Inhibition of the Biosynthesis of Prostaglandin E_2 By Low-Dose Aspirin: Implications for Adenocarcinoma Metastasis

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Abstract

Meta-analyses have demonstrated that low-dose aspirin reduces the risk of developing adenocarcinoma metastasis, and when colon cancer is detected during aspirin treatment, there is a remarkable 83% reduction in risk of metastasis. As platelets participate in the metastatic process, the antiplatelet action of low-dose aspirin likely contributes to its antimetastatic effect. Cycloxooxygenase-2 (COX-2)–derived prostaglandin E_2 (PGE_2) also contributes to metastasis, and we addressed the hypothesis that low-dose aspirin also inhibits PGE_2 biosynthesis. We show that low-dose aspirin inhibits systemic PGE_2 biosynthesis by 45% in healthy volunteers ($P < 0.0001$). Aspirin is found to be more potent in colon adenocarcinoma cells than in the platelet, and in lung adenocarcinoma cells, its inhibition is equivalent to that in the platelet. Inhibition of COX by aspirin in colon cancer cells is in the context of the metastasis of colon cancer primarily to the liver, the organ exposed to the same high concentrations of aspirin as the platelet. We find that the interaction of activated platelets with lung adenocarcinoma cells upregulates COX-2 expression and PGE_2 biosynthesis, and inhibition of platelet COX-1 by aspirin inhibits PGE_2 production by the platelet–tumor cell aggregates. In conclusion, low-dose aspirin has a significant effect on extraplatelet cyclooxygenase and potently inhibits COX-2 in lung and colon adenocarcinoma cells. This supports a hypothesis that the remarkable prevention of metastasis from adenocarcinomas, and particularly from colon adenocarcinomas, by low-dose aspirin results from its effect on platelet COX-1 combined with inhibition of PGE_2 biosynthesis in metastasizing tumor cells. *Cancer Prev Res; 9(11); 855–65. ©2016 AACR.*

Introduction

Both observational studies (1–5) and controlled clinical trials (6) support the concept that aspirin prevents mortality and metastasis from adenocarcinomas. Importantly, aspirin use reduced the development of metastases by 69% in patients who develop cancer while receiving aspirin (7). The antimetastatic benefit of aspirin occurred even at low doses in the ranges used for its antiplatelet effect (75 to <300 mg daily; refs. 7, 8).

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Clinical Trial Registration: This investigation has been registered at www.clinicaltrials.gov (identifier NCT 00761891).

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A convincing body of evidence supports the concept that platelets are integral to the process of metastasis, and this forms the basis for considering that the antiplatelet effect of low-dose aspirin contributes importantly to the prevention of metastasis (9–16).

In addition to the platelet, cyclooxygenase-2 (COX-2) and its product PGE_2 are known to contribute to both tumorigenesis and metastasis. COX-2 inhibitors reduce the incidence of colorectal adenomas (15, 16). PGE_2 exerts multiple effects that promote tumorigenesis and metastasis (17–19), including induction of the transition from epithelial to mesenchymal phenotype (20), increased angiogenesis and cell growth (21, 22), invasiveness (23), and downregulation of the immune response (24–27).

Given these effects of the COX-PGE_2 pathway on tumorigenesis and metastasis, an important question is whether low doses of aspirin can act directly on extraplatelet cyclooxygenases to inhibit PGE_2 biosynthesis in humans. The strong inhibition of platelet COX-1 by low doses of aspirin results from at least three determinants. First, there is virtually no turnover of COX-1 in platelets, and daily administration of aspirin inhibits platelet COX-1 cumulatively over the platelet life span (28). Second, because of presystemic clearance of aspirin, exposure of platelets to the high concentrations of the drug in the portal circulation leads to greater effects on the platelets than on systemic targets (29). Finally, aspirin is particularly potent in inhibiting COX-1 in the platelet (30).

We previously discovered that there is cellular selectivity in the potency of aspirin. High concentrations of hydroperoxides in cells accelerate redox cycling in the peroxidase site of the

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enzyme to generate a protoporphyrin radical, and in this higher oxidative state, the acetylation of the COXs by aspirin is inhibited (31). Thus, COXs in cells with robust antioxidant defenses and low levels of peroxides, such as the platelet, are highly sensitive to inhibition by aspirin. Epithelial cells of the lung (32–35) and colon (36–38) also are protected from elevated levels of peroxides by extensive antioxidant systems. Accordingly, in the research reported here, we tested the hypothesis that aspirin would potently inhibit COX activity in epithelial cancer cells *in vitro*. The potency of aspirin as a cyclooxygenase inhibitor in cancer cells was found to be as great (lung adenocarcinoma) or greater (colon adenocarcinoma) than the potency in platelets.

The hypothesis that low-dose aspirin also can inhibit the catalytic activity of extraplatelet cyclooxygenase *in vivo* was tested, demonstrating that aspirin 81 mg daily reduced the biosynthesis of PGE_2 and prostacyclin significantly in healthy humans.

In addition to the action of aspirin to directly inhibit PGE_2 biosynthesis in tumor cells, it is well established that aspirin also can reduce tumor cell PGE_2 biosynthesis indirectly by blocking the PGE_2 production that results from the interaction of platelets with colon cancer cells (16, 39). In those investigations, the interaction of platelets with colon cancer cells increased the expression of COX-2 and production of PGE2, and selective inhibition of the platelet cyclooxygenase was found to prevent the increase in PGE_2 biosynthesis (16, 39). Further, a PGE_2-dependent epithelial-to-mesenchymal transition (EMT) of these platelet-activated colon cancer cells was demonstrated, supporting the importance of PGE_2 in metastasis. To determine whether this effect of platelets to elicit PGE_2 biosynthesis could be generalized to other tumor types, we evaluated the effect of the interaction of platelets with lung adenocarcinoma cells on PGE_2 production. We found that, like colon cancer cells, PGE_2 biosynthesis is increased by the exposure of lung adenocarcinoma cells to platelets. Moreover, the expression of COX-2 protein, although necessary, was not sufficient to account for the increased PGE_2 production, and evidence is presented that is consistent with the hypothesis that platelet adherence initiates calcium-activated $cPLA_2$ in the tumor cells.

Materials and Methods

Materials

Human A549, HCC827, and H2122 were obtained from the American Type Culture Collection (ATCC). Human HCA-7 were a generous gift from Dr. Robert Coffey. Written informed consent was obtained from study participants, and human blood was obtained following a protocol approved by the Vanderbilt Institutional Review Board. Hanks' Balanced Salt Solution (HBSS), DMEM), FBS, aspirin (acetyl salicylic acid), Sepharose 2B column, tris(hydroxymethyl) aminomethane (Tris), and IL1β were purchased from Sigma-Aldrich. Silica gel 60A thin-layer chromatography plates were purchased from Whatman. Antibiotic antimycotic solution was obtained from Life Technologies. [$^2H_8$] Arachidonic acid (AA) was obtained from Perkin Elmer Life Sciences. Anti–COX-1 and COX-2 antibodies were obtained from Santa Cruz Biotechnologies, and anti-GADPH antibodies were obtained from Ambion.

