The Interaction of Platelets with Colorectal Cancer Cells
Inhibits Tumor Growth but Promotes Metastasis

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Platelets promote metastasis, however, their role in tumor growth remains controversial. Here, we investigated the effect of platelet interactions with colorectal tumor cells. Platelets extravasated into the tumor microenvironment and interacted with tumor cells in a cadherin-6-dependent manner. The interaction induced platelet spreading, release of their granule content, and the generation of three types of microparticles (iMP) that expressed platelet markers, tumor markers, or both. The presence of iMPs was confirmed in colorectal cancer tissue specimens. Platelets significantly reduced tumor growth and increased intratumoral macrophages. This was mediated by iMP recruitment of macrophages via the chemotaxants RANTES, MIF, CCL2, and CXCL12 and activation of their tumor cell killing capacity through IFNγ and IL4, which led to cell-cycle arrest of tumor cells in a p21-dependent manner. In contrast, in the bloodstream, iMPs activated endothelial cells and platelets and induced epithelial-to-mesenchymal transition of tumor cells, promoting metastasis. Altogether, these results indicate that depending on the environment, local or bloodstream, the consequences of the interactions between platelets and a tumor may promote or prevent cancer progression.

Significance: Tumor cell interaction with platelets produces chimeric extracellular vesicles that suppress primary tumor growth by activating tumor-eliminating macrophages, while promoting metastasis through EMT and endothelial activation.

Introduction

Platelets were first described as the major effectors of hemostasis and thrombosis. However, platelets may also actively participate in the progression of malignancies (1). Since pioneering studies performed in the early 1970s, increasing evidence has shown that the release of platelet agonists and the expression of procoagulant proteins, such as tissue factor (2–4), by tumor cells induce platelet activation and aggregation in the bloodstream and actively participate in the process of metastasis. Subsequently, numerous studies, including ours, have demonstrated that the inhibition of platelet activation by clopidogrel, ticagrelor, or daily aspirin impairs tumor metastasis in different animal models and in humans (5–7). In addition to their role in the development of metastasis, platelets have also been reported to participate in tumor growth, although the latter is still subject to controversy. On the one hand, studies have demonstrated that platelet-released factors promote tumor cell proliferation and prosurvival signaling (8, 9). On the other hand, other studies have shown that platelets and platelet-derived microparticles exert antiproliferative and cytotoxic effects on many tumor cells (10–12).

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Materials and Methods

Cell culture

A stable clone of murine colorectal cancer cells expressing GFP and luciferase named CT26GFP/Luci was obtained by infection of a CT26 WT cell line (ATCC CRL-2638, used at passage 7) with lentiviral particles (Amsbio LVP436-PBS) in the presence of polybrene (Life Technologies, 10 µg/mL) and with lentiviral particles (Amsbio LVP436-PBS) in the presence of polybrene (Life Technologies, 10 µg/mL). A CT26GFP/Luci clone was selected and cultured in RPMI-1640 supplemented by 10% of decomplemented FCS, 100 U/mL of penicillin, 100 mg/mL of streptomycin, 2 mmol/L of glutamine, 0.1% fungizone and 0.02 mg/mL of blasticidin. Human colorectal cancer cell line HT-29 (ATCC HTB-38) was cultivated in McCoy’s 5A medium supplemented by 10% of decomplemented FCS, 100 U/mL of penicillin, 100 mg/mL of streptomycin, 2 mmol/L glucose, and 0.1% fungizone. The cells were tested for Mycoplasma and were grown.
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**Mycoplasma**

10^6 CT26GFP/Luci cells were seeded into 6-well plates (Thermo-Fisher Scientific) for 18 hours. Washed human or murine platelets were added to tumor cells (50:1 ratio of platelets to tumor cells) in an appropriate prewarmed FCS-free medium. The interactions were stopped by extensive washing, and platelets interacting with tumor cells were fixed in 2% PFA or harvested to obtain total protein lysates. The interaction supernatants (called iSN) were collected, cellular debris was removed by centrifugation, and then the appropriate prewarmed FCS-free medium was added. Alternatively, interactions were performed in blocking conditions: platelets were incubated with cadherin-6 blocking antibody (20 or 40 μg/mL), RGDV (100 μmol/L), or both for 20 minutes before contact with cancer cells.

