) at 10 points on each well in a blinded fashion.

Western blot. Tissues or cultured cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (1 \times PBS added 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, protease inhibitors). Protein concentrations were measured using Bio-Rad (Hercules, CA) reagent. Proteins were then separated on 4% to 20% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were then blocked in 5% milk in TBS and 0.1% Tween 20 and incubated with primary antibody (EP4, 1:500; phosphorylated Akt, 1:2,000; Akt, 1:2,000; and β -actin or glyceraldehyde-3-phosphate dehydrogenase, 1:2,000 to 1:3,000) overnight at 4°C. The membranes were then treated with horseradish peroxidase-conjugated secondary antibody and developed using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Statistical analysis. The restricted maximum likelihood-based mixed effect model and ANOVA were used to analyze the differences of tumor growth. The rest data were analyzed by the Student's *t* test. All data were expressed as mean \pm SE, and differences were considered statistically significant when *P* < 0.05.

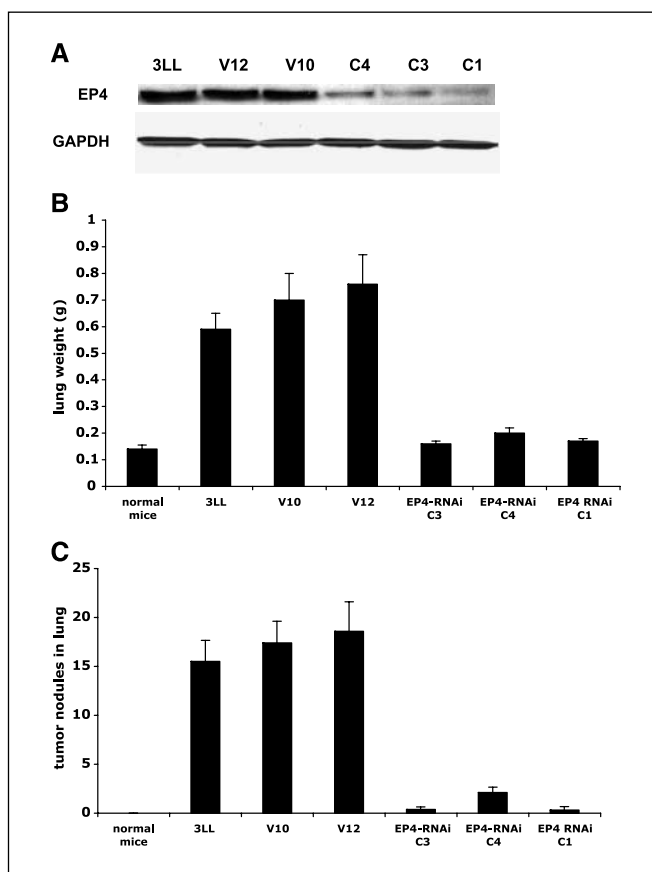
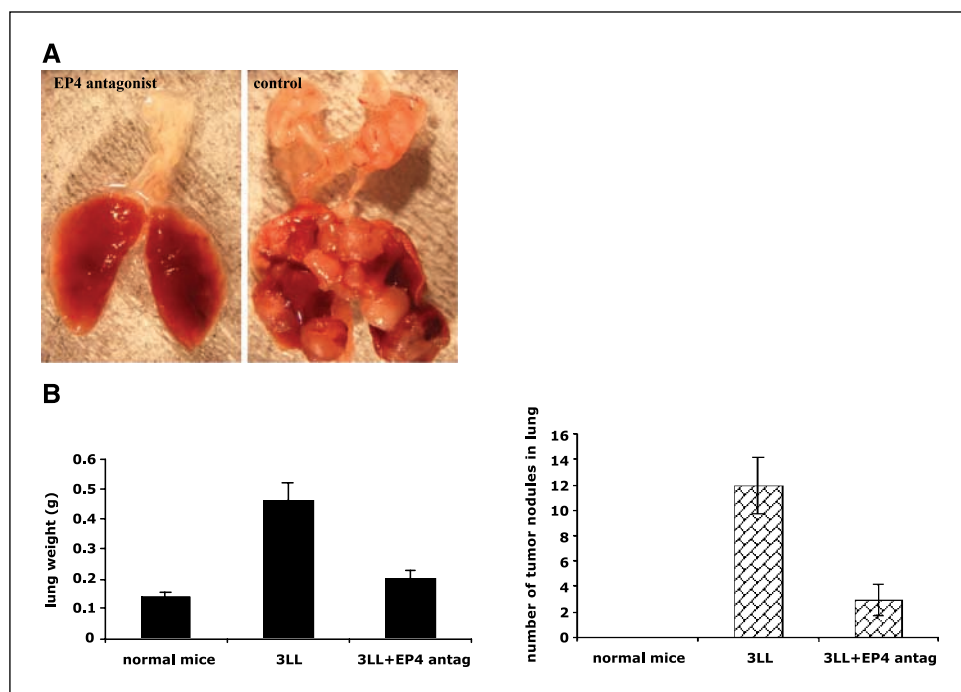