Participant blood sample procurement

Blood samples were obtained from healthy participants and collected from a peripheral vein using a 21-gauge needle into vacuum tubes containing 3.2% sodium citrate and centrifuged at 300 x *g* for 10 minutes at room temperature. The supernatant (platelet-rich plasma, PRP) was used to prepare washed platelets as previously described (40). Volunteers were enrolled only if they could confirm lack of aspirin or nonsteroidal inflammatory drug use for at least 10 days prior to sample procurement.

Preparation of washed platelets

PRP was acidified to pH 6.4 with 0.15 mol/L citric acid, and centrifuged at 1,000 x *g* for 10 minutes at room temperature. The resulting pellet was resuspended with 24.4 mmol/L sodium phosphate buffer, containing 113 mmol/L NaCl and 5.5 mmol/L glucose, pH 6.4, and platelets were purified on a Sepharose 2B column equilibrated with the same buffer. Platelets were counted with a Coulter counter and diluted with suspension buffer (110 mmol/L NaCl, 4 mmol/L K_2HPO_4, 8 mmol/L NaH_2PO_4, and 5 mmol/L glucose, pH 7.2) to a final concentration of 300,000 platelets/μL.

Cell culture

A549, HCC827, and H2122 non–small cell lung adenocarcinoma cells as well as HCA-7 and HT-29 colon cancer cells were cultured in 6-well plates at 37°C in a humid atmosphere containing 5% CO_2. Cells were maintained in DMEM media supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic–antimycotic solution. All cells except HCA-7 were activated with 1 ng/mL IL1β once they were 90% confluent to induce expression of COX-2 for 24 hours prior to experiment of interest.

Relation of aspirin concentration to inhibition of COX

To compare aspirin efficacy between cell types, washed platelets, or activated adenocarcinoma cell lines, prostanoid production was assayed using similar experimental conditions: (1) All cells were incubated in albumin-free media (41), (2) the same concentration of exogenous arachidonic acid was used for all experiments, (3) deuterated arachidonic acid was used to avoid confounding the results by contribution of prostanoids produced from endogenous arachidonic acid released during cell treatment, and (4) incubation time was the same for all cells. A detailed justification of these conditions is described in Supplementary Data. Washed platelets and activated adenocarcinoma cells were preincubated at 37°C for 30 minutes with aspirin at different concentrations: 0, 10, 20, 30, 50, and 100 μmol/L. Then, 2.0 μmol/L [$^2H_8$] AA was added and incubated for 15 minutes. The medium was harvested for GC/NICI/MS analysis.

Participant urine sample procurement

This study was approved by the Vanderbilt University Institutional Review Board and registered on ClinicalTrials.gov (NCT 00761891). Participants provided written-informed consent. Healthy nonsmoker volunteers over the age of 18 with no chronic medical illness or medications were enrolled. Exclusion criteria included concurrent use of other antiplatelet drugs, non-steroidal anti-inflammatory drugs (NSAIDs) or COX-2 inhibitors, history of coronary artery disease, uncontrolled hypertension (systolic blood pressure > 180 mmHg), significant gastrointestinal (GI) bleeding, hematocrit < 35%, or platelet count < 100,000/μL. *Volunteers* received a blister pack containing a 2-week supply of a daily dose of aspirin 81 mg (McNeil Pharmaceuticals) administered in the evening. The importance of strict adherence to therapy was emphasized, and participants were contacted by the research coordinator mid-study to assess and encourage

continued compliance. A pill count was performed at the conclusion of the study. Urine was collected for 24 hours starting after the first morning void on the day before the baseline and again prior to the 2-week visit.

GC/NICI/MS analysis of PGE$_2$ and TxB$_2$

PGE$_2$ and TxB$_2$ (the stable rearrangement product of TxA$_2$) were quantified by isotopic dilution by GC/NICI/MS monitoring—selected ions as previously described (42). The signals for different molecules are: TxB$_2$, $m/z = 614$; and internal standards [^{2}H$_4$] TxB$_2$, $m/z = 618$; [^{2}H$_4$] PGE$_2$, $m/z = 528$. To account for the deuterium–protium exchange at the position C12 of [^{2}H$_8$] PGE$_2$, the summation of the signals obtained at $m/z = 530$, 531, and 532 was performed (43).

Urinary metabolite analysis

PGE-M, the major PGE$_2$ urinary metabolite was quantified by isotopic dilution by LC/ESI/MS/MS as described previously (44). Detailed experimental protocol is described in Supplementary Data. It should be noted that PGD$_2$ is metabolized by a mechanism analogous to PGE$_2$, and metabolites for this eicosanoid are detected using the same m/z transitions as PGE-M. The methoximated metabolites of PGE$_2$ and PGD$_2$ are chromatographically separated using this method (Supplementary Fig. S1).

PGI-M, the PGI$_2$ urinary metabolite, was quantified by isotopic dilution using GC/NICI/MS as described previously (45).

Calculation of platelet-derived PGE$_2$

To determine the effect of low-dose aspirin on extra-platelet PGE$_2$ biosynthesis, it is necessary to account for its effect on the very small amount of platelet-derived PGE$_2$ production, assuming that this is inhibited almost completely by low-dose aspirin. PGE$_2$ is produced by the platelet in an amount that is approximately 6.4% that of TxB$_2$ (ref. 39 and Fig. 5). To estimate the fraction of PGE$_2$ that is derived from the platelet, the production rates for total PGE$_2$ and for platelet-derived PGE$_2$ in humans have been calculated. The production rates for PGE$_2$ in humans are 51 ng/mg creatinine in males and 22.7 ng/mg creatinine in females, where time is the amount of time in which 1 mg creatinine is excreted. This production rate is based on the determination that PGE-M represents 16% of infused tritium–labeled PGE$_2$ (46), and on the amount of PGE-M/mg Cr measured in this study.

Total PGE$_2$ production rate ng/mg Cr = [PGE-M ng/mg Cr]/0.16.

An estimate of platelet-derived PGE$_2$ is based on the fraction: PGE$_2$ produced by washed platelets/TxB$_2$ produced by washed platelets, and on the evidence that approximately 80% TxM is derived from platelets. Thus:

Extraplatelet PGE$_2$ production rate = (TxB$_2$ production rate × 0.064) × 0.8/PGE$_2$ production rate, where TxB$_2$ production rate is based on the determination that the normal excretion rate of the TxB$_2$ metabolite, 11-dehydrothromboxane B$_2$ (0.369 ng/mg Cr; ref. 47), represents 10.3% of the production rate (48). Thus, platelet-derived PGE$_2$ constitutes 0.36% of total PGE-M in males and 0.8% in females.