**Interactions between colorectal tumor cells and platelets**

The kinetics of the interaction were measured at several end points (10, 30, 60, 120, 180, and 360 minutes). Briefly, 1.9 × 10^5 HT29 or 1.7 × 10^5 CT26GFP/Luci cells were seeded into 6-well plates (Thermo-Nunc, Thermo Fisher Scientific) for 18 hours. Washed human or murine platelets were added to tumor cells (50:1 ratio of platelets to tumor cells) in an appropriate prewarmed FCS-free medium. The interactions were stopped by extensive washing, and platelets interacting with tumor cells were fixed in 2% PFA or harvested to obtain total protein lysates. The interaction supernatants (called iSN) were collected, cellular debris was removed by centrifugation, and then the appropriate prewarmed FCS-free medium was added. Alternatively, interactions were performed in blocking conditions: platelets were incubated with cadherin-6 blocking antibody (20 or 40 μg/mL), RGDV (100 μmol/L), or both for 20 minutes before contact with cancer cells.

**Purification and characterization of the iMPs contained in the iSN and in the plasma of patients with colorectal cancer**

iSN were centrifuged at 1,000 × g for 15 minutes, then centrifuged at 20,000 × g for 1 hour, and the iMP pellet was resuspended in FCS-free McCoy’s medium. The iSN or the purified iMPs were incubated with FITC-Annexin V (PharMingen), PE anti-human Epcam and APC anti-human CD41. The flow cytometry instrument settings and microparticle gating were performed with Megamix beads.

A total of 13 patients who underwent surgery for colorectal cancer in the Digestive Surgical Department of Timone Hospital were included in the study, and the samples were issued from our local collection database (DC2013-1815). Platelet-poor plasma containing MPs was obtained as previously described (17). The circulating iMPs contained in the PPP samples were analyzed using a Cytoflex flow cytometry instrument. The iMPs were detected with a combination of FITC-conjugated Annexin V, a PC7-conjugated CD41 antibody, and a PE-Dazzle-conjugated Epcam antibody. All analyses were performed with Kaluza software.

**Interactions between human colorectal tumor cells, platelets, and monocytes**

Interactions between HT29 and monocytes were examined in vitro on 20 × 20 objective on 20 fields per condition and per experiment. Human cytokine array

Human cytokine arrays were performed according to the manufacturer’s instructions (R&D Systems) with iSN and iMPs with or without permeabilizing conditions (1% Triton X-100).

**Ectopic syngeneic colorectal cancer mouse model**

Wild-type Balb/cByJRj mice were obtained from Janvier Elevage (Janvier Labs) and housed under standard conditions. All animal care and experimental procedures were performed as recommended by the European Community guidelines and were approved by local ethical committee number 14 (number 201803271342114). Six- to 8-week-old mice were anesthetized with 2% isoflurane gas. CT26GFP/Luci or CT26 shRNA cdh-6 clones were incubated with platelets or Tyrode buffer for 1 hour. Colorectal cancer cells were washed extensively before injection into the right flank of the mice (0.5 × 10^6 cells). Tumors were allowed to grow for 30 days and measured twice a week with an electronic caliper.

**Statistical analysis**

For in vitro experiments and tumor volume measurement, significance was determined using a two-tailed Student t test. The differences were significant at P < 0.05.

**Data sharing statement**

For original data, please contact christophe.dubois@univ-amu.fr

**Results**

Platelets were present in the tumor microenvironment and interacted with tumors through the expression of cadherin 6

To decipher the role played by platelets in the tumor microenvironment, we first determined if platelets were present apart from blood vessels, namely, within a colorectal tumor. We developed an ectopic colorectal cancer model using CT26 GFP/Luci cancer cells. Thirty days after the injection of the cells, when the tumors reached a volume of approximately 1,500 mm³, some platelets were detected apart from the tumor blood vessels (Fig. 1A) and directly interacting with the cancer cells (Fig. 1B). Intratumoral platelets were mostly detected at the periphery and not at the center of the tumors (Fig. 1C and D) and represented approximately 20% of the total platelets within the tumor (Fig. 1E). The presence of intratumoral platelets was confirmed in poorly differentiated colorectal human adenocarcinoma (Fig. 1F). We next studied the interactions of platelets with colorectal cancer cells in vitro, in the absence of plasma, to reproduce the intratumoral conditions. Interestingly, the colorectal cancer cell line HT29 did not induce aggregation in the presence of washed platelets (Supplementary...
Fig. S1), indicating that the intratumoral interactions of platelets with cancer cells are independent of the classic αIIbβ3-fibrinogen platelet aggregation pathway. This finding was confirmed by studying the kinetics of the interactions between the two cellular partners: single platelets, not aggregates, adhered to the cancer cells, and the number of adherent platelets increased over time to reach 200 platelets per field after 60 minutes (Fig. 2A and B). This result was confirmed under dynamic conditions at 6 dynes/cm². This interaction was specific for cancer cells: under the same conditions, platelets did not adhere to HUVECs (Fig. 2C and D).