Figure 1. Reduced lung metastasis after inhibition of the EP4 receptor expression with EP4 RNAi in 3LL cells. **A**, Western blot analysis of EP4 receptor expression. 3LL cells were transfected with EP4 RNAi or empty vector, and the cells were selected with puromycin and further cloned. 3LL, parental cell line; V12 and V10, vector control clones; C4, C3, and C1, EP4 RNAi clones. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B** and **C**, experimental metastasis of 3LL cells with EP4 RNAi and vector controls in C57BL/6 mice. Lung weight (**B**) and the number of tumor nodules in lung (**C**) in mice that received 3LL cells with EP4 RNAi 21 days after tail vein injection. One of the three independent experiments with similar results. The total number of animal tested: normal, *n* = 3; 3LL, *n* = 18; V10, *n* = 7; V12, *n* = 9; C4, *n* = 13; C3, *n* = 17; C1, *n* = 3. *P* < 0.05 between control versus EP4 RNAi.

Figure 2. Reduced lung metastasis of 3LL cells by EP4 receptor–specific antagonist treatment. **A**, photograph of the lungs from mice after tail vein injection of 3LL cells with or without treatment of the host with the EP4 antagonist ONO-AE3-208. Mice were pretreated with the drug 3 days before 3LL tail vein injection and 21 days after at 10 mg/kg/d orally in the drinking water. **B**, lung weight (*left*) and tumor nodules in lungs (*right*) from mice with or without EP4 receptor antagonist treatment. One of the two independent experiments with similar results. The number of animal tested: normal, $n = 5$; 3LL, $n = 11$; EP4, antagonist treatment, $n = 14$. $P < 0.001$.



Results

Targeting the EP4 receptor in lung cancer cells significantly inhibits their metastasis to lung. To test whether selective inhibition of EP4 receptor signaling inhibits tumor metastasis, we constructed EP4 RNAi vectors and transfected these constructs into the Lewis lung carcinoma tumor cell line 3LL. Expressing cells were selected with puromycin, and stably transfected cells were cloned. Each of several clones transfected with the EP4 RNAi vector (3LL-EP4 RNAi) as well as vector controls (3LL-vec) were subjected to Western blot analysis to determine the degree of EP4 receptor expression silencing (Fig. 1A). As shown in Fig. 1A, expression of the EP4 receptor in clones C1, C3, and C4 was significantly reduced, whereas expression of the EP4 receptor in vector control clones V10 and V12 is comparable with that of parental cell line 3LL. To determine whether reduction of tumor EP4 receptor expression reduced tumor metastasis, we assayed lung colonization by 3LL tumor cells after tail vein injection, a widely used model system for vascular metastasis. Syngeneic C57BL/6 mice were injected with the 3LL parental cell line, 3LL-EP4 RNAi clones, as well as vector control clones. Mice were sacrificed 3 weeks later when control mice become symptomatic, and lung weight and the number of tumor nodules in the lung were evaluated. We found that lung weight and the number of tumor nodules in mice injected with 3LL-EP4 RNAi cells were significantly decreased compared with that of controls (Fig. 1B and C).

To independently confirm these observations, we blocked EP4 receptor signaling with a systemically administered pharmacologic inhibitor, the EP4 receptor–specific antagonist ONO-AE3-208. The K_i values of AE3-208 obtained by radioligand competition-binding inhibition assays for the prostanoid receptors are 1.3, 30, 790, and 2,400 nmol/L for EP4, EP3, FP, and TP, respectively and >10,000 nmol/L for the other prostanoid receptors. AE3-208 was given (10 mg/kg/d) as a bolus orally in the drinking water, which showed specific EP4 receptor antagonistic effect, with a peak plasma concentration of 677 ng/mL (1,000 nmol/L) attained in 0.25 hour

after the administration with 18% of bioavailability. The plasma half-life of this compound measured in an experiment of i.v. injection was 0.2 hour (37, 38). Mice were pretreated with the EP4 antagonist ONO-AE3-208 3 days before 3LL tail vein injection and continuously afterward. We found that ONO-AE3-208 dramatically decreased lung weight and the number of tumor nodules, similar to the effect of knocking down EP4 receptor expression in 3LL using RNAi (Fig. 2A and B). These results showed that EP4 receptor signaling is important in 3LL cell metastasis to the lung and that

