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 PGI-M, thromboxane (TX) A₂, and prostaglandin (PG) E₂, assessing their urinary enzymatic metabolites, 2,3-dinor-6-keto PGF₁α, 11-dehydro-TXB₂ (TX-M), and 11-α-hydroxy-9,15-dioxyo-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. Moreover, 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
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) E₂ is a key prostanoid in tumorigenesis generated through the activity of coordinate expression of COX-2 and microsomal PGE₂ synthase-1 (mPGES-1), an enzyme downstream of COX-2 (Wang and Dubois, 2010). PGE₂ 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 prostanooids to colon cancer development is less clear. Thromboxane (TX) A₂ and prostacyclin (PGI₂) play important roles in cardiovascular (CV) homeostasis (Grosser et al., 2006). In particular, TXA₂, a major product of platelet COX-1, promotes platelet aggregation and vasoconstriction, whereas PGI₂, a major product of endothelial COX-2, inhibits platelet aggregation and promotes vasodilatation. It is noteworthy that it has been shown that enhanced TXA₂ and PGI₂ generation by the introduction of the downstream TXA₂ synthase (TXAS) and PGI₂ 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 PGI₂ 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 TXA₂ 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 PGI₂ 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 PGI₂ generated by COX-2 in endothelial cells, thus leaving unconstrained all mediators that stimulate platelets, elevate blood pressure, and accelerate atherosclerosis, including TXA₂ (Grosser et al., 2006).

In the present study, we investigated the biosynthesis of TXA₂, PGI₂, and PGE₂ 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 TXA₂ biosynthesis in patients with intestinal neoplasia. To exclude the possible contribution of clinically undetectable CV disease to enhanced TXA₂ 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 TXA₂ 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 PGI₂ by celecoxib would be associated with complex changes in circulating markers of angiogenesis possibly because PGI₂ 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-
For every experiment, 106 cells were cultured in McCoy’s 5A medium (Invitrogen, Milan, Italy) for 20 h. Hypertension was determined by a validated radioimmunoassay (RIA) technique (Patrono et al., 1980; Patrignani et al., 1994). A brief description of celecoxib (Sandoz, Vienna, Austria) and rofecoxib (Witega Laboratorien, Berlin, Germany) was given. 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 PGI-M, a sample size of nine individuals was chosen. The Spearman rank correlation coefficient (r) was calculated to quantify the statistical dependence between two variables. Linear multiple regression analysis of log10-transformed data was performed to test the relationship between PGI-M, PGE2, 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 celecoxib in patients with FAP were assessed using Wilcoxon matched-pairs test. All analyses were performed 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 or equal to 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

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**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>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± S.D., years</td>
<td>44 ± 11</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>Female, n, %</td>
<td>5, 55</td>
<td>5, 55</td>
</tr>
<tr>
<td>C-reactive protein, mg/l, mean ± S.D.</td>
<td>10 ± 19</td>
<td>4 ± 2</td>
</tr>
<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</td>
<td>0</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>Cytokine, mg/ml, mean ± S.D., median (range)</td>
<td>7.20 ± 19.60, 0.62 (0.40–59.40)</td>
<td>Not determined</td>
</tr>
<tr>
<td>TX-M, mg/mg creatinine, mean ± S.D., median (range)</td>
<td>1.19 ± 0.84, 0.93 (0.10–2.90)**</td>
<td>0.20 ± 0.10, 0.20 (0.09–0.40)</td>
</tr>
<tr>
<td>PGI-M, mg/mg creatinine, mean ± S.D., median (range)</td>
<td>0.12 ± 0.09, 0.09 (0.03–0.33)</td>
<td>0.12 ± 0.05, 0.10 (0.05–0.20)</td>
</tr>
<tr>
<td>PGE-M, ng/mg creatinine, mean ± S.D., median (range)</td>
<td>22.30 ± 16.20, 21.52 (2.82–55.40)**</td>
<td>10.30 ± 5.11, 12.42 (4–16)</td>
</tr>
<tr>
<td><strong>MYH mutation, n, %</strong></td>
<td>8, 88.9</td>
<td>Not determined</td>
</tr>
<tr>
<td><strong>APC mutation, n, %</strong></td>
<td>1, 11.1</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

*Data were compared by Student’s t test.