Dune and collaborators demonstrated that platelets express cadherin-6 (18), which is a type II cadherin that possesses an RGD motif, allowing it to interact in a homophilic manner with other cadherin-6 proteins and in a heterophilic manner with integrins through the RGD motif. To date, the functional roles of cadherins in platelet-cancer cell interactions have not yet been investigated. We hypothesize that cadherin-6 could be involved in the interactions of platelets with colorectal cancer cells. Flow cytometry analysis confirmed that platelets and HT29 cancer cells expressed cadherin-6 (Fig. 2E). The expression of cadherin-6 by HT29 was further confirmed by...
Figure 2.
Interactions of platelets with colorectal tumors are cadherin-6 dependent. A, Representative images of platelet-HT29 cell interactions at 10, 30, and 60 minutes. DiD-stained human washed platelets (hWP) were added to calcein-green-stained HT29 cells at a ratio of 50:1 (platelets: cancer cells) for 10, 30, and 60 minutes. HT29 cells are depicted in brightfield (left); DiD-stained human washed platelets, red (middle); merged image (right). Bars, 50 µm. B, Graph representing the mean number (± SD) of DiD-positive platelets in interaction with HT29 cells/field, with 10 fields analyzed per experiment (P < 0.05; n = 3). C, Top, representative images showing the DiD-stained human washed platelet and calcein-green-stained HT29 cell interaction after 60 minutes of interaction under dynamic conditions (6 dynes/cm²). Bottom, representative images showing the absence of an interaction between DiD-stained human washed platelets (red) and Hoechst 33342-stained endothelial cells (HUVEC, blue) after 60 minutes under dynamic conditions (6 dynes/cm²). D, The graph depicts the mean number (±SD) of DiD-positive platelets/field in interaction with HT29 cells or HUVECs. (P < 0.001; n = 3). E, Expression of cadherin-6 on HT29 cells and human washed platelets assessed by flow cytometry using indirect immunostaining with an unconjugated anti-human cadherin-6 IgG antibody (10 µg/mL) and AF649-conjugated anti-rabbit IgG secondary antibody (5 µg/mL). Cadherin-6-specific staining, green; matched isotype control, red. F, RT-PCR using SSVI retrotranscriptase and cadherin-6 forward and reverse primers on total extracted RNA from HT29 cells and Mo59K human malignant glioblastoma cells (ATCC CRL-2365, positive control). G, Cadherin-6 inhibition decreases the interaction of human washed platelets with HT29 cells. The human washed platelets were incubated with blocking antibody against cadherin-6 (40 µg/mL) and/or RGDV (100 µmol/L) or control IgG (40 µg/mL) for 20 minutes before their addition to the HT29 cells in dynamic conditions (6 dynes/cm²). The results are expressed as the mean percentage of the interaction between the human washed platelets and HT29 cells in blocking conditions in comparison with the control after 60 minutes of interaction ± SD. (P < 0.05; n = 4). Quantifications were performed on 10 fields per experiment using ImageJ software. Two-tailed Student t test: *, P < 0.05; ***, P < 0.001.
The interaction of platelets with cancer cells induced the generation of microparticles detected in patients with colorectal cancer

