Effects of Celecoxib on Prostanoid Biosynthesis and Circulating Angiogenesis Proteins in Familial Adenomatous Polyposis

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ABSTRACT

Vascular cyclooxygenase (COX)-2-dependent prostacyclin (PGI2) may affect angiogenesis by preventing endothelial activation and platelet release of angiogenic factors present in platelet α-granules. Thus, a profound inhibition of COX-2-dependent PGI2 might be associated with changes in circulating markers of angiogenesis. We aimed to address this issue by performing a clinical study with celecoxib in familial adenomatous polyposis (FAP). In nine patients with FAP and healthy controls, pair-matched for gender and age, we compared systemic biosynthesis of PGI2, thromboxane (TX) A2, and prostaglandin (PG) E2, assessing their urinary enzymatic metabolites, 2,3-dinor-6-keto PGF1a (PGI-M), 11-dehydro-TXB2 (TX-M), and 11α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M), respectively. The impact of celecoxib (400 mg b.i.d. for 7 days) on prostanoid biosynthesis and 14 circulating biomarkers of angiogenesis was evaluated in FAP. Intestinal tumorigenesis was associated with enhanced urinary TX-M levels, but unaffected by celecoxib, suggesting the involvement of a COX-1-dependent pathway, presumably from platelets. This was supported by the finding that in cocultures of a human colon adenocarcinoma cell line (HT-29) and platelets enhanced TXA2 generation was almost completely inhibited by pretreatment of platelets with aspirin, a preferential inhibitor of COX-1. In FAP, celecoxib profoundly suppressed PGE2 and PGI2 biosynthesis that was associated with a significant increase in circulating levels of most proangiogenesis proteins but also the antiangiogenic tissue inhibitor of metalloproteinase 2. Urinary PGI-M, but not PGE-M, was negatively correlated with circulating levels of fibroblast growth factor 2 and angiogenin. In conclusion, inhibition of tumor COX-2-dependent PGE2 by celecoxib may reduce tumor progression. However, the coincident depression of vascular PGI2, in a context of enhanced TXA2 biosynthesis, may modulate the attendant angiogenesis, contributing to variability in the chemopreventive efficacy of COX-2 inhibitors such as celecoxib.

Introduction

There is increasing appreciation of the role of platelets in tumor growth and metastatic dissemination (Gay and Felding-Habermann, 2011). Platelet activation can lead to the release of growth and angiogenesis factors present in α-granules into the tumor microenvironment (Italiano et al., 2008). Moreover, platelets and the factors they release can up-regulate cyclooxygenase (COX)-2, which is considered an early

ABBREVIATIONS: COX, cyclooxygenase; APC, adenomatous polyposis coli; AA, arachidonic acid; CV, cardiovascular; FAP, familial adenomatous polyposis; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; LT, leukotriene; PG, prostaglandin; mPGES-1, microsomal PGE2 synthase-1; PGI-M, 2,3-dinor-6-keto PGF1a; PGE-M, 11α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid; PGI2, prostacyclin; PDGF, platelet-derived growth factor; RIA, radioimmunoassay; sICAM, soluble intercellular adhesion molecule; TX, thromboxane; TXAS, TXA2 synthase; dinor-TX-M, 2,3-dinor-TXB2; TX-M, 11-dehydro-TXB2; TIMP, tissue inhibitor of metalloproteinases; UPLC/MS/MS, ultra-performance liquid chromatography/tandem mass spectrometry; VEGF, vascular endothelial growth factor.
event of cell transformation (Patrono et al., 2001). In colorectal cancer, COX-2 expression is induced early in stromal cells and subsequently at high levels in epithelial cells (Prescott, 2000), where it correlates with advanced tumor invasion and poor clinical outcomes (Sheehan et al., 1999).

Prostaglandin (PG) E$_2$ is a key prostanooid in tumorigenesis generated through the activity of coordinate expression of COX-2 and microsomal PGE$_2$ synthase-1 (mPGES-1), an enzyme downstream of COX-2 (Wang and Dubois, 2010). PGE$_2$ exerts its autocrine/paracrine effects on target cells by coupling to four subtypes of G protein-coupled receptors classified as EP1, EP2, EP3, and EP4 (E-series prostanooid receptors). We have shown previously that EP2 stimulation causes transactivation of the epidermal growth factor receptor signaling pathway to promote tumor cell proliferation and invasion (Donnini et al., 2007).