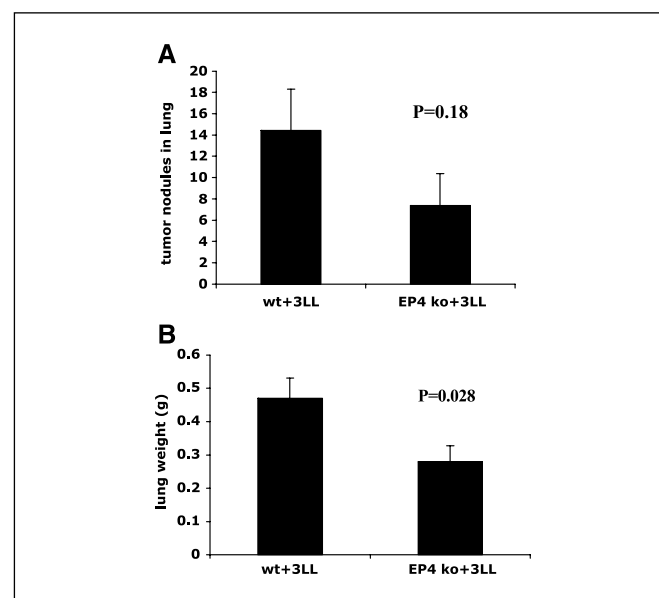
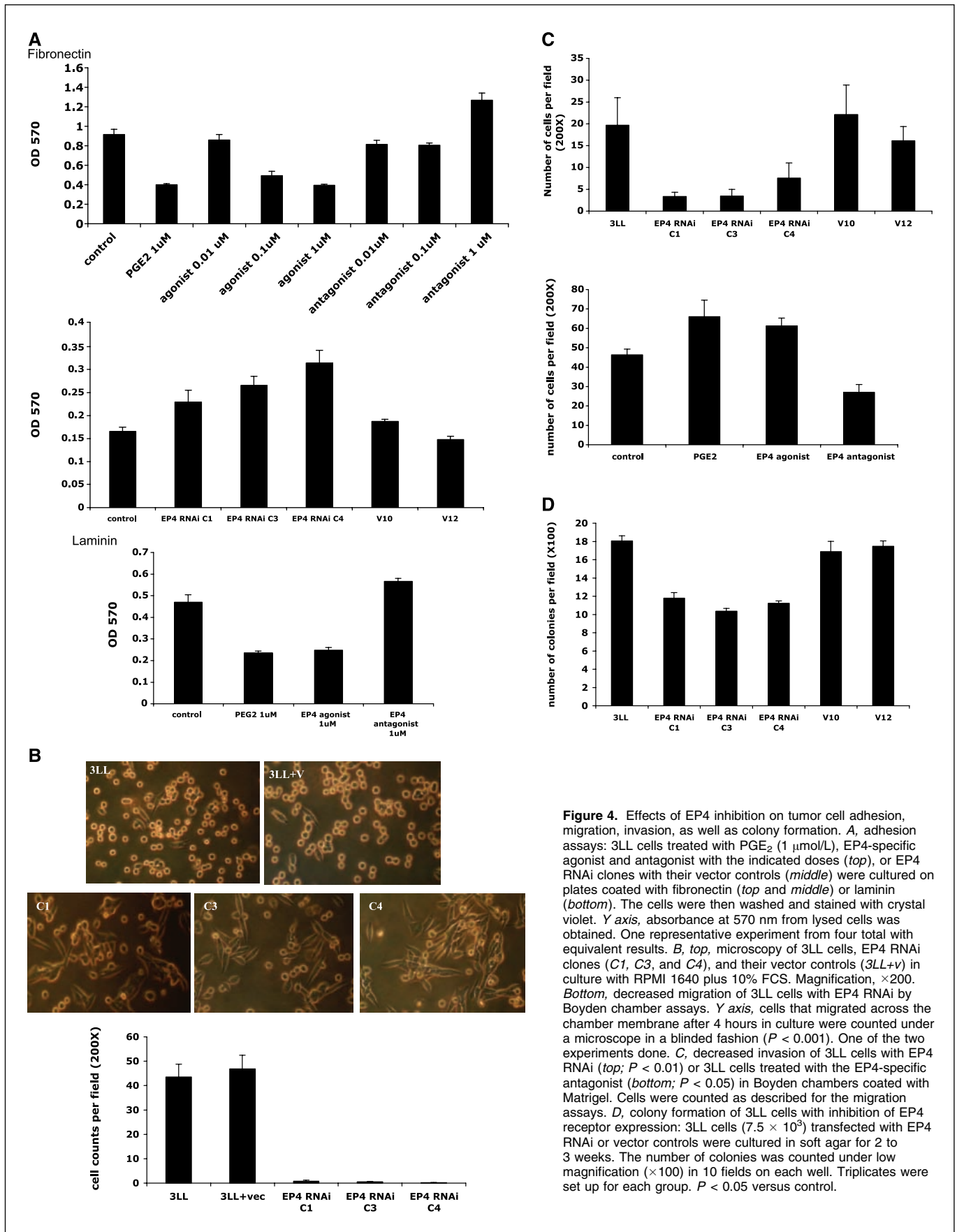


Figure 3. Contribution of host EP4 receptor to tumor metastasis. The number of tumor nodules in the lung (**A**) and lung weight (**B**) in EP4 knockout (*ko*) mice ($n = 7$) and their littermates ($n = 7$) were evaluated 21 days after 3LL tail vein injection, with $P = 0.18$ for the tumor nodules and $P = 0.028$ for lung weight. *wt*, wild-type.