**P < 0.05; **P < 0.01 vs. healthy controls.
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 biosynthesis in vivo in FAP, as shown in 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-
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 decreased.
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 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-
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 PGI2 may contribute to the variable response to celecoxib in this setting. In fact, PGI2 may control angiogenesis by preventing endothelial activa-

![Graphs showing effect of celecoxib on angiogenesis biomarkers](https://example.com/graphs)

**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.
The present study investigated the biosynthesis of PGI$_2$ and TXA$_2$, two key mediators of CV homeostasis (Grosser et al., 2006), and PGE$_2$, 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 PGI$_2$, TXA$_2$, and PGE$_2$ in vivo.

We found for the first time that intestinal tumorigenesis is associated with enhanced TXA$_2$ 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 TXA$_2$ 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 TXA$_2$ 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 TXA$_2$ 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 TXA$_2$ generation (Jurasz et al., 2004). Altogether, our results show that intestinal tumorigenesis is associated with enhanced TXA$_2$ generation through the COX-1-pathway. TXA$_2$ 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 TXA$_2$ 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 (Paciencia et al., 2008). In this scenario, vascular PGI$_2$ may play an important role by curbing platelet activation and the release of alpha-granules, which may act as angiogenesis regulatory proteins (Menter et al., 1987). This hypothesis was confirmed by our results showing that the inhibition of COX-2-dependent PGI$_2$ 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 PGE$_2$ biosynthesis by colon cancer cells (Yoshimatsu et al., 2001). This has been confirmed here by our observation of enhanced systemic biosynthesis of PGE$_2$ 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 TXA$_2$ generation in vivo contributed to COX-2-dependent PGE$_2$ 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 LTE$_4$ (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 TXA$_2$ is enhanced in colon tumorigenesis. In this setting, vascular COX-2-dependent PGI$_2$ 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 PGI$_2$, thus leaving enhanced TXA$_2$ 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 PGE$_2$ may reduce tumor progression (Steinbach et al., 2000), the coincident effects on vascular PGI$_2$ 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.
Authorship Contributions

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

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

Conferred new agents 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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rization of the cyclooxygenase activity of human blood prostaglandin endoperox-


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Effects of celecoxib on prostanoid biosynthesis and circulating angiogenesis proteins in familial adenomatous polyposis

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Supplementary Material and Methods

**Human Angiogenesis Protein Array**
The levels of human angiogenesis markers heparin-binding epidermal growth factor (HB-EGF), tissue inhibitor of metalloproteinases (TIMP)-1 and -2, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) A and D, interferon-inducible protein (IP)-10, platelet derived growth factor (PDGF)-BB, keratinocyte growth factor (KGF), soluble intercellular adhesion molecule (ICAM)-1, fibroblast growth factor (FGF)-2, angiogenin, and angiopoietin-1 and -2, were assessed in plasma of FAP patients before dosing and on the 7th day, 4 hr after the last morning dose of celecoxib, using an antibody protein array kit (US Biomax, Inc.) according to the vendors protocol.

**Assessment of 2,3-dinor-6-keto-PGF₁α (PGI-M) by HPLC-RIA**
The method consists of i) urine extraction (by using Sep-Pack C₁₈ cartridges, Waters Associates, Milan, Italy), ii) separation of PGI-M from 6-keto-PGF₁α by reversed-phase (RP)-HPLC and iii) quantification of PGI-M concentrations in HPLC fractions, corresponding to the retention time (RT) of PGI-M, by a sensitive RIA. The RIA used a commercially available polyclonal antibody (Assay Designs Inc, Ann Arbor, MI, USA) developed against PGI-M, but showing high cross-reactivity with 6-ketoPGF₁α (89%). Since the anti-PGI-M antibody showed a very low cross-reactivity with TXB₂ (<0.01%), we developed a RP-HPLC method where PGI-M co-eluted with TXB₂. Thus, recovery evaluation of endogenous PGI-M was performed by assessing [³H]-TXB₂ content (previously added to urine aliquots), in the same HPLC fractions that were analyzed for PGI-M by RIA.

Briefly, 3000 cpm of [³H]-TXB₂ (specific activity, 150mCi/mmol; GE Healthcare Life Science, Milan, Italy) was added to 10 ml aliquots of urine samples for recovery evaluation. After adjustment of urine pH to 4.0-4.5 with formic acid, prostanoids were extracted by using Sep-Pack C₁₈ cartridges and eluted with ethyl acetate and then dried with a Speed-Vac (Speed Vac Plus, Savant Instruments Inc, Farmingdale, NY, USA). All solvents were from Carlo Erba Reagents (Milan, Italy). After dryness, prostanoids were resuspended with 0.1ml water and 0.1 ml methanol, and injected into an HPLC system.

