Effects of Celecoxib on Prostanoid Biosynthesis and Circulating Angiogenesis Proteins in Familial Adenomatous Polyposis^[S]

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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 PGF_{1 α} (PGI-M), 11-dehydro-TXB₂ (TX-M), and 11- α -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.

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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 PGF_{1 α} ; PGE-M, 11- α -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 (Patrino 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 prostanoid 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 prostanoid 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 prostanoids 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 (Patrino 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 ability of PGI $_2$ to exert an antitumor effect, and if so, whether this occurs through direct inhibition of platelet activation (Grosser et al., 2006).

The selective COX-2 inhibitor celecoxib was approved by the Food and Drug Administration for the treatment of familial adenomatous polyposis (FAP) (400 mg b.i.d., which is a 4-fold higher dose than that recommended for analgesia). This decision was based on the results of the clinical study showing that in patients with FAP 6 months of treatment with celecoxib at 400 mg b.i.d., but not at 100 mg b.i.d., reduced the number of colorectal polyps by approximately one-third. However, marked variability in the response to celecoxib was noted, both at 100 and 400 mg b.i.d. (Steinbach et al., 2000). Thus, it is of clinical relevance to identify 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, nonsmokers, 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-

TABLE 1

Baseline characteristics of patients with FAP and healthy controls

Arterial hypertension is defined as current systolic/diastolic blood pressure $\geq 140/90$ mm Hg (referring to the 1999 World Health Organization criteria for the diagnosis of hypertension).

Variable	FAP Patients (n = 9)	Controls (n = 9)
Age, mean $\pm$ S.D., years ^a	44 $\pm$ 11	45 $\pm$ 10
Female, n, %	5, 55	5, 55
C-reactive protein, mg/l, mean $\pm$ S.D.	10 $\pm$ 19	4 $\pm$ 2
Median (range) ^b	3 (1.10–61.60)	3.30 (1.40–9.50)
Hypertension, %	0	0
Low-density lipoprotein cholesterol, mg/dl, mean $\pm$ S.D., median (range) ^b	105 $\pm$ 26, 97 (65–144)	106 $\pm$ 27, 103 (60–147)
Cotinine, ng/ml, mean $\pm$ S.D., median (range)	7.20 $\pm$ 19.60, 0.62 (0.40–59.40)	Not determined
TX-M, ng/mg creatinine, mean $\pm$ S.D., median (range) ^b	1.19 $\pm$ 0.84, 0.93 (0.10–2.90)**	0.20 $\pm$ 0.10, 0.20 (0.09–0.40)
PGI-M, ng/mg creatinine, mean $\pm$ S.D., median (range) ^b	0.12 $\pm$ 0.09, 0.09 (0.03–0.33)	0.12 $\pm$ 0.05, 0.10 (0.05–0.20)
PGE-M, ng/mg creatinine, mean $\pm$ S.D., median (range) ^b	22.30 $\pm$ 16.20, 21.52 (2.82–55.40)*	10.30 $\pm$ 5, 11.22 (4–16)
APC mutation, n, %	8, 88.9	Not determined
MYH mutation, n, %	1, 11.1	Not determined

^aData were compared by Student's *t* test.^bData were compared by Mann-Whitney test.**P* < 0.05; ***P* < 0.01 vs. healthy controls.

lecular 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-PGF_{1 α} (PGI-M), a major enzymatic metabolite of PGI₂, by reversed-phase high-performance liquid chromatography/RIA technique [validated by comparison with ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) (Song et al., 2007) (Supplementary Fig. S1 and Supplemental Materials and Methods)]. PGI-M and TX-M are indexes of PGI₂ and TXB₂ generation in vivo (FitzGerald et al., 1983). Moreover, in the same urine collections we assessed the levels of 11- α -hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostanoic acid (PGE-M) by UPLC/MS/MS (Song et al., 2007), an index of PGE₂ generation in vivo (Murphey et al., 2004) which has been used as a biomarker for risk assessment of colorectal cancer (Csiki et al., 2005). Metabolite levels were corrected for urinary creatinine assessed by UPLC/MS/MS.

Inhibition of Human Whole Blood COX-1 and COX-2 by Celecoxib In Vitro. It was assessed using previously published whole blood assays (Patrino et al., 1980; Patrignani et al., 1994). A brief description is reported in Supplemental Materials and Methods.

Studies in *Apc(Min/+)* Mice. In vivo prostanoid 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 μ l/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): PGE-M, 2,3-dinor-thromboxane B₂ (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, 1×10^6 cells were seeded in six-multiwell plates containing 2 ml of McCoy 5A supplemented with 0.5% fetal bovine serum and 10 μ g/ml of Polimyxin B sulfate (Sigma-Aldrich, Milan, Italy) for 20 h, alone or cocultured with platelets. Human platelets were freshly isolated from leukocyte con-

centrates obtained from Stadtische Kliniken Höchst (Frankfurt, Germany) as reported previously (Albert et al., 2002) (briefly described in Supplemental Materials and Methods). HT-29 cells (1×10^6 cells) were cultured alone or with washed human platelets (1×10^8 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 (Patrino 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 μ M 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 μ M; Witega Laboratorien, Berlin, Germany) on prostanoid biosynthesis by HT-29 cells cultured alone or with platelets for 20 h were evaluated.