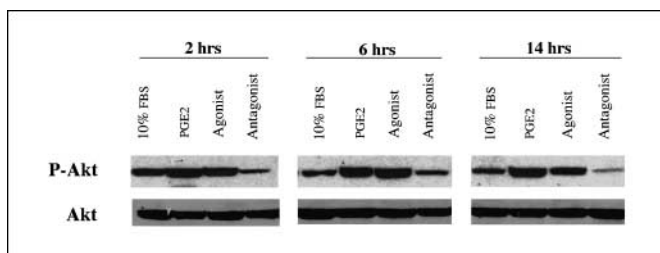


Figure 5. Western blot analysis of total and phosphorylated Akt from 3LL cells treated with PGE₂ and EP4-selective agonist and antagonist. 3LL cells were treated with the drugs each at 1 μ M/L concentration, harvested at indicated time points, and lysed with RIPA buffer. Proteins were then subjected to Western blot analysis with primary antibodies for phosphorylated Akt (P-Akt) and Akt.

knocking down EP4 receptor expression significantly reduces the metastatic potential of these cells.

The contribution of host EP4 receptor to tumor metastasis.

We next assayed the contribution of host EP4 receptor function on tumor metastasis and growth using EP4 knockout mice. 3LL cells were injected into the tail vein of the EP4 knockout mice, and their littermates and these mice were observed for \sim 3 weeks before sacrifice. Lung weight and tumor nodules in the lung were evaluated. We found that, although there was no significant difference in the number of tumor nodules between EP4 knockout and their wild-type controls ($P = 0.18$; Fig. 3A), there was a significant difference in the lung weights between the two groups ($P = 0.028$; Fig. 3B). Together with the results we obtained with the systemic EP4 antagonist and tumor cell small interfering RNA knockdown experiments, these data suggest that inhibition of tumor EP4 may affect the establishment of the metastasis and the host EP4 receptor, in contrast, may play an important role in promoting metastatic growth.

EP4 receptor signaling modulates tumor cell adhesion, migration, invasion, as well as colony formation. Tumor metastasis is a dynamic multistep process involving decreased adhesion to the primary host site, increased migration, increased invasive potential, as well as the establishment of the tumor colony at the distant metastasis site. Thus, we next examined the roles of EP4 receptor signaling in each of these steps using cell culture systems. We coated culture plates with fibronectin, one of the important components of the extracellular matrix, and tested the adhesion of 3LL cells to these plates after treatment with PGE₂ and an EP4-specific agonist and antagonist. We found that cells treated with the antagonist were significantly more adherent than agonist controls (Fig. 4A). These data were confirmed using the 3LL-EP4 RNAi cells, which were more adherent than the vector controls (Fig. 4A, middle). We also observed similar results when the culture plates were coated with laminin (Fig. 4A, bottom). We next examined the migration of these cells and observed morphologic changes (Fig. 4B, top) as well as significantly decreased migration of RNAi-transfected cells across Boyden chamber filters compared with vector controls or parental 3LL cells (Fig. 4B, bottom).

The ability of tumor cells to invade is also one of the hallmarks of the metastatic phenotype. We examined whether the EP4 receptor signaling affects the invasive phenotype of 3LL cells in a Matrigel assay. Matrigel was added to the Boyden chamber serving as the matrix barrier. 3LL-EP4 RNAi showed significantly reduced invasive potential compared with controls (Fig. 4C).

As we observed significantly decreased lung colonization of 3LL tumor cells when animals were treated with the specific EP4

receptor antagonist, we investigated whether this could be recapitulated using colony formation assays *in vitro*. We found that the number of colonies derived from 3LL stably transfected with EP4-RNAi was significantly decreased when compared with vector controls (Fig. 4D). It is also interesting to note that the colonies formed from 3LL-EP4 RNAi were much smaller in size (data not shown). This was unique to anchorage-independent growth, as proliferation assays using tritium thymidine (³H) did not show significant difference of proliferation rate between EP4 RNAi clones compared with their vector controls when grown on plastic (data not shown).

Increased Akt phosphorylation with enhanced EP4 receptor signaling. We next investigated the intracellular mechanisms mediated by EP4 receptor signaling. PI3K-Akt pathway is important in tumor cell survival and metastasis, so we examined whether EP4 receptor affects activation of Akt in our system. As shown in Fig. 5, 3LL cells treated with PGE₂ or EP4 receptor agonist showed increased phosphorylation of Akt, whereas when the cells were treated with EP4-specific antagonist there was a clear decrease of phosphorylated Akt after 2 hours of treatment (Fig. 5).

EP4 receptor antagonism significantly reduced colon cancer metastasis. As the EP4 receptor and other EP receptors have been previously implicated in colorectal malignancies (41, 42), we then investigated the effect of ONO-AE3-208 on colon cancer metastasis to liver. Liver metastases were induced by injecting 5×10^4 of MC26 cells (in 100 μ L PBS) into the spleen of syngeneic BALB/c mice followed by splenectomy 10 minutes after injection. The mice were pretreated with ONO-AE3-208 (10 mg/kg/d, orally in the drinking water) 3 days before MC26 cell injection and during the experiment until control animals showed signs of clinical distress related to tumor burden (\sim 4 weeks). The weight of the liver and tumor nodules in liver from all groups of animals were then evaluated. We found that there was a profound reduction of liver weight and in the number of tumor nodules in the liver in mice receiving ONO-AE3-208 treatment compared with control mice (Fig. 6A). Together, our data show that EP4 receptor signaling seems to play an important role in the metastasis of colorectal cancer cells to the liver.