The possible contribution of other prostanooids to colon cancer development is less clear. Thromboxane (TX) A$_2$ and prostacyclin (PGI$_2$) play important roles in cardiovascular (CV) homeostasis (Grosser et al., 2006). In particular, TXA$_2$, a major product of platelet COX-1, promotes platelet aggregation and vasoconstriction, whereas PGI$_2$, a major product of endothelial COX-2, inhibits platelet aggregation and promotes vasodilatation. It is noteworthy that it has been shown that enhanced TXA$_2$ and PGI$_2$ generation by the introduction of the downstream TXA$_2$ synthase (TXAS) and PGI$_2$ synthase, respectively, into murine colon cancer cells modified tumor growth in vivo via differential effects on tumor angiogenesis (Pradono et al., 2002). Tumors derived from cells expressing TXAS grew faster and exhibited more abundant vasculature, whereas tumors from PGI$_2$ synthase-expressing cells produced the opposite effects (Pradono et al., 2002; De Bock et al., 2011).

Aspirin, even at low doses (such as 75 mg daily, recommended for the prevention of heart disease, which preferentially inhibits platelet COX-1) (Charman et al., 1993), reduces the incidence and mortality of colorectal cancer (Rothwell et al., 2011). This is consistent with the hypothesis that the antiplatelet effect of aspirin is central to its antitumor efficacy (Patrono et al., 2001). Enhanced systemic biosynthesis of TXA$_2$ is mainly from platelet COX-1 and is suppressed by low-dose aspirin in colorectal cancer (Sciulli et al., 2005). Based on this, a critical question is centered on the potential mechanistic contributors to this variability in response. It is noteworthy that the development of biomarkers predictive of response will allow one to avoid exposure of patients unlikely to benefit from chemoprevention to the CV hazard from this drug. Drugs, such as celecoxib, suppress vascular PGI$_2$ generated by COX-2 in endothelial cells, thus leaving unconstrained all mediators that stimulate platelets, elevate blood pressure, and accelerate atherogenesis, including TXA$_2$, (Grosser et al., 2006).

In the present study, we investigated the biosynthesis of TXA$_2$, PGI$_2$, and PGE$_2$ in vivo, by the measurement of their major urinary enzymatic metabolites, in patients with FAP and healthy controls, pair-matched for gender and age, who were nonsmokers and without clinically detectable CV risk factors. In patients with FAP, we performed an open-label study with a clinically relevant dose of celecoxib (400 mg b.i.d. for 7 days) to verify the COX isozyme involved in TXA$_2$ biosynthesis in patients with intestinal neoplasia. To exclude the possible contribution of clinically undetectable CV disease to enhanced TXA$_2$ biosynthesis detected in FAP, we assessed urinary levels of a major enzymatic metabolite of TX in a mouse model of colon tumorigenesis, i.e., Apc(Min/+ ) mice (Moser et al., 1995). The hypothesis that tumor cells and their released products or microvesicles are the trigger of platelet activation and enhanced TXA$_2$ biosynthesis was verified by performing coculture studies of human HT-29 colon cancer cells and platelets. Finally, we verified the hypothesis that in FAP a profound inhibition of COX-2-dependent PGI$_2$ by celecoxib would be associated with complex changes in circulating markers of angiogenesis possibly because PGI$_2$ may constrain platelet release of angiogenesis factors present in platelet α-granules (Menter et al., 1987).

**Materials and Methods**

**Clinical Study of Patients with FAP, Design, and Assessments.** Nine patients with FAP, recruited from the national hereditary colorectal tumor registry (National Cancer Institute of Milan, Italy), and nine healthy controls, matched for gender and age, non-smokers, and without clinically detectable CV risk factors (Table 1), were enrolled to participate in the study, after providing informed consent. In the nine patients with FAP, we performed an open-label study with celecoxib (Pfizer, New York, NY) (400 mg b.i.d. for 7 consecutive days) that was previously approved by the institutional ethical committee (National Cancer Institute of Milan, Italy). Patients with FAP had not had a complete colorectal resection and had five or more polyps, 2 mm or more in diameter, that could be assessed endoscopically. Exclusion criteria included a history of colectomy within the previous 12 months; use of nonsteroidal anti-inflammatory drugs or aspirin, a minimum of one or two times a week within 3 months of enrollment; abnormal results of serum laboratory tests (complete blood count and liver function and renal function tests); a history of myocardial infarction, stroke, coronary artery bypass graft, invasive coronary revascularization, or new-onset angina within the previous 6 months or electrocardiogram evidence of recent silent myocardial ischemia; intolerance or allergy to nonsteroidal anti-inflammatory drugs; and pregnancy.