During the first 30 minutes following the interactions of single platelets with colorectal cancer cells, we did not observe any changes in the platelet morphology. However, starting at 60 minutes postinteraction, nondiscoid platelets with an area greater than 10 μm², corresponding to spread platelets, were detected on the surface of cancer cells (Fig. 3A and B). The interaction of platelets with cancer cells also led to the generation of microparticles (MP). Platelet-derived microparticles (PMP) were observed in the supernatant after 60 minutes of interaction between platelets and cancer cells (SN) and was significantly increased at 360 minutes to 2,000 MPs/μL. This generation of PMPs was not due to the apoptosis of platelets since, in the absence of cancer cells, the control platelets generated fewer than 100 MPs/μL at 360 minutes (Fig. 3C, left). Tumor-derived microparticles (TMP) were also detected at a concentration of 420 MPs/μL after 360 minutes of interaction. In this case, however, the number of MPs was not significantly different from the number of MPs detected in the absence of platelets (Fig. 3C, middle), mainly because the tumor cells constantly generated MPs (19). Interestingly, the interaction of platelets with tumor cells also led to the production of MPs expressing both CD41 and Epcam at their surface (T+PMPs) with a concentration of 200 MPs/μL at 360 minutes (Fig. 3C, right). We excluded the possibility that T+PMPs were MP aggregates by performing serial dilutions of the sample. We indeed observed a direct correlation between the dilution of the sample performed and the quantity of T+PMPs detected (r² = 0.9288). Thus, the interaction of platelets with cancer cells induced the production of 3 different types of MPs: 76% PMPs, 16% TMPs, and 8% T+PMPs. We named these MPs (PMPs, TMPs, and T+PMPs) iMPs for platelet- and tumor cell-interacting microparticles.

We next determined whether the T+PMPs could be detected at the surface of cancer cells interacting with platelets by confocal microscopy. As illustrated in Fig. 3D, vesicles expressing Annexin V, CD41, and Epcam were identified on the surface of cancer cells interacting with platelets. Spread platelets, by secreting their granule contents into the cancer cells, might have induced a membrane redistribution that was involved in the production of T+PMPs. To test this hypothesis, we injected fluorescent fibrinogen into recipient mice. After 1 day, platelets isolated from these mice were found to express fluorescent fibrinogen in their granules (Fig. 3E). After interaction with tumor cells, a signal corresponding to fluorescent fibrinogen was detected in the cancer cells apart from the platelets (Fig. 3F), which indicated that platelet granule contents were indeed released into the cancer cells and, consequently, that the platelet and cancer cell membranes were fused. We next determined whether T+PMPs are indeed generated in patients with colorectal cancer. MPs expressing both Epcam and CD41 were detected in the plasma of all 13 patients studied, with concentrations ranging from 20 to 257 and a median of 70 MPs/μL of plasma (Fig. 3G). These T+PMPs were also detected in the tumor microenvironment of human colorectal tumors (Fig. 3H).

All together, these results indicated that upon interacting with cancer cells, the platelets spread, released their granules into the cancer cells and secreted MPs. The fusion of platelet and cancer cell membranes led to the production of iMPs, including MPs expressing markers of both platelets and cancer cells, which were detected in patients with colorectal cancer.

To characterize the sizes of the iMPs generated in greater detail, the different populations of iMPs produced after 6 hours of interactions between platelets and cancer cells were isolated using a cell sorter (Fig. 4A and B) and analyzed by electron microscopy (Fig. 4C and D). More than 50% of the iMPs had a size between 100 and 300 nm. None of them was greater in size than 700 nm. All three types of iMPs had similar size distributions; however, 34% of T+PMPs had a size between 200 and 300 nm, versus 24% and 27% for the PMPs and TMPs, respectively. Additionally, 12% of TMPs had a size between 500 and 600 nm versus 4% for PMPs and T+PMPs.

The generated iMPs recruited monocytes and contained two cytokines (IFNγ and IL4) involved in activating the tumoricidal functions of macrophages