We also tested the effect of ONO-AE3-208 on colon tumor growth. Colorectal cancer cells MC26 were injected into syngeneic BALB/c mice *s.c.* These mice were treated 3 days before the injection and continuously thereafter with the EP4 antagonist ONO-AE3-208. Tumor volume was determined by direct measurement of tumor dimensions using calipers and calculated as follows: volume = [length \times (width)²] \times 0.5. We observed significantly reduced tumor growth ($P < 0.05$) in mice treated with ONO-AE3-208 compared with the control group, including tumor volume and tumor growth rate (Fig. 6B-D). Although this inhibition only occurred in later stages of tumor growth after the tumor reached \sim 600 mm³ (Fig. 6B). Very interestingly, 30% of mice treated with ONO-AE3-208 did not develop any tumors (Fig. 6D compared with Fig. 6C), suggesting that EP4 receptors modulate the ability of tumors to establish themselves at the site of implantation, which agrees with the effect of the EP4 receptor antagonist on tumor metastasis.

Discussion

The EP4 receptor is one of many mediators of the effects of the products of COX-2 and one of four receptors mediating PGE₂ signaling. The EP4 receptor is overexpressed in several

cancers (30–32), and enhanced EP4 receptor signaling has been previously shown to correlate with the invasion of several different cancer types (26–29, 32, 33).

Specific blockade of the EP4 receptor signaling rather than blocking all of the COX-2 products with COX inhibitors may retain the antitumor properties of other COX-2 products, such as prostacyclin. The overexpression of prostacyclin in mice exhibited significantly reduced lung tumor incidence (22), and it is beneficial for cardiovascular function (43, 44). Downstream targeting of COX-2 pathway, such as selective EP4 antagonism, may allow more effective therapeutic targeting while potentially avoiding off-target

adverse or toxic effects associated with the COX inhibitors. Our data showed an important role of PGE₂-EP4 signaling in lung and colon cancer metastasis. We studied the effects of a systemically administered selective antagonist on this process in two completely different model systems. We found that metastasis to the lung was significantly reduced when mice were treated with a specific EP4 antagonist ONO-AE3-208 or when EP4 receptor expression was knocked down using RNAi technology. This effect of EP4 antagonism is equally profound in treatment of mice with liver metastasis. Because metastasis is a dominant cause of morbidity and mortality in many types of cancer, improved

