Cyclooxygenase-2 Inhibitor Induces Apoptosis in Breast Cancer Cells in an In vivo Model of Spontaneous Metastatic Breast Cancer

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Abstract
Cyclooxygenase-2 (COX-2) inhibitors are rapidly emerging as a new generation of therapeutic drug in combination with chemotherapy or radiation therapy for the treatment of cancer. The mechanisms underlying its antitumor effects are not fully understood and more thorough preclinical trials are needed to determine if COX-2 inhibition represents a useful approach for prevention and/or treatment of breast cancer. The purpose of this study was to evaluate the growth inhibitory mechanism of a highly selective COX-2 inhibitor, celecoxib, in an in vivo oncogenic mouse model of spontaneous breast cancer that resembles human disease. The oncogenic mice carry the polyoma middle T antigen driven by the mouse mammary tumor virus promoter and develop primary adenocarcinomas of the breast. Results show that oral administration of celecoxib caused significant reduction in mammary tumor burden associated with increased tumor cell apoptosis and decreased proliferation in vivo. In vivo apoptosis correlated with significant decrease in activation of protein kinase B/Akt, a cell survival signaling kinase, with increased expression of the proapoptotic protein Bax and decreased expression of the antiapoptotic protein Bcl-2. In addition, celecoxib treatment reduced levels of proangiogenic factor (vascular endothelial growth factor), suggesting a role of celecoxib in suppression of angiogenesis in this model. Results from these preclinical studies will form the basis for assessing the feasibility of celecoxib therapy alone or in combination with conventional therapies for treatment and/or prevention of breast cancer.

Introduction
In the United States, breast cancer is the second most common cancer and contributes to 40,000 deaths in a year. If confined within the breast, the tumor can be surgically removed with an increased survival rate. However, primary tumors that metastasize to distant sites such as lymph nodes, lungs, liver, and brain correlate with poor prognosis. Complications from metastatic disease are the leading cause of cancer-related deaths. Mean survival for patients with metastatic breast cancer is 18 to 24 months. Response to chemotherapy or endocrine therapy in metastatic breast cancer patients is ~50% (1). Clearly, a need for development of novel therapies to enhance the existing triad of surgery, radiation, and chemotherapy is evident. Cyclooxygenase-2 (COX-2), the inducible form of the COX enzymes, catalyzes conversion of arachidonic acid to prostaglandin H2, which is further converted to several other prostaglandins with diversified functions. Deregulation of COX-2 activity and downstream prostaglandins plays a vital role in carcinogenesis, inflammation, and tissue damage (2-5). COX-2 is overexpressed in many cancers including breast cancer, and the major functional prostaglandin in breast cancer is prostaglandin E2 (PGE2). Overexpression of COX-2 protein and PGE2 during carcinogenesis is implicated in proliferation, invasion, apoptosis, immune suppression, and angiogenesis. COX-2 is induced by a variety of factors including tumor promoters, cytokines, growth factors, and hypoxia. Importantly, selective inhibition of this enzyme reduces adenocarcinoma formation and cancer progression in preclinical animal models (6-8). The first direct evidence of COX-2 function in cancers came from the study by Eberhart et al. (9), documenting significant elevations in COX-2 expression in 85% of human colorectal carcinomas and 50% of colorectal adenomas. COX-2 overexpression has since been found in many other human cancers including breast (10, 11), esophageal (12, 13), lung (14, 15), prostate (16, 17), bladder (18, 19), skin (20, 21), and pancreas (22, 23).

Studies with specific inhibitors of COX-2 enzyme have shown significant effects in reducing the incidence and progression of tumors in both animal models and in treatment of cancer patients (6-8). Studies to evaluate effects of COX-2-specific inhibitors in the treatment of breast cancer have started recently; therefore, data are limited. In animal studies, COX-2 inhibitors have shown promising results. In rat models of chemical carcinogenesis, COX-2 inhibitors significantly reduced incidence and size of mammary tumors (31, 32). COX-2 inhibitors were also effective in retarding tumor progression and metastasis in mouse models of injected breast cancer cell lines and in xenograft models of human breast cancer cells in nude mice (24, 33, 34). Clinically, COX-2 inhibitors have been...
used in combination with other anticancer drugs or radiation therapy to treat solid tumors, mostly focusing on colon and colorectal cancers. Reports emerging from these studies strongly suggest that COX-2 inhibitors may emerge as a new generation of therapeutic drugs for cancer therapy. A recent report indicated that regular nonsteroidal anti-inflammatory drug use for 5 to 9 years was associated with a 21% reduction in the incidence of breast cancer and regular use for >10 years was associated with 28% reduction (35). This area of research is underexplored and more thorough preclinical trials are needed to further determine if COX-2 inhibition represents a useful approach to treatment of breast cancer.