The HPLC system consisted of a Beckman model HPLC pump (Beckman, Fullerton, CA, USA), a Beckman 126 programmable solvent delivery module and a Beckman 168 programmable ultraviolet detector, with a Beckman System GOLD 32 Karat Software. A reversed-phase column was used, i.e. Novapak C₁₈ (3.9x150mm) (Waters Associates, Milan, Italy). The mobile phase consisted of A, water/acetic acid [100:0.1 (v/v)] and B, acetonitrile/acetic acid [100:0.1 (v/v)] as follows: 79% and 21% respectively, at a flow rate of 1 ml/min for 18 minutes. Then, the mobile phase was changed into A 50% and B 50%, at a flow rate of 1.5ml/min for the following 7 minutes. The absorbance was assessed at 195 nm. In these conditions, the retention times (RTs) of authentic 6-keto-PGF₁α, TXB₂ and PGI-M (Cayman Chemical, Ann Arbor, MI, USA) were 14.5, 20.3 and 20.5 min, respectively. One-minute- HPLC fractions were collected and dried with the Speed-Vac and then resuspended in 0.5ml of phosphate buffer (0.02M, pH7.4). HPLC fractions eluted from 18 to 22 minutes were mixed, evaluated for [³H]-TXB₂ content (to calculate the recovery that was 50%) and then stored at –80°C until the measurement of PGI-M by RIA. The assay used 2500cpm of [³H]-6-keto-PGF₁α (specific activity, 185mCi/mmol; GE Healthcare Life Science, Milan, Italy) and an anti-PGI-M polyclonal antibody (at the final dilution of 1:100000) in a final volume of 1.5 ml of phosphate buffer (0.02M, pH 7.4) and was incubated for 18-24 h at 4°C. Separation of the antibody-bound from free [³H]-6-keto-PGF₁α was carried out by rapidly adding 0.05ml of bovine serum albumin and 0.1 ml of a charcoal suspension (100 mg/ml) followed by centrifugation at 3000 rpm/10min at 4°C. The detection limit of the RIA was 2 pg/ml. We assessed the intra-assay and inter-assay coefficients of variation (CV) by evaluating PGI-M concentrations in urine samples (collected from 3 healthy volunteers treated with Naproxen 500 mg - to suppress endogenous PGI₂
generation - after providing their informed consent and approval by the institutional Ethical Committee) spiked with known concentrations of PGI-M: Low Quality Control (QC) (200 pg/ml added), Mid QC (400 pg/ml added) and High QC (800 pg/ml added). The intra-assay CVs (n=6) were 9, 6, and 9%, for low QC, mid QC and high QC, respectively. The inter-assay CVs (n=3) were 13, 7 and 11%, for low QC, mid QC and high QC, respectively.

**Inhibition of human whole blood COX-1 and COX-2 by celecoxib in vitro**

Approval from Ethics Committee of “G. d’Annunzio” University, Chieti, Italy, was obtained for peripheral whole blood collection from 3 healthy male volunteers (age range: 25-29 years) and informed consent was obtained from each subject. The same healthy volunteers were studied on different occasions. Celecoxib (1.9-19,000 mg/ml, kindly provided by Searle, USA) was dissolved in DMSO, and 2 µl aliquots of the solutions were pipetted directly into test tubes to give final concentrations of 3.8-38,000 ng/ml in 1 ml of whole blood. The effect of celecoxib on inducible monocyte PGE$_2$ synthesis was assessed by incubating the drug with heparinized peripheral venous blood samples, in the presence of lipopolysaccharide (LPS, 10 µg/ml) for 24 hr at 37 ºC, as previously described (Patrignani et al., 1994). The contribution of platelet COX-1 activity was suppressed by pretreating the subjects with 300 mg of aspirin 48 hr before sampling. The effect of celecoxib on platelet COX-1 activity was assessed by incubating the drug with 1 ml of whole blood samples (drawn from the same donors in aspirin-free periods) and then they were allowed to clot for 1 hr at 37 ºC (Patrano et al., 1980). Plasma PGE$_2$ and serum TXB$_2$ were measured by validated RIA (Patrignani et al., 1994; Patrano et al., 1980).