Celecoxib compliance was monitored by means of pill count and review of diaries completed by patients and the assessment of celecoxib plasma levels (Schönberger et al., 2002). Before and after treatment, blood and urine samples were collected for the assessment of different molecular and biochemical analyses. Heparinized blood samples were collected before dosing and on the seventh day, 4 h after the last dose of celecoxib, to assess the levels of 14 circulating angiogenesis proteins by using an angiogenesis antibody array kit (U.S. Biomax, Inc., Rockville, MD; the list of proteins analyzed is reported in Supplemental Materials and Methods and Supplemental Table S1). Plasma cotinine levels were determined by using an enzyme immunoassay kit (Cozart Biosciences, Oxford, UK). The presence of mutations in the APC gene, the earliest detectable mo-
lue abnormality in colorectal cancer (Powell et al., 1992), and in the MutY human homolog (MYH), gene which is associated with a recessive form of polyposis (Sieber et al., 2003), was assessed as described previously (Gismondi et al., 2004). Overnight urine samples (from 8:00 PM to 8:00 AM) were collected before treatment and on the eighth day after the last dose of the drug to evaluate the urinary excretion of 11-dehydro-TXB2 (TX-M), a major enzymatic metabolite of TXA2, by a validated radioimmunoassay (RIA) technique (Ciabattoni et al., 1987), and 2,3-dinor-6-keto-PGF1α (PGI-M), a major enzymatic metabolite of PGI2, by reversed-phase high-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) (Song et al., 2007) (Supplemental Materials and Methods). Metabolite levels were corrected for urinary creatinine as a biomarker for risk assessment of colorectal cancer (Csiki et al., 2005).

Table 1: Baseline characteristics of patients with FAP and healthy controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>FAP Patients (n = 9)</th>
<th>Controls (n = 9)</th>
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<tbody>
<tr>
<td>Age, mean ± S.D., years</td>
<td>44 ± 11</td>
<td>45 ± 10</td>
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<tr>
<td>Female, n, %</td>
<td>5, 55</td>
<td>5, 55</td>
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<tr>
<td>C-reactive protein, mg/l, mean ± S.D.</td>
<td>10 ± 19</td>
<td>4 ± 2</td>
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<tr>
<td>Median (range)</td>
<td>3 (1.10–61.60)</td>
<td>3.30 (1.40–9.50)</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>0.12 ± 0.09, 0.09 (0.03–0.33)</td>
<td>Not determined</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol, mg/dl, mean ± S.D., median (range)</td>
<td>105 ± 26, 97 (65–144)</td>
<td>106 ± 27, 103 (60–147)</td>
</tr>
<tr>
<td>Cotinine, ng/ml, mean ± S.D., median (range)</td>
<td>7.20 ± 19.60, 0.62 (0.40–59.40)</td>
<td>1.19 ± 0.84, 0.93 (0.10–2.90)**</td>
</tr>
<tr>
<td>TX-M, ng/mg creatinine, mean ± S.D., median (range)</td>
<td>0.12 ± 0.09, 0.09 (0.03–0.33)</td>
<td>0.20 ± 0.10, 0.20 (0.09–0.40)</td>
</tr>
<tr>
<td>PGI-M, ng/mg creatinine, mean ± S.D., median (range)</td>
<td>22.30 ± 16.20, 21.52 (2.82–55.40)**</td>
<td>0.12 ± 0.05, 0.10 (0.05–0.20)</td>
</tr>
<tr>
<td>APC mutation, n, %</td>
<td>8, 88.9</td>
<td>Not determined</td>
</tr>
<tr>
<td>MYH mutation, n, %</td>
<td>1, 11.1</td>
<td>Not determined</td>
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*A data were compiled by Student’s t test.
*B data were compiled by Mann-Whitney test.
**P < 0.05; ***P < 0.01 vs. healthy controls.