Preclinical studies must precede clinical trials, and use of appropriate mouse models is key to the development of efficient therapeutic strategies. We have used in this study the oncogenic mice that carry the polyoma virus middle T antigen (MTag) driven by the mouse mammary tumor virus (MMTV) long terminal repeat promoter. These mice develop spontaneous tumors of the breast, which metastasize to the lungs and bone marrow. All mice are congenic on the C57BL/6 background to eliminate strain-specific modifier effects. In the MTag mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus MTag driven by the MMTV promoter (36). MTag specifically associates with and activates the tyrosine kinase activity of several c-src family members, eliciting tumors when a threshold level of gene product has been attained. In these mice, the MMTV promoter is transcriptionally active throughout all stages of mammary gland development, which results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Focal atypical lesions can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from ~60 days onward. Tumor progression is quite rapid, reaching 10% of body weight by ~20 to 24 weeks. All of the female mice get tumors. Tumors arise with synchronous kinetics and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast (37, 38). These mice exhibit metastasis in the lungs (60%) and micrometastasis in the bone marrow by 4 months of age (39). Therefore, the MTag mouse model is an appropriate model for human metastatic breast cancer in which to evaluate therapeutic strategies and to understand the mechanisms associated with therapy-induced growth inhibition. This is the first study to evaluate the efficacy and growth inhibitory mechanisms of celecoxib in an in vivo model of spontaneous metastatic breast cancer.

Results

Celecoxib Treatment Caused Significant Reduction in Primary Mammary Tumor Burden

Ten-week-old tumor-bearing female MTag mice were gavaged daily for 4 weeks with celecoxib at 5, 10, or 20 mg/kg body weight. In mice, at 10 and 20 mg/kg dose, the concentration of celecoxib in the plasma ranges from 6.5 to 13 μmol/L at 2 hours and from 4.2 to 8 μmol/L at 4 hours post-celecoxib treatment (40). This dose is attainable clinically and sufficient to inhibit PGE₂ (41). At 10 weeks, mice have small palpable tumors (1-2 tumors, ~0.1-0.5 mg tumor weight). One hundred percent of the MTag mice have hyperplastic mammary glands by 6 to 8 weeks (starting at time of puberty). Because the MTag is a strong oncogene, driven by the MMTV promoter, 100% of the MTag mice develop multifocal tumors with palpable tumors in at least 1 to 2 glands (of 10 mammary glands in mice) by 10 weeks. Every gland is hyperplastic by this time and every gland has palpable tumors by 14 weeks. Complete blood count analysis including hemoglobin levels was done to determine cytopenia and/or anemia post-celecoxib treatment. Regardless of the celecoxib dose, there was no detectable change in their complete blood count or hemoglobin levels (data not shown) as compared with untreated MTag mice. Flow cytometric analysis of T cells, B cells, and natural killer cells revealed no change in treated versus control MTag mice, nor were there any signs of weight loss in treated mice (data not shown). This suggested that celecoxib was well tolerated in these mice with no detectable signs of toxicity. Mice were sacrificed at 14 weeks of age, tumors were removed, and serum was collected. Tumor burden in MTag mice treated with 10 and 20 mg/kg dose was significantly reduced (P < 0.003 for 10 mg/kg and P < 0.01 for 20 mg/kg; Fig. 1). Note that in this study we started the treatment at 10 weeks when the mice had established tumors. The purpose of this study was to focus on the short-term effect of celecoxib on breast cancer cells in vivo at early times during tumor development and evaluate the mechanism of action of the drug on primary breast cancer cells. All mice were terminated at 14 weeks of age. The cumulative palpable tumors from 10 mammary glands at 14 weeks of age for individual mouse are presented in Fig. 1. Because metastasis in the MTag mice only develop between 19 and 24 weeks of age, we evaluated 6 MTag mice that received 20 mg/kg celecoxib and 10 vehicle-treated MTag mice between 20 and 24

![FIGURE 1. Reduced tumor burden in 14-week-old MTag mice post-celecoxib treatment. MTag mice were palpated weekly for presence of mammary tumors. Tumor weights plotted represent total tumor burden (including all mammary glands) per mouse at 14 weeks of age (n = 9 mice for vehicle and 10 and 20 mg/kg celecoxib and n = 6 mice for 5 mg/kg celecoxib).](image)
weeks of age. Gross microscopic examination of lungs revealed that the celecoxib-treated mice did not develop metastasis (0 of 6), whereas 6 of the 10 control mice developed lung metastasis (data not shown). These results are preliminary and we need to enroll more mice to the study to achieve statistical significance.