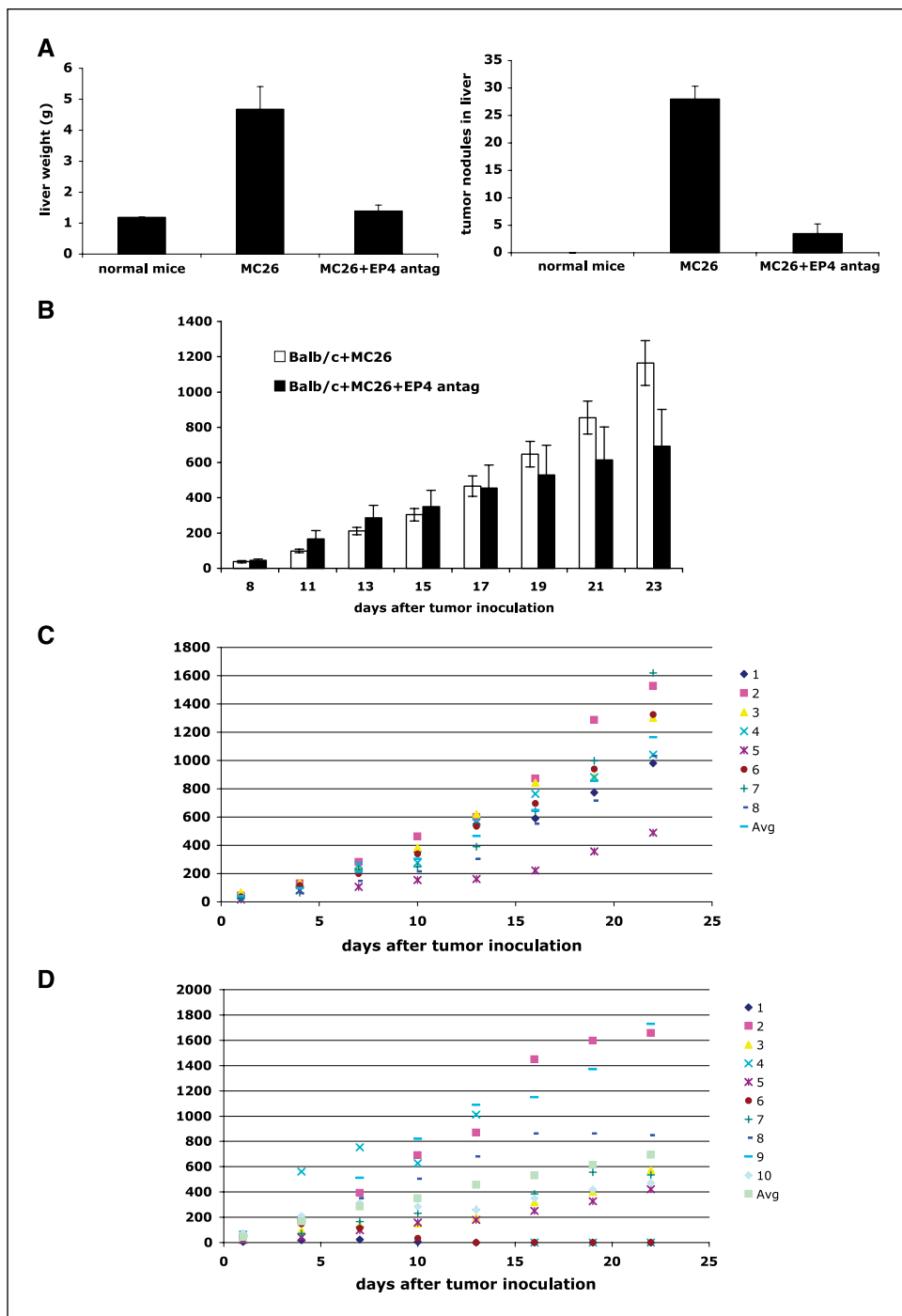


Figure 6. Effects of selective EP4 receptor antagonist on colorectal cancer growth and metastasis. **A**, effect of ONO-AE3-208 on liver metastasis of MC26 after intrasplenic injection. Liver metastasis was established by intrasplenic injection of MC26 followed by splenectomy. Liver weights (*left*) and tumor nodule numbers in liver (*right*) of mice with or without ONO-AE3-208 treatment. One of the two independent experiments with similar results. Number of mice tested: normal, $n = 3$; MC26, $n = 8$; MC26 + EP4 antagonist, $n = 9$. **B to D**, effect of ONO-AE3-208 on MC26 growth. **B**, growth curves of colorectal cancer MC26 with or without ONO-AE3-208 treatment. BALB/c mice were injected with MC26 cells s.c. and treated with EP4 antagonist ONO-AE3-208 3 days before the injection and for 23 days afterward. Y axis, tumor volume was determined every 2 to 3 days by direct measurement of tumor dimensions using calipers and calculated as follows: volume = [length \times (width)²] \times 0.5. **C to D**, tumor growth of each mouse, with control mice in (**C**) without treatment ($n = 8$) and EP4 receptor antagonist treated mice in (**D**) ($n = 10$). Three of the 10 treated mice showed no tumor or complete regression of the tumor.

understanding of its mechanisms and the development of specific targeted agents could be of dramatic clinical use.

Metastasis is a complex multistep process that is regulated by multiple mechanisms. We found that EP4 receptor antagonism significantly affected many of the known mechanisms related to metastatic potential, including tumor cell adhesion, motility, invasion, and colony formation. Our *in vitro* data showed a direct and specific effect of EP4 receptor antagonism on the 3LL tumor cells, including decreased migration and invasion, decreased potential to form colonies, and survival. We also observed that antagonism of EP4 significantly decreased the level of Akt phosphorylation, whereas a selective agonist increased phosphorylation of Akt (Fig. 5), consistent with other published reports (24, 25, 45, 46), suggesting that this agent may affect other Akt-mediated tumor properties, such as chemosensitivity. β -Arrestin 1, an important adaptor protein known to regulate the activities of G protein-coupled adrenergic receptor and adrenergic receptor-Src protein kinase (47), may act as an important mediator in this process (46). It associates with EP4 receptor and c-Src to form a signaling complex, resulting in the transactivation of the epidermal growth factor receptor (EGFR) and downstream Akt signaling (46). Other signaling pathways affected by the EP4 receptor include the mitogen-activated protein kinase signaling cascades (48) and the EGFR through a c-Src-dependent mechanism (8, 46). Other studies have shown that PGE₂ signaling and PGE₂-EP4 receptor signaling may affect tumor cell expression MMPs (26, 48). Consistent with our observations, a very recent publication showed that EP4 antagonism inhibits breast cancer metastasis (49). However, our study significantly extends these data with several important respects. We not only confirm the effect in two established models of lung and colon cancers but also establish the specificity of the observation using RNAi against EP4 excluding nonspecific drug effects. We show effects on Akt phosphorylation, invasion, adhesion, and soft agar colony formation as well as migration. Perhaps most importantly, we establish that knockout of EP4 in the host alone resulted in significantly reduced metastatic growth.

It should be noted that, when compared with the profound reduction of tumor metastasis, the effect of the EP4 receptor antagonist on tumor growth in both 3LL (not significant; data not shown) and MC26 (Fig. 6B-D) is modest. Proliferation assays using

tritium thymidine (³H) did not show a significant difference in the proliferation rate between EP4 RNAi clones compared with vector controls (data not shown). Our data indicate that the effect of the EP4 antagonism is via the dynamic processes that are specifically important for the establishment of metastasis (tumor cell motility, invasion, colony formation, and survival) but not directly on tumor growth (such as cell proliferation). EP4 antagonism is also well tolerated, as mice receiving the drug treatment appeared healthy, with normal activity and appearance and without weight loss.