Studied in Apc(Min/+), mice. In vivo prostanooid generation was accomplished by using Apc(Min/+), mice, an established model of FAP (Moser et al., 1995). Five female Apc(Min/+) mice and their wild-type C57BL/6J littermates at 11 weeks of age were used for each group. Urine samples (100 µl/mouse) were manually collected and immediately frozen. Systemic production of PGE2, TXA2, and PGI2 was evaluated by UPLC/MS/MS quantification of their major urinary metabolites (Song et al., 2007): PGE2-M, 2,3-dinor-thromboxaneB2 (dinor-TX-M), and PGI-M, respectively. All animals were maintained in a pathogen-free animal facility, and experiments were approved by the Institutional Animal Use and Care Committee (Center for Colon Cancer Research, University of South Carolina, Columbia, SC).

Coculture Experiments with a Human Colon Adenocarcinoma Cell Line (HT-29) and Isolated Human Platelets. HT-29 cells were cultured in McCoy’s 5A medium (Invitrogen, Milan, Italy) containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. For every experiment, 1 × 10⁶ cells were seeded in six-multiwell plates containing 2 ml of McCoy 5A supplemented with 0.5% fetal bovine serum and 10 µg/ml of Polymyxin B sulfate (Sigma-Aldrich, Milan, Italy) for 20 h, alone or cocultured with platelets. Human platelets were freshly isolated from leukocyte concentrates obtained from Stadttische Kliniken Hochst (Frankfurt, Germany) as reported previously (Albert et al., 2002) (briefly described in Supplemental Materials and Methods). HT-29 cells (1 × 10⁶ cells) were cultured alone or with washed human platelets (1 × 10⁶ cells) for 20 h at 37°C in a humidified mixture of 5% CO2 in air. In the culture medium, PGE2 and TXB2 were measured by validated RIAs (Patrone et al., 1980; Patrignani et al., 1994), whereas in cell lysates, COX-1, COX-2, mPGES-1, and TXAS were assessed by Western blot (Di Francesco et al., 2009) (Supplemental Materials and Methods).

Platelets, obtained by preincubating platelet-rich plasma with 300 µM aspirin for 30 min at room temperature before washing, were used as indicated. The effects of the highly selective COX-2 inhibitor rofecoxib (0.3 µM; Witega Laboratorien, Berlin, Germany) on prostanooid biosynthesis by HT-29 cells cultured alone or with platelets for 20 h were evaluated.

Statistical Analyses. Values were reported as mean ± S.D. and median (range) as appropriate. A P value <0.05 was assumed to be significant. The data were compared by parametric tests (Student’s t test or analysis of variance) or nonparametric tests when they did not pass the Kolmogorov-Smirnov normality test. The Spearman rank correlation coefficient (r) was calculated to quantify the statistical dependence between two variables. Linear multiple regression analysis of log10-transformed data were performed to test the relationship between PGI-M, PGE2-M, TX-M, and circulating angiogenesis proteins. Multicollinearity of biomarkers was verified by assessing the individual r² and variance inflation factor. Variables with r² values more than 0.75 (so variance inflation factor was more than 4.0) were excluded for multicollinearity. Comparisons of urinary and plasma biomarker levels between baseline and celexib in patients with FAP were assessed by the Wilcoxon matched-pairs test. All analyses were performed by using InStat (GraphPad Software Inc., San Diego, CA). In the clinical pharmacology study, the primary hypothesis was that celexib would cause 60% reduction of urinary PGI-M. Assuming an intersubject coefficient of variation of 22% for urinary excretion of PGI-M (McAdam et al., 2005), six volunteers would allow detecting at least 46% change in its measurement between predrug and postdrug with a power of 90% based on two-tailed tests with P values less than the type I error rate of 0.05. Thus, we chose a sample size of nine individuals. Concentration-response curves of celexib were fitted (using Prism; GraphPad Software Inc.), and IC50 (drug concentration required for obtaining 50% of inhibition) values were calculated.

Results

Biosynthesis of Prostanoids In Vivo in Patients with FAP at Baseline. The baseline characteristics of patients...
with FAP and healthy controls, pair-matched for gender and age, are reported in Table 1. All individuals were nonsmokers without significant CV risk factors. Despite the fact that all patients with FAP claimed to be nonsmokers, one of the nine exhibited plasma cotinine levels (59.4 ng/ml) compatible with a moderate smoking habit (Binnie et al., 2004). Eight patients were carriers of the APC (adenomatous polyposis coli) mutation, and one was carrier of the MutY human homolog mutation.

