

Pharmacological Inhibition of Platelet-Tumor Cell Cross-Talk Prevents Platelet-Induced Overexpression of Cyclooxygenase-2 in HT29 Human Colon Carcinoma Cells[§]

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ABSTRACT

Cyclooxygenase (COX)-2-derived prostanoids can influence several processes that are linked to carcinogenesis. We aimed to address the hypothesis that platelets contribute to aberrant COX-2 expression in HT29 colon carcinoma cells and to reveal the role of platelet-induced COX-2 on the expression of proteins involved in malignancy and marker genes of epithelial-mesenchymal transition (EMT). Human platelets cocultured with HT29 cells rapidly adhered to cancer cells and induced COX-2 mRNA expression, but not protein synthesis, which required the late release of platelet-derived growth factor and COX-2 mRNA stabilization. Platelet-induced COX-2-dependent prostaglandin E₂ (PGE₂) synthesis in HT29 cells was involved in the down-regulation of p21^{WAF1/CIP1} and the upregulation of cyclinB1 since these effects were prevented by rofecoxib (a selective COX-2 inhibitor) and rescued by exogenous PGE₂. Galectin-3, which is highly expressed in HT29 cells, is unique among galectins

because it contains a collagen-like domain. Thus, we studied the role of galectin-3 and platelet collagen receptors in platelet-induced COX-2 overexpression. Inhibitors of galectin-3 function (β -lactose, a dominant-negative form of galectin-3, Gal-3C, and anti-galectin-3 antibody M3/38) or collagen receptor-mediated platelet adhesion (revacept, a dimeric platelet collagen receptor GPVI-Fc) prevented aberrant COX-2 expression. Inhibition of platelet-cancer cell interaction by revacept was more effective than rofecoxib in preventing platelet-induced mRNA changes of EMT markers, suggesting that direct cell-cell contact and aberrant COX-2 expression synergistically induced gene expression modifications associated with EMT. In conclusion, our findings provide the rationale for testing blockers of collagen binding sites, such as revacept, and galectin-3 inhibitors in the prevention of colon cancer metastasis in animal models, followed by studies in patients.

Introduction

Platelet-tumor cell interactions within the bloodstream play an important role in the metastatic dissemination of epithelial tumors. Platelets may contribute to metastasis through several mechanisms (Gay and Felding-Habermann, 2011): 1) the formation of platelet aggregates surrounding

tumor cells, which may support tumor cell survival and protection from immune elimination; 2) enhancement of the adhesion of tumor cells to the endothelium, thus leading to tumor cell arrest and extravasation; and 3) synthesis of lipid products [such as thromboxane (TX)_{A2}] and the release of proteins from α -granules [such as transforming growth factor (TGF)- β and platelet-derived growth factor (PDGF)] during platelet activation, which may affect tumor vascularization and facilitate tumor cell dissemination into the bloodstream. Labelle et al. (2011) showed that the direct interaction of platelets with tumor cells synergizes with platelet-released TGF- β to induce the expression of genes for epithelial-mesenchymal-like-transition (EMT), thus increasing the invasive potential of tumor cells and their capacity to colonize the lung.

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ABBREVIATIONS: AA, arachidonic acid; COX, cyclooxygenase; CRD, carbohydrate recognition domain; EGF, epidermal growth factor; EMT, epithelial-mesenchymal-like-transition; EP, E-series prostanoid receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HuR, human antigen R; NHE, Na⁺/H⁺ exchanger; PGE₂, prostaglandin E₂; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PRP, platelet-rich plasma; TGF, transforming growth factor; TX, thromboxane; VEGF, vascular endothelial growth factor; ZEB1, zinc finger E-box binding homeobox.

The direct interaction of platelets with tumor cells seems to be a crucial mechanism in regulating the initial steps in the metastatic process. Thus, the elucidation of platelet receptors and tumor cell plasma membrane components that participate in cell-cell interaction will facilitate progress in cancer treatment strategies. Different platelet adhesion receptors may govern platelet-tumor cell interactions depending on cell-type-specific dysregulated expression of distinct membrane components induced by neoplastic transformation (Boukerche et al., 1989; Mannori et al., 1995). Galectin-3 is a member of a family of carbohydrate-binding proteins, but it uniquely consists of a C-terminal carbohydrate recognition domain (CRD), a collagen-like internal R domain, and the N-terminal domain, is highly elevated in malignancies, including colon cancer (Yang et al., 2008). It is localized inside the cells but also on cell surfaces, where it mediates cell-cell and cell-matrix interactions by binding to glycoconjugates that contain β -galactosides via the CRD. Galectin-3 is unique among galectins because it has the collagen-like domain, which is suggestive of the role of galectin-3 in platelet-cancer cell cross-talk through interaction with platelet collagen receptors. Several different receptors for collagen have been identified on platelets, including glycoprotein Ib, integrin $\alpha 2\beta 1$, and a dimeric platelet collagen receptor, GPVI. In humans, there is growing evidence for the dimeric platelet collagen receptor GPVI as the major collagen receptor for platelet activation (Nieswandt and Watson, 2003).

Another important question to unravel is the role played by other platelet-released factors, in addition to TGF- β , on tumor cell acquisition of a disseminating phenotype. PDGF, a major component of platelet α -granules, is released during platelet activation (Coppinger et al., 2007), and it induces cell signaling pathways that stimulate EMT (Yang et al., 2006). Moreover, the contribution of platelet TXA₂, a proaggregatory lipid mediator generated from arachidonic acid (AA) by the activity of cyclooxygenase (COX)-1 (Hamberg et al., 1975), is indirectly suggested by the finding from randomized clinical trials with aspirin showing that the drug reduced by 40–50% the likelihood that cancers would spread to distant organs (Algra and Rothwell, 2012; Rothwell et al., 2012a,b). The efficacy of aspirin is also detected at the low doses used for the prevention of atherothrombosis, which act by causing the selective inhibition of platelet COX-1 activity (Patrono et al., 2005). Thus, it has been proposed that the antiplatelet effect of aspirin plays a central role in its efficacy against cancer (Patrono et al., 2001; Dovizio et al., 2013).

Platelets might reprogram cancer cells to a more malignant phenotype through the induction of aberrant expression of COX-2, a hallmark of tumor invasion, metastasis, and poor prognosis in colorectal cancer and other types of cancer (Prescott, 2000; Patrono et al., 2001). COX-2 mediates these effects by enhancing the generation of prostaglandin E₂ (PGE₂), which triggers tumorigenic signals in target cells by coupling to four subtypes of receptors, classified as EP1, EP2, EP3, and EP4 (E-series prostanoid receptors), expressed on the plasma membrane and/or the nuclear envelope (Bhattacharya et al., 1998; Regan, 2003; Cha and DuBois, 2007). COX-2 overexpression, in both cancer and inflammation, is associated with altered expression and cytoplasmic accumulation of *trans*-acting factors that bind to adenylate-uridylylate-rich elements of COX-2 mRNA and influence its stability, such as the

mRNA-stability factor human antigen R (HuR) (Dixon et al., 2006; Young et al., 2009).

In this study, we aimed to characterize the role played by tumor and platelet surface molecules and released products on the expression of COX-2 and other proteins associated with malignancy in HT29 cells. Our findings show that platelet-tumor cell interaction, mediated by platelet collagen receptors and galectin-3, and the release of PDGF from activated platelets play a central role in the aberrant expression of COX-2 in cancer cells. The cellular interactions conferred an increased mitogenic potential to human colon adenocarcinoma HT29 cells through the downregulation of p21^{WAF1/CIP1} associated with upregulation of cyclin B1. Direct platelet-tumor cell interaction and platelet-induced COX-2 overexpression synergistically induced the expression of genes for EMT in HT29 cells. Our findings that revacept, a novel antiplatelet drug that inhibits collagen-mediated platelet adhesion (Ungerer et al., 2011), prevented these changes provides the basis for further investigation of agents that block collagen-induced platelet function for colon cancer chemotherapy.

Materials and Methods

Coculture Experiments with Human Colon Carcinoma Cell Line HT29 and Isolated Human Platelets. The HT29 cell line, obtained from European Collection of Cell Cultures (ECC, Salisbury, UK), was cultured in McCoy's 5A medium (Invitrogen, Milan, Italy) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine, as previously described (Dovizio et al., 2012). In all experiments, 1×10^6 cells were seeded in six multiwell plates containing 2 ml of McCoy's 5A supplemented with FBS 0.5% and polymyxin B sulfate 10 μ g/ml (Sigma-Aldrich, Milan, Italy). HT29 cells and platelets were cultured either alone or together for different lengths of time (2, 4, 8, 12, 16, or 20 hours); then conditioned media were collected and centrifuged at 10,000g for 2 minutes to discard cell debris, and the levels of TXB₂, PGE₂, PDGF-BB, epidermal growth factor (EGF), and TGF- β 1 were evaluated by specific immunoassays. The levels of COX-2, p21^{WAF1/CIP1} and cyclin B1 were assessed in HT29 cell lysates (at 4, 8, 12, 16, and 20 hours). Since changes in these protein levels in HT29 cells cocultured with platelets occurred with different kinetics, but were significantly modulated at 20 hours, the levels of other proteins, such as proliferating cell nuclear antigen, cyclin D1, Bcl-2, ornithine decarboxylase, galectin-3, and galectin-4, and the effects of pharmacological inhibitors were evaluated at this time point. Human platelets were freshly isolated from leukocyte concentrates obtained from Städtische Kliniken Hoechst (Frankfurt, Germany) as previously described (Dovizio et al., 2012). In brief, venous blood was collected from healthy adult donors and leukocyte concentrates were prepared by centrifugation (4000g, 20 minutes, 20°C). Leukocyte concentrate was sedimented in 5% dextran solution (Sigma-Aldrich), and the supernatant was stratified in lymphocyte separation medium (PAA Laboratories GmbH, Pasching, Austria) by centrifugation (800g, 10 minutes, room temperature). After centrifugation, platelet-rich plasma (PRP) was obtained. PRP was then mixed with phosphate-buffered saline (PBS), pH 5.9 (3:2, v/v) and centrifuged (2000g, 15 minutes, room temperature); pelleted platelets were resuspended in PBS, pH 5.9/0.9% NaCl (1:1, v/v), washed by centrifugation (2000g, 10 minutes, room temperature), and finally resuspended in McCoy's 5A medium containing FBS 0.5% and polymyxin B sulfate 10 μ g/ml. Fifty microliters of platelet suspension containing 1×10^8 cells was added to HT29 cells (1×10^6) and incubated for different times. As control conditions, HT29 cells were incubated with 50 μ l of culture medium and cultured alone. In some experiments, platelets were pretreated with aspirin before incubation

with HT29 cells; briefly, PRP was incubated with aspirin 300 μM for 30 minutes at room temperature; then platelets were isolated, washed twice, and either cultured alone or cocultured with HT29 cells. In experiments where we aimed to evaluate the effect of COX-2 inhibition, the highly selective COX-2 inhibitor rofecoxib (0.3 μM ; Witega Laboratorien, Berlin, Germany) was used. The drug was used to assess the contribution of HT29 COX-2 to PGE₂ released in the culture medium. Thus, in coculture experiments performed with HT29 cells and platelets (untreated with aspirin), rofecoxib was added 20 minutes before the addition of platelets and the incubation continued for 20 hours; then conditioned media were collected and centrifuged at 10,000g for 2 minutes to discard cell debris, and supernatants were analyzed for PGE₂ levels. In some experiments, we aimed to verify whether platelet-induced COX-2 was catalytically active even at 20 hours of coculture. Thus, HT29 cells were cocultured with aspirin-treated platelets for 20 hours (under these conditions, COX-2 was upregulated). Platelets were pretreated with aspirin to eliminate the contribution of platelet COX-1 to PGE₂ levels detected in the culture medium. Then the medium was changed with a fresh one containing rofecoxib (0.3 μM) or dimethylsulfoxide vehicle, and the cells were incubated for 1 hour before the addition of exogenous AA (Sigma-Aldrich) 0.5 μM (to start COX product formation); the incubation continued for 1 hour. At the end of the incubation, conditioned media were harvested and centrifuged at 10,000g for 2 minutes to discard cell debris, and supernatants were analyzed for PGE₂ levels. In other experiments, HT29 cells cocultured with platelets for 20 hours in the presence of rofecoxib were treated with exogenous PGE₂ (1.5 and 15 ng/ml) (Cayman Chemical, Ann Arbor, MI).