To determine the role of iMPs, we primed mouse colorectal cancer cells (CT26GFP/Luci) with platelets before injecting them into the right flank of wild-type mice. When we compared the size of the tumors in the presence and absence of platelets, we observed, starting at day 18, that both the tumor volumes and the bioluminescent signal at day 28 were significantly lower in the group of mice that received CT26GFP/Luci cells primed with platelets than in the control group (Fig. 5A). When cadherin 6 was blocked from cancer cells using shRNA (Fig. 5B and C), the priming of the cancer cells with platelets no longer affected the growth of the tumor (Fig. 5A), indicating that cadherin 6-dependent interactions of platelets with cancer cells were involved in the decreased tumor size. Interestingly, the shutdown of cadherin 6 in cancer cells by shRNA almost completely blocked the inhibitory role of platelets in colon cancer cell progression in vivo. However, cadherin 6 knockdown inhibited platelet adhesion only slightly in vitro (Supplementary Fig. S3A and S3B). To understand this apparent discrepancy, we next examined the generation of iMPs following the interaction of platelets with cancer cells not expressing cadherin 6. We observed that the shutdown of cadherin 6 by shRNA significantly diminished the concentration of iMPs generated following interaction with platelets and mostly abolished the production of PMPs (Fig. 5D), suggesting that cadherin 6 expressed by cancer cells is involved in the activation of platelets leading to the generation of PMPs. We concluded that cadherin 6 expressed by cancer cells plays a key role in the generation of iMPs.

When comparing the cellular composition of the primary tumor in the presence and absence of platelets, no difference was observed in the presence of intratumoral neutrophils in the two groups of mice. However, the F4/80 fluorescent signal corresponding to macrophages was significantly higher in the tumor microenvironment of mice injected with CT26GFP/Luci cells primed with platelets than in the control mice (Fig. 5E). The presence of platelets significantly increased the number of intratumoral macrophages (Fig. 5E).

Using a Transwell assay, we observed that monocytes transmigrated from the upper to the lower chamber when the iSN or the purified iMPs were present in the lower chamber. In contrast, the supernatants from platelets alone or cancer cells alone containing PMPs and TMPs, respectively, were significantly less effective than iMPs in attracting...
Figure 3.
The interaction of platelets with tumor cells induces the generation of microparticles. A, Representative images of human washed platelets spreading on HT29 cells. CD41, red; CD29, green. Confocal microscopy, 100 ×. B, Quantification of platelet spreading. The results are presented as the mean percentage value of spread platelets relative to the total number of platelets ± SD (n = 4). Spread platelets were defined by their form (nondiscoid platelets) and their area (above 10 μm²). C, Quantification of the generation of MPs in the supernatant of the interaction (iSN), control platelet supernatant, or control tumor cell supernatant. The results are expressed as the mean concentration of the MPs released in the supernatants in MPs/µL relative to the total number of platelets CD41, red; CD29, green. Confocal microscopy, 100 ×. D, Representative images showing the formation of iMPs at 6 hours. CD41, red; Epcam, blue; AnV, green; colocalization, white. Confocal microscopy, oil immersion; 0.2 μm. E, Flow cytometry characterization (left) and immunofluorescence (right) of fibrinogen (FGN)-positive washed platelets 24 hours after the intravenous injection of AF488-conjugated fibrinogen in recipient mice. Fibrinogen is present in the platelet granules. CD41, red. F, Representative images demonstrating the presence of iMPs (AnV⁺, CD41⁺, and Epcam⁺ MPs) in plasma from patients with colorectal cancer. G, Representative images demonstrating the presence of MPs in the microenvironment of human colorectal tumors. Frozen tissue slides containing 5-μm sections from a poorly differentiated, human colorectal adenocarcinoma. Right, crops corresponding to the white rectangular region. CD41, red; Epcam, green. Confocal microscopy, 60 ×. Two-tailed Student t test; *, P < 0.05; ***, P < 0.001; ns, nonsignificant.
monocytes (Fig. 5F). Taken together, these results indicated that the iMPs produced following the cadherin 6–dependent interaction of platelets with cancer cells could attract and activate monocytes, whereas PMPs or TMPs alone could not. These results strongly suggested that the T+PMPs were directly involved in this process.

To determine how iMPs attract macrophages at the site of a primary tumor, we next performed a cytokine array on the supernatant of platelets alone and of cancer cells alone, on the iSN and on purified iMPs (Fig. 5G). RANTES and MIF, two cytokines known to be involved in the recruitment of leukocytes, were detected in the iSN and in the iMPs. Of note, only one cytokine, MIF, was detected in the supernatant of cancer cells. Interestingly, various cytokines were present in iMPs only when the iSN and the iMPs were permeabilized. These cytokines included CXCL12 and CCL2 (involved in the attraction of leukocytes), IFNγ and IL4 (known to activate macrophages and to be involved in macrophage tumoricidal activity; ref. 20). These results indicated that cytokines in iMPs can attract macrophages (CCL2 and CXCL12) and activate their tumoricidal capacity (IFNγ and IL4). Furthermore, based on the expression of iNOS and Arg1, we found that 89% (C6/34) of the macrophages were polarized into an M1 phenotype in the tumor primed with platelets versus 38% (C6/32) in the control mice (Fig. 5H).