**Culture of human colon adenocarcinoma cell line (HT-29)**

HT-29 cell line was obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK) and the quality control and authentication procedures were performed by ECACC. Cells were routinely tested for mycoplasma contamination by the authors using a PCR approach. HT-29 cells were cultured in McCoy’s 5A medium (Invitrogen, Milan, Italy) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and L-glutamine 2 mM. For every experiment, 1x10$^6$ cells were seeded in 6-multiwell containing 2 ml of McCoy 5A supplemented with fetal bovine serum (FBS) 0.5% and 10 µg/ml of Polymyxin B sulphate (Sigma-Aldrich, Milan, Italy) for 20 hr, alone or co-cultured with platelets.

**Preparation of washed human platelets**

Human platelets were freshly isolated from leukocyte concentrates obtained from Stadtische Kliniken Hochst (Frankfurt, Germany) as previously described (Albert et al., 2002). In brief, venous blood was collected from healthy adult donors and leukocyte concentrates were prepared by centrifugation (4000xg, 20 min, 20 ºC). Leukocyte concentrate was sedimented in 5% dextran solution (Sigma-Aldrich) and the supernatant was stratified in lymphocytes separation medium (PAA) by centrifugation (800xg, 10 min at room temperature). After centrifugation, platelet-rich plasma (PRP) was obtained. PRP was then mixed with PBS, pH 5.9 (3:2, v/v), centrifuged (2000xg, 15 min, room temperature), and pelleted platelets were resuspended in PBS, pH 5.9/0.9% NaCl (1:1, v/v), washed by centrifugation (2000xg, 10 min, room temperature), and finally re-suspended in McCoy 5A medium containing 0.5% FBS and 10 µg/ml of Polymyxin B sulphate.

**Western blot analyses**

As previously described (Di Francesco et al., 2009), cells were lysed in Triton 1% with 1 mM of PMSF and 50 µg of total proteins were loaded onto 4-9% Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Separated proteins were transferred to nitrocellulose membranes (GE Healthcare, Milan, Italy) and incubated with anti-COX-2 (1:1000, mouse monoclonal, Cayman Chemical, USA), anti-COX-1 (1:1000, rabbit polyclonal, Cayman
Chemical), anti-mPGES-1 (1:500, rabbit polyclonal, Cayman Chemical), anti-TXAS (1:1000, rabbit polyclonal, Cayman Chemical) and anti-β-actin (1:2000, goat polyclonal, Santa Cruz, USA) overnight at 4°C. Then, the membranes were washed in TBS-Tween-20 and incubated with the secondary antibodies. Detection of bands was performed using a LI-COR Odyssey two-color Western detection system (LI-COR Biosciences, Biosciences, Lincoln, NE, USA), according to the instructions of the manufacturer.
Supplementary Table S1. Circulating angiogenesis proteins detected before and after dosing with celecoxib, in 9 FAP patients

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post-treatment</th>
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</thead>
<tbody>
<tr>
<td><strong>HB-EGF</strong></td>
<td>624±379, 666(106-1033)</td>
<td>1248±1807, 387(121-5304)</td>
</tr>
<tr>
<td><strong>TIMP-1</strong></td>
<td>4494±1107, 4525(2424-6001)</td>
<td>3675±1121, 3645(2053-5892)</td>
</tr>
<tr>
<td><strong>TIMP-2</strong></td>
<td>2986±2255, 2892(667-8203)</td>
<td>10144±1227, 10021(8821-12126)**</td>
</tr>
<tr>
<td><strong>HGF</strong></td>
<td>555±356, 489(91-1232)</td>
<td>659±303.5, 648(51-1099)</td>
</tr>
<tr>
<td><strong>Angiopoietin-1</strong></td>
<td>1012±899, 983(112-2906)</td>
<td>1084±608.5, 1375(279-1811)</td>
</tr>
<tr>
<td><strong>Angiopoietin-2</strong></td>
<td>3515±2197, 2480(1589-7385)</td>
<td>5799±3727, 5479(2168-11516)*</td>
</tr>
<tr>
<td><strong>Angiogenin</strong></td>
<td>890±583, 715(196-2216)</td>
<td>1996±381, 1993(1287-2487)**</td>
</tr>
<tr>
<td><strong>IP-10</strong></td>
<td>670±225.5, 538(437-1032)</td>
<td>887±483, 808(264-1490)</td>
</tr>
<tr>
<td><strong>PDGF-BB</strong></td>
<td>1380±981, 944(511-3550)</td>
<td>703±834, 271(70-2103)*</td>
</tr>
<tr>
<td><strong>KGF</strong></td>
<td>241±126, 224(65-484)</td>
<td>271±188, 322(28-572)</td>
</tr>
<tr>
<td><strong>VEGF-A</strong></td>
<td>123±137, 76(1-432)</td>
<td>298±227, 216(23-803)**</td>
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<tr>
<td><strong>VEGF-D</strong></td>
<td>757±377, 956(122-1145)</td>
<td>1429±848, 1188(739-3539)*</td>
</tr>
<tr>
<td><strong>sICAM-1</strong></td>
<td>5642±3043, 5394(4-9614)</td>
<td>4.9±2.6, 5(2-9)**</td>
</tr>
<tr>
<td><strong>FGF-2</strong></td>
<td>32±46, 14(4-143)</td>
<td>77±104, 44(4-315)*</td>
</tr>
</tbody>
</table>