Statistical Analyses. Values were reported as mean $\pm$ 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_s*) 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, PGE-M, TX-M, and circulating angiogenesis proteins. Multicollinearity of biomarkers was verified by assessing the individual *r*² and variance inflation factor. Variables with *r*² 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 celecoxib 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 celecoxib 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 celecoxib 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 $\pm$ 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 $\pm$ 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 $58 \pm 9\%$ (Fig. 2b) and PGE₂ by $48 \pm 31\%$ (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 (mean $\pm$ 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

Fig. 1. Biosynthesis of prostanoids in vivo in *Apc*(Min/+) mice versus wild type (WT). Urinary excretion of dinor-TX-M (a), PGI-M (b), and PGE-M (c) in *Apc*(Min/+) mice and wild-type mice. Data are expressed as the mean $\pm$ S.E.M. Five mice were used for each group. Metabolite levels were corrected for urinary creatinine and expressed as ng/mg creatinine. *, $p < 0.05$; **, $p < 0.01$ versus wild-type mice using Student's *t* test.

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. 2. Biosynthesis of prostanoids in patients with FAP before and after celecoxib treatment (400 mg b.i.d., for 7 consecutive days). Systemic production of TXA₂ (a), PGI₂ (b), and PGE₂ (c) was assessed by measuring their enzymatic urinary metabolites, i.e., TX-M, PGI-M, and PGE-M, respectively, in samples collected before treatment (from 8:00 PM to 8:00 AM) and on the eighth day after the last dose of the drug. Data were presented as box and whiskers, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and whiskers represent the highest and lowest values ($n = 9$). Metabolite levels were corrected for urinary creatinine and expressed as ng/mg creatinine. *, $p < 0.05$; **, $p < 0.01$ versus baseline using Wilcoxon matched-pairs test.

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 experi-

mental 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 $\pm$ 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

Fig. 3. Prostanoid biosynthesis and protein expression in human adenocarcinoma cell line HT-29, human platelets, and HT-29 cocultured with platelets. **a**, TXB₂ generation by HT-29 alone (HT), HT-29 cocultured with platelets (HT+PLT), and platelets alone (PLT) for 20 h. In some experiments, the effects of platelets pretreated with 300 μM aspirin was studied. Mean ± S.E.M. of 5–10 different experiments are shown. **, $p < 0.01$ versus HT-29 alone and platelets alone, \$, $p < 0.01$ versus untreated platelets, #, $p < 0.01$ versus HT-29 cocultured with untreated platelets). Repeated-measures analysis of variance was used for statistical analysis. **b**, the effect of rofecoxib (0.3 μM) on TXB₂ generation by HT-29 alone, platelets alone, and HT-29 cocultured with platelets for 20 h. Means of two different experiments performed in triplicate are shown. **c**, the effect of rofecoxib (0.3 μM) on PGE₂ generation by HT-29 alone. *, $p < 0.05$ versus dimethyl sulfoxide (DMSO) (vehicle). Student's *t* test was used for statistical analysis. **d**, COX-1, COX-2, TXAS, and mPGES-1 protein levels were assessed by Western blot.

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 regres-

sion analysis of log₁₀-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 (β : 0.80; S.E.M.: 0.27; $P = 0.02$), whereas PGI-M was positively related to TX-M (β : 0.50; S.E.M.: 0.17; $P = 0.01$) and inversely related to angiogenin (β : -0.44; S.E.M.: 0.16; $P = 0.02$) and FGF-2 (β : -0.24; S.E.M.: 0.10; $P = 0.03$) in a statistically significant fashion. PGE-M was significantly correlated with TX-M (β : 0.77; 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 PGE₂ 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-

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.

proximately 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 PGI_2 may contribute to the variable response to celecoxib in this setting. In fact, PGI_2 may control angiogenesis by preventing endothelial activa-

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 (Jurasz 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 ($r_s = -0.86$; $p < 0.01$), 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 prostanoids, 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>).

eu/docs/en_GB/document_library/Press_release/2011/05/WC500106524.pdf).

Authorship Contributions

Participated in research design: Maier, Bertario, Dixon, Steinhilber, and Patrignani.

Conducted experiments: Dovizio, Tacconelli, Ricciotti, Bruno, Anzellotti, Di Francesco, Sala, Signoroni, and Dixon.

Contributed new reagents or analytic tools: Ricciotti, Maier, and Lawson.

Performed data analysis: Dovizio, Tacconelli, Bruno, Anzellotti, Di Francesco, Bertario, FitzGerald, and Patrignani.

Wrote or contributed to the writing of the manuscript: Dovizio, Tacconelli, Ricciotti, FitzGerald, and Patrignani.

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