In FAP, urinary levels of TX-M at baseline were 1.19 ± 0.84, 0.93 (0.10–2.90) ng/mg creatinine [mean ± S.D., median (range)] and resulted in significantly \( p < 0.01 \) higher than the values detected in healthy controls [0.20 ± 0.10, 0.20 (0.09–0.40) ng/mg creatinine] (Table 1). Baseline urinary PGI-M levels were not significantly different in FAP and healthy controls (Table 1). In patients with FAP, PGE-M baseline levels were significantly \( p < 0.05 \) higher than in healthy controls [22.30 ± 16.20, 21.52 (2.82–55.40) and 10.30 ± 5.02, 11.22 (4–16) ng/mg creatinine, respectively; Table 1]. They were similar to the values found in patients with recurrent nonsmall cell lung cancers (27.2 ± 213.5 ng/mg creatinine) (Csiki et al., 2005).

**Biosynthesis of Prostanoids In Vivo in Apc(Min+) Mice.** To exclude the possible contribution of clinically undetectable CV disease to enhanced TXA2 biosynthesis in vivo in FAP, we assessed urinary levels of a major enzymatic metabolite of TX in a mouse model of colon tumorigenesis, i.e., Apc(Min+) mice (Moser et al., 1995). In Apc(Min+) and wild-type C57BL/6J mice, we also assessed urinary levels of PGI-M and PGE-M.

In Apc(Min+) mice, a significant increase of urinary dinor-TX-M was detected versus wild-type mice (mean ± S.D. 84.3 ± 26 versus 48.2 ± 23 ng/mg creatinine, respectively, \( p < 0.05 \)) (Fig. 1a). Average PGI-M was higher in Apc(Min+) than in wild-type mice, but the differences were not statistically significant (Fig. 1b). Urinary PGE-M was increased in Apc(Min+) versus wild-type mice (5.3 ± 1 versus 2.5 ± 1.3 ng/mg creatinine, respectively, \( p < 0.01 \)) (Fig. 1c). These results confirm the data in FAP showing that enhanced in vivo generation of TXA2 and PGE2 is associated with multiple intestinal neoplasia.

**Effects of Celecoxib on the Biosynthesis of Prostanoids In Vivo in Patients with FAP.** Celecoxib administered for 7 consecutive days did not significantly affect urinary TX-M in patients with FAP (Fig. 2a). In contrast, the drug caused profound and significant \( p < 0.01 \) inhibition of PGI2 biosynthesis by 58 ± 9% (Fig. 2b) and PGE2 by 48 ± 31% (Fig. 2c). The finding that celecoxib did not affect TX biosynthesis in vivo suggests a COX-1-dependent pathway for enhanced TX generation, presumably from platelets.

**Prostanoid Biosynthesis by Coculture of Human Adenocarcinoma Cell Line HT-29 and Human Platelets.** To address whether epithelial tumorigenesis triggers platelet activation and enhances TXA2 biosynthesis, we performed an in vitro study by coculturing HT-29 cells with platelets for 20 h. As shown in Fig. 3a, very low concentrations of TXB2 were generated by HT-29 cells with platelets for 20 h. As shown in Fig. 3a, very low concentrations of TXB2 were generated by HT-29 cells (mean ± S.D. 0.02 ± 0.02 ng/ml). Unstimulated platelets cultured alone for 20 h released 8 ± 3 ng/ml of TXB2. When unstimulated platelets were cocultured with HT-29 cells for 20 h, TXB2 generation was significantly enhanced \( p < 0.01; 57 ± 32 \) ng/ml. This finding suggests that colon cancer cells triggered platelet activation. TXB2 generation was profoundly reduced by pretreatment of platelets with aspirin under these experimental conditions, which is consistent with the enhanced TXB2 bio-

![Fig. 1. Biosynthesis of prostanoids in vivo in Apc(Min+) mice versus wild type (WT).](https://example.com)
synthesis in cocultures of the two cell types deriving mainly from platelet COX-1 activity (Fig. 3a). This notion was further supported by the finding that, under the same experimental conditions, a selective inhibitor of COX-2 activity, rofecoxib, did not affect TXB2 levels, either in platelets cocultured with HT-29 cells or platelets cultured alone (Fig. 3b). By contrast, rofecoxib significantly inhibited PGE2 generation as detected in the culture medium of treated HT-29 cells (Fig. 3c), suggesting that colon cancer cells generated PGE2 principally via the COX-2 pathway. These differences in prostanoid generation can be attributed to the relative expression levels of COX-1, COX-2, TXAS, and mPGES-1 observed in HT-29 cells and platelets (Fig. 3d). Altogether, these results suggest that intestinal neoplasia is associated with COX-2/mPGES-1-dependent PGE2 generation and enhanced platelet COX-1-dependent TX generation may be triggered by tumor cell constituents, their released products, and/or microvesicles.

