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

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Running title: *Celecoxib and angiogenesis biomarkers in FAP*

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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 9 FAP patients and healthy controls, pairmatched for gender and age, we compared systemic biosynthesis of PGI₂, thromboxane (TX)A₂ and prostaglandin (PG)E₂ (assessing urinary enzymatic metabolites, PGI-M, TX-M and PGE-M, respectively). The impact of celecoxib (400mg/BID 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, unaffected by celecoxib; thus suggesting the involvement of a COX-1-dependent pathway, presumably from platelets. This was supported by the finding that in co-cultures of human colon adenocarcinoma cell line (HT-29) and platelets, enhanced TXA₂ generation was almost completely inhibited by pre-treatment 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 pro-angiogenesis proteins but also the anti-angiogenic TIMP-2. Urinary PGI-M, but not PGE-M, was negatively correlated with circulating levels of FGF-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 \( \alpha \)-granules into the tumor microenvironment (Italiano et al., 2008). Moreover, platelets and the factors which they release can upregulate cyclooxygenase (COX)-2, considered an early event of cell transformation (Patruno 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) \( \text{E}_2 \) is a key prostanoid in tumorigenesis generated through the activity of coordinate expression of COX-2 and mPGES-1 (microsomal PGE\(_2\) synthase-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). Recently, we have shown 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) \( \text{A}_2 \) 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. Interestingly, 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 (PGIS), 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 PGIS-expressing cells resulted in opposite effects (Pradono et al., 2002; De Bock et al., 2011).

Aspirin, even at low-doses (such as 75mg daily, recommended for the prevention against 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 FDA for the treatment of familial adenomatous polyposis (FAP) [400mg/BID, 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, six months of treatment with celecoxib 400mg/BID, but not celecoxib 100mg/BID, reduced the number of colorectal polyps by roughly one third. However, marked variability in the response to celecoxib was noted, both at 100mg and at 400mg/BID (Steinbach et al., 2000). Thus, it is of clinical relevance to identify potential mechanistic contributors to this variability in response. Importantly, 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 atherogenesis, 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 FAP patients and healthy controls,
pairmatched for gender and age, who were nonsmokers and without clinically detectable CV risk factors. In FAP patients, we performed an open-label study with a clinically relevant dose of celecoxib (400mg/BID for 7 days) to verify the COX-isozyme involved in TXA₂ biosynthesis in patients with intestinal neoplasia. In order 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 co-culture 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).

Methods

Clinical Study in FAP patients, design and assessments

Nine patients with FAP, recruited from the national hereditary colorectal tumor registry, National Cancer Institute (Milan, Italy), and 9 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 9 patients with FAP, we performed an open-label study with celecoxib (Pfizer) (400mg/BID for 7 consecutive days) that was previously approved by the institutional ethical committee. FAP patients had not had a complete colorectal resection and had 5 or more polyps, 2mm or more in diameter, that could be assessed endoscopically. Exclusion criteria included a history of colectomy within the previous 12 months; use of non steroidal anti-inflammatory drugs (NSAIDs) 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 ischaemia; intolerance or allergy to NSAIDs; pregnant women.

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 7th day, 4 hr after the last dose of celecoxib, to assess the levels of 14 circulating angiogenesis proteins using an angiogenesis antibody array kit (US Biomax, Inc., Rockville, MD, USA; the list of proteins analyzed is reported in Supplementary material and methods and Supplementary Table S1). Plasma cotinine levels were determined using an enzyme immunoassay kit (Cozart Biosciences, Oxford, UK). The presence of mutations in the APC gene, the earliest detectable molecular abnormality in colorectal cancer (Powell et al., 1992) and in the MutY human homologue (MYH) gene, associated with a recessive form of polyposis (Sieber et al., 2003), was assessed as previously described (Gismondi et al., 2004). Overnight urine samples (from 8pm to 8am) were collected before treatment and on the 8th 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-HPLC-RIA technique [(validated by comparison with ultra performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) (Song et al., 2007) (Supplementary Figure S1 and Supplementary material 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 11alpha-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M) by UPLC/MS/MS (Song et al., 2007), an index of
PGE\textsubscript{2} generation \textit{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.

\textit{Inhibition of human whole blood COX-1 and COX-2 by celecoxib in vitro}

It was assessed using previously published whole blood assays (Patrignani et al., 1994; Patrono et al., 1980) and a brief description is reported in the Supplementary material and methods.