Pharmacological Treatments. The nonselective COX inhibitor aspirin (Sigma-Aldrich), the selective COX-2 inhibitor rofecoxib (Witega Laboratorien), the inhibitor of RNA synthesis actinomycin D (Sigma-Aldrich), the PDGF receptor (PDGFR) antagonist imatinib (Cayman Chemical), the P-selectin antagonist (galloyllyl-N-gaba-WVDV-OH; Calbiochem, Merck KGaA, Darmstadt, Germany), the inhibitor of protein kinase C (PKC) δ rottlerin (Calbiochem, Merck KGaA), and the inhibitor of phosphoinositide 3-kinase (PI3K) wortmannin (Sigma-Aldrich) were dissolved in dimethylsulfoxide; the competitive antagonist of P2-purinceptors suramin (Calbiochem, Merck KGaA) and the pan-galectin inhibitor β -lactose (Sigma-Aldrich) were dissolved in distilled water. The protein obtained by fusing the extracellular domain of GPVI with the human immunoglobulin fragment (Fc) domain (Ungerer et al., 2011) (revacept; provided by Dr. Münch, CorImmun GmbH, Munich, Germany) was dissolved in PBS/4% mannitol/1% sucrose. The neutralizing antibody anti-PDGF (R&D Systems, Minneapolis, MN) was dissolved in PBS; the M3/38 purified anti-mouse/human galectin-3 monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX) was dissolved in PBS; the truncated form of galectin-3 lacking 107 amino acids from the N terminus, Gal-3C (John et al., 2003) (provided by Dr. John, Mandal Med, Inc., San Francisco, CA) was dissolved in PBS containing lactose as a stabilizer, which was removed before use by a biodialyser system with cellulose acetate membrane for 24 hours (Sigma Aldrich); and the inhibitor of Na⁺/H⁺ exchanger (NHE) dm-amiloride [5-(N,N-dimethyl) amiloride hydrochloride; (Sigma-Aldrich)] was dissolved in methanol. All the compounds or the appropriate vehicles were added to HT29 cells 20 minutes before the addition of platelets and cocultured for 20 hours. Revacept or vehicle was added to HT29 cells cultured alone for 1 hour, and then the medium was changed, replaced with platelet cell suspension, and incubated up to 20 hours.

Biochemical Analyses. TXB₂ (the stable hydrolysis product of TXA₂) and PGE₂ levels were measured in cell culture media by previously described, validated, and specific radioimmunoassay techniques (Patrono et al., 1980; Patrignani et al., 1994). PDGF-BB, EGF, and active TGF- β 1 levels released in the medium were determined by enzyme-linked immunosorbent assay (R&D Systems) with detection limits of 2, 0.7, and 4 pg/ml, respectively. In addition, vascular endothelial growth factor levels were measured by enzyme-linked

immunosorbent assay (Pierce, Rockford, IL) (detection limit of 4 pg/ml), according to the manufacturer's protocol.

Western Blot Analysis. Western blot analysis of cell lysates from HT29 cells cultured alone or cocultured with platelets was performed as described (Dovizio et al., 2012). Detailed methods are reported in Supplemental Methods.

mRNA Analysis. Total RNA was extracted from 1×10^6 HT29 cells using EZNA-Total RNA KIT (Omega Bio-Tek, Inc., Norcross, GA) according to the manufacturer's protocols. Two micrograms of total RNA was treated with DNase kit (Fermentas, St. Leon-Rot, Germany) and subsequently reverse-transcribed into cDNA using Iscript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocols.

One hundred nanograms of cDNA was used for the reaction mixture, and the amplification of COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using TaqMan Polymerase Kit (Fermentas) and these couples of primers: COX-2-fwd: 5'GCTCAGCCATACAGCAAATCC; rev: 5'CCAAAATCCCCTT-GAAGTGGG; GAPDH- fwd: 5'TCACCAGGGCTGCTTTTAAC; rev: 5'GACAAGCTTCCCGTCTCAG, using a MyQ Single Color Real-Time PCR Detection System (Bio-Rad). COX-2 mRNA expression levels were normalized with GAPDH levels. Gene expression assays were performed by relative quantification with comparative Ct using MyQ Optical System Software 1.0 (Bio-Rad).

The stability of COX-2 mRNA was assessed in HT29 cells cultured alone or cocultured for 4 hours or 20 hours with platelets; then the transcription was stopped by the addition of actinomycin D (2.5 $\mu\text{g}/\text{ml}$) for 1 and 4 hours. After actinomycin D treatment, RNA was isolated and subjected to quantitative polymerase chain reaction analysis.

Regarding the expression of EMT markers, 100 ng of cDNA was used for the reaction mixture and the amplification of the zinc finger E-box binding homeobox 1 (ZEB1), TWIST1, vimentin, and E-cadherin was performed using TaqMan gene expression assays (Hs00232783_m1, Hs01675818_s1, Hs00185584_m1, Hs01023894_m1) (Applied Biosystems, Foster, City, CA) according to the manufacturer's instructions using a 7900HT Real-Time PCR system (Applied Biosystems). The mRNA expression levels of EMT markers were normalized with GAPDH levels (Rn99999916_s1) (Applied Biosystems). Gene expression assays were performed by relative quantification with comparative cycle threshold (Ct) using ABI Prism, SDS 2.4 software (Applied Biosystems).

Immunofluorescence. HT29 cells (0.5×10^6 cells) were cultured alone or with platelets (0.5×10^8) for 2, 4, and 20 hours; then culture media were collected and cells were washed twice with PBS and fixed in cold acetone/methanol solution (40:60) for 20 minutes at room temperature. COX-2, COX-1, HuR, and GAPDH immunostaining were detected as previously reported (Dixon et al., 2006), and a detailed description is reported in Supplemental Methods.

Statistics. All values were reported as mean \pm S.E.M. Statistical analysis was performed using GraphPad Prism Software (version 5.00 for Windows; GraphPad, San Diego, CA). Briefly, Student's *t* test was used to compare the means of two independent groups to each other, whereas one-way analysis of variance followed by Newman-Keuls post-test was used to compare the means of more than two independent groups. Linear multiple regression analysis was performed to test the relationship between PGE₂ levels and COX-2 protein levels, and the coefficient of determination (*r*²) was calculated using GraphPad Prism Software (version 5.00 for Windows; GraphPad). Values of *P* < 0.05 were considered statistically significant.

Results

Platelet-HT29 Cell Interaction Triggers Platelet Activation and COX-2 Protein Overexpression in HT29 Cells. In freshly isolated unstimulated platelets (100×10^6 cells) and HT29 cells (1×10^6), cultured alone or cocultured up to 20 hours, we studied the time course of TXB₂ release, the

growth factors vascular endothelial growth factor (VEGF), EGF, and PDGF-B, and the pleiotropic cytokine TGF- β 1 (Fig. 1, A and B; Supplemental Fig. 1, A–C). HT29 cells cultured alone did not release detectable levels of TXB₂ (Fig. 1A). The incubation of platelets with HT29 cells was associated with a time-dependent increase in TXB₂ levels in the medium. TXB₂ concentrations were significantly increased 2 hours after the addition of platelets to HT29 cells, and they continued to increase for up to 4 hours at a fast rate; thereafter, TXB₂ continued to be released in a time-dependent manner, up to 20 hours, albeit at a slower rate (Fig. 1A). Platelets cultured alone released significantly lower levels of TXB₂ than those detected in platelet-HT29 cell cocultures at each time point (at 20 hours: 7 ± 0.7 and 116 ± 12 ng/ml, respectively, $n = 5$ –8) (Fig. 1A).

HT29 cells cultured for 20 hours released VEGF (766 ± 180 pg/ml, $n = 4$), which was not affected by the cocubation with platelets (Supplemental Fig. 1A). Platelets cultured alone did not release detectable levels of VEGF (not shown).

The EGF levels measured in conditioned medium of HT29 cells cultured alone were almost undetectable (roughly 5 pg/ml), whereas platelets cultured alone released EGF in a time-dependent fashion (73 ± 7 and 117 ± 9 pg/ml, at 4 and 20 hours, respectively, $n = 3$) (Supplemental Fig. 1B). EGF concentrations were not significantly enhanced by the cocubation of platelets with HT29 cells, but instead they decreased with time (Supplemental Fig. 1B).

Platelets, but not HT29 cells, released low concentrations of active TGF- β 1 in a time-dependent fashion (at 20 hours, 116 ± 20 pg/ml, $n = 3$) (Supplemental Fig. 1C). In cocultures of HT29 cells and platelets, TGF- β 1 levels were released at a low rate for up to 4 hours, and they were not significantly higher than the levels measured in the conditioned medium of platelets or HT29 cells cultured alone; at 8 hours of coculture, a rapid increase in TGF- β 1 release (378 ± 80 pg/ml, $n = 3$) was observed, and then it declined in a time-dependent fashion. At 20 hours, TGF- β 1 levels detected in the medium were not significantly different from those released by platelets cultured alone (Supplemental Fig. 1C).

As shown in Fig. 1B, PDGF-BB levels, measured in the conditioned medium of HT29 cells cultured alone, were very low (5 ± 2 pg/ml, $n = 5$). Platelets, cultured alone, time dependently released low levels of PDGF-BB (at 20 hours, 292 ± 37 pg/ml, $n = 8$). In HT29 cells and platelets cocultured for 4 hours, PDGF-BB levels were not significantly different from those detected in platelets cultured alone. A large increase in PDGF-BB levels was detected at 8 hours (818 ± 16 pg/ml, $n = 5$), and the levels time dependently increased up to 20 hours (1450 ± 144 pg/ml, $n = 5$) (Fig. 1B). The levels of PDGF-BB detected in the conditioned medium at 8 and 20 hours of platelet-HT29 cell cocultures were roughly 2- and 8-fold higher than those of TGF- β 1, respectively.

Together, these results showed that platelets activated by the interaction with HT29 cells rapidly released TXB₂ (Fig. 1A), whereas a substantial release of PDGF-BB and TGF- β 1 from α -granules began after a lag time of 4 hours (Fig. 1B; Supplemental Fig. 1C). PDGF-BB was a major protein released by platelets when cocultured with HT29 cells, and its levels continuously accumulated up to 20 hours (Fig. 1B). In cocultures of HT29 cells and platelets, EGF and TGF- β 1, after being released, decreased in a time-dependent manner,

suggesting their possible cellular reuptake (Supplemental Fig. 1, B and C).

As shown in Fig. 1C, in cocultures of HT29 cells and platelets, we detected a time-dependent increase in COX-2 protein levels, which occurred in parallel with the release of PDGF-BB by activated platelets. However, the onset of COX-2 synthesis was delayed compared with PDGF-BB secretion (Fig. 1B).

The temporal dynamic of platelet-HT29 cell interactions and COX-2 induction in HT29 cells was studied by performing double-immunofluorescence staining of COX-1 (red) in platelets and HT29 cells and COX-2 (green) in HT29 cells, followed by confocal microscopy analysis (Fig. 1D). Both COX-1 and COX-2 proteins were constitutively expressed in HT29 cells (Fig. 1D). At 2 and 4 hours of cocultures, small platelet aggregates (expressing only COX-1) surrounded the HT29 cells (Fig. 1D). In contrast, at prolonged incubation times, most platelet aggregates were dissociated from the HT29 cells (Fig. 1D). Long-term incubation with platelets was associated with upregulation of COX-2, but not COX-1, protein expression in HT29 cells (Fig. 1D).

To elucidate the importance of direct platelet-tumor cell interaction in the platelet release of TXB₂ and PDGF, we performed coculture experiments incubating HT29 cells with platelets for 20 hours in the absence and in the presence of a transwell cell insert with a pore size of 0.4 μ m, which avoids direct contact between the two cell types but permits the passage of soluble factors. As shown in Fig. 1, E and F, the increased release of TXB₂ and PDGF, respectively, was completely prevented if the two cell types were separated by the transwell.