Effects of Circulating Celecoxib Concentrations on the Activity of Monocyte COX-2 and Platelet COX-1 in Human Whole Blood In Vitro. We measured celecoxib levels in plasma of patients with FAP 4 h after the last morning dose and assessed the degree of inhibition on whole-blood COX-1 and COX-2 activities in vitro produced by these concentrations. These experiments allowed us to verify whether circulating concentrations of celecoxib detected in patients with FAP after dosing with 400 mg b.i.d. were sufficient to suppress completely COX-2 activity in vivo because it has been suggested that the chemopreventive effects of celecoxib at high concentrations may occur through COX-2-independent pathways (Schiffmann et al., 2009). At 4 h after the last dose of celecoxib, plasma concentrations ranged from 955 to 3566 ng/ml (1871 ± 946.8 ng/ml mean ± S.D.). In Supplemental Fig. S2, the sigmoidal dose-response curves of celecoxib for the inhibition of whole-blood COX-1 and COX-2 in vitro are shown. Celecoxib inhibited lipopolysaccharide-induced monocyte COX-2 and platelet COX-1 activities in a concentration-dependent fashion, with IC50 values of 128 (72–228) and 3444 (2116–5604) ng/ml [mean (95% confidence intervals), respectively. Individual plasma celecoxib concentrations detected in patients with FAP were associated with inhibition by 90 to 95% of monocyte COX-2 activity in vitro and 25 to 55% of platelet COX-1 in vitro (Supplemental Fig. S2). These results indicate that circulating concentrations of celecoxib were appropriate to almost completely inhibit monocyte COX-2 activity and they only modestly affected platelet COX-1 activity.

Effects of Celecoxib on Circulating Angiogenesis Biomarkers in Patients with FAP. We assessed the effects of celecoxib treatment for 1 week on circulating levels of 14 angiogenesis proteins [heparin-binding epidermal growth factor, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, interferon-inducible protein 10, platelet-derived growth factor (PDGF)-BB, keratinocyte growth factor, angiogenin, angiopoietin-1, angiopoietin-2, vascular endothelial growth factor (VEGF)-A, VEGF-D, soluble intercellular adhesion molecule (sICAM)-1, fibroblast growth factor (FGF)-2, and growth factor (HGF)] (Figs. 4 and 5; Supplemental Table S1). Celecoxib caused a significant increase in the proangiogenic markers FGF-2, VEGF-D, VEGF-A, and angiogenin (Fig. 4) and angiopoietin-2 (Fig. 5a). However, PDGF-BB was significantly reduced by celecoxib treatment (Fig. 5b). TIMP-2, an inhibitor of angiogenesis through mechanisms involving mainly the inhibition of matrix metalloproteinase activity (Stetler-Stevenson and Seo, 2005), was significantly increased.
increased ($p < 0.01$) (Fig. 5c), whereas sICAM-1, which is believed to play a role in tumor cell resistance to cell-mediated cytotoxicity (Fiore et al., 2002), was significantly inhibited ($p < 0.01$) by celecoxib (Fig. 5d).

Relationships among Urinary Biomarkers of Prostanoid Biosynthesis In Vivo and Circulating Angiogenesis Proteins in Patients with FAP. A statistically significant inverse Spearman’s rank correlation was detected between the urinary PGI-M, assessed at baseline and after dosing with celecoxib, and the two growth factors, HGF ($r_s = 0.48; p < 0.05$) and FGF-2 ($r_s = -0.47; p < 0.05$) (Supplemental Fig. S3). We did not find any correlation among urinary PGE-M or TX-M and circulating angiogenic proteins.

Celecoxib caused an increase in six circulating angiogenesis proteins (both proangiogenic and antiangiogenic) (Figs. 4 and 5; Supplemental Table S1). Thus, we tested the relationship among PGI-M, TX-M, and PGE-M and these circulating proteins (Supplemental Table S2). In linear multiple regression analysis of log$_{10}$-transformed data, among the $X$ variables that resulted independent of each other, the only one that was significantly related to TX-M was PGI-M ($\beta = 0.80; \text{S.E.M.: } 0.27; P = 0.02$), whereas PGI-M was positively related to TX-M ($\beta = 0.50; \text{S.E.M.: } 0.17; P = 0.01$) and inversely related to angiogenin ($\beta = -0.44; \text{S.E.M.: } 0.16; P = 0.02$) and FGF-2 ($\beta = -0.24; \text{S.E.M.: } 0.10; P = 0.03$) in a statistically significant fashion. PGE-M was significantly correlated with TX-M ($\beta = 0.77; \text{S.E.M.: } 0.20; P = 0.003$), but not with any of the angiogenesis markers.