\textit{Studies in Apc\textsuperscript{Min/+} mice}

\textit{In vivo} prostannoid generation was accomplished using Apc\textsuperscript{Min/+} mice, an established model of FAP (Moser et al., 1995). Five female Apc\textsuperscript{Min/+} mice and their wild-type C57BL/6J littermates at 11 weeks of age, were used for each group. Urine samples (100 \textmu l/mouse) were manually collected and immediately frozen. Systemic production of PGE\textsubscript{2}, TXA\textsubscript{2} and PGI\textsubscript{2} was evaluated by UPLC/MS/MS quantification of their major urinary metabolites (Song et al., 2007): PGE-M, 2,3-dinor-thromboxaneB\textsubscript{2}(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.

\textit{Co-culture 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 (FBS), 1% penicillin/streptomycin and L-glutamine 2mM. For every experiment, 1x10\textsuperscript{6} cells were seeded in 6-multiwell containing 2 ml of McCoy 5A supplemented with FBS 0.5% and 10 \textmu g/ml of Polimyxin B sulphate (Sigma-Aldrich, Milan, Italy) for 20 hr, alone or co-cultured with platelets. Human platelets were freshly isolated from leukocyte concentrates obtained from Stadtische Kliniken Hochst (Frankfurt, Germany), as previously reported (Albert et al., 2002) (briefly described in Supplementary material and methods). HT-29 cells (1x10\textsuperscript{6} cells) were cultured alone or with washed human platelets (1x10\textsuperscript{8} cells) for 20 hr, at 37°C in a humidified mixture of 5% CO\textsubscript{2} in air. In the culture
medium, PGE\textsubscript{2} and TXB\textsubscript{2} were measured by validated RIAs (Patrignani et al., 1994; Patrono et al., 1980), while in cell lysates, COX-1, COX-2, mPGES-1 and TXAS were assessed by Western blot (Di Francesco et al., 2009)(Supplementary material and methods). Platelets, obtained by preincubating platelet rich plasma (PRP) with aspirin 300\textmu M for 30min at room temperature before washing, were used as indicated. The effects of the highly selective COX-2 inhibitor rofecoxib(0.3\textmu M, Witega Laboratorien, Berlin, Germany) on prostanoid biosynthesis by HT-29 cells cultured alone or with platelets for 20hr were evaluated.

**Statistical analyses**

Values were reported as mean±SD 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 ANOVA) 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 log\textsubscript{10} transformed data was 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 VIF (Variance Inflation Factor). Variables with $r^2$ values greater than 0.75 (so VIF was greater than 4.0) were excluded for multicollinearity. Comparisons of urinary and plasma biomarker levels between baseline and celecoxib in FAP patients were assessed by the Wilcoxon matched pairs test. All analyses were performed using GraphPad, InStat (San Diego, CA, USA). 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), 6 volunteers would allow detecting at least 46\% change in its measurement between pre- and post-drug 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 choose a sample-size of 9 individuals. Concentration-response curves of celecoxib were fitted (using PRISM,
GraphPad) and IC$_{50}$(drug concentration required for obtaining 50% of inhibition) values were calculated.

**Results**

**Biosynthesis of prostanoids in vivo in FAP patients at baseline**

The baseline characteristics of FAP patients and healthy controls, pairmatched for gender and age, are reported in Table 1. All individuals were nonsmokers without significant CV risk factors. Despite all FAP patients claiming to be nonsmokers, 1 out of the 9 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 MYH mutation.

In FAP, urinary levels of TX-M, at baseline, were $1.19\pm0.84$, $0.93(0.10-2.90)$ng/mg creatinine[mean±SD, median(range)], and resulted significantly($p<0.01$) higher than the values detected in healthy controls[i.e., $0.20\pm0.10$, $0.20(0.09-0.40)$ng/mg creatinine](Table 1). Baseline urinary PGI-M levels were not significantly different in FAP and healthy controls(Table 1). In 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 non-small cell lung cancers(27.2±213.5ng/mg creatinine) (Csiki et al., 2005).

