Abstract. Background: Manipulating prostaglandin (PG) production modulates tumor development. Elevated PGI2 production prevents murine lung cancer, while decreasing PGE2 content protects against colon cancer. PGE2 receptor subtype 2 (EP2)-deficient mice were hypothesized to be resistant to lung tumorigenesis. Materials and Methods: EP2 null BALB/c mice and their wild-type littermates were exposed to an initiation-promotion carcinogenesis protocol and lung tumorigenesis was examined. Chronic lung inflammation was induced to determine whether EP2 ablation influenced inflammatory cell infiltration. Results: Tumor multiplicity in EP2 null mice was 34% lower than in their wild-type littermates (21.9±3.0 vs. 14.5±2.9 tumors/mouse, p<0.001). The lung tumor burden, an indicator of growth rate, also declined (57%, p<0.05). All the mice exhibited similar inflammatory cell infiltration. Conclusion: PGE2, acting through EP2, enhanced lung tumorigenesis through a mechanism that may be distinct from its pro-inflammatory activity. Thus, EP2 is a potential target for novel chemoprevention strategies.

Lung cancer is the leading cause of cancer death in the United States, and the majority of lung cancers are diagnosed in former smokers (1). The large number of current and former smokers, coupled with poor 5-year survival rates, emphasizes the need for effective chemopreventive agents. Prostaglandins are lipid mediators derived from arachidonic acid by the action of cyclooxygenase (COX) enzymes and are important in cancer biology. The need for better lung cancer chemopreventive strategies beyond smoking cessation, coupled with the impressive reduction in colon cancer observed with cyclooxygenase (COX) inhibition, has stimulated considerable interest in manipulating the pulmonary prostaglandin content to modify lung cancer risk.

Prostaglandin E2 (PGE2) mediates several hallmarks of cancer. During early neoplastic transformation, PGE2 activates the epidermal growth factor receptor (EGFR) to enhance cell proliferation (2) and up-regulates survivin concentrations in tumors to decrease apoptosis (3). At later stages of progression, PGE2 up-regulates the biosynthesis of vascular endothelial growth factor (VEGF) expression, which stimulates angiogenesis (4), increases IL-10 biosynthesis to inhibit adaptive antitumor immune responses (5, 6) and augments protease expression, thereby promoting invasion (7). Many cancers exhibit increased expression of the cyclooxygenase enzymes, COX1 and COX2, that provide the PGH2 substrate for PGE2 synthase (PGES) (8). Non-specific COX inhibitors, including conventional non-steroidal anti-inflammatory drugs (NSAIDs) and COX2-specific inhibitors, have been applied to pre-clinical models of various cancers and are used therapeutically against a hereditary form of colon cancer (9, 10). Clinical lung cancer trials in which celecoxib is applied along with conventional platinum and taxane cytotoxic agents are on-going (11-14). However, recent studies using a mouse model of adenocarcinoma (AC), the most common form of human lung cancer, suggest that downstream targeting of the PGE2 pathways may be more effective. Celecoxib administration did not reduce the multiplicity of chemically-induced mouse lung tumors and actually enhanced their growth rate (15). This may be, at least in part, because PGH2 is also the substrate for prostaglandin...
I2 synthase (PGI2 synthase, PGIS). Mice that overexpress PGIS are protected from lung tumor induction by both chemical carcinogens and cigarette smoke (16, 17). Cell lines derived from mouse lung tumors produce more PGE2 and less PGIS than non-tumorigenic cell lines (18). The PGIS content in human AC is usually down-regulated while the PGES content rises but, importantly, a small subset of AC patients whose lung tumors retained PGIS expression exhibited significantly improved survival (19). These results suggest that specifically interfering with PGE2 function downstream from COX, while maintaining PGI2 production, would be an effective chemoprevention strategy for human lung cancer.