Discussion

Several lines of evidence support the role of COX-2-dependent PGE$_2$ in colon tumorigenesis (Wang and Dubois, 2010). Thus, in FAP, the administration of the selective COX-2 inhibitor celecoxib (400 mg/b.i.d.) was associated with a significant reduction of the number of colorectal polyps by ap-
approximately one-third (Steinbach et al., 2000). However, marked variability in the response to celecoxib was noted (Steinbach et al., 2000). We hypothesized that the inhibition of vascular COX-2-dependent PGI₂ may contribute to the variable response to celecoxib in this setting. In fact, PGI₂ may control angiogenesis by preventing endothelial activa-

Fig. 4. Effect of the administration of celecoxib on circulating angiogenesis biomarkers [FGF-2 (a), VEGF-D (b), VEGF-A (c), and angiogenin (d)] in patients with FAP. Heparinized blood samples were collected before dosing and on the seventh day 4 h after the last morning dose of celecoxib to assess the levels of circulating angiogenesis proteins. Data are presented as box and whiskers (n = 9); *, p < 0.05; **, p < 0.01 versus baseline using Wilcoxon matched-pairs test.

Fig. 5. Effect of the administration of celecoxib on circulating angiogenesis biomarkers [angiopoietin-2 (a), PDGF-BB (b), TIMP-2 (c), and sICAM-1 (d)] in patients with FAP. Heparinized blood samples were collected before dosing and on the seventh day 4 h after the last morning dose of celecoxib to assess the levels of circulating angiogenesis proteins. Data are presented as box and whiskers (n = 9); *, p < 0.05; **, p < 0.01 versus baseline using Wilcoxon matched-pairs test.
tion and platelet release of angiogenic factors present in α-granules (Menter et al., 1987). Thus, we performed the present study to investigate the biosynthesis of PGI2 and TXA2, two key mediators of CV homeostasis (Grosser et al., 2006), and PGE2, a well known mediator of inflammation and tumorigenesis (Wang and Dubois, 2010) in intestinal neoplasia. We aimed to explore the impact of selective inhibition of COX-2 by celecoxib on circulating biomarkers of angiogenesis in vivo in FAP and correlate them with the biosynthesis of PGI2, TXA2, and PGE2 in vivo.

We found for the first time that intestinal tumorigenesis is associated with enhanced TXA2 biosynthesis in vivo that was not inhibited by the administration of the selective COX-2 inhibitor celecoxib. This finding suggests a COX-1-dependent pathway for enhanced TX generation, presumably from platelets. To exclude the possible contribution of clinically undetectable CV disease to enhanced TXA2 biosynthesis in vivo in FAP, we assessed urinary levels of a major enzymatic metabolite of TX in a mouse model of colon tumorigenesis, i.e., Apc(Min/+) mice (Moser et al., 1995). It is noteworthy that we detected increased generation of TXA2 in vivo compared with wild-type mice. To address the hypothesis that epithelial tumorigenesis is associated with platelet activation, we performed an in vitro study using cocultures of a human colon adenocarcinoma cell line (HT-29 cells) and isolated human platelets. It is quite interesting that HT-29 cells triggered platelet TXA2 generation, which was almost completely inhibited by pretreatment of platelets with aspirin. As reported previously there is multiplicity of molecular mechanisms that can be used by cancer cells to activate platelets and enhance TXA2 generation (Jurazs et al., 2004). Altogether, our results show that intestinal tumorigenesis is associated with enhanced TXA2 generation through the COX-1-pathway. TXA2 has been reported to be involved in the angiogenesis and development of tumor metastasis (Honn, 1983). Thus, pharmacological inhibition of TXAS has been shown to significantly inhibit tumor cell growth, invasion, metastasis, and angiogenesis in a range of experimental models (Honn, 1983). Moreover, in a recent study, aspirin reduced the incidence and mortality of colorectal cancer, at doses of at least 75 mg daily (Rothwell et al., 2011), which is recommended for prevention against heart disease (Patrono et al., 2005), consistent with inhibition of platelet TXA2 being central aspirin’s efficacy in cancer prevention (Patrono et al., 2001).