In summary, these data suggest that HT29 cells activate platelets through a direct cell-cell interaction associated with a rapid generation of TXA₂, which may amplify the activation response and recruit additional platelets. At prolonged incubation times, platelet aggregates detach from HT29 cells and release α -granule content, with PDGF-BB being more abundant than TGF- β 1.

Prolonged Incubation with Platelets Induces COX-2 mRNA Stabilization in HT29 Cells. To clarify the mechanism involved in HT29 cell COX-2 protein overexpression induced by prolonged incubation with platelets, we compared protein and mRNA levels of COX-2 at short-term (2–4 hours) and long-term (20 hours) incubation times with platelets.

At 2–4 hours of incubation, COX-2 protein was not upregulated, whereas COX-2 transcript levels were increased (Fig. 2, A and B). At long-term incubation (20 hours), both COX-2 protein and mRNA levels were increased (Fig. 2, A and B). Increased levels of COX-2 mRNA detected at 2, 4, and 20 hours of platelet-HT29 cell cocultures were not different from each other in a statistically significant fashion. Interestingly, COX-2 mRNA levels remained significantly elevated at 20 hours of incubation, even when platelet aggregates dissociated from HT29 cells (Fig. 2B).

A possible mechanism to explain the discrepancy between mRNA accumulation and protein synthesis of COX-2 is that platelets switched on a stabilization pathway of COX-2 mRNA (Dixon et al., 2006). This hypothesis was addressed by comparing COX-2 mRNA levels in HT29 cells cocultured with platelets for 20 hours versus 4 hours or in HT29 cells cultured alone in the presence of actinomycin D to stop transcription. As seen in Fig. 2C, a 4-hours exposure to actinomycin D

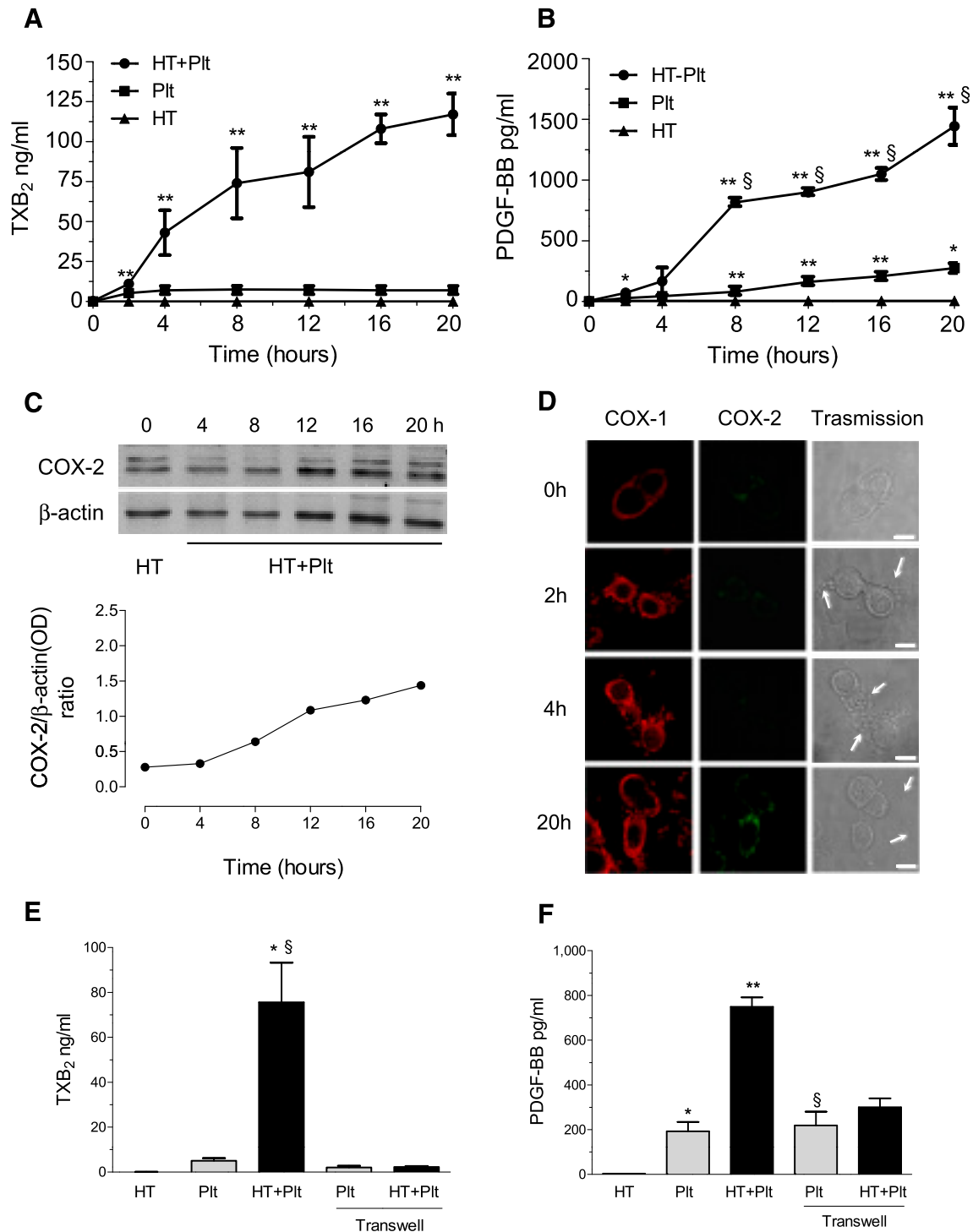


Fig. 1. Platelet-HT29 cell interaction triggers platelet activation and overexpression of COX-2 in tumor cells. HT29 cells (1×10^6) (HT) or isolated unstimulated platelets (100×10^6) (Plt) were cultured alone or cocultured (HT + Plt) for up to 20 hours. The release of TXB₂ (the stable hydrolysis product of TXA₂) (A) and PDGF-BB (B) were assessed in the culture medium, and values are reported as mean \pm S.E.M. ($n = 5-8$, panel A and $n = 3-8$, panel B). (A) $**P < 0.01$ versus Plt alone at same time points; (B) $*P < 0.05$; $**P < 0.01$ versus HT at the same time points; $\S P < 0.01$ versus Plt alone at the same time points. (C) COX-2 protein expression was detected by Western blot technique in HT29 cells cultured alone (HT) or cocultured with platelets (HT + Plt) for up to 20 hours and normalized to β -actin expression. Data are reported as mean of two separate experiments as a ratio between optical density (OD) values of COX-2 immunoreactive bands to that of β -actin bands. (D) Immunofluorescence analysis of COX-1 (in red) and COX-2 (in green) was performed in HT29 cells cultured alone (0 hour) or with platelets for 2, 4, and 20 hours. Arrows indicate platelet aggregates; scale bars, 10 μ m. (E and F) HT29 cells and platelets were cocultured for 20 hours using a Transwell (pore size, 0.4 μ m), and TXB₂ and PDGF-BB, respectively, were assessed in the medium. HT29 cells were cultured in the lower chamber of the Transwell unit, and platelets were in the upper chamber of the Transwell unit. Data are reported as mean \pm S.E.M. ($n = 3$). (E) $*P < 0.05$ versus Plt; $\S P < 0.01$ versus HT + Plt (Transwell); (F) $*P < 0.05$ versus HT; $**P < 0.01$ versus Plt and HT + Plt (Transwell).

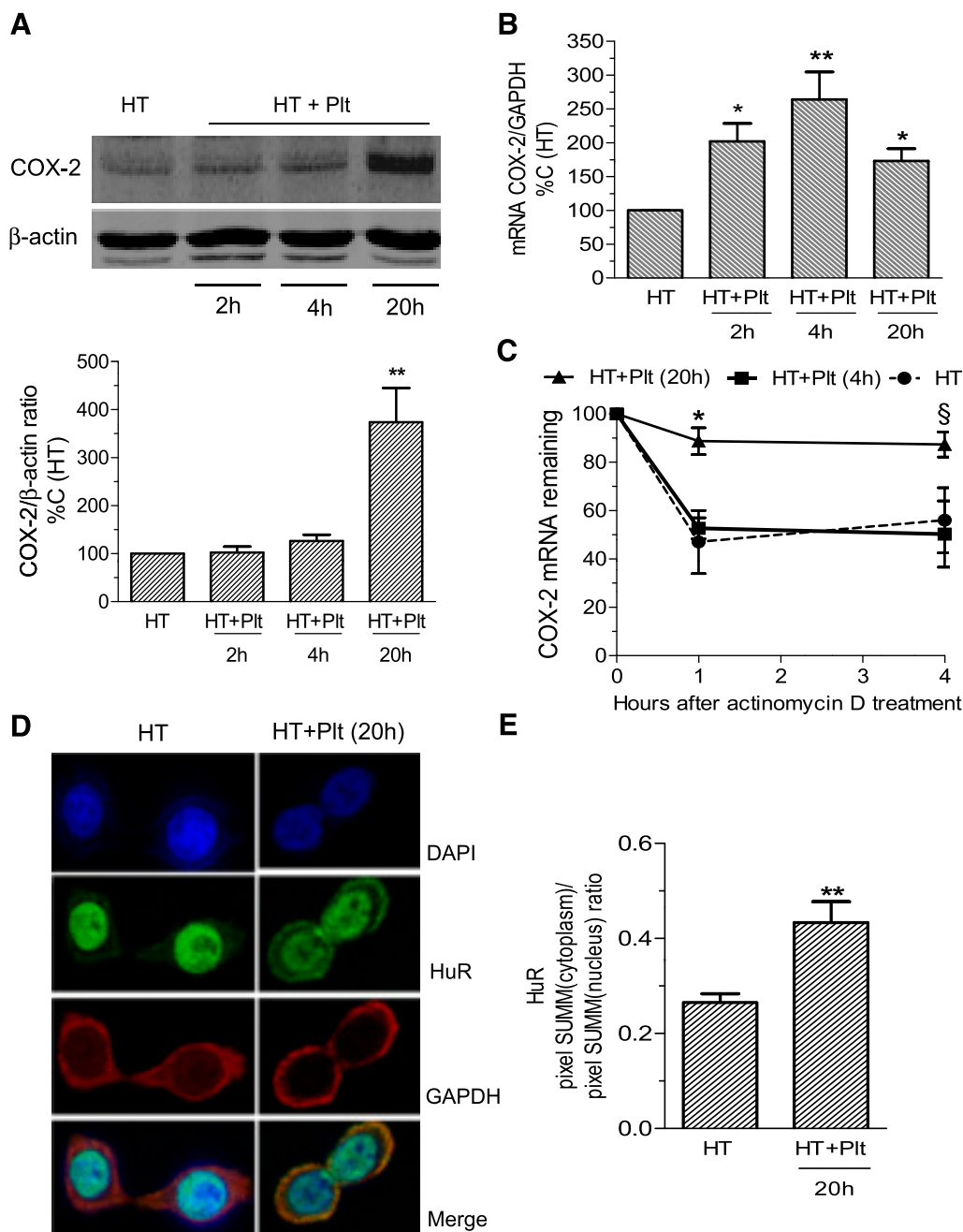


Fig. 2. Prolonged incubation with platelets modulates COX-2 protein synthesis and mRNA stability in HT29 cells. (A) Levels of COX-2 protein (normalized to β -actin levels) were detected by Western blot in HT29 cells cultured alone (HT) or with platelets (HT + Plt) for up to 20 hours. Data are reported as mean \pm S.E.M. ($n = 6-11$) as a percent of control (HT29 cells cultured alone). $**P < 0.01$ versus HT. (B) In the same experimental conditions, COX-2 mRNA expression (normalized to GAPDH) in HT + Plt versus HT alone was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Data are reported as mean \pm S.E.M. ($n = 5-6$) as a percent of control (HT29 cells cultured alone). $*P < 0.05$; $**P < 0.01$ versus HT. (C) Assay of COX-2 mRNA stability. Actinomycin D ($2.5 \mu\text{g/ml}$) was added to HT29 cells cultured alone (HT), and platelet-HT29 cells were cocultured for 4 hours [HT + Plt (4 hours)] or for 20 hours [HT + Plt (20 hours)]. COX-2 mRNA levels were assessed by qRT-PCR after 1 and 4 hours of treatment with actinomycin D. Data are reported as mean \pm S.E.M. ($n = 3$ to 4). $*P < 0.05$ and $\S P < 0.05$ versus HT + Plt (4 hours) and HT. (D) HuR localization was assessed by confocal microscopy analysis in HT29 cells alone (HT) or incubated with platelets (HT + Plt) for 20 hours. Immunostaining of HuR is shown in green, GAPDH (cytoplasmic protein) in red, and DAPI (4',6-diamidino-2-phenylindole) (nuclear marker) in blue. Merge images are shown (merge). (E) Ratios between the pixel sum of HuR staining in cytoplasm and pixel sum of HuR staining in the nucleus were calculated using LAS AF software, 2.2.1. Data are expressed as mean \pm S.E.M. ($n = 3$), $**P < 0.01$ versus HT.

resulted in a substantial decrease in the amount of mRNA present in HT29 cells that had been previously cultured alone or with platelets for only 4 hours. In contrast, the actinomycin D treatment led to a much smaller decrease in mRNA levels in HT29 cells previously cultured with platelets for 20 hours.