Overexpression of the EP4 receptor has been reported in several cancers, including glioblastoma, gallbladder, and ovarian carcinoma (32, 33). One recent report showed that EP4 expression is regulated by the activation of peroxisome proliferator-activated receptor β/δ and PI3K signals with increased CCAAT/enhancer-binding protein β binding to the promoter region of the EP4 gene (50). There are as yet no convincing data on the correlation of EP4 expression in tumors with tumor behavior.

The challenges of clinical development of an agent that is primarily anti-invasive or antimetastatic are great, however, in that clinical metastases may be established before the cancer has been detected. However, it is possible that these agents could be useful chemopreventatives in high-risk patients or that treatment in the immediate perioperative period could reduce the ultimate incidence of metastasis. Additional work is needed to assess the effectiveness of EP4 inhibition in these clinical situations. Nevertheless, we show here that EP4 receptor antagonism is very effective in inhibiting lung and colon cancer metastasis, and its ability to inhibit the phosphorylation of Akt suggests additional potential use to increase sensitivity to chemotherapy or radiation-induced apoptosis.

Acknowledgments

Received 4/6/2006; revised 7/14/2006; accepted 8/4/2006.

Grant support: National Cancer Institute grants NCIP50CA 90949 (D.P. Carbone) and GM 15431, P30 DK58404, and Spanish Ministry of Health "Instituto de Salud Carlos III" (R. Porta).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Yu Shyr for statistical analysis in the study and the Mouse Pathology and Immunohistochemistry Core Labs at Vanderbilt for technical assistance.

References

- Eberhart CE, Coffey RJ, Radhika A, et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994;107:1183-8.
- Kargman SL, O'Neill GP, Vickers PJ, et al. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res* 1995;55:2556-9.
- Hida T, Yatabe Y, Achiwa H, et al. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* 1998;58:3761-4.
- Sonoshita M, Takaku K, Sasaki N, et al. Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc^{Δ716} knockout mice. *Nat Med* 2001;7:1048-51.
- Tsujii M, Kawano S, Tsuji S, et al. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998;93:705-16.
- Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci U S A* 1997;94:3336-40.
- Sheng H, Shao J, Washington MK, et al. Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. *J Biol Chem* 2001;276:18075-81.
- Buchanan FG, Wang D, Bargiacchi F, et al. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem* 2003;278:35451-7.
- Sheng H, Shao J, Morrow JD, et al. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res* 1998;58:362-6.
- Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* 1995;83:493-501.
- DuBois RN, Shao J, Tsujii M, et al. G₁ delay in cells overexpressing prostaglandin endoperoxide synthase-2. *Cancer Res* 1996;56:733-7.
- Huang M, Stolina M, Sharma S, et al. Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. *Cancer Res* 1998;58:1208-16.
- Yang L, Yamagata N, Yadav R, et al. Cancer-associated immunodeficiency and dendritic cell abnormalities mediated by the prostaglandin EP2 receptor. *J Clin Invest* 2003;111:727-35.
- Koehne CH, Dubois RN. COX-2 inhibition and colorectal cancer. *Semin Oncol* 2004;31:12-21.
- Sandler AB, Dubinett SM. COX-2 inhibition and lung cancer. *Semin Oncol* 2004;31:45-52.
- Brown JR, DuBois RN. Cyclooxygenase as a target in lung cancer. *Clin Cancer Res* 2004;10:4266-9s.
- Arun B, Goss P. The role of COX-2 inhibition in breast cancer treatment and prevention. *Semin Oncol* 2004;31:22-9.
- Lieberman R. Chemoprevention of prostate cancer: current status and future directions. *Cancer Metastasis Rev* 2002;21:297-309.
- Ray WA, Stein CM, Daugherty JR, et al. COX-2 selective non-steroidal anti-inflammatory drugs and risk of serious coronary heart disease. *Lancet* 2002;360:1071-3.
- Fitzgerald GA. Coxibs and cardiovascular disease. *N Engl J Med* 2004;351:1709-11.
- Csiki I, Morrow JD, Sandler A, et al. Targeting