**Celecoxib Induces Apoptosis in Breast Cancer Cells**

**In vivo**

We have reported recently that celecoxib induces growth inhibition of human and mouse breast cancer cells *in vitro* by simultaneously activating tumor cell apoptosis and inhibiting proliferation (42). Apoptosis of primary MTag tumor cells was determined by Annexin V/propidium iodide staining and flow cytometry. Data revealed significant increase in apoptotic cell population at 10 and 20 mg/kg celecoxib dose as compared with control MTag mice (39% in control mice versus 65% in 10 mg/kg dose, *P* < 0.05; 59% in 20 mg/kg dose, *P* < 0.05). The lowest dose (5 mg/kg) did not have a significant effect (Fig. 2A). Tumor cells from untreated MTag mice gave similar percentage of apoptotic cells (~35-40%) as vehicle-treated mice (data not shown). The high baseline apoptosis level in vehicle-treated and untreated mice is likely due to the method of isolating single cells. However, the 1.5- to 1.7-fold increase following celecoxib treatment was reproducibly observed.

We also evaluated celecoxib-induced apoptosis *in situ* by detection of DNA fragmentation using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (43). We observed an increase in TUNEL-positive cells in celecoxib-treated tumor sections *in situ* as compared with control tumor sections, confirming the flow cytometry data (Fig. 2B). Representative immunohistochemical images of vehicle-treated and celecoxib-treated MTag tumor sections are shown at 100× magnification, demonstrating considerable TUNEL positivity in celecoxib-treated versus control MTag tumor sections.

**Increased Bax and Decreased Bcl-2 in Tumor Lysate Derived from Celecoxib-Treated MTag Mice**

The downstream signaling pathways involved in COX-2-induced apoptosis are not well understood, but at least three pathways have been suggested: Bcl-2-mediated pathway, nitric oxide pathway, and production of ceramide (44). Because it has been shown previously in cell lines that celecoxib-induced apoptosis is associated with decreased Bcl-2 (an antiapoptotic protein) and increased Bax (a proapoptotic protein), we evaluated the levels of Bcl-2 and Bax by Western blot analysis of whole MTag tumor lysate post-celecoxib treatment. Treatment with celecoxib at 10 and 20 mg/kg induced increased expression of Bax (inducer of apoptosis) in all five mice tested as compared with vehicle-treated tumors (Fig. 3A). The increase was most pronounced at the 10 mg/kg dose of celecoxib. Simultaneously, there was decrease in Bcl-2 (inhibitor of apoptosis) protein expression in the 10 and 20 mg/kg dose of celecoxib (Fig. 3A). Untreated MTag tumor lysate was prepared from 21-week-old MTag tumors, whereas the treated mice were at 14 weeks of age. This could explain the difference in protein expression observed.
between vehicle-treated and untreated MTag tumors. Tumor lysate from 14-week-old MTag mice have similar Bcl-2 and Bax levels as vehicle-treated tumors (data not shown). Densitometric analysis of the Western blots indicates significant increase in Bax protein levels between vehicle-treated and 10 mg/kg ($P < 0.05$) and 20 mg/kg ($P < 0.06$) celecoxib-treated tumor lysates. Similarly, significant decrease in Bcl-2 was observed between vehicle-treated and 5 mg/kg celecoxib–treated mice versus 10 mg/kg ($P < 0.05$) and 20 mg/kg ($P < 0.05$) celecoxib-treated groups. All comparisons are between 14-week-old vehicle-treated tumors and age-matched celecoxib-treated tumors. Thus, data suggest that celecoxib-induced apoptosis in MTag tumor cells in vivo is associated with an elevated expression of Bax and reduced expression of Bcl-2 proteins. These results give further credence to the flow cytometry and TUNEL data, confirming that celecoxib induces apoptosis in vivo in a highly aggressive and metastatic breast cancer model.

**Reduced Phosphorylation of Akt in Tumor Lysate Derived from Celecoxib-Treated MTag Mice**

Protein kinase B/Akt is a serine/threonine protein kinase that is involved in promoting cell survival signals through the phosphatidylinositol 3-kinase (PI3K) pathway leading to inactivation of a series of proapoptotic proteins. These kinase activities are frequently deregulated in human disease including cancer (45). Akt represents a key signaling component in cell survival by activating downstream proapoptotic proteins and caspases (46-48). Celecoxib has been shown recently to induce apoptosis of cancer cells by blocking Akt activation in cultured prostate cancer cells (49, 50). To explore whether inhibition of Akt activation may be linked to the observed in vivo apoptosis in MTag tumors, we determined the effect of in vivo celecoxib administration on phosphorylation of Akt (at Ser$^{473}$ in the carboxyl terminus) in MTag tumors. Data show that celecoxib substantially suppresses phosphorylation of Akt in MTag tumors. Two of five mice in the 5 mg/kg dose showed reduced phosphorylation, whereas four of five in 10 mg/kg and five of five mice in 20 mg/kg dose showed reduced Akt activation (Fig. 4). Densitometric analysis clearly indicates significant down-regulation of Akt phosphorylation in celecoxib-treated tumors as compared with vehicle-treated tumors ($P < 0.05$ for 10 and 20 mg/kg celecoxib). All tumors showed approximately equivalent levels of the Akt protein as shown in Fig. 4 (bottom). This result clearly suggested the involvement of the Akt pathway in induction of apoptosis in vivo in our mouse model of spontaneous breast cancer. Akt represents a key signaling component in cell survival by activating downstream proapoptotic proteins and caspases (46-48). Because we observed a decrease in Akt phosphorylation and increase in proapoptotic protein (Bax), we determined if caspases were activated post-celecoxib treatment. MTag tumor cells treated with celecoxib in vitro (20, 40, and 60 μmol/L) were analyzed for activation of effector caspase-3 and caspase-7. Most apoptotic signals induce intracellular cleavage of caspase-3 and caspase-7 and convert them into active forms. Caspase activity is presented as fluorescence emission, which is directly proportional to caspase-3/7 activities. Increase in fluorescence emission was observed with increasing dose of celecoxib, which correlates with increase in active forms of caspase-3 and caspase-7. Table 1 illustrates the fluorescence emission for untreated versus