These results show that prolonged incubation of platelets with HT29 cells induced COX-2 mRNA stabilization.

We investigated whether platelet-induced COX-2 mRNA stabilization in HT29 cells occurred by increasing the cytoplasmic accumulation of HuR. In HT29 cells cocultured

with platelets for 2–4 hours, HuR levels detected by Western blot in the cytoplasmic fraction were not different from those detected in cancer cells cultured alone (Supplemental Fig. 2A). In contrast, enhanced HuR levels were detected in the cytoplasm of HT29 cells after prolonged incubation times with platelets by Western blot and confocal microscopy analyses (Fig. 2, D and E, respectively; Supplemental Fig. 2A).

Involvement of Tumor Galectin-3 and Platelet Collagen Receptors, but not P-Selectin, in Platelet-Induced COX-2 Upregulation in HT29 Cells. Next we aimed to

characterize the cell surface constituents involved in platelet-tumor cell interactions that translated into COX-2 overexpression in cancer cells. HT29 cells express high levels of different galectins, whose involvement in cell-cell and cell-extracellular matrix adhesion is well established (Satelli et al., 2008). Among the galectins highly expressed in HT29 cells, galectin-3 (Fig. 3A) is unique among the galectin family of lectins because it contains a “collagen-like” domain (Nangia-Makker et al., 2008). HT29 cell galectin-3 levels were not affected by incubation with platelets for 20 hours

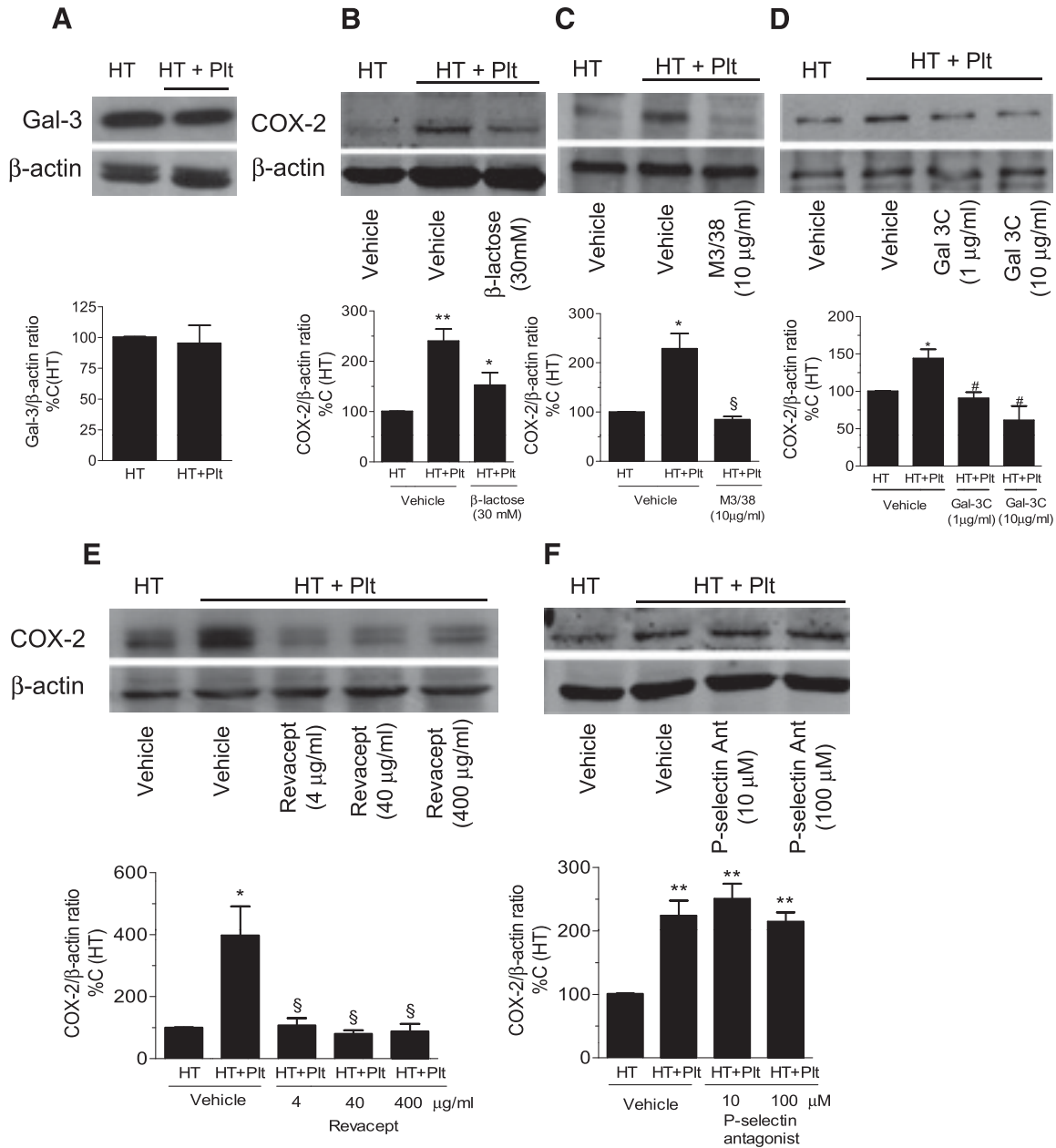


Fig. 3. Tumor galectin-3 and platelet collagen receptors, but not P-selectin, regulate COX-2 upregulation in HT29 cells. Galectin-3 (A) and COX-2 protein expression (B–F) were assessed by Western blot in HT29 cells cultured alone (HT) or cocultured for 20 hours with platelets (HT + Plt) and in the presence of vehicle or different pharmacological agents, i.e., (B) the pan-galectin inhibitor β-lactose (30 mM), (C) The monoclonal antibody that binds the N terminus of galectin-3 (M3/38; 10 μg/ml), (D) an N terminally truncated form of galectin-3 that acts as a dominant negative inhibitor (Gal-3C; 1 μg/ml and 10 μg/ml), (E) revacept (dimeric glycoprotein VI-Fc; 4, 40 and 400 μg/ml), or (F) the P-selectin antagonist (Galloyl-N-gaba-WVDV-OH peptide; 10 and 100 μM). Data are expressed as mean ± S.E.M. ($n = 3$ to 4). (B) ** $P < 0.01$ versus HT; * $P < 0.05$ versus HT + Plt (vehicle); § $P < 0.05$ versus HT + Plt (vehicle); (D) * $P < 0.05$ versus HT; # $P < 0.05$ versus HT + Plt (vehicle); (E) * $P < 0.05$ versus HT; § $P < 0.05$ versus HT + Plt (vehicle); (F) ** $P < 0.01$ versus HT.

(Fig. 3A). To address whether galectin-3 plays a role in platelet-induced COX-2 overexpression in HT29 cells, we used three different pharmacological tools: 1) β -lactose, which competitively binds to CRD of galectins; 2) M3/38, an antibody that inhibits galectin-3 function by binding to its N-terminal domain (Fukushi et al., 2004); and 3) Gal-3C, an N terminally truncated galectin-3 that contains the entire CRD and retains the ability to bind to carbohydrate-containing ligands such as glycoproteins containing glycans but is unable to cross-link them, thus acting as a dominant-negative inhibitor of full-length galectin-3 (Markowska, 2010).

As shown in Fig. 3B, β -lactose caused a significant reduction of 63% of enhanced COX-2 protein expression in HT29 cells cocultured for 20 hours with platelets. M3/38 antibody

and Gal-3C completely prevented platelet-induced COX-2 overexpression in HT29 cells (Fig. 3, C and D). Based on these results, we hypothesized that platelet collagen receptors could play a role in the direct interaction with HT29 cells. To address this question, we used the novel antiplatelet drug revacept obtained by fusing the extracellular domain of the platelet collagen receptor GPVI with the human immunoglobulin Fc domain (Ungerer et al., 2011). Human platelet GPVI represents the major signaling receptor for collagen on platelets (Nieswandt and Watson, 2003). The N terminus of revacept, constituted from the human GPVI extracellular region, binds to the ligands collagen and fibronectin in atherosclerotic plaques, either stable or ruptured, thus preventing platelet adhesion mediated by GPVI and possibly