It has been shown that tumor cell-derived products may cause endothelial dysfunction and increase vascular permeability (Padua et al., 2008). This phenomenon may facilitate the interaction of platelets with tumor constituents that are capable of inducing platelet activation (Pacienza et al., 2008). In this scenario, vascular PGI2 may play an important role by curbing platelet activation and the release of α-granules, which segregate angiogenesis regulatory proteins (Menter et al., 1987). This hypothesis was confirmed by our results showing that the inhibition of COX-2-dependent PGI2 by celecoxib was associated with complex changes in circulating markers of angiogenesis with enhanced levels of growth and angiogenesis factors (Figs. 4 and 5). It is noteworthy that in multiple regression analysis circulating levels of angiogenin, an inducer of angiogenesis present in platelets and released in response to agonist stimulation (Coppinger et al., 2007), were inversely related to urinary PGI-M. There was also an inverse Spearman’s correlation between urinary excretion of PGI-M and plasma levels of two mediators of angiogenesis and tumor progression, i.e., FGF-2 (Hanahan and Folkman, 1996) and HGF (Jiang et al., 1999) (Supplemental Fig. S3). However, we found a coincident increase of the plasma levels of a matrix metalloproteinase inhibitor TIMP-2 (Stetler-Stevenson and Seo, 2005), and its concentrations inversely correlated with circulating levels of sICAM (rs = -0.86; p < 0.01), considered a hallmark of tumor cell evasion of immune surveillance (Fiore et al., 2002).

A COX-2/mPGES-1 pathway has been implicated in PGE2 biosynthesis by colon cancer cells (Yoshimatsu et al., 2001). This has been confirmed here by our observation of enhanced systemic biosynthesis of PGE2 in patients with FAP, which was profoundly reduced by celecoxib. It is noteworthy that we did not find any correlation between urinary PGE-M and circulating angiogenic factors, suggesting the origin of these circulating proteins to be outside of the tumor proper. However, in multiple linear regression analysis, we found that PGE-M was significantly correlated with TX-M (Supplemental Table S2), which may suggest that enhanced TXA2 generation in vivo contributed to COX-2-dependent PGE2 generation in colon tumorigenesis.

In addition to COX-2-dependent prostanooids, emerging data suggest that leukotrienes (LTs), generated from arachidonic acid (AA) through the activity of 5-lipoxygenase, can have a role in carcinogenesis (Wang and Dubois, 2010). Both COX-2 and 5-lipoxygenase use AA as the substrate for eicosanoid biosynthesis, thus free AA accumulation, as a consequence of COX-2 inhibition by celecoxib, might lead to the increase of LT biosynthesis. Indeed, Duffield-Lillico et al. (2009) have shown that urinary levels of LTE4 (the end product of the cysteinyl LT metabolism) (Wang and Dubois, 2010) is increased in celecoxib-treated smokers with elevated COX-2 activity manifested by high baseline PGE-M levels. Whether this phenomenon occurs in patients with FAP treated with celecoxib was not assessed in the present study, but it deserves to be investigated in a specific study.

In summary, we show that COX-1-dependent TXA2 is enhanced in colon tumorigenesis. In this setting, vascular COX-2-dependent PGI2 may play a protective role by restraining the release of growth and angiogenesis factors from platelets and the generation of angiogenesis mediators from different cell types (Supplemental Fig. S4). The administration of a selective COX-2 inhibitor, such as celecoxib, caused a profound inhibition of COX-2-dependent PGI2, thus leaving enhanced TXA2 generation unconstrained. This may explain the complex changes in circulating markers of angiogenesis with enhanced levels of both proangiogenesis and antiangiogenesis factors. Although the inhibition of COX-2-dependent PGE2 may reduce tumor progression (Steinbach et al., 2000), the coincident effects on vascular PGI2 may have undesirable effects, predisposing to thrombosis (Grosser et al., 2006) and modulating angiogenesis. These contrasting effects may contribute to the marked variability in the reduction of the number of colorectal polyps detected in patients with FAP by chronic treatment with celecoxib.

It is noteworthy that recently Pfizer has voluntarily withdrawn celecoxib’s indication for reduction of colorectal polyps in patients with FAP. The sponsor was unable to provide further efficacy data, as a result of slow enrollment in an ongoing clinical trial (http://www.ema.europa.
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