Cell lysates and Western blot analysis

Adenocarcinoma cells lysates were obtained by washing cells with ice-cold PBS containing a protease inhibitor (PI) cocktail and then scraping them off the well surface. Cells were pelleted

by centrifugation at 300 x g for 10 minutes at room temperature. Cells were resuspended in 100 μL of PBS containing PI cocktail and protein were quantified using the BCA assay.

Proteins (80 μg) were separated by LDS-PAGE (NuPAGE) and transferred on nitrocellulose membranes for analysis by Western blot. Nonspecific binding sites were blocked with Tris buffer saline with Tween-20 (TBST) buffer (50 mmol/L Tris, 150 mmol/L NaCl, and 0.2% (v/v) Tween-20, pH 7.5) containing 5% (w/v) non-fat dry milk for 1 hour at room temperature. After blocking, membranes were incubated overnight at 4°C with 1:1,000 dilution of appropriate primary antibody: COX-2 goat polyclonal IgG (sc-1747; Santa Cruz Biotechnologies), COX-1 (sc-1752, Santa Cruz Biotechnologies), and GADPH goat polyclonal IgG (AM4300, Ambion). Bands of interest were detected using horseradish peroxidase–conjugated donkey anti-goat IgG polyclonal antibodies (1:5,000 dilution; sc-2020; Santa Cruz Biotechnologies) and enhanced chemiluminescence kit. Quantification of signal in Western blots was performed using Image J downloaded from NIH website.

A549 and activated platelets studies

A549 cells were grown to 90% confluence and starved overnight in serum-free DMEM. Two batches of washed platelets were prepared as follow. Whole blood was incubated with 100 μmol/L aspirin (aspirin-treated) or not (no aspirin) for 40 minutes at room temperature. Washed platelets were then prepared as described separately from each batch as described above. Washed platelets (resuspended concentration of 600,000 platelets/μL) were activated with 50 μmol/L ADP for 2 minutes at 37°C prior to addition to A-549 cells at a ratio of about 180 platelets per adenocarcinoma cell. Washed platelets were activated by ADP because we found that (1) activating platelets with ADP yielded much less variability than without activation, and (2) ADP does not directly activate A549 cells. Cell and platelet mixture were then incubated at 37°C for 2 hours with no addition of exogenous arachidonic acid. The medium was harvested for analysis of PGE$_2$ by GC/NICI/MS as described above. The cells were then scraped from the wells, lysates obtained, and protein quantified using BSA assay. Western blots were performed for COX-1, COX-2, and GADPH proteins as described above. Each experiment was performed in triplicates, and a total of three experiments were performed ($n = 9$).

To confirm that the reduction in PGE$_2$ production was mediated by the effect of aspirin to inhibit platelet COX-1 and not due to direct inhibition of COX-2 expressed by A549 cells by residual aspirin present in washed platelets, we performed an additional control experiment in which A549 cells were incubated with IL1β overnight to induce COX-2 expression. A549 cells were then incubated for 2 hours at 37°C with either PBS buffer, the supernatant from washed platelets, or supernatant from washed platelets prepared from aspirin-treated whole blood. COX-2 catalytic activity in the platelet/A549 cell aggregates was assessed by measuring [^{2}H$_8$]-PGE$_2$ production for 15 minutes following the addition of [^{2}H$_8$]-AA.

Fluorescence microscopy

The ability of aspirin to block COX-2 active site has been further demonstrated using a fluorophore conjugated to indomethacin as previously described (49) In short, A549 cells were grown to 90% confluence, and expression of COX-2 was induced as described above. Cells were incubated in 2.0 mL of a

1:1 dilution of HBSS and modified Tyrode's buffer (20 mmol/L HEPES, 10 mmol/L glucose, 150 mmol/L NaCl, 6 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, pH 7.4) containing 50 nmol/L of the fluorescent probe, fluorocoxib A, for 30 minutes at 37°C. The cells were then washed briefly three times with PBS, and a washout was performed by incubating the cells in HBSS/Tyrode's solution for 30 minutes at 37°C. Following the washout period, the cells were imaged in 2.0 mL fresh HBSS/Tyrode's on a Zeiss Axiovert 25 Microscope with the propidium iodide filter (0.5–1.0 seconds exposure, gain of 2). All treatments were performed in duplicate dishes in at least three separate experiments ($n = 6$). To block the COX-2 active site, the cells were preincubated with 10 µmol/L NS-398 for 30 minutes at 37°C prior to the addition of fluorocoxib A. To test the ability of aspirin to block the COX-2 active site, the cells were preincubated with 100 µmol/L aspirin for 1 hour at 37°C prior to the addition of fluorocoxib A.

Measurement of diastolic calcium

A549 cells were incubated for 2 hours at 37°C with washed platelets which were preincubated with ADP (50 µmol/L) or aspirin (100 µmol/L) or vehicle for 5 minutes. A549 cells were incubated with 2 µmol/L Fura-2 acetoxymethyl ester (Fura-2 AM) for 8 minutes at room temperature to load the calcium indicator in the cytosol. A549 cells were washed twice for 10 minutes with Tyrode's solution containing 250 µmol/L probenecid to retain the indicator in the cytosol. A minimum of 30 minutes was allowed for de-esterification before the cells were imaged. All the experiments were conducted in Tyrode's solution containing the following: 1 mmol/L $CaCl_2$, 134 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 10 mmol/L HEPES, pH adjusted to 7.4 with NaOH. The final concentration of Ca^{2+} was 1 mmol/L. Baseline of Fura-2 fluorescence ratio was used as intracellular Ca^{2+} levels. Data were analyzed using commercially available data analysis software (IonWizard, IonOptix).

Statistical analysis

Statistical analysis was performed using Prism 4 from Graph-Pad Software, Inc. Paired analyses with Wilcoxon signed ranked test were used to determine significant differences between PGI-M and PGE-M values before and after aspirin therapy. The significance of differences between PGE_2 and COX-2 production in A549 cells that were untreated, treated with activated platelets, and treated with activated platelets preincubated with aspirin was determined using one-way ANOVA with the Tuckey multiple comparisons test. Differences among the groups in diastolic calcium measurements were analyzed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Low-dose aspirin inhibits nonplatelet biosynthesis of prostaglandins

The effect of aspirin 81 mg daily for 2 weeks on urinary metabolites of PGE_2 (PGE-M) and prostacyclin (PGI-M) was quantified in healthy volunteers who had no medical history of chronic inflammatory conditions that could predispose them to elevated prostaglandin levels. Detailed medication history was reviewed prior to enrollment to ensure that those did not interfere with the investigation. Demographics of study population are described in Table 1. Volunteers were given 2 weeks of aspirin

Table 1. Demographics of study population

	All Subjects ($N = 54$)
Age (y)	33.5 [26.8, 44.0]
Female sex	30 (56%)
Race – White	38 (70%)
Black	9 (17%)
Asians	5 (9%)
Other	2 (4%)
BMI (kg/m^2)	25.0 [22.8, 27.3]
Platelet count ($\times 10^3$/mL)	249 [215, 293]

NOTE: Values are median [IQR] or counts (%).
Abbreviation: BMI, body mass index.