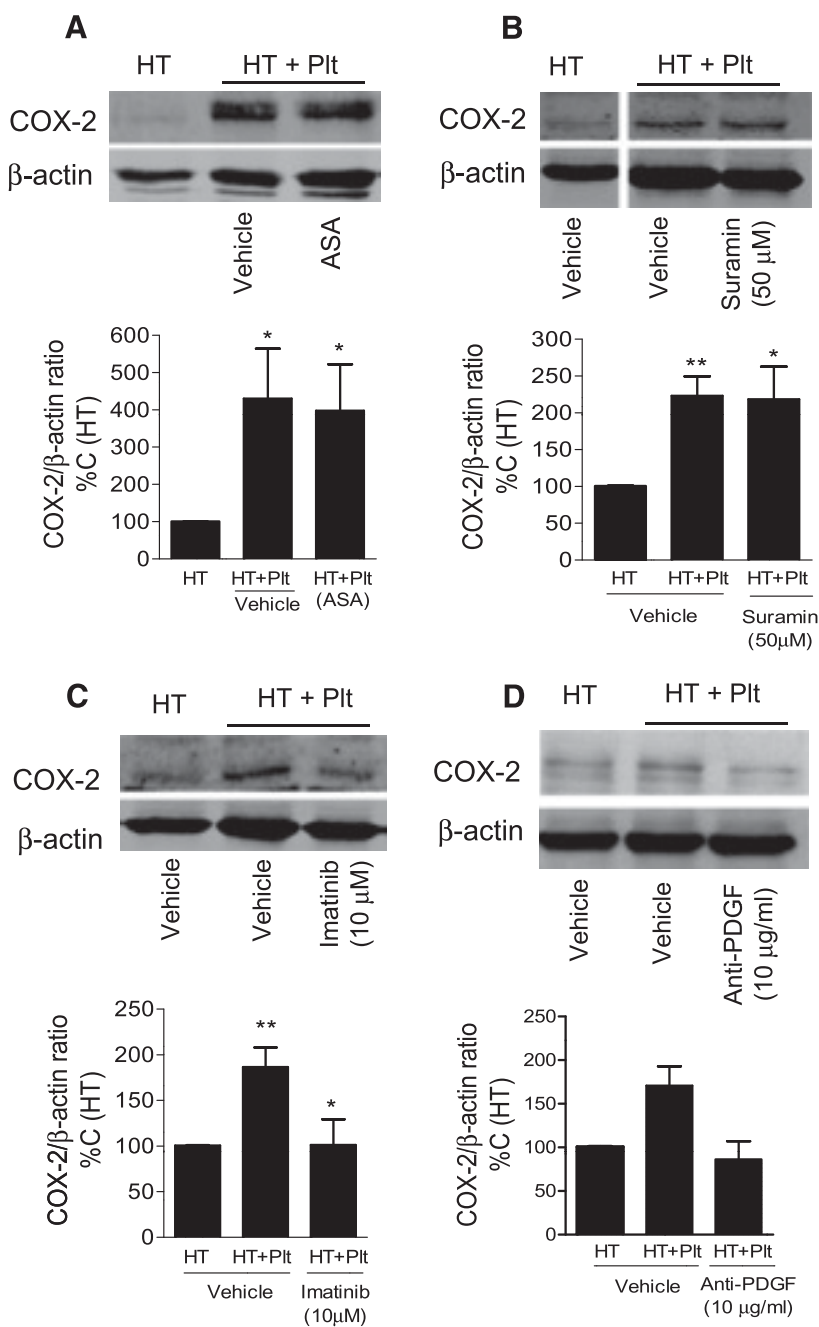


Fig. 4. Platelet-derived PDGF, but not TXA₂ and ADP/ATP regulates COX-2 expression in HT29 cells cocultured with platelets. COX-2 protein levels (normalized to β -actin levels) were assessed by Western blot in HT29 cells cultured alone (HT) or cocultured for 20 hours with platelets (HT + Plt) in the presence of vehicle or different pharmacological agents. (A) HT29 cells were incubated with aspirin-(ASA)-treated platelets. Moreover, HT29 cells were cocultured with platelets in the presence or absence of (B) suramin (50 μ M, a P2-purinoceptor antagonist), (C) imatinib (10 μ M, a PDGFR antagonist), and (D) a PDGF-neutralizing antibody (anti-PDGF, 10 μ g/ml). The values were reported as percent of control (HT29 cells cultured alone). Data are expressed as mean \pm S.E.M. from three or four (A-C) or two separate experiments (D). (A) * P < 0.05 versus HT; (B) ** P < 0.01 versus HT; * P < 0.05 versus HT; (C) ** P < 0.01 versus HT; * P < 0.05 versus HT + Plt (vehicle).

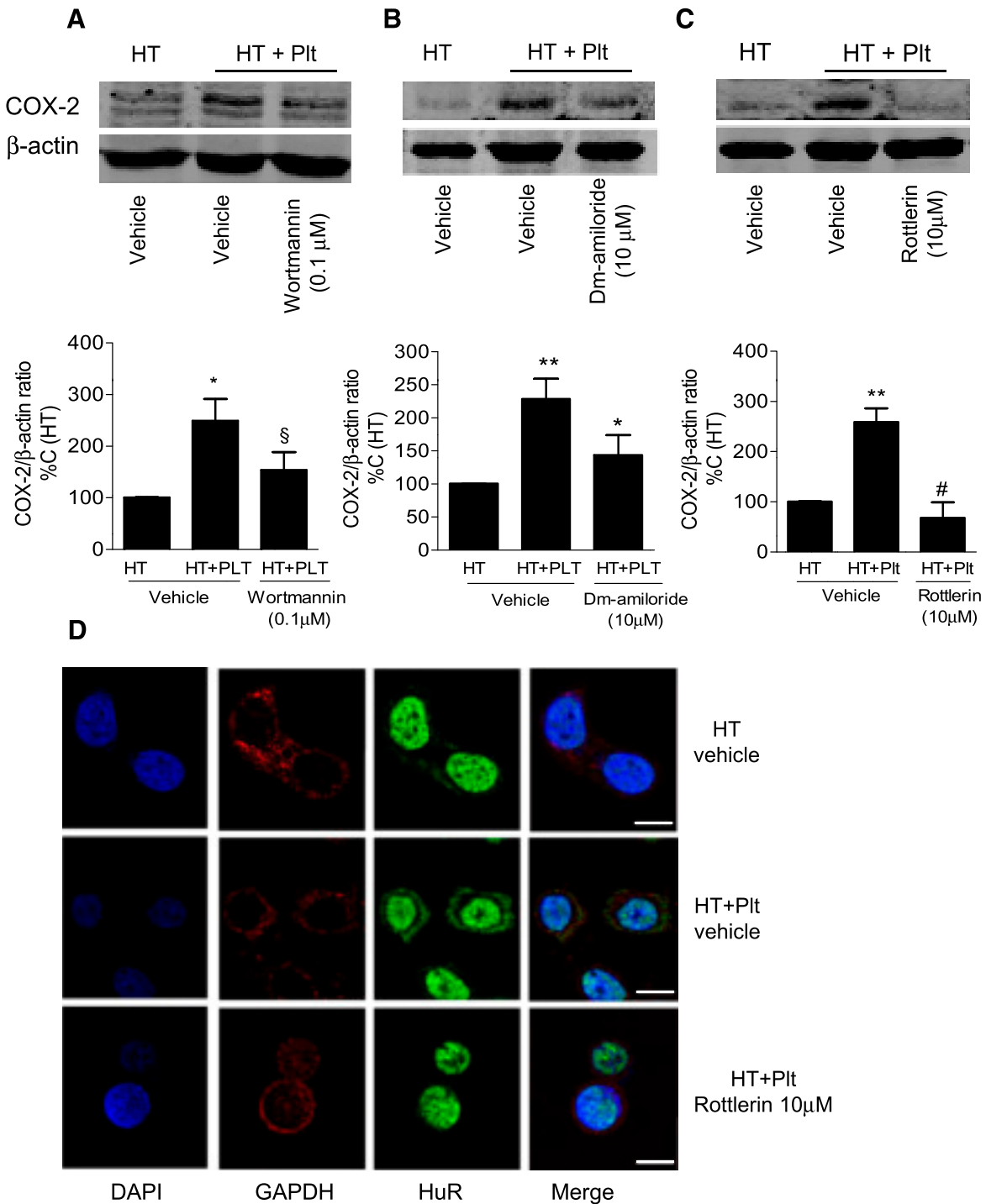


Fig. 5. Activation of PDGFR signaling regulates platelet-dependent COX-2 expression in HT29 cells. HT29 cells were cultured alone (HT) or cocultured for 20 hours with platelets (HT + Plt) in the presence of vehicle or (A) wortmannin (0.1 μM), an inhibitor of PI3K; (B) dm-amiloride (10 μM), an inhibitor of NHE; or (C) rottlerin (10 μM), a specific inhibitor of PKCδ isoform. (A–C) COX-2 protein expression was detected by Western blot analysis, and quantification of optical density (OD) of different specific bands was calculated and normalized to the OD of β-actin. The values were reported as percent of control (i.e., HT29 cells cultured alone). Data are expressed as mean ± S.E.M. ($n = 3$). (A) * $P < 0.05$ versus HT; $^{\S}P < 0.05$ versus HT + Plt (vehicle); (B) ** $P < 0.01$ versus HT; * $P < 0.05$ versus HT + Plt (vehicle); (C) ** $P < 0.01$ versus HT; $^{\#}P < 0.01$ versus HT+Plt (vehicle). (D) Immunofluorescence localization of HuR was examined by confocal microscopy analysis in HT29 cells cultured alone (HT) or incubated with platelets (HT + Plt) for 20 hours in the presence of vehicle or rottlerin (10 μM). Immunostaining of HuR is shown in green; GAPDH (cytoplasmic protein) is shown in red, and DAPI (4',6-diamidino-2-phenylindole) (nuclear marker) is shown in blue. Merge images are shown (merge). Scale bars, 10 μm.

by other platelet receptors for collagen (i.e., $\alpha 2/\beta 1$ and glycoprotein Ib) (Ungerer et al., 2011). We showed that the drug, at clinically relevant concentrations (Ungerer et al., 2011), completely prevented the platelet-induced upregulation of

COX-2 in HT29 cells (Fig. 3E). In contrast, experiments performed with a P-selectin antagonist (Appeldoorn et al., 2003) led us to exclude its role in platelet-HT29 cell interactions (Fig. 3F).

Role of Platelet-Derived Soluble Mediators in COX-2 Upregulation in HT29 Cells. In a further set of experiments, the role of platelet released products, such as TXA₂, ADP/ATP, and PDGF, on COX-2 overexpression in HT29 cells was assessed by a pharmacological approach. As shown in Fig. 4A, platelets pretreated with aspirin (300 μM) to suppress completely and persistently COX-1-dependent TXB₂ generation retained the capacity to induce COX-2 expression in HT29 cells. Similarly, blockage of P2-purinoceptors by suramin (Hourani et al., 1992) did not affect platelet-induced COX-2 expression in HT29 cells (Fig. 4B).

In contrast, imatinib, a nonselective PDGFR inhibitor (Buchdunger et al., 2002) or a PDGF neutralizing antibody completely prevented platelet-dependent induction of COX-2 protein expression (Fig. 4, C and D, respectively). Together, these results suggest involvement of the concurrent action of direct platelet/tumor cell contact and platelet-released PDGF in the aberrant expression of COX-2 in HT29 cells.

Role of Downstream Effectors of PDGFR Signaling on Platelet-Dependent Induction of COX-2 in HT29 Cells. We studied the involvement of downstream effectors of PDGFR (i.e., PI3K, NHE, and PKCδ) (Heldin and Westermark, 1999) by using pharmacological tools affecting their activity (i.e., wortmannin, dm-amiloride, and rottlerin) (Doller et al., 2011), respectively. As shown in Fig. 5, A and B,

wortmannin and dm-amiloride reduced by 63 and 66%, respectively, platelet-dependent COX-2 protein induction. In contrast, rottlerin completely suppressed platelet-induced COX-2 upregulation (Fig. 5C).

These data may place PKCδ downstream of PI3K and NHE in mediating platelet-induced COX-2 expression. PKCδ may modulate COX-2 expression by controlling intracellular HuR localization and function (Doller et al., 2011). As shown in Fig. 5D, rottlerin, at a concentration shown to be selective for PKCδ (Doller et al., 2011), prevented platelet-induced cytoplasmic shuttling of HuR in HT29 cells detected at 20 hours of coculture.

Platelet-HT29 Cell Interaction Triggers PGE₂ Release. We assessed the time course of PGE₂ release by HT29 cells and platelets cultured alone or cocultured for up to 20 hours. As shown in Fig. 6A, HT29 cells released low levels of PGE₂ (at 20 hours, 45 ± 15 pg/ml, *n* = 3). Platelets cultured alone released higher concentrations of PGE₂. The levels of PGE₂ reached the maximum within 4 hours and stayed at the plateau for the remaining incubation period (Fig. 6A). PGE₂ concentrations detected at each time point, from 4 to 20 hours, were not significantly different from each other. In platelet-HT29 cell cocultures, PGE₂ levels released in the medium for up to 8 hours were comparable to those released by platelets cultured alone; at 12 hours, PGE₂ levels started to be higher