Data were reported as pg/ml of mean±SD, median(range).

*P<0.05, **P<0.01 vs baseline; Wilcoxon matched pairs test.
Supplementary Table S2. Linear multiple regression analysis among urinary biomarkers of prostanoid biosynthesis in vivo and circulating angiogenesis proteins in FAP patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SEM**</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGI-M</td>
<td>0.80</td>
<td>0.27</td>
<td>0.02</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>0.25</td>
<td>0.31</td>
<td>0.44</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>0.31</td>
<td>0.24</td>
<td>0.23</td>
</tr>
<tr>
<td>FGF-2</td>
<td>0.03</td>
<td>0.16</td>
<td>0.83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SEM**</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX-M</td>
<td>0.50</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>0.26</td>
<td>0.24</td>
<td>0.29</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>-0.44</td>
<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>FGF-2</td>
<td>-0.24</td>
<td>0.10</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SEM**</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX-M</td>
<td>0.77</td>
<td>0.20</td>
<td>0.003</td>
</tr>
<tr>
<td>PGI-M</td>
<td>0.50</td>
<td>0.26</td>
<td>0.07</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>-0.47</td>
<td>0.23</td>
<td>0.066</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>-0.20</td>
<td>0.19</td>
<td>0.32</td>
</tr>
<tr>
<td>FGF-2</td>
<td>0.13</td>
<td>0.12</td>
<td>0.29</td>
</tr>
</tbody>
</table>

*β: standardized coefficient; **SEM: mean standard error
Supplementary Figure S1. Comparison of measurements of major urinary enzymatic metabolites of PGI$_2$ and TXB$_2$, i.e. 2,3-dinor-6-keto-PGF$_{1\alpha}$ (PGI-M) (a) and 11-dehydro-TXB$_2$, (TX-M) (b), respectively, by specific radioimmuno assay (RIAs) and ultra performance liquid chromatography tandem mass spectrometric (UPLC/MS/MS). Urinary samples (n=18) collected from FAP patients before and after dosing with celecoxib were assessed for the levels of PGI-M and TX-M using specific RIAs [PGI-M analysis is described in Supplementary Methods, while TX-M analysis was performed by a previously described and validated technique (Ciabattoni et al., 1987)] or UPLC/MS/MS (Song et al., 2007). The least-squares line and coefficient of determination r$^2$ were calculated by linear regression analysis using PRISM (GraphPad, San Diego, California).
Supplementary Figure S2. Effects of celecoxib on platelet COX-1 and monocyte COX-2 activities in human whole blood. Concentration-response curves for inhibition of COX-1 (▲) and COX-2 (Δ) by celecoxib in vitro were plotted. Celecoxib caused a concentration-dependent inhibition of COX-1 and COX-2 in vitro with IC$_{50}$ values of 3444(2116-5604) and 128(72-228)ng/ml (mean, 95%CI), respectively. The range of plasma concentrations detected at 4 hr after dosing with celecoxib in FAP patients was reported.
Supplementary Figure S3. Statistical dependence between urinary levels of PGI-M and angiogenesis proteins in FAP patients. The Spearman’s rank correlation coefficient ($r_s$) was calculated to quantify the statistical dependence between urinary levels of PGI-M and angiogenesis proteins in FAP patients. A statistically significant inverse Spearman's rank correlation was found between the urinary levels of PGI-M, assessed at baseline and after dosing with celecoxib, and HGF and FGF-2 (Panel a and b, respectively).
Supplementary Figure S4. Possible mechanisms involved in the regulation of the generation and release of angiogenesis markers by PGI\(_2\) from endothelial cells (EC) and platelets.