81 mg daily, and the urinary metabolites of PGE_2 (11α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid) was measured using LC/MS/MS. Because the average PGE-M excretion is greater in males (8.2 ng/mg creatinine) than in females (3.6 ng/mg creatinine), the effect of aspirin was determined separately by gender. Aspirin 81 mg daily was sufficient to reduce urinary PGE-M levels in healthy females by 56% and in healthy males by 39%. When correction is made for the estimated small contribution of platelet-derived PGE_2 to total PGE_2 biosynthesis (see Methods), the reduction in extraplatelet PGE_2 production by aspirin is 55.4% in females and 38.6% in males (Fig. 1). The same aspirin dose also inhibited urinary PGI-M by 37% ($P < 0.0001$, $n = 54$; Supplementary Fig. S2). Serum thromboxane B_2 levels were reduced by 96.6%. These results demonstrate that low-dose aspirin can inhibit nonplatelet prostaglandin production.

### Aspirin is a potent inhibitor of PGE_2 biosynthesis in adenocarcinoma cells

To examine the effect of aspirin on PGE_2 biosynthesis in lung adenocarcinoma cells, we tested a range of concentrations of aspirin on PGE_2 biosynthesis in the non–small cell lung adenocarcinoma cell lines, H2122, HCC827, and A549. As shown in Fig. 2A, all three adenocarcinoma cell lines express COX-2 after overnight stimulation with IL1β, as well as various levels of COX-1. We compared the effect of the same concentrations of aspirin on COX-1–derived biosynthesis of thromboxane A_2 in human washed platelets. Platelets were utilized as a comparator in this *in vitro* study, because the dose range designated as "low dose aspirin" in humans is defined as the lowest dose of aspirin that nearly completely inhibits platelet COX-1.

In these experiments, the same concentration of isotopically labeled arachidonic acid (2 µmol/L) was added to all cells as substrate and the correspondingly labeled product analyzed. In all three cell lines, PGE_2 biosynthesis was markedly inhibited by aspirin (IC_{50}s are 22.5 ± 3.2 µmol/L, 7.2 ± 1.1 µmol/L, and 15.0 ± 1.3 µmol/L for HCC827, A549, and H2122 cells, respectively). By comparison, the IC_{50} for aspirin inhibition of platelet TxB_2 biosynthesis assayed in the same conditions was 19.8 ± 1.5 µmol/L (31).

Based on this evidence that aspirin potently inhibits PGE_2 biosynthesis in lung adenocarcinoma cells, its effect as a COX inhibitor in colon cancer cells also was determined (Fig. 2C). The IC_{50} for inhibition of PGE_2 biosynthesis in the colon cancer cells, HCA-7 and HT-29, was 3.9 ± 0.7 µmol/L and 8.9 ± 1.2, respectively. This demonstrates that aspirin is more potent as an inhibitor of COX in cells from colon adenocarcinomas than in the platelet ($P < 0.0001$). Thus, the catalytic activity of the cyclooxygenases in this group of lung and colon adenocarcinoma cells is inhibited by aspirin to the same or greater extent than COX-1 in the platelet.

MALE

FEMALE

Figure 1.
Daily low-dose aspirin inhibits prostaglandin E$_2$ biosynthesis in humans. Aspirin (81 mg) was administered daily to healthy participants for 2 weeks. PGE-M, the urinary metabolite of prostaglandin E$_2$, was measured by LC/MS before and after aspirin therapy. Data are presented for males and females. Paired analysis comparisons were performed using Wilcoxon-ranked tests. Low-dose aspirin inhibited the biosynthesis of PGE$_2$ by 45% ($P < 0.0001$, $n = 54$).
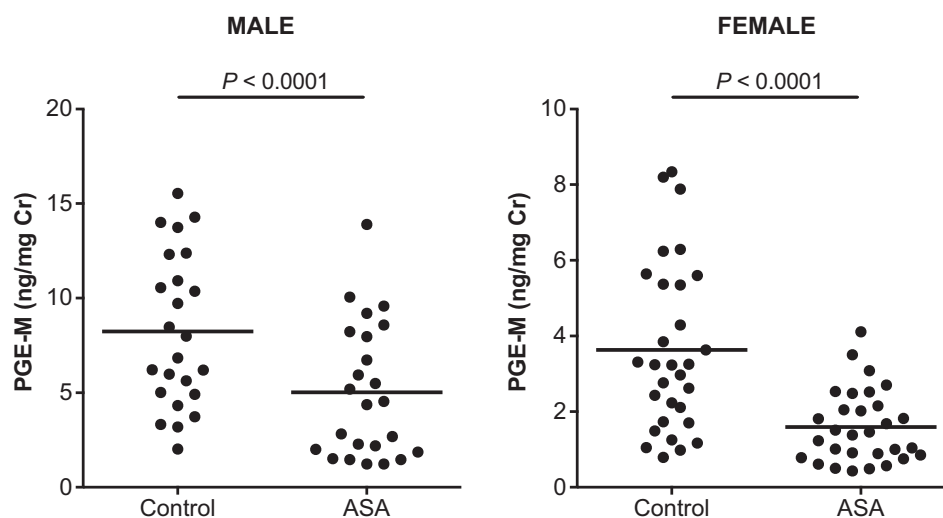
We have previously demonstrated that aspirin equally inhibits both COX isoforms (31). Interestingly, our results show that each of the five adenocarcinoma cells express various levels of the two isoforms. A549 cells, for example, mainly express COX-2, whereas HT 29 mainly express COX-1. Our results show that aspirin effectively inhibits prostanoid synthesis in all these cell lines irrespective of the isoforms expressed demonstrating that it is not selective for platelet COX-1. To further confirm that aspirin is not isoform specific, we measured prostanoid production before and after preincubation with the COX-2–specific inhibitor valde-

coxib. As shown in Supplementary Fig. S4, 100 nmol/L valdecoxib only inhibited 60% of PGE$_2$ synthesis in HCA-7, a concentration that fully inhibited PGE$_2$ synthesis in A549 and H2122. The same concentration of valdecoxib had no effect on TxB$_2$ synthesis by washed platelets (Supplementary Fig. S4).