- cyclooxygenase-2 in recurrent non-small cell lung cancer: a phase II trial of celecoxib and docetaxel. *Clin Cancer Res* 2005;11:6634-40.
22. Keith RL, Miller YE, Hoshikawa Y, et al. Manipulation of pulmonary prostacyclin synthase expression prevents murine lung cancer. *Cancer Res* 2002;62:734-40.
 23. Keith RL, Miller YE, Hudish TM, et al. Pulmonary prostacyclin synthase overexpression chemoprevents tobacco smoke lung carcinogenesis in mice. *Cancer Res* 2004;64:5897-904.
 24. Fujino H, Xu W, Regan JW. Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. *J Biol Chem* 2003;278:12151-6.
 25. Regan JW. EP2 and EP4 prostanoid receptor signaling. *Life Sci* 2003;74:143-53.
 26. Dohadwala M, Batra RK, Luo J, et al. Autocrine/paracrine prostaglandin E2 production by non-small cell lung cancer cells regulates matrix metalloproteinase-2 and CD44 in cyclooxygenase-2-dependent invasion. *J Biol Chem* 2002;277:50828-33.
 27. Ohshiba T, Miyaura C, Ito A. Role of prostaglandin E produced by osteoblasts in osteolysis due to bone metastasis. *Biochem Biophys Res Commun* 2003;300:957-64.
 28. Timoshenko AV, Xu G, Chakrabarti S, et al. Role of prostaglandin E2 receptors in migration of murine and human breast cancer cells. *Exp Cell Res* 2003;289:265-74.
 29. Timoshenko AV, Lala PK, Chakraborty C. PGE2-mediated upregulation of iNOS in murine breast cancer cells through the activation of EP4 receptors. *Int J Cancer* 2004;108:384-9.
 30. Jabbour HN, Milne SA, Williams AR, et al. Expression of COX-2 and PGE synthase and synthesis of PGE(2) in endometrial adenocarcinoma: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. *Br J Cancer* 2001;85:1023-31.
 31. Raza SM, Fuller GN, Rhee CH, et al. Identification of necrosis-associated genes in glioblastoma by cDNA microarray analysis. *Clin Cancer Res* 2004;10:212-21.
 32. Spinella F, Rosano L, Di Castro V, et al. Endothelin-1-induced prostaglandin E2-2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion. *J Biol Chem* 2004;279:46700-5.
 33. Asano T, Shoda J, Ueda T, et al. Expressions of cyclooxygenase-2 and prostaglandin E-receptors in carcinoma of the gallbladder: crucial role of arachidonate metabolism in tumor growth and progression. *Clin Cancer Res* 2002;8:1157-67.
 34. Hull MA, Ko SC, Hawcroft G. Prostaglandin EP receptors: targets for treatment and prevention of colorectal cancer? *Mol Cancer Ther* 2004;3:1031-9.
 35. Kitamura T, Itoh M, Noda T, et al. Combined effects of prostaglandin E receptor subtype EP1 and subtype EP4 antagonists on intestinal tumorigenesis in adenomatous polyposis coli gene knockout mice. *Cancer Sci* 2003;94:618-21.
 36. Sales KJ, Katz AA, Davis M, et al. Cyclooxygenase-2 expression and prostaglandin E(2) synthesis are up-regulated in carcinomas of the cervix: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. *J Clin Endocrinol Metab* 2001;86:2243-9.
 37. Kabashima K, Saji T, Murata T, et al. The prostaglandin receptor EP4 suppresses colitis, mucosal damage, and CD4 cell activation in the gut. *J Clin Invest* 2002;109:883-93.
 38. Kabashima K, Sakata D, Nagamachi M, et al. Prostaglandin E2-4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells. *Nat Med* 2003;9:744-9.
 39. Segi E, Sugimoto Y, Yamasaki A, et al. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. *Biochem Biophys Res Commun* 1998;246:7-12.
 40. Kobayashi T, Narumiya S. Function of prostanoid receptors: studies on knockout mice. *Prostaglandins Other Lipid Mediat* 2002;68-9:557-73.
 41. Mutoh M, Watanabe K, Kitamura T, et al. Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis. *Cancer Res* 2002;62:28-32.
 42. Pozzi A, Yan X, Macias-Perez I, et al. Colon carcinoma cell growth is associated with prostaglandin E2/EP4 receptor-evoked ERK activation. *J Biol Chem* 2004;279:29797-804.
 43. Cheng Y, Austin SC, Rocca B, et al. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science* 2002;296:539-41.
 44. Egan KM, Lawson JA, Fries S, et al. COX-2-derived prostacyclin confers atheroprotection on female mice. *Science* 2004;306:1954-7.
 45. Fujino H, Salvi S, Regan JW. Differential regulation of phosphorylation of the cAMP response element-binding protein after activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *Mol Pharmacol* 2005;68:251-9.
 46. Buchanan FG, Gorden DL, Matta P, et al. Role of β -arrestin 1 in the metastatic progression of colorectal cancer. *Proc Natl Acad Sci U S A* 2006;103:1492-7.
 47. Luttrell LM, Ferguson SS, Daaka Y, et al. β -Arrestin-dependent formation of β 2 adrenergic receptor-Src protein kinase complexes. *Science* 1999;283:655-61.
 48. Pai R, Soreghan B, Szabo IL, et al. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* 2002;8:289-93.
 49. Ma X, Kundu N, Rifat S, et al. Prostaglandin E receptor EP4 antagonism inhibits breast cancer metastasis. *Cancer Res* 2006;66:2923-7.
 50. Han S, Ritzenthaler JD, Wingerd B, et al. Activation of peroxisome proliferator-activated receptor β/δ (PPAR β/δ) increases the expression of prostaglandin E2 receptor subtype EP4. The roles of phosphatidylinositol 3-kinase and CCAAT/enhancer-binding protein β . *J Biol Chem* 2005;280:33240-9.