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**FIGURE 3.** Increase in the proapoptotic protein Bax and decrease in the antiapoptotic protein Bcl-2 post-celecoxib treatment. Western blot analysis of Bax and Bcl-2 protein levels in mammary gland tumor lysates from vehicle and celecoxib (5, 10, and 20 mg/kg)–treated MTag mice; 100 μg of protein were loaded per lane. *n* = 5 individual mice. Numbers below each lane, percentage of protein expression compared with MTag lysate, which was set to equivalent of 100% as determined by densitometric analysis. Average percentage expression for each treatment group (*n* = 5 mice). $P$, significant difference between treatment groups and vehicle control. β-Actin is used as the protein loading control for all tumor lysates.
celecoxib-treated MTag tumor cells. Significant increase in fluorescence emission was observed in 40 and 60 μmol/L celecoxib-treated cells compared with untreated or vehicle-treated cells (P < 0.05 and 0.01, respectively).

**Celecoxib Inhibits Tumor Cell Proliferation**

Antiproliferative effect of in vivo celecoxib treatment was determined by in situ immunohistochemical analysis of MTag tumor sections stained with proliferating cell nuclear antigen (PCNA). A representative light microscope image suggests inhibition of proliferation in MTag tumors in vivo with 10 and 20 mg/kg celecoxib treatment (Fig. 5). PCNA protein levels peak during the S phase of the cell cycle and is almost undetectable in other phases of the cycle. Vehicle and 5 mg/kg celecoxib treatments show almost every cell expressing PCNA, indicative of highly proliferative cells (Fig. 5A and B). With 10 and 20 mg/kg treatment, fewer cells expressed PCNA staining, suggestive of fewer cells undergoing proliferation (Fig. 5C and D). A lymph node within an untreated mammary tumor section shows only a few proliferating cells, confirming the specificity of the stain (Fig. 5E). Staining specificity is further confirmed with second antibody control (Fig. 5F).

**Celecoxib Treatment Significantly Reduced Serum Levels of PGE2 in vivo**

Next, we analyzed sera and tumor lysate from celecoxib-treated and vehicle-treated MTag mice for PGE2 levels to assess COX-2 activity in vivo. COX-2 converts arachidonic acid to bioactive prostaglandins. It has been shown that COX-2-derived PGE2 is the major prostaglandin produced by breast cancer cells and may be required for the angiogenic switch leading to initiation and progression of mammary cancer in a MMTV-COX-2 transgenic mouse model (51). Production of secreted PGE2 is an appropriate measure of COX-2 activity in the MTag mouse model. PGE2 is unstable in vivo and

![FIGURE 4. Decreased phosphorylation of protein kinase B/Akt post-celecoxib treatment. Western blot analysis of phospho-Akt (pAkt) and Akt levels in mammary gland tumor lysates from vehicle and celecoxib (5, 10, and 20 mg/kg)–treated MTag mice: 100 μg of protein were loaded per lane. n = 5 individual mice. Numbers below each lane, percentage of protein expression compared with mouse expressing the most protein, which was set equivalent to 100% as determined by densitometric analysis. Average percentage expression for each treatment group (n = 5 mice). P, significant difference between treatment groups and vehicle control.](image)

![FIGURE 5. Celecoxib-induced inhibition of tumor cell proliferation in vivo in a dose-dependent manner. Light microscopy images of PCNA staining of mammary tumor sections from vehicle-treated (A) and celecoxib (5, 10, and 20 mg/kg)–treated (B-D) MTag mice. All images are representative of five standardized fields from six separate experiments. Inhibition of proliferation is most evident at 10 and 20 mg/kg dose of celecoxib. Lymph node section (E) and second antibody staining (F) are shown as controls. Magnification, ×200.](image)

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<thead>
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<th>Treatment</th>
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<tr>
<td>Vehicle</td>
<td>1 × 10^6 ± 2.0 × 10^5</td>
</tr>
<tr>
<td>Celecoxib (20 μmol/L)</td>
<td>2 × 10^6 ± 1.6 × 10^5</td>
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<tr>
<td>Celecoxib (40 μmol/L)</td>
<td>4 × 10^6 ± 2.2 × 10^5</td>
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<tr>
<td>Celecoxib (60 μmol/L)</td>
<td>5 × 10^6 ± 2.0 × 10^6</td>
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NOTE: Spectrofluorometric analysis of lysates prepared from 48-hour vehicle-treated and celecoxib (20, 40, and 60 μmol/L)–treated MTag cells. Activity of caspase-3 and caspase-7 was monitored by enzymatic cleavage using a fluorescence microplate reader with excitation at 485 ± 10 nm and emission detection at 530 ± 12.5 nm. Mean ± SD of three experiments.