PGF2a exhibits potent immunosuppressive effects (5, 6) through distinct G protein coupled E-prostanoid receptors, EP1-4. EP receptors can increase cellular cAMP (EP2 and EP4), decrease intracellular cAMP (EP3), or mobilize intracellular calcium (EP1) (20). EP receptors are differentially expressed based on the cell type and regulate B and T cell development (21, 22), macrophage cytokine release (23) and modify the post-surgical immune response (24). Genetically modified mice that lack specific EP receptors have led to an improved understanding of their specific roles (reviewed in (25)). In a murine colon cancer model, EP1 and EP4 receptor knockout mice developed fewer and smaller tumors, while EP2 and EP3 ablation did not affect the tumor number or size (26, 27). EP3 is also believed to play a role in lung carcinogenesis by reinforcing the activation of the ras signaling pathway (28) and Src signaling in cultured A549 (lung adenocarcinoma) cells (29). However, the effects of genetic ablation of individual receptors are organ-specific. For example, EP2 null mice develop fewer skin tumors in response to chemical carcinogens than do their wild-type littermates (30). In pancreatic cancer cell lines, PGE2 induced VEGF production via EP2 signaling (4). Down-regulation of EP2 in immortalized human keratinocytes reduced paxillin expression, thereby decreasing progression to an invasive phenotype (31). In the lung, EP2 is important in bronchodilation (32) and fibroproliferative lung disease (33, 34). While the role of PGE2 receptors in human lung cancer is unknown, growth inhibition of human non-small lung cancer (NSCLC) cells by peroxisome proliferator-activated receptor gamma (PPAR-γ) ligands is mediated through inhibiting EP2 expression (35). We therefore hypothesized that mice null for EP2 would exhibit impaired lung tumor development when subjected to a chemical carcinogenesis model. A defective EP2 receptor mutation was bred into BALB/c mice (36), an inbred strain highly susceptible to two-stage carcinogenesis initiated by the tobacco carcinogen, 3-methylcholanthrene (MCA) and promoted by the food additive, butylated hydroxytoluene (BHT) (37). Phenotypically, these EP2 null mice exhibit salt-sensitive hypertension and decreased fertility (38).

We report here, fewer lung tumors in EP2 null mice compared to their wild-type littermates, as well as a significant reduction in the rate of tumor growth. The EP2 mutation did not affect leukocyte infiltration in response to chronic BHT administration, suggesting that PGE2-mediated inflammation is not integral to the protumorigenic mechanism mediated by EP2.

**Materials and Methods**

**Tumorigenesis experiments.** EP2 null mice and their wild-type littermates were bred in the animal care facility at Vanderbilt University, USA, and genotyped by Southern analysis, as previously published (38). The mice were transferred to the Denver VAMC animal care facility at 8 weeks of age for the tumorigenesis experiments. The mice were maintained in a 12-h light/dark cycle on hardwood bedding, and fed standard chow and water ad libitum. The mice used in the inflammation studies were bred at the Denver VAMC animal facility and genotyped and maintained as described above.

Seventeen wild-type and 16 EP2-null mice (10-12 weeks old) were injected intraperitoneally (i.p.) with 15 mg 3-methylcholanthrene (MCA)/kg body weight dissolved in Mazola® corn oil. In nine wild-type and eight EP2 null mice, this was followed by six weekly i.p. injections of butylated hydroxytoluene (BHT, 150 mg BHT/kg body weight administered week 1; 200 mg/kg thereafter) dissolved in corn oil. The remaining wild-type and EP2 null mice received six weekly corn oil injections. Twenty weeks after MCA exposure, the mice were sacrificed by lethal pentobarbital injection and their lungs were excised. Lung tumors were counted under a dissecting microscope (5X magnification) and their diameters measured using digital calipers. The volume of each individual tumor was determined using the formula "Volume = 4/3πr^3." The tumor burden per animal was calculated as the sum of these volumes. To confirm the pulmonary adenoma pathology, tumors and surrounding uninvolved lung were paraffin-embedded and sectioned, followed by H&E staining. All slides were reviewed with a pathologist to confirm findings.

**Chronic inflammation studies.** Homozygous EP2 (−/−, n=6), heterozygous (+/−, n=15), and wild-type (+/+ , n=13) male and female littermates (8-10 weeks of age) were exposed to four weekly i.p. BHT injections (150 mg BHT/kg body weight administered week 1; 200 mg/kg thereafter) to induce a pulmonary inflammatory response, as described previously in BALB mice (39). The control mice received four weekly vehicle injections. Six days after the last treatment, the mice were euthanized and bronchoalveolar lavage (BAL) performed. The trachea was cannulated with an 18g angiocatheter and three serial 1 ml aliquots of 10 mM K2HPO4 (pH 7.4), 150 mM NaCl, 0.6 mM EDTA instilled and aspirated back into the syringe. Cells from all three aliquots were pooled, quantified, cytospun onto glass slides and sent for routine cell counts with differentials (performed by the Clinical Laboratory at the University of Colorado Hospital, USA).