Aspirin blocks the COX-2 binding site in the COX-2–expressing A549 cell line

The experiments above examined aspirin's inhibition of PGE$_2$ biosynthesis in adenocarcinoma cells. To further support the

Figure 2.
Aspirin inhibits COX-derived PGE$_2$ in adenocarcinoma cells as potently as it inhibits COX-1–dependent TxB$_2$ in washed platelets. Three lung and two colon adenocarcinoma cell lines were used to examine the potency of aspirin to inhibit PGE$_2$ synthesis. **A,** Western blots analysis of COX-1 and COX-2 expression in H2122, HCC827, A549, and HT 29 cells after cell activation with IL1β 1 ng/mL. HCA = 7 was not activated prior analysis. Protein expressions are expressed as a ratio against housekeeping protein GAPDH. $n = 3$. **B,** washed platelet biosynthesis of TxB$_2$ and lung adenocarcinoma cell line biosynthesis of PGE$_2$ were measured by GC/MS. **C,** washed platelet biosynthesis of TxB$_2$ and biosynthesis of PGE$_2$ in the colon adenocarcinoma cell lines, HCA-7 and HT-29, were measured by GC/MS. Aspirin IC$_{50}$ for the different cell types is indicated. Values represent mean ± SEM ($n = 6$).
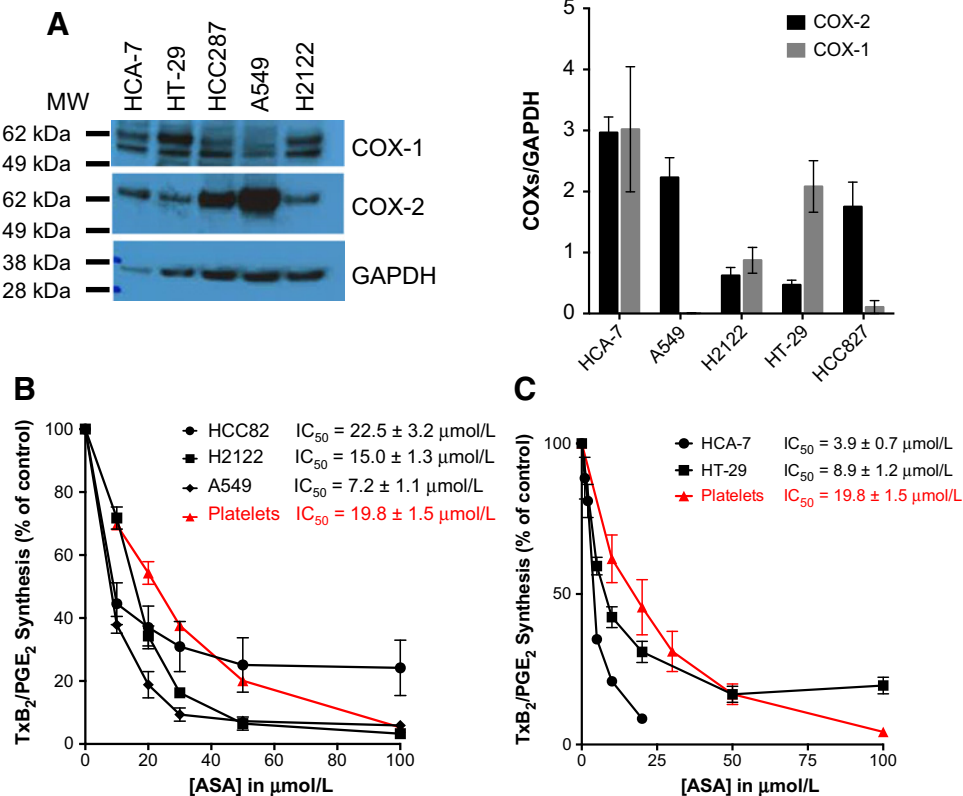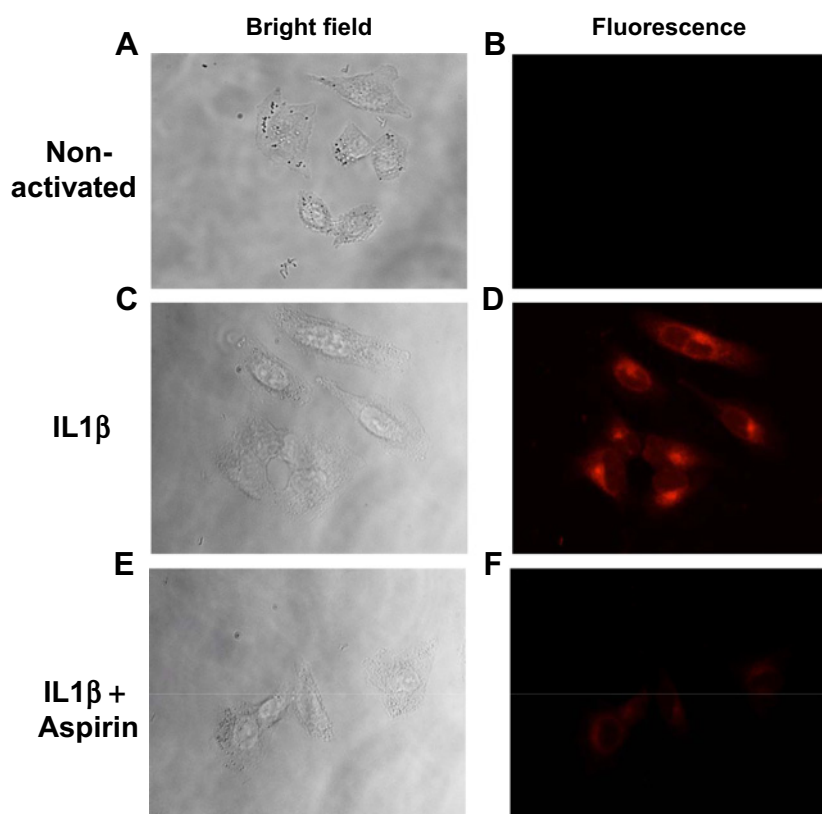
Figure 3.
Aspirin inhibits COX-2 labeling by fluorocoxib A in A549 lung adenocarcinoma cells. **A, C,** and **E,** bright field images of A549 cells. **B,** nonactivated A549 cells were treated with 200 nmol/L of rhodamine-conjugated indomethacin (fluorocoxib A) for 30 minutes. **D,** IL1β-activated A549 cells were treated with 200 nmol/L of rhodamine-conjugated indomethacin (fluorocoxib A) for 30 minutes. **F,** IL1β-activated A549 cells were pretreated with 100 μmol/L aspirin for 30 minutes before adding fluorocoxib A. Pretreatment with aspirin inhibited COX-2–associated fluorocoxib A fluorescence.

hypothesis that aspirin can inhibit PGE_2 production by blocking the COX-2 active site, we analyzed the effectiveness of aspirin at preventing the binding of a COX-2–specific fluorescent probe (49) using fluorescence microscopy. A549 cells were chosen because COX-1 expression is negligible (8%) compared with that of COX-2 when activated with IL1β (Fig. 2A and Supplementary Fig. S3). A rhodamine conjugated-indomethacin probe, fluorocoxib A (49), was used to label the active site of COX-2 (Fig. 3). As expected, the probe did not label the nonactivated A549 that are devoid of COX-2 (Fig. 3B) but strongly labeled IL1β-activated cells (Fig. 3D). Importantly, aspirin effectively blocked COX-2 labeling by fluorocoxib A (Fig. 3F), providing direct evidence that aspirin blocks the COX-2 active site. These results support the concept that inhibition of PGE_2 production by aspirin in A549 cells (Fig. 2) is caused by COX-2 inhibition and not by cyclooxygenases-independent effects of aspirin.