*P < 0.01, significant difference between vehicle control and celecoxib treatment.
measurement of the metabolites is necessary to provide a reliable estimate of actual PGE2 production. Thus, we measured PGE2 metabolite (PGEM; i.e., 13,14-dihydro-15-keto prostaglandin A2) using a commercially available ELISA. A significant reduction in serum PGEM is observed in 10 and 20 mg/kg celecoxib-treated MTag mice as compared with pretreatment and vehicle-treated mice (2,000 pg/mL in vehicle-treated mice versus <1,000 pg/mL in 10 mg/kg celecoxib-treated mice, P < 0.01; Fig. 6A). Similar reduction in PGEM was observed in tumor lysates (data not shown). This suggests that, although celecoxib was partially effective in reducing PGEM levels, treatment was not sufficient to completely reverse the up-regulation of PGE2 levels in MTag mice because these mice were not completely tumor free.

To test if celecoxib had a direct effect on COX-2 protein expression in the tumor, we evaluated COX-2 protein expression in tumor lysates from vehicle-treated and celecoxib-treated MTag tumors. MTag tumors from untreated and vehicle-treated mice expressed higher levels of COX-2 (Fig. 6B) as compared with celecoxib-treated (10 and 20 mg/kg) tumors, indicating that celecoxib has a direct effect on COX-2 protein expression in vivo. Densitometric analysis showed some difference between vehicle-treated and 10 or 20 mg/kg celecoxib-treated tumors (P < 0.07). However, the direct effect of celecoxib on COX-2 protein expression was less significant (P < 0.07) than its effect on COX-2 activity as measured by PGE2 levels.

Celecoxib Treatment Reduced Vascular Endothelial Growth Factor Levels in vivo

It has been shown recently that COX-2-induced PGE2 stimulated the expression of angiogenic regulatory genes including vascular endothelial growth factor (VEGF) in mammary tumor cells isolated from COX-2 transgenic mice and that treatment with indomethacin (nonspecific COX inhibitor) suppressed the expression of these genes in vitro (51). We therefore evaluated levels of in vivo VEGF protein levels in the tumor microenvironment of MTag tumors post-celecoxib treatment. Treatment with celecoxib (10 or 20 mg/kg) reduced VEGF levels in the tumor lysate in four of six treated mice as compared with vehicle-treated MTag tumors (P < 0.05; Fig. 7). No reduction was observed in mice treated with 5 mg/kg celecoxib. Untreated MTag tumor lysate had similar levels as vehicle-treated MTag mice (data not shown). Similar reduction in circulating VEGF levels was also
observed. Serum levels of VEGF in untreated or vehicle-
treated 14-week-old MTag mice were found to be between
150 and 400 pg/mL, whereas in the mice treated with celecoxib
(10 or 20 mg/kg) the levels ranged from 20 to 90 pg/mL.
In some of the treated mice, the VEGF levels were too low
to be detected by ELISA. Preliminary histologic evaluation
also suggests the presence of fewer blood vessels in the
celecoxib-treated tumor sections versus control tumor using
the Masson’s trichrome staining. A representative picture of
vehicle, 5, 10, and 20 mg/kg celecoxib-treated MTag tumor
is shown in Fig. 7B.

Discussion
We show for the first time that in vivo treatment with
celecoxib causes significant reduction in mammary gland tumor
burden in a mouse model of spontaneous breast cancer.
Recently, we have evaluated 6 MTag mice that received
20 mg/kg celecoxib and 10 control MTag mice between 20 and
24 weeks of age. None of the celecoxib-treated mice developed
lung metastasis, whereas 5 of the 10 control mice developed
lung metastasis (data not shown).

Tumor reduction was associated with induction of tumor cell
apoptosis in vivo. Investigation into the potential molecular
pathway revealed that treatment with celecoxib caused
reduction in activation of antiapoptotic/prosurvival kinase
(Akt). Increased apoptosis was associated with increased
expression of the proapoptotic protein Bax and decreased
expression of the antiapoptotic protein Bcl-2. Concurrently, we
observed decreased tumor cell proliferation and decreased
synthesis of VEGF in mammary gland tumors treated with
celecoxib in vivo, most probably associated with decreased
PGE2 synthesis.