In tumorigenesis, endothelial dysfunction and increased vascular permeability occur (Padua et al., 2008). This can facilitate the interaction of platelets with tumor components, such as galectin(GAL)-1, belonging to the galectin family of endogenous lectins(Pacienza et al., 2008) and/or extracellular matrix and basement membrane proteins, such as collagen. Activated platelets generate TXA\(_2\) which participates to the release of ADP from dense granules. In platelets, the activation of TXA\(_2\) receptor(TP), ADP receptor(P2Y\(_{12}\)), collagen receptor(GPVI) and the binding of GAL-1 to surface components, lead to the release of \(\alpha\)-granule content, rich in angiogenic proteins (both pro- and anti-angiogenic factors) (Italiano et al., 2008; Coppinger et al., 2007).

Endothelial PGI\(_2\), by interacting with PGI\(_2\) receptor(IP), restrains platelet activation and \(\alpha\)-granule release (Menter et al., 1987). Moreover, in endothelial cells, PGI\(_2\) can restrain HGF and FGF-2 expression by interfering with the binding of CREB(cAMP responsive element binding protein) and phospho-CREB to the CRE of their promoters (Kothapalli et al., 2003). This might occur through CREB phosphorylation at Ser142 which has been shown to block the formation of CREB-CBP(CREB binding protein) complexes and the activation of target genes (Sun et al. 1994). In the presence of the inhibition of endothelial COX-2-dependent PGI\(_2\), enhanced FGF-2 can participate in increased circulation levels of VEGF-A, VEGF-D and Angio(angiopoietin)-2. In fact, it has been reported that expression of both VEGF and Angio-2 is induced by FGF-2 (Seghezzi et al., 1998; Fujii and Kuwano, 2010). Angio-2 concentrations have been shown to be a marker of a poor prognosis in different types of cancers, i.e. breast cancer, non-small cell lung cancer and colorectal cancer (Shim et al., 2007). Different mechanisms may explain these findings: i) up-regulation of Angio-2 causes the exposure of the capillary endothelium to other angiogenic growth factors, including VEGF, that promote new vessel growth (Lobov et al., 2002); ii) enhanced levels of
Angio-2 may chemoattract pro-angiogenic Tie-2 expressing monocyte/macrophages and stimulate them to express tumor promoting factors (Lewis et al., 2007). In the presence of vascular COX-2-dependent PGI_2 inhibition, platelet activation participates to the increase of circulating levels of pro-angiogenic factors, such as VEGF, PDGF and angiogenin (Folkman et al., 2007), but also anti-angiogenic factors, such as TIMP-2. The release of TIMP-2 may lead to the inhibition of MMP(matrix metallo-proteinase) activity in tumor cells thus preventing ICAM-1 shedding from tumor cells (Fiore et al., 2002). This effect is important to restrain tumor cell evasion of immune surveillance. In fact, ICAM-1-mediated cell-cell adhesion is essential for various immunologic functions, such as natural killer(NK) cell-mediated cytotoxicity (Fiore et al., 2002).

PDGF-BB, a major PDGF-isoform stored in α-granules of platelets, governs cancer cell proliferation and survival and plays a role in angiogenesis (Heldin and Westermark, 1990). In a tumor-bearing mouse, it has been shown that platelets may uptake PDGF and a sustained and persistent elevation of PDGF levels in platelets is detected (Klement et al. 2009). The decrease of circulating levels of PDGF-BB observed after PGI_2 inhibition by celecoxib detected in FAP patients, in the present study, suggests that this prostanoid influences the uptake/release machinery operating in platelets which control α-granule content. Alternatively, it can be hypothesized that depression of circulating levels of PDGF-BB may be dependent on the increase of circulating levels of adiponectin, which is able to bind PDGF-BB. In fact, adiponectin, abundantly present in human plasma, can bind directly to PDGF-BB and regulates postreceptor signal in vascular smooth muscle cells (Arita et al., 2002). Interestingly, it has been found an inverse association between total adiponectin and colorectal adenoma (Wei et al., 2005).
Supplementary References