The interaction of platelets with lung adenocarcinoma cells induces PGE_2 biosynthesis via a calcium-mediated mechanism

The effect of the interaction of platelets with adenocarcinoma cells on PGE_2 production by COX-2 was evaluated in A549 cells because they produce low levels of PGE_2 at baseline (Fig. 4). Washed platelets from a normal volunteer were activated with ADP and subsequently incubated with A549 cells, and COX-2 expression and PGE_2 production were measured. Both COX-2 expression and PGE_2 production were significantly increased (Fig. 4). We then determined whether inactivation of platelet COX-1 by aspirin would prevent COX-2 expression and/or PGE_2 production by the platelet-A549 cell aggregates. We first

incubated whole blood with 100 μmol/L aspirin for 40 minutes and then prepared washed platelets from the aspirin-treated blood. This was done to ensure that all residual aspirin was removed from the platelet preparation and that no free aspirin was available for direct inhibition of A549's COX-2. Platelets derived from aspirin-treated blood were activated using ADP and subsequently incubated with A549 cells. COX-2 protein expression in A549 cells was still upregulated by aspirin-inhibited platelets (Fig. 4A), but PGE_2 production was significantly inhibited (Fig. 4B), suggesting that PGE_2 synthesis is induced by activated platelets by a mechanism independent of the level of expression of COX-2.

To confirm that the reduction in PGE_2 production was mediated by the effect of aspirin to inhibit platelet COX-1 and not due to direct inhibition of COX-2 expressed by A549 cells by residual aspirin present in washed platelets, we performed an additional control experiment in which COX-2 catalytic activity was measured in IL1β-activated A549 cells incubated with either PBS buffer, the supernatant from washed platelets, or supernatant from washed platelets prepared from aspirin-treated whole blood. PGE_2 production was no different in all three groups (3.5 ± 0.2, 3.3 ± 0.2, and 3.3 ± 0.3 ng/mL, respectively; $n = 3$). These results demonstrate that there is insufficient residual aspirin present in aspirin-treated platelets washed as described in our protocol to directly inhibit COX-2-mediated PGE_2 biosynthesis by A549 cells.

Consideration was given to the finding that platelet COX-1 inhibition by aspirin inhibits PGE_2 production without affecting COX-2 expression (Fig. 4). This could occur only if the increase in PGE_2 elicited by the interaction of the A549 cells

Figure 4.
Activated platelets induce COX-2 expression and PGE₂ production in lung adenocarcinoma cell line A549. Washed platelets were activated with 50 μmol/L ADP and subsequently incubated with A549 cells. Incubation of A549 cells with activated platelets increased COX-2 protein expression (**A**), PGE₂ production (**B**), and intracellular calcium mobilization (**C**) from baseline. Pretreatment of blood with aspirin (100 μmol/L) prior to isolation of washed platelets and their activation with ADP effectively blocked PGE₂ production by A549 cells (**B**) and intracellular calcium mobilization (**C**) but not COX-2 protein upregulation (**B**). **A,** **** indicate $P < 0.0001$ versus A-549 alone, $n \geq 9$; **B** and **C,** **** indicate $P < 0.0001$ versus all other conditions, * indicates $P < 0.05$ versus A-549 alone ($n \geq 9$).
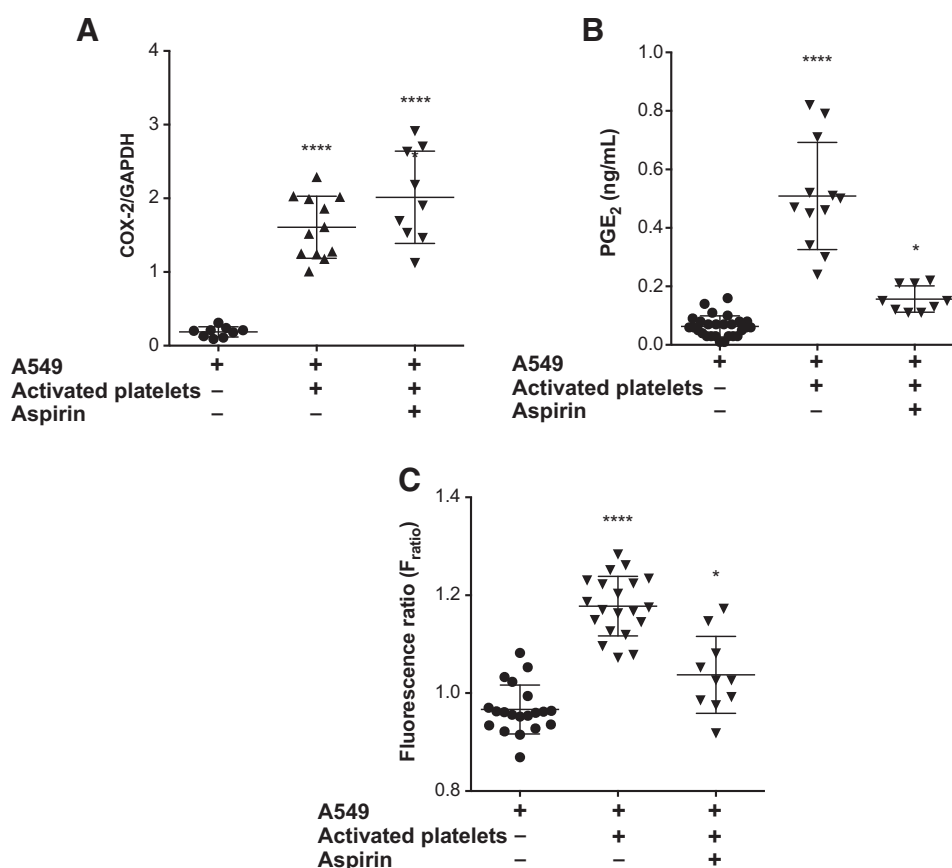
with platelets resulted from release of the COX substrate, arachidonic acid, from phospholipids by a phospholipase. In A549 cells, the phospholipase is cytosolic phospholipase A₂ (cPLA₂ or Group IVA PLA₂; refs. 50, 51). cPLA₂ is activated by an increase in cytosolic calcium (52, 53), which binds to the C2 domain of the enzyme and increases its hydrophobicity, thereby initiating penetration of the lipid bilayer. In A549 cells, ionophore-induced increases in cytosolic calcium have been shown to increase production of PGE₂ in a cPLA₂-dependent manner (50). Accordingly, we measured cytosolic calcium in A549 cells in response to incubation with ADP-activated washed platelets inhibited or not by aspirin. Cytosolic calcium levels were quantified for the following conditions: A549 cells incubated with (1) vehicle, (2) incubated with ADP-activated platelets, and (c) incubated with aspirin-pretreated ADP-activated platelets. Cytosolic calcium (expressed as fluorescence ratios) was increased by 21.9% following incubation of A549 cells with ADP-activated platelets (Fig. 4C, $n = 20$), but this effect was abrogated when the platelets had been preincubated with aspirin (7.3% increase, $n = 10$). These data indicate that platelets inhibited by aspirin are unable to trigger the mobilization of cytosolic calcium necessary for initiating PGE₂ synthesis by A549 cells. Interestingly, this calcium signal does not appear to be necessary for COX-2 induction.