**Biosynthesis of prostanoids in vivo in Apc$^{Min/+}$ mice**

In order 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). In Apc$^{Min/+}$ and wild-type C57BL/6J mice, we assessed also urinary levels of PGI-M and PGE-M.
In Apc\textsuperscript{Min/+} mice, a significant increase of urinary dinor-TX-M vs wild-type mice was detected [mean±SD: 84.3±26 vs 48.2±23ng/mg creatinine, respectively, \(p<0.05\), Figure 1a]. Average PGI-M was higher in Apc\textsuperscript{Min/+} than in wild-type mice, but the differences were not statistically significant (Figure 1b). Urinary PGE-M was increased in Apc\textsuperscript{Min/+} vs wild-type mice [5.3±1 vs 2.5±1.3ng/mg creatinine, respectively, \(p<0.01\), Figure 1c]. These results confirm the data in FAP showing that enhanced \textit{in vivo} generation of TXA\textsubscript{2} and PGE\textsubscript{2} is associated with multiple intestinal neoplasia.

**Effects of celecoxib on the biosynthesis of prostanoids in vivo in FAP patients**

Celecoxib, administered for 7 consecutive days did not significantly affect urinary TX-M in FAP patients(Figure 2a). In contrast, the drug caused profound and significant(\(p<0.01\)) inhibition of PGI\textsubscript{2} biosynthesis by 58±9\%(Figure 2b) and PGE\textsubscript{2} by 48±31\%(Figure 2c).

The finding that celecoxib did not affect TX biosynthesis \textit{in vivo} suggests a COX-1-dependent pathway for enhanced TX generation, presumably from platelets.

**Prostanoid biosynthesis by co-culture of human adenocarcinoma cell line HT-29 and human platelets**

To address whether epithelial tumorigenesis triggers platelet activation and enhances TXA\textsubscript{2} biosynthesis, we performed an \textit{in vitro} study by co-culturing HT-29 cells with platelets for 20hr. As shown in Figure 3a, very low concentrations of TXB\textsubscript{2} were generated by HT-29 cells(mean±SD: 0.02±0.02ng/ml). Unstimulated platelets cultured alone for 20hr released 8±3 ng/ml of TXB\textsubscript{2}. When unstimulated platelets were co-cultured with HT-29 cells for 20 hr, TXB\textsubscript{2} generation was significantly(\(p<0.01\)) enhanced (57±32ng/ml). This finding suggests that colon cancer cells triggered platelet activation. TXB\textsubscript{2} generation was profoundly reduced by pre-treatment of platelets with aspirin under these experimental conditions, which is consistent with the enhanced TXB\textsubscript{2} biosynthesis in co-cultures of the 2 cell types deriving mainly from platelet COX-1 activity(Figure 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 co-cultured with HT-29 cells or in platelets cultured alone (Figure 3b). By contrast, rofecoxib significantly inhibited PGE₂ generation as detected in the culture medium of treated HT-29 cells (Figure 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 (Figure 3d). Altogether these results suggest that intestinal neoplasia is associated with COX-2/mPGES-1-dependent PGE₂ generation and that 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 FAP patients 4hr after the last morning dose and we 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 FAP patients after dosing with 400mg BID were sufficient to suppress completely COX-2 activity *in vivo* as it has been suggested that the chemopreventive effects of celecoxib at high concentrations may occur through COX-2-independent pathways (Schiffman et al., 2009). At 4hr after the last dose of celecoxib, plasma concentrations ranged from 955 to 3566 ng/ml (1871±946.8 ng/ml, mean±SD). In Supplementary Figure S2, the sigmoidal dose-response curves of celecoxib for inhibition of whole blood COX-1 and COX-2 *in vitro* were shown. Celecoxib inhibited LPS-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, CI)], respectively. Individual plasma celecoxib concentrations detected in FAP patients were associated with inhibition by 90-95% of monocyte COX-2 activity *in vitro* and by 25-55% of platelet COX-1, *in vitro* (Supplementary Figure S2). These results indicate that circulating concentrations of celecoxib...
were appropriate to inhibit almost completely monocyte COX-2 activity and that they only modestly affected platelet COX-1 activity.

**Effects of celecoxib on circulating angiogenesis biomarkers in FAP patients**

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

**Relationships among urinary biomarkers of prostanoid biosynthesis in vivo and circulating angiogenesis proteins in FAP patients**

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 2 growth factors HGF (r_s=-0.48, p<0.05) and FGF-2 (r_s=-0.47, p<0.05) (Supplementary Figure S3). We did not find any correlation amongst urinary PGE-M or TX-M and circulating angiogenic proteins.