The importance of this study lies in the use of a mouse
model system that resembles human disease in many aspects
of tumor progression. The MTag tumors start as hyperplasia, like
early proliferative lesions seen in the human breast; show
indication of histologic progression to malignant mammary
adenocarcinomas and metastasis; are heterogenous with respect
their malignant potential; and trigger signaling pathways
inactive in normal breast epithelium (38). One of the pathways
that is activated in these mice is the arachidonic acid/COX-2
pathway (52), similar to that described in many human breast
cancers. Furthermore, we have shown recently that COX-2
protein and its downstream product PGE2 were highly elevated
in human breast tumors and lymph node metastasis compared
with normal tissue, with the highest expression being observed
in lymph node metastasis (53). There was a direct correlation
between increased COX-2 and PGE2 expression with impaired
immune cell function in newly diagnosed stage I and II breast
cancer patients (53). Our observations are similar to the reports
that have shown significant elevation of COX-2 protein levels
in 43% of human invasive breast cancers and 63% of ductal
carcinomas in situ (11, 54). Thus, the MTag model offers
the potential to evaluate chemoprevention with a highly specific
COX-2 inhibitor, celecoxib.

Celecoxib has been shown to target multiple pathways of
tumorigenesis including proliferation, apoptosis, angiogenesis,
invasion, and tumor-induced immune suppression in various
breast tumor cell lines. The current report by Chang et al. (51)
supports the concept that COX-2 may provide an early target
for breast cancer prevention. We show that early intervention
with celecoxib causes reduced primary tumor burden in the
MTag model (Fig. 1). We further show that reduced PGE2
synthesis (Fig. 6A) and reduced PI3K/Akt kinase activation
(Fig. 4) post-celecoxib treatment may be the mechanism(s)
underlying enhanced tumor cell apoptosis (Fig. 2) and reduced
tumor cell proliferation (Fig. 5) in vivo. Our data are in line
with the recent in vitro study in prostate cancer cell lines, where
it was shown that celecoxib induces apoptosis by blocking or suppressing Akt activation (50). The PI3K/Akt pathway is typically activated in response to oncogenes that bind to receptor kinases at the plasma membrane and lead to the activation of PI3K (55, 56). Activated Akt targets multiple factors involved in cell proliferation, migration, and survival/apoptosis. Mechanistically, activated Akt is known to trigger several cyclins including cyclin D1 that affects all stages of the cell cycle and induces downstream proliferation (56). Preliminary data suggest decreased levels of cyclin D1 in tumor lysates of mice treated with celecoxib, with significant arrest of the mammary tumor cells at the G2-M checkpoint phase of cell cycle (data not shown). Thus far, our results implicate the PI3K/Akt pathway to be critical in the celecoxib-induced apoptosis and inhibition of tumor cell proliferation. However, other pathways such as the Raf/mitogen-activated protein kinase-extracellular signal-regulated kinase/mitogen-activated protein kinase pathway may also be affected by celecoxib, and future studies will be designed to evaluate these pathways in vivo in the MTag mouse model. One potential mechanism that has been associated with PGE2-related inhibition of apoptosis is that PGE2 reduces the basal apoptotic rate by increasing the level of antiapoptotic proteins such as Bcl-2 (54, 57). Our in vivo data support this concept, because inhibiting PGE2 production by targeting COX-2 activity in the MTag tumors led to decrease in Bcl-2 protein levels and concurrent increase in the proapoptotic protein Bax (Fig. 3A and B) as well as activate effector caspase-3 and caspase-7 (Table 1).

Finally, angiogenesis plays a crucial role in tumor development and progression. COX-2-dependent PGE2 is a potent inducer of angiogenesis in vivo and induces expression of angiogenic regulatory proteins such as VEGF (51, 58, 59). It has been shown recently that overexpression of COX-2 in the mammary gland by MMTV promoter induces mammary carcinogenesis and that the major prostaglandin that is produced in these tumors is PGE2 (51, 54). These authors further defined the role of COX-2-dependent PGE2 production in transforming local tumors to invasive cancer by triggering a so-called angiogenic switch by increasing expression of proangiogenic mediators such as VEGF and its receptors. Thus, we examined whether celecoxib treatment in vivo was effective in reducing the exaggerated VEGF levels observed in MTag tumors and in the serum. Significant decrease in levels of VEGF in the mammary gland tumors accompanied by fewer blood vessels in the celecoxib-treated tumor sections versus control was observed (Fig. 7), once again suggesting a role of COX-2 and PGE2 in mediating angiogenesis in the polyoma virus MTag-induced breast tumors. Although additional mechanisms are involved in mediating the angiogenic effects of COX-2, our data suggest that COX-2 influences angiogenesis at least in part by enhancing VEGF secretion by tumor endothelial cells. Additional studies are needed to fully elucidate the complex events involved in COX-2-mediated angiogenesis in our model. Our data clearly show extensive down-regulation of PGE2 in serum (Fig. 6A) post-celecoxib treatment in vivo. PGE2 binds to cell surface receptors that belong to the family of seven-transmembrane domain G protein-coupled receptors, designated EP1, EP2, EP3, and EP4 (54, 60). Future studies will determine the pattern of prostanoid receptor distribution in the MMTV-MTag mice and whether COX-2 inhibitors can modulate prostanoid receptor expression and its activation state. Although we suggest that PGE2 down-regulation may be in part responsible for the reduced VEGF levels, we fully recognize that much work is required to define a direct relationship between PGE2 and VEGF and that other pathways and angiogenic markers may be involved. We also acknowledge that the effect of celecoxib in the MTag mice may be COX independent; indeed, we do not see a dramatic down-regulation of COX-2 expression in celecoxib-treated compared with vehicle-treated mice (Fig. 6B). However, we must point out that we have published that the MTag tumors overexpress COX-2 and the expression increases as tumors progress and that the PGE2 levels are significantly decreased with celecoxib treatment.