The cellular origin of the increased PGE₂ in the platelet/A549 cell aggregates was investigated by employing the COX-2 inhibitor, NS-398, at 100 nmol/L. We first demonstrated that this concentration of NS-398 that does not block the production of PGE₂ or thromboxane B₂ (TxB₂) in washed

platelets (Fig. 5B and Supplementary Fig. S5) but fully blocks PGE₂ production by IL1β-activated A549 (Supplementary Fig. S4). When washed platelets were incubated together with A549 cells, the increased PGE₂ production was reduced by NS-398 by only 48%, indicating that approximately half of the increase in PGE₂ in the platelet/A549 aggregates was derived from the platelets, either by increased synthesis of PGE₂ in the platelet and/or intercellular transfer of PGH₂ from the platelet to the A549 cells. Thus, when platelets adhere to the cancer cells, both the platelets and the cancer cells are activated and both contribute to the increased biosynthesis of PGE₂.

Also, 31% of the increase in TxB₂ that resulted from exposure of platelets to A549 cells was blocked by NS-398 (Fig. 5B). Because no TxB₂ is formed by A549 cells in the absence of platelets, it may be inferred that intercellular transport of PGH₂ from A549 cells to the platelet contributes to the increase in TxB₂ that results from the interaction of platelets with A549 cells.

Discussion

We have demonstrated that aspirin 81 mg daily for 2 weeks inhibits the biosynthesis of PGE₂ in humans by an average of 45%. Thus, even at this low dose, extraplatelet COX is inhibited significantly. To place this in the context of the effect of the nonsteroidal anti-inflammatory drugs, our group found previously that ibuprofen 800 mg 4 times daily reduced PGE₂ production by 63% (44).
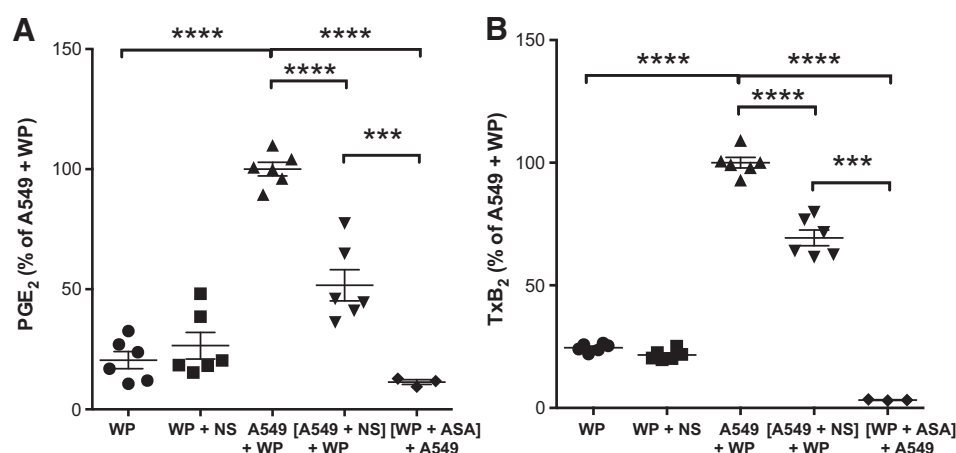
Figure 5.
Both platelet COX-1 and tumor cell COX-2 contribute to PGE$_2$ and TxB$_2$ production when platelets interact with the lung adenocarcinoma cells, A549. Washed platelets obtained from blood treated with aspirin (WP + ASA) or vehicle (WP) were activated with 50 μmol/L ADP and subsequently incubated with A549 cells. PGE$_2$ production (**A**) and TxB$_2$ production (**B**) were measured by GC/NICI/MS. The COX-2–specific inhibitor, NS-398, inhibited PGE$_2$ production by 48% and platelet TxB$_2$ production by 31%. NS-398 (100 nmol/L) treatment of platelets in the absence of A549 cells did not inhibit TxB$_2$ production (WP + NS). **** indicates $P < 0.0001$, and *** indicates $P \leq 0.001$.

Metastasis is facilitated by cyclooxygenase-derived PGE$_2$ (54–57). PGE$_2$ enhances the metastatic process via multiple mechanisms that in combination yield a strong prometastatic effect. The effects of PGE$_2$ that promote metastasis include upregulation of the cell adhesion receptor, CD44 (56, 58, 59), induction of a transition from an epithelial to a mesenchymal phenotype (16, 20, 60), activation of matrix metalloproteinases (23, 54, 61), enhanced tumor cell migration (62), increased angiogenesis (63), and disruption of the immune surveillance of tumor cells (24, 26, 64–66).

Prostacyclin biosynthesis also reflects oxygenation of arachidonic acid by extraplatelet COX. Previous studies with small sample size (67, 68) suggested that low-dose aspirin also can inhibit prostacyclin biosynthesis. Here, we clearly demonstrate a 37% reduction in prostacyclin biosynthesis by aspirin 81 mg daily.

The potency of low-dose aspirin as an inhibitor of COX-1 in platelets is one of the factors contributing to its antiplatelet efficacy even when administered at low doses. We found that aspirin inhibited COX isoforms expressed in lung adenocarcinoma cells as potently as its inhibition of platelet COX-1.