Celecoxib caused an increase in 6 circulating angiogenesis proteins (both pro- and anti-angiogenic) (Figure 4, 5 and Supplementary Table S1). Thus, we tested the relationship among PGI-M,
TX-M and PGE-M and these circulating proteins (Supplementary Table S2). In linear multiple regression analysis of $\log_{10}$ transformed data, among the X variables which resulted independent of each other, the only one that was significantly related to TX-M was PGI-M ($\beta$: 0.80; SEM: 0.27; $P$ = 0.02), while PGI-M was positively related to TX-M ($\beta$: 0.50; SEM: 0.17; $P$ = 0.01), and inversely related to angiogenin ($\beta$: -0.44; SEM: 0.16; $P$ = 0.02), and FGF-2 ($\beta$: -0.24; SEM: 0.10; $P$ = 0.03), in a statistically significant fashion. PGE-M was significantly correlated with TX-M ($\beta$: 0.77; SEM: 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/BID) was associated with a significant reduction of the number of colorectal polyps by roughly one third (Steinbach et al., 2000). However, marked variability in the response to celecoxib was noted (Steinbach et al., 2000). We hypothesized that 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 activation and platelet release of angiogenic factors present in $\alpha$-granules (Menter et al., 1987). Thus, we performed the present study to investigate 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. Importantly, we aimed to explore the impact of selective inhibition of COX-2 by celecoxib on circulating biomarkers of angiogenesis *in vivo* in FAP and to correlate them with the biosynthesis of PGI$_2$, TXA$_2$ and PGE$_2$ *in vivo*. 

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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. In order 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). Interestingly, we detected increased generation of TXA$_2$ *in vivo* as compared to wild-type mice. To address the hypothesis that epithelial tumorigenesis is associated with platelet activation, we performed an *in vitro* study using co-cultures of 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 pre-treatment of platelets with aspirin. As previously reported there is multiplicity of molecular mechanisms that can be utilized by cancer cells to activate platelets and to 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 angiogenesis and development of tumor metastasis (Honn, 1983). Thus, pharmacological inhibition of TXAS has been shown significantly to 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 75mg daily (Rothwell et al., 2011), recommended for the 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 which are capable of inducing platelet activation (Pacienza 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 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 (Figure 4,5). Interestingly, 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) (Supplementary Figure S3). However, we found a coincident increase of the plasma levels of a MMP inhibitor TIMP-2 (Stetler-Stevenson and Seo, 2005) and, interestingly, 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 FAP patients which was profoundly reduced by celecoxib. Interestingly, we did not find any correlation between urinary PGE-M and circulating angiogenic factors, suggesting the origin of these circulating proteins to be outside the tumor proper. However, in multiple linear regression analysis, we found that PGE-M was significantly correlated with TX-M (Supplementary 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 also leukotrienes (LTs), generated from arachidonic acid (AA) through the activity of 5-lipoxygenase (5-LO), can have a role in carcinogenesis (Wang and Dubois, 2010). Both COX-2 and 5-LO 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 recently 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 FAP patients 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 (Supplementary Figure 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 pro- and anti-angiogenesis factors. Despite 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 enrolment in an ongoing clinical trial (European Medicine Agency, 2011).
Authorship Contributions:

Participated in research design: Bertario, Dixon, Steinhilber, Patrignani

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

Contributed new reagents or analytic tools: Ricciotti, Lawson

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

Contributed to the writing of the manuscript: Dovizio, Tacconelli, Ricciotti

Wrote the manuscript: FitzGerald, Patrignani


Footnotes

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Authors declare no conflict of interest.

MD, ST and ER contributed equally

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Legends for Figures

**Figure 1. Biosynthesis of prostanoids *in vivo* in Apc<sup>Min/+</sup> mice vs wild-type.** Urinary excretion of dinor-TX-M(Panel a), PGI-M(Panel b) and PGE-M(Panel c) in Apc<sup>Min/+</sup> mice and wild-type(WT) mice. Data were expressed as the mean±SEM. 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 vs wild-type mice using Student’s t-test.