**Statistical analysis.** All values are expressed as means±SEM. For tumor multiplicity and cell counts, the data were normally distributed and statistical analyses were performed using GraphPad Prism 4.0 for Windows (GraphPad Software for Science Inc., San Diego, CA, USA). Data was considered significant at the p<0.05 level. Groups were compared by ANOVA followed by Student Newman Keuls post-hoc analysis.
Results

Tumor growth. Tumor multiplicities and burdens from wild-type and EP2 null mice induced with MCA or MCA + BHT were determined (Figure 1A, 1B). EP2 null mice developed 34% fewer tumors in response to the two-stage MCA + BHT protocol (21.9±3.0 vs. 14.5±2.9 tumors/mouse, p<0.001). Both groups developed a small number of tumors in response to MCA alone (1.1±0.4 (EP2 null) vs. 1.8±0.4 (wild-type) tumors/mouse, p=NS). No significant differences in tumor incidence between MCA-treated EP2 null and wild-type mice (6/8 vs. 7/8) were observed and all mice exposed to MCA + BHT developed tumors (100% incidence).

The tumors in EP2 null mice were significantly smaller than in their wild-type littermates (0.69±0.03 vs. 0.81±0.03 mm tumor diameter, p<0.05). EP2 null animals exhibited a nearly three-fold reduction in tumor burden compared to their wild-type littermates (5.1±2.1 vs. 14.3±4.2 mm³/mouse, p<0.05) (Figure 1B). The tumor burden reflects growth rate.

Chronic inflammation. To determine if loss of functional EP2 altered the pulmonary inflammatory response and, thereby, accounted for the diminished tumor multiplicity and growth, homozygous and heterozygous EP2 knockouts and wild-type littermates were exposed to four weekly injections of BHT. This insult typically results in a substantial influx of macrophages (39). Differential cell counts were performed on BAL fluid to determine the number and types of inflammatory cells present. Macrophage cell counts were similar in EP2 null, EP2 heterozygote and wild-type mice (Figure 2). No differences in lymphocyte, neutrophil, or eosinophil counts were observed (data not shown).

Discussion

Preclinical modeling of lung cancer is critical for evaluating novel prevention strategies and to better predict outcomes in human trials. Orthologous gene expression studies of human and chemically-induced murine adenocarcinomas revealed significant similarities (19), validating the use of murine models. Based on the many pro-tumorigenic properties of PGE2, we hypothesized that EP2 null mice would be less susceptible to chemical lung carcinogenesis. When EP2 null mice and their wild-type littermates were subjected to a widely used initiation-promotion lung tumor model, the genetically modified mice exhibited significant reductions in tumor multiplicity and burden (Figures 1A and 1B).

In this lung carcinogenesis model, chronic pulmonary inflammation mediates tumor formation (40). Inbred strains such as BALB/c that are sensitive to BHT-tumor promotion, are also characterized by a macrophage predominant influx following BHT treatment (39). When pulmonary macrophage infiltration during MCA/BHT carcinogenesis in BALB mice
was reduced, tumor multiplicity decreased (40). PGE2 is a potent mediator of inflammation, and EP3 receptor null mice exhibit attenuated inflammation when exposed to topical arachidonic acid (41). We hypothesized that one explanation for differences in tumor multiplicity may have been altered responses to a lung inflammatory insult. EP2 null, heterozygous (to assess for the presence of a critical level of EP3 receptor expression), and their wild-type littermates did not differ in BAL macrophage cell counts (Figure 2), however. Therefore, the effects of EP2 ablation on tumorigenesis may be explained by alternative mechanisms. EP2 may suppress the immune response to lung cancer by altering the activity of T regulatory cells (Treg). Tumor-derived PGE2 induces expression of Foxp3, a Treg-specific transcription factor, and Foxp3 expression is ablated in the absence of EP2 (42). A decreased production of these immune suppressor cells upon ablating EP2 may explain observed differences in tumor number and growth rate. The EP2-independent activities of PGE2 include PGE2 stimulating colon cancer growth by inactivating and releasing glycogen synthase 3β from its complex with axin, which leads to the activation of β-catenin (43).

Efforts are currently underway to systematically examine lung tumor formation in BALB/c mice null for EP1, EP3 and EP4, since specific antagonists and agonists that act at each of these receptors are available. Modulating PGE2 pro-tumorigenic activities by inhibiting individual EP receptors may complement other lung chemopreventive strategies, especially those that up-regulate the PGI2 pathway. Since EP2 is downstream from COX, the pleiotropic effects of COX inhibition can thereby be avoided. EP3 is thus an attractive target, singly or in combination with other preventive strategies, for future pre-clinical and human trials.

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References


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