In summary, celecoxib treatment may exert its antiproliferative, antiangiogenic, and proapoptotic effects by regulating the PGE2-prostanoid receptor–associated pathways and by decreasing PI3K/Akt phosphorylation. This leads to significant reduction in primary breast tumor burden. Furthermore, this effect may or may not be dependent on down-regulation of COX-2 protein expression in the tumor. Thus, we believe that COX-2 inhibitors not only represents a future therapeutic option for the treatment of human breast cancer in combination with standard therapies but also may be considered as a potent chemopreventive agent for individuals with high risk of developing breast cancer and for individuals with high risk of disease relapse.

Materials and Methods

Generation of MTag Mouse Model

MTag oncogenic mice was originally a kind gift from Dr. W.J. Muller (McGill University, Toronto, Ontario, Canada; ref. 36). MTag male mice were mated to C57BL/6 mice to maintain the MTag mice as heterozygous. Approximately 50% of the pups carry the oncogene, and in these pups, ~50% are females that develop mammary gland adenocarcinomas and are used for the experiments. PCR was used to routinely identify the MTag oncogene. PCR was carried out as described previously (39). Primer pairs for MTag transgene are 5′-AGTCCACTGCT-ACTGCACCCCAG-3′ (282-302 bp) and 5′-CTCTTCTCAGTC-TCCCTGCTCC-3′ (817-837 bp). The amplification program for MTag consisted of 1 cycle of 5 minutes at 95°C and 40 cycles of 30 seconds at 95°C, 1 minute at 61°C, and 30 seconds at 72°C followed by 1 cycle of 10 minutes at 72°C. The PCR product was analyzed by size fractionation through a 1% agarose gel. Amplification of MTag gene results in a 480-bp fragment. All mice are congenic on the C57/BL6 background at n ≥ 10. All mice were bred and maintained in specific pathogen-free conditions in the Mayo Clinic Scottsdale Natalie Schafer Transgenic Animal Facility. All experimental procedures were conducted according to Institutional Animal Care and Use Committee guidelines.

Celecoxib Treatment

Celecoxib was purchased from Pharmacia Pharmaceuticals (Skokie, IL) as 100-mg capsules. Drug was prepared for p.o. administration according to the manufacturer’s recommendation.
Briefly, the drug was dissolved in DMSO, rotated at low speed in a 37°C hot room for 12 hours, and centrifuged at 1,800 rpm for 10 minutes, and the supernatant was collected and stored at 4°C as stock solution of 20 mg/mL. Ten-week-old female MTag mice were gavaged p.o. with 20-gauge barrel tip feeding needles (Fine Science Tools, Foster City, CA) at 5, 10, or 20 mg/kg body weight daily (5 days on with 2 days off) for 4 weeks. Control mice were gavaged with DMSO. Six mice per treatment group were used. Following 4 weeks of treatment, mice were sacrificed and mammary tumors dissected and divided into three parts: (a) to generate single cell suspension for flow cytometry, (b) to prepare tumor lysate for Western blot analysis and ELISA, and (c) to fix in formalin and embedded in paraffin blocks for immunohistochemical analysis. Serum was collected for ELISA. A dose range of 5 to 20 mg/kg body weight was used in our spontaneous mouse model based on previous reports in the literature (24, 34). These doses correspond to physiologic dose of celecoxib and are clinically relevant because the doses of COX-2 inhibitors recommended to patients are in the range of 5 to 20 mg/kg body weight (29).