We next determined whether platelets and macrophages could indeed affect cancer cell proliferation or apoptosis. The interaction of platelets alone (Fig. 6A–C) or monocytes alone (Fig. 6B) with cancer cells did not induce cancer cell apoptosis. The expression of P21 (Waf1/Cip1) was unchanged in cancer cells after platelet interaction (Fig. 6D and E). However, the interaction of monocytes alone or
Figure 5.
Platelet–tumor cell interactions decrease tumor growth in vivo via the attraction of tumoricidal macrophages. A, Tumor growth curve of 60 minutes ± platelet-primed colorectal tumors expressing (sh RNA Mock) or not (sh RNA Cdh6) cadherin 6. Tumor volumes are expressed in mm³ and are presented as the mean value ± SD (P < 0.05; n = 11 per group). B, RT-PCR using SSVI retrotranscriptase and cadherin-6 forward and reverse primers on total extracted RNA from the CT26 shRNA control and the CT26 shRNA cdh6-6. C, Western blot analysis of the cadherin-6 protein expression on total protein lysates from the CT26 shRNA control and the CT26 shRNA cdh-6. D, Percentage MPs generated (iMPs, TMPs, PMPs, and T+PMPs) following 60-minute interaction of platelets when cadherin 6 is shut down (shRNA Cdh6) from CT26 cancer cells in comparison with control (shRNA Mock). E, Quantification of the fluorescent signal corresponding to F4/80 in tumor or in tumor with platelets (P < 0.01; n = 3). F, Representative graph of the monocyte transwell assay in the presence of free FCS McCoy’s 5A medium (medium control), 6-hour platelet supernatant control (Plts SN control), TNF-α (100 U/mL), iSN, and purified MPs. The results are expressed as the mean percentage of transmigrating monocytes in comparison with that in the medium control (P < 0.05; n = 3). G, Quantification of cytokine and chemokine levels in the iSN and the purified MPs under permeabilized conditions (0.1% Triton X100) or nonpermeabilized conditions. The graph depicts the normalized level of cytokines relative to reference spots using ImageJ software. H, Quantification of the fluorescent signal of arginase 1 (Arg1; right) and inducible nitric oxide synthase (iNOS; left) colocalizing with F4/80 signal in platelet-primed CT26 cell tumor microenvironment and in control CT26 cell tumor microenvironment. Two-tailed Student t test: *, P < 0.05; **, P < 0.01; *** P < 0.001; ns, nonsignificant. hWPs: iSN, 6-hour hWP-HT29 interaction supernatant; IMP, iSN-purified MPs.
Figure 6.
Platelets and their MPs affect tumor cell proliferation in the presence of monocytes. A, Western blot analysis of the cleavage of caspase-3: noncleaved caspase-3 (35 kDa) and cleaved caspase-3 (17 and 12 kDa). The positive control was a treatment of HT29 cells with 200 μmol/L camptothecin (CPT) for 24 hours. B, Western blot analysis of the cleavage of caspase-3 induced by monocytic interaction. C, Flow cytometry analysis of the apoptosis of HT29 cells. The graph depicts the mean percentage of AnV-positive and 7-AAD-negative HT29 cells at different times of interaction relative to the total number of analyzed HT29 cells ± SD (P < 0.05; n = 3, Kaluza software). D, Western blot analysis of the P21 expression in total protein lysates from HT29 cells that interacted with human washed platelets. E, Relative protein expression level of P21 in HT29 cells at different times of interaction with human washed platelets ± SD (ns, nonsignificant; n = 3). F, Western blot analysis of the P21 expression in total protein lysates from HT29 cells that interacted with human purified monocytes, or human purified monocytes and human washed platelets. Kinetic measurements of the interaction between human HT29 cells and human purified monocytes in the presence or absence of human washed platelets from the 60-minute time point to the 360-minute time point were performed. G, The relative protein expression level of P21 in HT29 cells at different times of interaction with human purified monocytes in the presence or absence of human washed platelets ± SD (P < 0.05; n = 3). H, Top, Western blot analysis of the PRb 807/811 expression in total protein lysates from HT29 cells that interacted with human washed platelets for 5 hours, followed by 5 hours of interactions with purified human monocytes. Bottom, the relative protein expression level is presented (P < 0.05; n = 3, ImageJ software). Two-tailed Student t test: * P < 0.05.
Platelets and iMPs facilitated the interaction of cancer cells with the endothelium