**Figure 2. Biosynthesis of prostanoids in FAP patients before and after celecoxib treatment (400mg/BID, for 7 consecutive days).** Systemic production of TXA<sub>2</sub>, PGI<sub>2</sub>, and PGE<sub>2</sub> was assessed by measuring their enzymatic urinary metabolites, ie TX-M, PGI-M and PGE-M (Panels a, b and c, respectively) in samples collected before treatment(from 8pm to 8am) and on the 8<sup>th</sup> day after the last dose of the drug. Data were presented as box and whiskers, where the boxes represent the 25<sup>th</sup> to 75<sup>th</sup> 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 vs baseline using Wilcoxon matched pairs test.

**Figure 3. Prostanoid biosynthesis and protein expression in human adenocarcinoma cell line HT-29, human platelets and HT-29 co-cultured with platelets.** Panel a shows TXB<sub>2</sub> generation by HT-29 alone(HT), HT-29 co-cultured with platelets(HT+PLT) and platelets alone(PLT) for 20hr. In some experiments, the effects of platelets pre-treated with aspirin 300μM was studied. Mean±SEM of 5-10 different experiments are shown. **p<0.01 vs HT-29 alone and platelets alone; § p<0.01 vs untreated platelets; #p<0.01 vs HT-29 co-cultured with untreated platelets(HT+PLT). Repeated measures ANOVA was used for statistical analysis.

Panel b shows the effect of rofecoxib(0.3μM) on TXB<sub>2</sub> generation by HT-29 alone, platelets alone and HT-29 co-cultured with platelets for 20hr. Means of 2 different experiments performed in triplicate are
shown. Panel c shows the effect of rofecoxib (0.3 μM) on PGE2 generation by HT-29 alone. *p<0.05 vs DMSO (vehicle). Student’s t-test was used for statistical analysis.

In panel d, COX-1, COX-2, TXAS and mPGES-1 protein levels were assessed by Western blot.

**Figure 4. Effect of the administration of celecoxib on circulating angiogenesis biomarkers (FGF-2, VEGF-D, VEGF-A and angiogenin) in FAP patients.** Heparinized blood samples were collected before dosing and on the 7th day 4 hr after the last morning dose of celecoxib to assess the levels of circulating angiogenesis proteins. Data were presented as box and whiskers (n=9). *p<0.05; **p<0.01 vs baseline using Wilcoxon matched pairs test.

**Figure 5. Effect of the administration of celecoxib on circulating angiogenesis biomarkers (Angiopoietin-2, PDGF-BB, TIMP-2, sICAM-1) in FAP patients.** Heparinized blood samples were collected before dosing and on the 7th day 4hr after the last morning dose of celecoxib to assess the levels of circulating angiogenesis proteins. Data were presented as box and whiskers (n=9). *p<0.05; **p<0.01 vs baseline using Wilcoxon matched pairs test.
Table 1. Baseline characteristics of FAP patients 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±SD), y§</td>
<td>44±11</td>
<td>45±10</td>
</tr>
<tr>
<td>Female, %</td>
<td>5, 55</td>
<td>5, 55</td>
</tr>
<tr>
<td>CRP, mg/l, mean±SD, Median(range)†</td>
<td>10±19, 3(1.10-61.60)</td>
<td>4±2, 3.30(1.40-9.50)</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl, mean±SD, median(range)†</td>
<td>105±26, 97(65-144)</td>
<td>106±27, 103(60-147)</td>
</tr>
<tr>
<td>Cotinine, ng/ml, mean±SD, median(range)</td>
<td>7.20±19.60, 0.62(0.40-59.40)</td>
<td>Not determined</td>
</tr>
<tr>
<td>TX-M, ng/mg creatinine, mean±SD, 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, ng/mg creatinine, mean±SD, 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±SD, median(range)†</td>
<td>22.30±16.20, 21.52(2.82-55.40)</td>
<td>10.30±5, 11.22(4-16)</td>
</tr>
<tr>
<td>APC mutation, %</td>
<td>8, 88.9</td>
<td>Not determined</td>
</tr>
<tr>
<td>MYH mutation, %</td>
<td>1, 11.1</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

Arterial hypertension, defined as current systolic/diastolic blood pressure ≥140/90 mm Hg (referring to the 1999 World Health Organization criteria for the diagnosis of hypertension). §Data were compared by Student’s t-test; †data were compared by Mann-Whitney test; *p<0.05 vs healthy controls; **p<0.01 vs healthy controls.
Figure 1
Figure 2
Figure 3
Figure 4