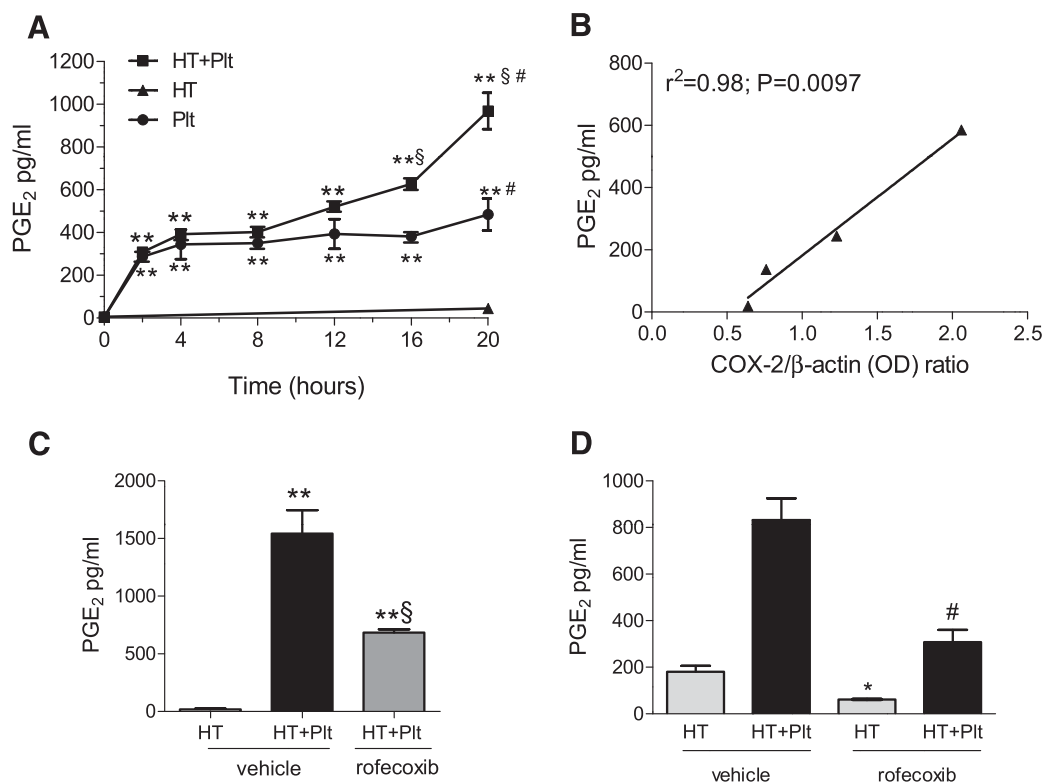


Fig. 6. Platelet-HT29 cell interaction triggers PGE₂ generation. (A) HT29 cells (HT) or unstimulated platelets (Plt) were cultured alone or cocultured (HT + Plt) for up to 20 hours, and the release of PGE₂ was assessed in the culture medium. Values are reported as mean ± S.E.M. (*n* = 3–6). ***P* < 0.01 versus time (0); §*P* < 0.01 versus Plt at same time points; #*P* < 0.01 versus HT at the same time point. (B) PGE₂ levels released from 8 to 20 hours, subtracted from the PGE₂ levels generated by platelets alone, linearly correlated with COX-2 protein levels detected at the same time points in HT29 cell lysates by Western blot. The least-squares line and the coefficient of determination *r*² were calculated by linear regression analysis using PRISM software. (C) Effect of rofecoxib (0.3 μM) or vehicle on PGE₂ released by HT29 cells cultured with platelets for 20 hours. (D) Effect of rofecoxib on AA-induced COX activity of HT29 cells cultured alone or with aspirin-treated platelets. HT29 cells were cultured alone or with aspirin-treated platelets for 20 hours; the medium was changed, and vehicle or rofecoxib was added for 1 hour; then the incubation was continued for 1 hour in the presence of AA (0.5 μM). At the end of the incubation, the medium was collected and assayed for PGE₂. Data are expressed as mean ± S.E.M. (*n* = 3). (C) ***P* < 0.01 versus HT (vehicle); §*P* < 0.01 versus HT + Plt (vehicle). (D) **P* < 0.05 versus HT (vehicle); #*P* < 0.05 versus HT + Plt (vehicle).

than those released by platelets cultured alone and then continued to rise for up to 20 hours (Fig. 6A). PGE₂ levels released from 8 to 20 hours, subtracted from the PGE₂ levels generated by platelets alone, linearly correlated with COX-2 protein levels detected at same time points in cell lysates by Western blot ($r^2 = 0.98$; $P = 0.0097$) (Fig. 6B). This result indirectly suggests that COX-2 induced in HT29 cells by the interaction with platelets contributed to the enhanced levels of PGE₂ detected at late time points. In contrast, the early generation of PGE₂ detected in the medium of platelet-HT29 cell cocultures was derived mainly from platelets.

To obtain direct evidence of the contribution of COX-2 overexpression to the enhanced PGE₂ detected at 20 hours of HT29 cell-platelet cocultures, we studied the effect of rofecoxib (added 20 minutes before platelets) at a concentration (0.3 μ M) that profoundly inhibits the COX-2 activity of HT29 cells without affecting COX-1 activity (Dovizio et al., 2012). As shown in Fig. 6C, PGE₂ accumulation in the medium of HT29 cells and platelets cocultured for 20 hours was significantly reduced (57%) by rofecoxib. To confirm that overexpressed COX-2 was functionally active even at late time points, we studied the effect of rofecoxib on the activity of COX-2 expressed at 20 hours. In this experiment, HT29 cells were incubated with platelets that were pretreated with aspirin (to suppress completely platelet COX-1 activity) and

extensively washed (to eliminate the drug) before the addition to cancer cells. At 20 hours, the medium was changed with a fresh one containing rofecoxib or vehicle and the incubation continued for 1 hour; then AA (0.5 μ M) (to start COX catalysis) was added for a further 1 hour, and PGE₂ levels were measured in the conditioned medium. As shown in Fig. 6D, under these experimental conditions, substantial PGE₂ levels were generated and were profoundly reduced by the treatment with rofecoxib. Together, these results showed that HT29 cells had a low capacity to release PGE₂ from endogenous AA and that COX-2 overexpression induced by platelets enables the generation of increased levels of this prostanoid in these cancer cells.

Effects of Platelet-HT29 Cell Cross-Talk on the Expression of Malignancy-Related Proteins. We studied whether platelet-HT29 cell cross-talk caused changes in phenotypic biochemical markers of malignancy in HT29 cells and whether these changes were dependent on the activity of COX-2. As shown in Fig. 7, A–G, among several proteins analyzed known to be involved in proliferation, apoptosis, or differentiation, p21^{WAF1/CIP1} (Fig. 7A) was downregulated and cyclin B1 (Fig. 7C) was upregulated in HT29 cells cocultured for 20 hours with platelets.

We studied the kinetics of p21^{WAF1/CIP1} and cyclin B1 expression changes induced in HT29 cells by the interaction

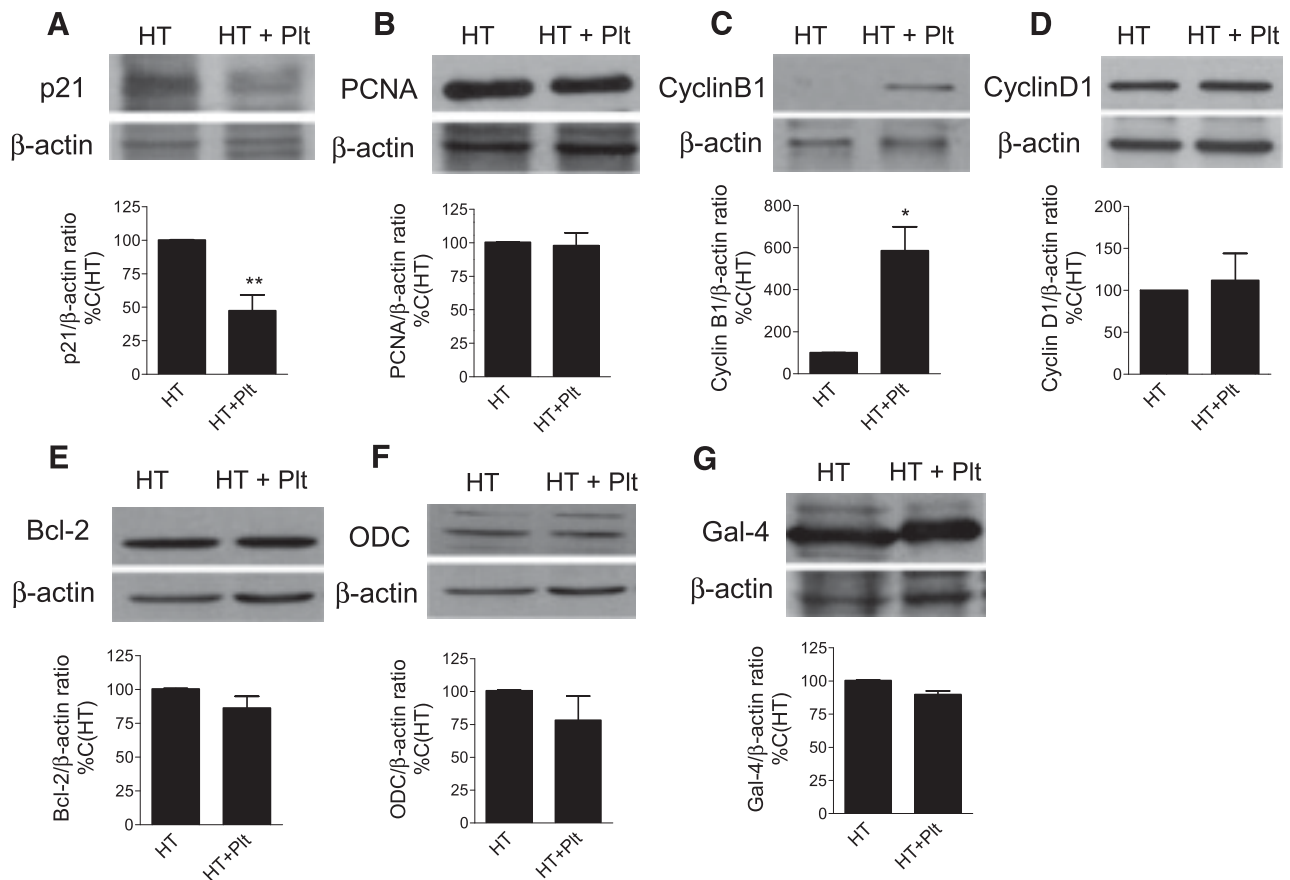


Fig. 7. Platelet-HT29 cell cross-talk induces changes in the expression of proteins involved in malignancy. In HT29 cells cultured alone (HT) or cocultured for 20 hours with platelets (HT + Plt), Western blot analysis was performed to assess the expression levels of several proteins: (A) p21^{WAF1/CIP1} (p21), (B) proliferating cell nuclear antigen (PCNA), (C) cyclin B1, (D) cyclin D1, (E) Bcl-2, (F) ornithine decarboxylase (ODC), (G) galectin-4 (Gal-4). The expression level of different proteins was assessed as a ratio of their optical density (OD) normalized to the OD of β -actin and shown as a percent of control (HT29 cells cultured alone). Data are expressed as mean \pm S.E.M. ($n = 3-6$). (A) $*P < 0.01$ versus HT; (C) $*P < 0.05$ versus HT.

with platelets. As shown in Fig. 8, p21^{WAF1/CIP1} was rapidly induced after the addition of platelets to HT29 cells but returned to baseline levels at 8 hours; later, p21^{WAF1/CIP1} levels decreased in a time-dependent fashion. Incubation of HT29 cells with platelets was associated with cyclin B1 induction only at late time points (i.e., 20 hours) (Fig. 8).

To demonstrate the role of COX-2-derived PGE₂ in p21^{WAF1/CIP1} and cyclin B1 changes caused by the interaction of platelets with HT29 cells, we studied the effects of rofecoxib (0.3 μM). As shown in Fig. 9, A and B, rofecoxib reverted the reduction of p21^{WAF1/CIP1} and the induction of cyclin B1 in HT29 cells cocultured with platelets. These effects of rofecoxib were completely abrogated by the exogenous addition of PGE₂ at concentrations generated in the cocultures of HT29 cells and platelets (Fig. 9C). Together, these results convincingly support the role of COX-2-derived PGE₂ induced by the interaction of platelets with HT29 cells in the reduction of p21^{WAF1/CIP1} and the induction of cyclin B1.

Platelets Induce EMT Markers in HT29 Cells: Role of Direct Cell-Cell Interaction and COX-2 Overexpression. We assessed the effects of platelets on mRNA expression of EMT-inducing transcription factors, such as ZEB1 and TWIST1, the mesenchymal marker vimentin, and the epithelial marker E-cadherin in HT29 cells. First, we studied the kinetics of mRNA changes of these EMT markers in

cocultures of HT29 cells and platelets. As shown in Fig. 10A, in HT29 cells, platelets induced a time-dependent increase in ZEB1 and TWIST1 associated with increased expression of vimentin and a decrease in the levels of E-cadherin. A significant increase in ZEB1 and TWIST1 mRNAs were detected at 4 hours, and this increase persisted up to 20 hours. Vimentin mRNA was significantly increased at 4 hours and further increased at 20 hours. E-cadherin was significantly decreased (by 35%) in HT29 cells at 20 hours of cocultivation with platelets.