**Tumor Burden**

From 10 weeks of age until sacrifice, control and celecoxib-treated mice were palpated weekly for presence of mammary tumors. Palpable tumors were measured by calipers and tumor weight was calculated according to the following formula: 

$$\text{g} = L \times W / 2$$

**Analysis of Apoptosis by Flow Cytometry**

Part of the tumor tissue was dissociated to generate single cell suspension by incubating in 5 mmol/L EDTA solution for 1 hour at 37°C. Apoptosis was determined by staining single cells (1 x 10^6) with Annexin V and propidium iodide using the BD PharMingen (San Diego, CA) apoptosis kit following the manufacturer’s protocol. Cell staining was determined by flow cytometry using the CellQuest program. Percentage apoptotic cells were determined by CellQuest statistical analysis program as the cumulative percentage cells that were stained positive for both propidium iodide and Annexin V (upper right quadrant) and cells that were stained for Annexin V only (lower right quadrant).

**Analysis of Apoptosis, Proliferation, and Blood Vessels by Immunohistochemistry**

Part of the tumor was formalin fixed [10% neutral-buffered formalin (pH 6.8-7.2), Fisher Scientific, Pittsburgh, PA] and paraffin embedded and 5-μm sections were prepared by the Mayo Clinic Scottsdale Histology Core Facility. Immunohistochemistry was done using the ApoTag Peroxidase In situ Apoptosis Detection kit (Serologicals Corp., Norcross, GA). 3,3′-Diaminobenzidine was used as the chromogen and hematoxylin was used as counterstain. TUNEL-positive cells were examined under light microscopy and representative images taken at 200×. For PCNA staining, paraffin-embedded and 5-μm sections were subjected to antigen retrieval using the DAKO Target Retrieval (Carpinteria, CA) at 95°C for 40 minutes. Primary antibody (PCNA antibody, BD Biosciences, San Jose, CA) was used at 5 μg/mL at 4°C overnight and DAKO anti-mouse secondary conjugated to horseradish peroxidase was used at 1:200 for 2 hours at room temperature. 3,3′-Diaminobenzidine was used as the chromogen and hematoxylin was used as counterstain. Histologic evaluation of vascularity was determined by Masson’s trichrome staining (61). This method stains fibrous tissue and stroma green. Blood vessels containing RBC stain bright red.

**Assay for Caspase-3 and Caspase-7**

Primary MTag tumor cells derived from 17-week-old MTag mice were treated with increasing concentrations (20-60 μmol/L) of celecoxib or DMSO (vehicle) in medium supplemented with 5% FCS for 48 hours. To evaluate if celecoxib treatment can induce activation of caspase-3 and caspase-7, we detected levels of active forms of caspase-3 and caspase-7 in freshly prepared cell lysates from treated and untreated MTag tumor cells using the EnzChek Caspase-3/7 Assay Kit (Molecular Probes, Eugene, OR) following the manufacturer’s protocol. In principle, active caspase-3 or caspase-7 will cleave a fluorogenic substrate releasing the fluorochrome, and the fluorescence was detected and quantified by spectrofluorometry using UV excitation of 380 nm and detected at an emission wavelength range of 430 to 460 nm. Fluorescence emission is an indication of caspase-3 and caspase-7 activity. Thus, apoptotic cell lysates containing active caspase-3 and caspase-7 yield considerable emission as compared with nonapoptotic lysates that do not contain the active forms of the enzymes.

**ELISA for PGE2 and VEGF**

PGE2 and VEGF enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI for PGE2 and Oncogene Research Products, La Jolla, CA for VEGF) were used to assay the levels of PGE2 and VEGF in tumor lysates and serum derived from treated and control mice. All tumor lysates were made in tissue lysis buffer containing 20 mmol/L HEPES, 0.15 mol/L NaCl, and 1% Triton X-100 supplemented with 80 μL/mL phosphatase inhibitor cocktail II (Sigma P-5726, St. Louis, MO) and 10 μL/mL complete protease inhibitor cocktail (Boehringer Mannheim GmbH, Indianapolis, IN). The PGE2 and VEGF assays were done according to the manufacturer’s recommendation. Lysates were diluted appropriately to ensure that readings were within the limits of accurate detection. Results are expressed as picogram of PGE2 or VEGF per milliliter of serum or per microgram protein of tumor lysate for individual mice.

**Western Blot Analysis for COX-2, Phospho-Akt, Bax, and Bcl-2**

Tumor lysates from treated and untreated mice prepared as stated previously were resolved by SDS-PAGE on 10% to 15% resolving gels. Tumor lysate (100 μg) was loaded per lane. Gels were blotted and probed for COX-2 (p70, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Akt and Akt protein (p60, 1:1,000, Cell Signaling, Beverly, MA), Bax-horseradish peroxidase conjugated (p23, 1:200, Santa Cruz Biotechnology), and Bcl-2 (p26, 1:1,000, Trevigen, Gaithersburg, MD). Mammary gland tumor lysates from 20- to 22-week-old MTag
mouse are used as positive control for COX-2. Jurkat T lymphoma cell lysate was used as positive control for the other proteins. Individual animal protein expression data are shown.

Statistical Analysis
All data are expressed as means ± SD. Statistically significant difference between experimental groups was assessed by one-way ANOVA with Dunnett adjustment.

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