Our results indicated that in the tumor microenvironment, platelets play an important role in reducing tumor growth through the generation of iMPs and the recruitment of tumoridical macrophages. However, different studies, including ours, have demonstrated that activated platelets may participate in the process of metastasis. To understand this apparent discrepancy, we next studied the role of platelets in cancer cell–endothelium interactions, a key step involved in metastasis. We first investigated the adhesive properties of HT29 colorectal cancer cells to the endothelium in the presence of absence of platelet priming in dynamic conditions (4 dynes/cm²). As illustrated in Fig. 7A, compared with control levels, a ten-fold increase in the adhesion of cancer cells to the endothelium was observed when cancer cells were primed with platelets. Interestingly, during priming, platelets released and transferred integrin subunit β3-positive (CD61) vesicles to the cancer cells (Fig. 7B), and these vesicles might have participated in the interactions with the endothelium. As an alternative to this mechanism, a few studies have demonstrated that platelet–cancer cell interactions are facilitated by the transfer of cancer cells by inducing EMT. To determine whether platelet interaction with HT29 colorectal cancer cells induced EMT, we assessed the expression of E-cadherin and Snail1, two known markers involved in EMT. The interaction of platelets with cancer cells decreased the expression of E-cadherin and increased the expression of snail1 in a time-dependent manner (Fig. 7C). These results were confirmed in vivo using a model of experimental metastasis (Supplementary Fig. S3C; ref. 21). The priming of cancer cells with platelets significantly increased the number of metastatic modules detected in the lungs.

To better understand the transfer of biological material from platelets to cancer cells, we next compared the expression of more than 200 mRNA involved in inflammation and metastasis in platelets alone, cancer cells alone, and cancer cells primed with platelets (Fig. 7D). Interestingly, some mRNAs that were absent in cancer cells alone were overexpressed when cancer cells were primed with platelets. For example, consistent with a previous publication (22), the mRNA PTGS2, coding for COX2, is overexpressed following the interaction of platelets with cancer cells. Other mRNAs are shut down following the interaction of cancer cells with platelets, including the mRNAs coding for SerpinB5, also known as Maspin, and NF-2, coding for two proteins reported to function as tumor suppressors, that suppress the ability of cancer cells to invade and metastasize to other tissues and are thus described as playing important roles in metastasis. We also confirmed that platelet interactions with cancer cells play an important role in EMT because the expression of E-cadherin was diminished, whereas the mRNA CTNNAL1, coding for β-catenin, MMP10 and MMP2 (SNAIL and ZEB families) and fibronectin (SNAIL and TWIST families) were overexpressed in cancer cells primed with platelets.

We next investigated the ability of iMPs to prime the endothelium and facilitate the adhesion of cancer cells at the site of metastasis. Purified iMPs activated HUVECs, as attested by the induction of the expression of ICAM-1 at the surface of the endothelial cells (Fig. 7E). In a dynamic assay, at 4 dynes/cm², purified iMPs, as well as the interaction supernatant, significantly increased the percentage of HT29 in interaction with HUVECs in comparison with the control (Fig. 7F). Altogether, these results indicate that platelets and iMPs may contribute to metastasis by favoring interaction between cancer cells and the endothelium.