The inhibition of platelet adhesion to HT29 cells by revacept completely prevented the induction of EMT markers in HT29 cells at 20 hours of incubation with platelets (Fig. 10B). In contrast, E-cadherin mRNA levels were significantly higher in revacept-treated cells than in those treated with vehicle. These results may suggest that a direct platelet-HT29 cell interaction induces cancer cell transition to an invasive mesenchymal-like phenotype.

Platelet-induced COX-2 overexpression in HT29 cells might emanate mitogenic and survival signaling pathways to stabilize gene signature in cells undergoing EMT. Thus, we studied the effect of rofecoxib on mRNA changes of EMT markers in platelet-HT29 cell cocultures at 20 hours. As shown in Fig. 10B, in the presence of rofecoxib, the expression of ZEB1, TWIST1, and vimentin was significantly reduced, whereas that of E-cadherin tended to increase.

The inhibitory effect of revacept on ZEB1, TWIST1, and vimentin expression was higher than that caused by rofecoxib, although only ZEB1 reduction was different in a statistically significant fashion (Fig. 10B). These results may suggest that direct platelet-tumor cell interaction and platelet-induced COX-2 overexpression synergistically activate HT29 cell transition to mesenchymal-like phenotype.

Discussion

In the present study, we show that platelet-cancer cell cross-talk led to enhanced COX-2-dependent PGE₂ generation in HT29 cells, which contributed to downregulation of p21^{WAF1/CIP1} and upregulation of cyclin B1, together with the induction of gene expression signatures associated with EMT. We used a pharmacological approach to characterize the molecular partners involved in the direct platelet-HT29 cell interactions and the platelet-derived soluble factors contributing to the aberrant COX-2 overexpression in cancer cells. The role of platelet GPVI, and possibly other collagen receptors, was confirmed using revacept, which is a soluble form of the platelet GPVI receptor that has been shown to bind specifically to collagen at sites of vascular damage, thus inhibiting platelet adhesion and aggregation (Ungerer et al., 2011). The use of three different inhibitors of galectin-3 function [(β-lactose, Gal-3C) (John et al., 2003) and anti-galectin-3 antibody M3/38 (Fukushi et al., 2004)] showed the involvement of this galectin in platelet-induced COX-2 expression in HT29 cells. The activity of both lactose and Gal-3C are dependent on the carbohydrate-mediated functions of galectin-3. The M3/38 antibody binds to the N-terminal domain of galectin-3, which contains a collagen-like domain (Nangia-Makker et al., 2008). The results suggest that galectin-3 may act as a binding partner of platelet collagen receptors and, interestingly, imply that the binding is dependent on carbohydrate binding mediated by CRD and

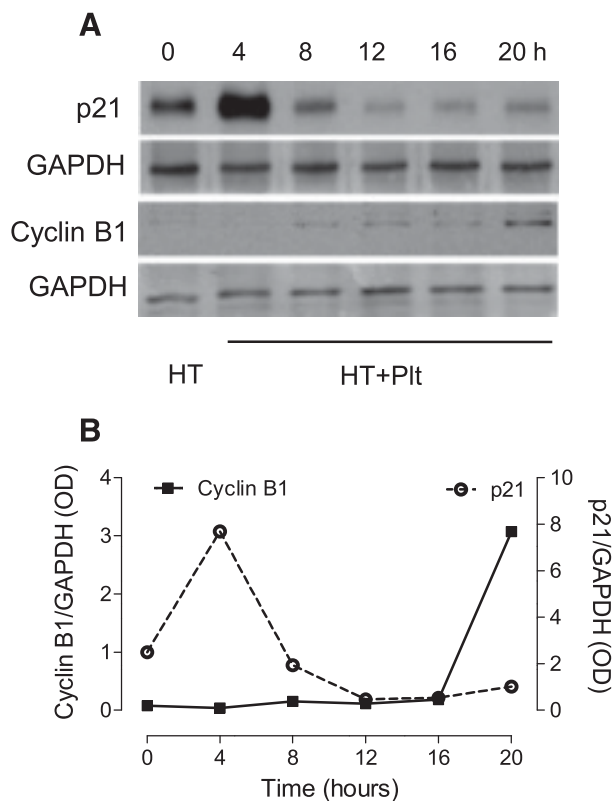


Fig. 8. Time-related expression of p21^{WAF1/CIP1} and cyclin B1 proteins in HT29 cells cocultured with platelets. (A) HT29 cells were cultured alone (HT) or cocultured with platelets (HT + Plt) for different time points up to 20 hours; p21^{WAF1/CIP1} (p21), cyclin B1, and GAPDH (as loading control) protein levels were assessed by Western blot technique. (B) Quantification of optical density (OD) of different immunoreactive bands of p21^{WAF1/CIP1} and cyclin B1 was calculated and normalized to the OD of GAPDH. Data are reported as mean of two separate experiments.

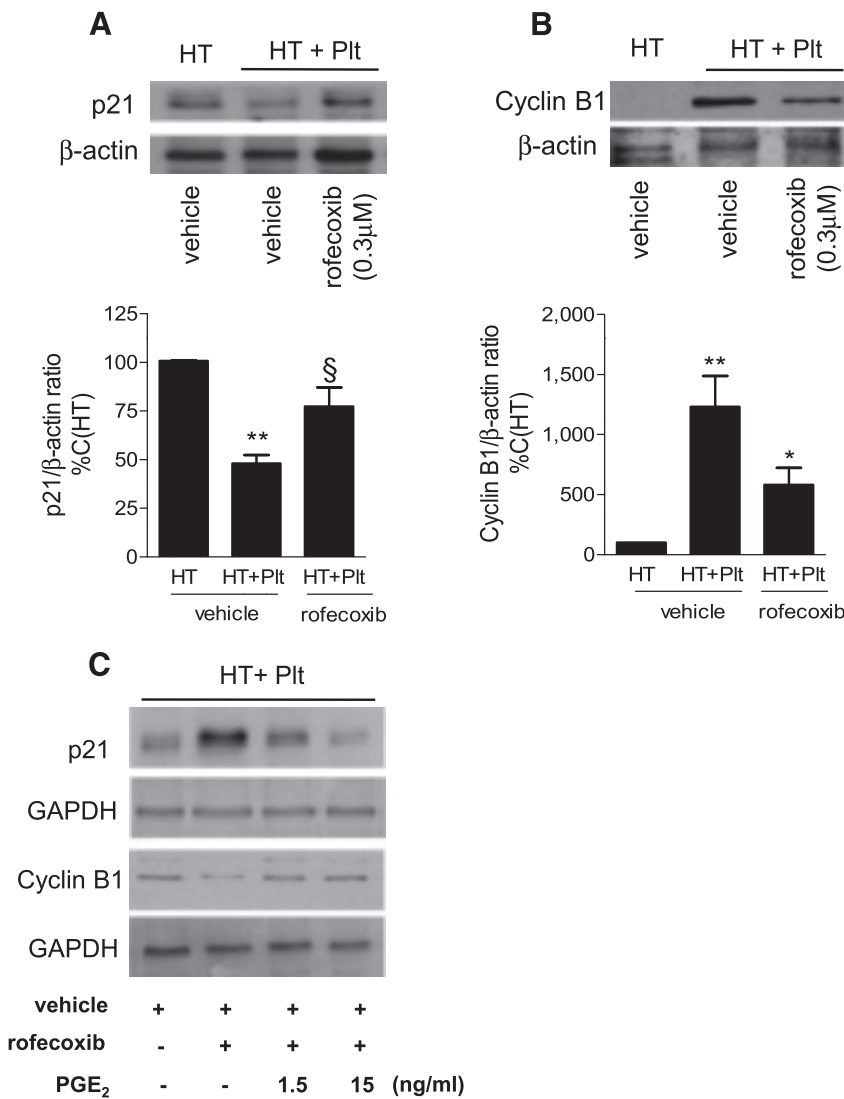


Fig. 9. Platelet-induced COX-2 activity regulates p21^{WAF1/CIP1} and cyclin B1 protein levels in HT29 cells. (A and B) The effect of a selective COX-2 inhibitor, rofecoxib (0.3 μM), on protein levels of p21^{WAF1/CIP1} and cyclin B1, respectively, in HT29 cells cultured with platelets for 20 hours was assessed by Western blot. Data are expressed as mean ± S.E.M. from three experiments, as % of control (i.e., HT29 cells cultured alone). (A) ** $P < 0.01$ versus HT; § $P < 0.05$ versus HT + Plt (vehicle); (B) ** $P < 0.01$ versus HT; * $P < 0.05$ versus HT + Plt (vehicle). (C) The effects of two different concentrations of exogenous PGE₂ (1.5 and 15 ng/ml) on the expression levels of p21^{WAF1/CIP1} (p21) and cyclin B1, in HT29 cells cocultured with platelets for 20 hours in the presence of rofecoxib (0.3 μM) or vehicle, were assessed by Western blot technique.

by homologous or heterologous protein-protein binding mediated by the collagen-like domain. Our results showing that Gal-3C prevented platelet-induced COX-2 overexpression in cancer cells also suggest that this could be a mechanism of its anticancer activity, as demonstrated in animal models (John et al., 2003; Mirandola et al., 2011). The contribution of PDGF, released by activated platelets, to the induction of COX-2 in HT29 cells was disclosed by blocking PDGFR activation with imatinib (Buchdunger et al., 2002) or by using a PDGF-neutralizing antibody.

Herein, we provide evidence that COX-2-derived PGE₂ induced by the interaction of platelets with HT29 cells was involved in the reduction of p21^{WAF1/CIP1} and the induction of cyclin B1, typical changes that allow progression through the G₂-M checkpoint (Wang et al., 2009). In fact, the changes of these two proteins were prevented by rofecoxib and rescued by the addition of exogenous PGE₂.

Platelet-HT29 cell cocultivation was associated with a rapid release of PGE₂ derived from platelets and a delayed release of this prostanoid dependent on COX-2 upregulation in HT29 cells. The efficacy of rofecoxib to prevent platelet-induced changes of p21^{WAF1/CIP1} and cyclin B1 supports the dominant

role of COX-2-dependent PGE₂, which may activate EP receptors localized, similarly to COX-2, in the nuclear compartment (Morita et al., 1995; Bhattacharya et al., 1998, 1999; Schlötzer-Schrehardt et al., 2002; Konger et al., 2005). As shown in Supplemental Fig. 3, EP1, EP2, and EP4 were detected by Western blot in both extranuclear and nuclear compartments of HT29 cells.