Inhibition of both COX isoforms expressed in cells from adenocarcinomas of the colon by aspirin is significantly greater than inhibition of platelet COX-1. Hepatic metastases from colon cancers derive from circulating tumor cells that lodge in the liver where they are exposed to the same high concentrations of aspirin that are so effective in inhibiting platelet COX-1 (Fig. 6), and are exposed to portal vein concentrations of aspirin constantly, whereas circulating platelets are in the portal circulation only about 20% to 25% of the time. Our demonstration of the potency of aspirin in colon cancer cells, therefore, supports a hypothesis that low-dose aspirin produces a high degree of inhibition of both COX isoforms in tumor cells from colon cancers that are sequestered in the hepatic portal vasculature. Given the evidence that PGE$_2$ is prometastatic, substantial inhibition of the biosynthesis of PGE$_2$ in tumor cells by low-dose aspirin could be hypothesized to contribute to the antimetastatic effect in cancers with venous drainage into the portal circulation. Indeed, the examination of metastasis in the meta-analysis of randomized controlled clinical trials of aspirin demonstrated exceptional prevention of metastasis from colon cancer. During the trials while randomized aspirin was being administered, if patients had no clinical
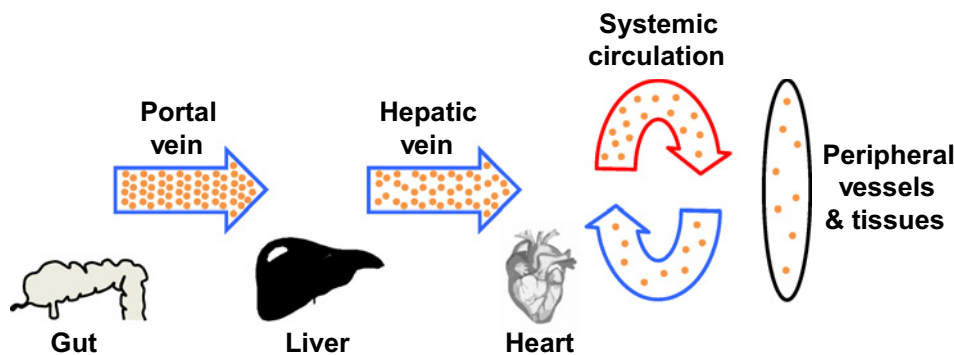

Figure 6.
Aspirin concentration in the different circulation compartments. Aspirin concentration is represented by the density of the orange dots. Aspirin concentration decreases as it travels through the liver and is further diluted in the systemic circulation. The highest plasma concentrations are in the portal vein and in the liver where colon cancer metastases will be exposed to aspirin.

evidence of metastasis at the time of diagnosis of colon cancers, the HR for later metastasis was 0.13 (95% CI, 0.03–0.56, $P = 0.007$; ref. 7). For adenocarcinomas other than those of the colon, the HR was 0.47. The profound inhibition of metastasis from colon adenocarcinomas by low-dose aspirin is consistent with its marked inhibition of the biosynthesis of PGE_2 by COX-2 in the circulating tumor cells acting in concert with inhibition of platelet COX-1.

Circulating tumor cells from cancers with venous drainage into the systemic circulation would be exposed to lower concentrations of aspirin than cells released from colon adenocarcinomas. Consequently, less than complete inhibition of COX in these cells by low-dose aspirin would be expected, as exemplified by the 45% reduction in total body PGE_2 biosynthesis The potential biological importance of a partial reduction in PGE_2 biosynthesis is contingent on whether the effect of PGE_2 on its receptor approximates a log-linear function of the concentration of the agonist. The explosive release of TxA_2 within seconds after platelet activation (69) produces concentrations of this agonist that are supramaximal relative to receptor response. In contrast to the platelet, PGE_2 release progresses slowly from tumor cells (70). Inhibition of PGE_2 biosynthesis by ibuprofen in humans by only 63% (44) occurs at doses that are associated with both efficacy and gastrointestinal bleeding, and an effect of aspirin on tumor volume in 4-(methyl-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)-treated mice is observed even at doses inhibiting PGE_2 levels by less than 50%. Accordingly, it is highly likely that a partial inhibition of PGE_2 biosynthesis by aspirin would reduce receptor-mediated responses and, importantly, could be additive with the antiplatelet effects of aspirin on metastasis. However, adding an intervention that achieves a more complete and selective inhibition of PGE_2 biosynthesis or action, e.g., an mPGES-1 inhibitor or an EP antagonist, conceivably could produce an antimetastatic effect in noncolonic cancers that is superior to aspirin alone.

Adherence of platelets to circulating tumor cells (12, 13, 71) is important to the process of implantation at the metastatic site (14, 15) via interaction of platelet P-selectin with P-selectin glycoprotein ligand-1 on cancer cells (71, 72). The phenotype of tumor cells is reprogrammed by their interaction with platelets to a more metastatic phenotype (20). Recently, Patrignani and colleagues, Dovizio et al. (39) and Guillem-Lobat et al. (16), demonstrated that tumor cell–platelet interaction upregulates COX-2 expression and PGE_2 release in the colon cancer cells, and provide convincing evidence that blockade of platelet COX-1 by aspirin inhibits the PGE_2 release, inhibits EMT, and also abrogates metastasis to the lung. We now provide evidence that adherence of platelets to lung adenocarcinoma cells also leads to PGE_2 production, indicating that the phenomenon they describe in colon cancer cells is generalizable to other cell types. We further demonstrate in lung adenocarcinoma cells that the release of PGE_2 is linked to a platelet-induced increase in cytosolic calcium in the tumor cells. Treatment of platelets with aspirin prevents calcium mobilization in the tumor cells and PGE_2 release, but does not alter the expression of COX-2. We propose that interaction of activated platelets with lung adenocarcinoma cells leads to calcium-mediated activation of $cPLA_2$ (52, 53), which yields the COX-2 substrate, arachidonic acid, from membrane phospholipids. Inhibition of platelet activation with aspirin reduces calcium mobilization in the tumor cells, thereby inhibiting

formation of PGE_2. The mechanism of thromboxane-dependent calcium mobilization in A549 remains to be characterized. However, it has been recently demonstrated that A549 cells express the thromboxane receptor (TP; ref. 73). Activation of TP is well known to trigger calcium mobilization via G_q activation, a mechanism that could possibly be involved in the effective PGE_2 production observed in our experiments.

In conclusion, low-dose aspirin has a significant effect on extraplatelet cyclooxygenases. It inhibits systemic biosynthesis of PGE_2 in humans by 45%, while blocking platelet TxB_2 production by 96.6%, and therefore is only relatively selective for the platelet. Aspirin inhibits COX catalytic activity and PGE_2 biosynthesis in lung and colon adenocarcinoma cells with a potency as great or greater than that for COX-1 in platelets.

These findings provide a basis for considering that even partial inhibition of tumor COX activity by low-dose aspirin could reduce the biosynthesis of PGE_2 by these cells. In the context of the convincing body of data that PGE_2 facilitates metastasis, it can be hypothesized that a reduction in tumor cell PGE_2 could be additive with its antiplatelet effect, and thereby contribute to the reduction of mortality and metastasis from adenocarcinomas. Low-dose aspirin is likely to produce a high degree of inhibition of COX in tumor cells arising from colon cancer that are sequestered in the portal circulation where they are exposed to the same high concentration of aspirin as the platelet. This forms the basis for a hypothesis that the remarkable prevention of metastasis from colon cancer by low-dose aspirin results from its well-recognized effect on platelet COX-1 combined with strong inhibition of PGE_2 biosynthesis in potentially metastatic tumor cells sequestered in the liver. A conclusion that PGE_2 contributes to colon cancer metastasis also implies that the metastatic potential of cancer cells that are not sequestered in the portal circulation could be further inhibited by combining aspirin with an inhibitor of PGE_2 production or action.

Disclosure of Potential Conflicts of Interest

P.E. Lammers is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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