The kinetics of biologic events induced by platelet-HT29 cell interactions, which contributed to changes in proteins associated with malignant progression, was dissected by recording (every 4–20 hours) simultaneously the release of platelet-derived products, i.e., TXB₂, growth factors PDGF-BB and EGF and the cytokine TGF-β1, and protein levels of COX-2, p21^{WAF1/CIP1}, and cyclin B1 in HT29 cell lysates. In the first 4 hours, platelets adhered to HT29 cells, and they were activated and released large amounts of TXA₂, which may recruit and activate additional platelets. In contrast, a substantial release of PDGF-BB and TGF-β1 from platelet α-granules began after a lag time of 4 hours. However, PDGF-BB levels were higher than those of TGF-β1, and they continuously increased in a time-dependent fashion up to 20 hours. In contrast, TGF-β1, after being released, decreased

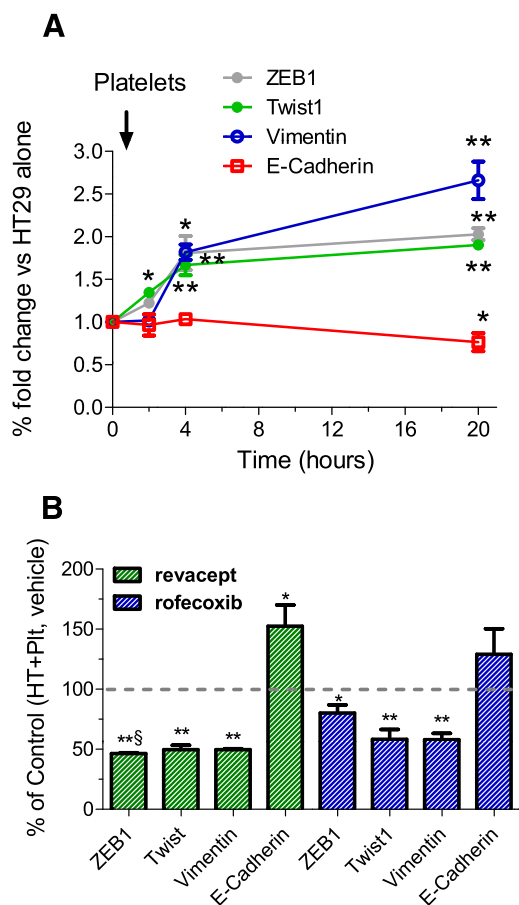


Fig. 10. Platelet adhesion to HT29 cells and upregulation of COX-2 protein expression are involved in the induction of genes involved in the epithelial-mesenchymal transition. (A) HT29 cells were cultured alone (0 hour) or cocultured with platelets for different time points up to 20 hours. The mRNA levels of ZEB1, Twist1, vimentin, and E-cadherin were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to GAPDH levels. Data are reported as mean \pm S.E.M. ($n = 3$) of fold change versus HT29 cells cultured alone (0 hour). (B) The effects of revacept (400 μ g/ml) and rofecoxib (0.3 μ M) on the expression of these genes were assessed in HT29 cells cocultured for 20 hours with platelets. Data are reported as mean \pm S.E.M. ($n = 3$), as a percent of control (vehicle); * $P < 0.05$; ** $P < 0.01$ versus vehicle; § $P < 0.05$ versus rofecoxib.

in a time-dependent manner, suggesting its possible cellular reuptake. Platelets cultured alone released low concentrations of EGF, which were not significantly enhanced by the cocultivation with HT29 cells; instead, they decreased over time. These results show that PDGF-BB was the dominant growth factor continuously released by cancer cell-activated platelets.

The activation of platelet collagen receptor GPVI during the adhesion phase of platelets to HT29 cells may explain the delay in the release of α -granule proteins, such as PDGF. In fact, GPVI signaling is known to regulate negatively platelet granule secretion (Chari et al., 2009). Interestingly, in platelet-HT29 cell cocultures, we detected a time-dependent release of soluble GPVI (Supplemental Fig. 4), which seemed to parallel the detachment of platelets from HT29 cells and the release of PDGF-BB into the medium. In platelets, GPVI activation may trigger proteolytic pathways, leading to GPVI ectodomain shedding that results in

the release of a soluble approximately 55-kDa fragment into the platelet supernatant (Gardiner et al., 2004; Bender et al., 2010).

During the adhesion phase of platelets to HT29 cells, the levels of COX-2 mRNA rapidly increased and remained stable up to 20 hours. In contrast, COX-2 protein synthesis began to increase after a lag time of 8 hours, and then it continued to rise, in a time-dependent manner, even when platelets were detached from cancer cells. These results show that HT29 cells can be primed for COX-2 mRNA expression by a transient interaction with platelets. However, soluble factors released by platelets at later time points may contribute to post-transcriptional regulation of COX-2 through the stabilization of COX-2 mRNA. In this study, we provide several lines of evidence that platelet PDGF may be involved in this phenomenon: 1) the onset of PDGF-BB secretion occurred earlier than that of COX-2 protein synthesis and COX-2-dependent PGE₂ release, 2) the time-dependent increase in PDGF-BB levels was accompanied by a parallel upregulation of COX-2 protein, 3) imatinib and a specific anti-PDGF antibody prevented the induction of COX-2 protein, and 4) pharmacological inhibition of downstream effectors of PDGFR (i.e., PI3K, NHE, and PKC δ) by using wortmannin, dm-amiloride, and rottlerin, respectively (Doller et al., 2011) reduced COX-2 protein induction.

Prolonged incubation of platelets with HT29 cells was associated with nucleo-cytoplasmic translocation of HuR, which has been reported to control both mRNA decay and protein translation of COX-2 (Young et al., 2009). The role of PKC δ in the export of HuR from the nuclear compartment was evidenced using rottlerin, which has been shown to interfere specifically with PKC δ -triggered HuR phosphorylation, in a cell-free HuR phosphorylation assay (Doller et al., 2011).

TGF- β 1, released by cancer cell-activated platelets, seems to have a negligible, if any, role in COX-2 upregulation detected in HT29 cells. In fact, this human colon cancer cell line is resistant to TGF- β 1-mediated growth inhibition and apoptosis (Winesett et al., 1996; Tong et al., 2009) as a result of inactivated TGF- β type II receptor and deficient expression of RUNX3 and Smad4, which are involved in the TGF- β signaling pathway. TGF- β has been shown to promote cancer cell growth through the activation of alternative signaling pathways, but at concentrations (i.e., ≥ 5 ng/ml) (Halder et al., 2005) that are more than 10-fold higher than those detected, transiently, in the medium of HT29 cell-platelet cocultures (Supplemental Fig. 1C).

The adhesion of platelets to HT29 cells was associated with a transient upregulation of p21^{WAF1/CIP1} protein, possibly through the activation of transcriptional or post-transcriptional regulatory mechanisms (Wang et al., 2012), which necessitate further investigation. This effect was unlikely due to released TGF- β 1 because 1) the cytokine was released later than p21^{WAF1/CIP1} protein induction, and 2) HT29 cells have been previously shown to be insensitive to p21^{WAF1/CIP1} induction after TGF- β treatment (Li et al., 1995). In prolonged cocultures of HT29 cells with platelets, p21^{WAF1/CIP1} levels decreased in parallel to enhanced COX-2-dependent PGE₂ generation. The induction of cyclin B1 was a late response that probably required a profound down-regulation of p21^{WAF1/CIP1} to emerge since this protein has been previously shown to target cyclin B1 for degradation (Gillis et al., 2009).

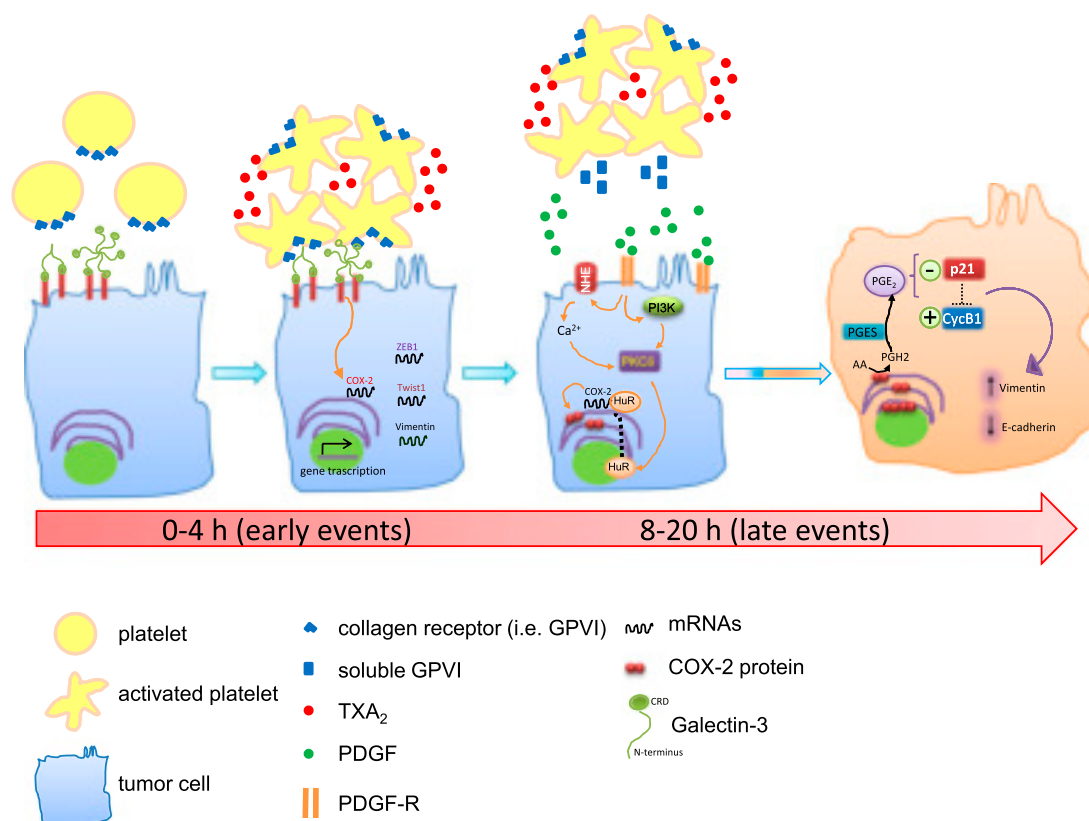


Fig. 11. Proposed mechanism of COX-2 overexpression in tumor cells by the interaction with platelets. Unstimulated platelets interact rapidly with tumor cells through the binding of platelet collagen receptors (in particular, GPVI) and tumor components, such as galectin-3. This early event translates into platelet activation, as demonstrated by enhanced generation of TXA₂. Direct platelet-tumor cell interaction is associated with enhanced mRNA expression of COX-2 (but not COX-2 protein) and EMT-inducing transcription factors, such as ZEB1 and Twist1, and the mesenchymal marker vimentin. Later, platelet aggregates detach from tumor cells, possibly as a consequence of the shedding of platelet GPVI receptors, and acquire the capacity to release their α -granule products, such as PDGF. The release of PDGF is associated with COX-2 mRNA stabilization via NHE-PI3K/PKC δ -dependent nucleocytoplasmic translocation of the mRNA-stabilizing protein HuR and COX-2 protein synthesis. In HT29 cells, overexpressed COX-2 and enhanced generation of PGE₂ emanate mitogenic and survival signaling pathways through the downregulation of p21^{WAF1/CIP1} and the upregulation of cyclin B1 as well as of EMT-inducing transcription factors and mesenchymal markers, such as vimentin, in association with repression of epithelial markers, such as E-cadherin.

Platelet adhesion to HT29 cells caused an increased expression of genes involved in the EMT, such as the EMT-inducing transcription factors ZEB1 and TWIST1 and the mesenchymal marker vimentin (Kalluri and Weinberg, 2009). These changes persisted at long incubation times, when platelets detached from cancer cells, in association with a reduced expression of the epithelial marker E-cadherin. The finding that rofecoxib prevented mRNA changes of EMT markers in platelet-HT29 cell cocultures at 20 hours, although less efficiently than revacept, suggests that direct platelet-tumor cell interaction and platelet-induced COX-2 overexpression synergistically activate the HT29 cell transition to a mesenchymal-like phenotype.

In the present study, we show that selective inhibition of platelet COX-1 activity by aspirin did not significantly affect platelet-induced upregulation of COX-2 in HT29 cells. HT29 cells may be insensitive to an enhanced release of TXA₂ by activated platelets as a result of the absence of TXA₂ receptor (TP) isoforms (Supplemental Fig. 5). TP signaling seems to play a critical role in tumor colonization (Matsui et al., 2012). Our results provide the rationale for studying whether TP expression in circulating tumor cells can identify individuals who are responders to aspirin chemotherapy.

In conclusion, we have unraveled the role of platelets in inducing COX-2 upregulation in HT29 cells, considered a key event in carcinogenesis (Prescott, 2000; Young et al., 2009).

This program of malignancy is primed by transient platelet-cancer cell contact involving tumor galectin-3 and platelet collagen receptors and the release of platelet PDGF (Fig. 11). These findings also reveal that inhibitors of this program, such as blockers of collagen binding sites, such as revacept, and galectin-3 may represent an innovative strategy in colon cancer chemotherapy that should be tested in experimental animals, followed by randomized clinical trials in colon cancer patients.

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Authorship Contributions

Participated in research design: Patrignani, Dovizio, Steinhilber.
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Wrote or contributed to the writing of the manuscript: Patrignani, Steinhilber, Sgambato, Suess, Dovizio.

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