Discussion

The role of platelets and platelet activation in tumor growth has been subject to controversy in the literature. Some studies have demonstrated that the use of drugs that inhibit platelet activation, such as ticagrelor and clopidogrel, reduces tumor growth and metastasis in vivo in pancreatic cancer (1, 23). Other studies performed on breast, colorectal, and prostate cancers did not observe any effect of clopidogrel or prasugrel alone on the tumor growth (24, 25). The activation of platelets might play different roles in tumor behavior and metastasis in different cancers. We previously reported that in pancreatic cancer, inhibition of platelets decreases tumor growth and metastasis (5). In this case, cancer cells strongly activate platelets, leading to TCIPA, which is mainly dependent on αiβ3 interactions and not cadherin-6. Here, in a colorectal model, we observed a paradoxical role of platelets (Supplementary Fig. S4). Platelets mainly interacted with the cancer cells through cadherin-6, independent of TCIPA. Although our results indicate that cadherin 6 plays a crucial role in generating iMPs, other receptors expressed by platelets, including GPV1 or P-selectin, may also be involved in the interaction of platelets with cancer cells (22). A deep characterization of the role played by platelets depending on the type of cancer and its stage is needed before using antiplatelet drugs in the management of cancer and cancer-associated thrombosis.

The kinetics of platelet activation and spread on colorectal cancer cells was different from what is usually described, with the activation of platelets starting as late as 60 minutes following their interaction with cancer cells. Steele and colleagues demonstrated that the canonical Wnt/β-catenin signaling components are present in platelets and negatively regulate platelet functions (including platelet αiβ3 activation, platelet adhesion and spread, and dense granule secretion) by regulating small GTPase activity (26, 27). Later, Maguire and colleagues demonstrated that platelet β-catenin is strongly associated with cadherin adherent junctions including cadherin-6 (28). It is possible that the cadherin-6–dependent interactions between platelets and tumor cells are responsible for the absence of TCIPA and the observed kinetics of platelet spreading through activation of the Wnt/β-catenin signaling pathway. Of note, consistent with previous publications (29, 30), the tumor size in the Cdh6 knockout was smaller than that in the control group, indicating that cadherin 6 expressed by cancer cells is involved in the proliferation of colorectal cancer cells. Because we observed that the shutdown of cadherin 6 by shRNA strongly affected the generation of iMPs, it is possible that the role played by cadherin 6 expressed by cancer cells in tumor growth is mainly mediated through its interactions with platelets and the generation of iMPs.

For a few years, platelets have been considered effectors of inflammation and immunity, mainly based on the fact that platelet α-granules contain a plethora of chemokines, including RANTES, MIF, CCL-3, CCL5, and CXCL12, which are all known to attract leukocytes in different pathologic conditions such as bacterial infections and atherosclerosis (31, 32). One study also suggests that platelets alone can exert directly cytotoxic effects on tumor cells (12). This...
Figure 7.
Platelets and iMPs facilitate the interaction of tumor cells with the endothelium. **A**, Left, representative images of the HUVEC-HT29 tumor cell interaction in dynamic conditions (4 dynes/cm²). Calcein-green–stained HT29 cells, green; HUVEC monolayer (nuclei, blue). Right, the graph depicts the mean number of HT29 cells interacting with HUVECs/field ± SD (n = 3, 10 field/condition). **B**, Representative images of the β3 integrin subunit (green), platelet vesicles (white arrow), and HT29 cells (F-actin, red). Confocal microscopy, 100×. **C**, Western blot analysis of E-cadherin and Snail-1 expression in total lysate proteins from HT29 cells that interacted with human washed platelets. **D**, mRNA expression was compared in platelets (PLTs), HT29 cells (HT29), and HT29 cells preincubated with platelets (HT29PLTs) using the Applied Biosystems TaqMan Human Inflammation or Tumor Metastasis Array Plates. Results were analyzed with the manufacturer’s online software and are represented using a color code: black, no expression; green, low expression; red, strong expression of the mRNA. **E**, ICAM-1 expression on HUVECs following 3 hours of control McCoy’s 5A medium, iSN, iMPs, platelet control supernatant [cSN(Plts)] and HT29 control supernatant [cSN(HT29)] treatments. TNFa (10 ng/mL) was used as a positive control for endothelial cell activation and degranulation. Endothelial cells were stained with Hoechst 33342 (nuclei, blue) and PE-conjugated ICAM-1 mouse IgG antibody (green). **F**, The graph depicts the mean percentage of HT29 cells interacting with the treated HUVEC monolayer in dynamic conditions (4 dynes/cm²) relative to the control medium ± SD (P < 0.01; n = 3). Two-tailed Student t test: **,** P < 0.01; ***,** P < 0.001. iSN, 6-hour hWP-HT29 interaction supernatant; iMPs, iSN-purified MPs.
cytotoxicity is dependent on the type of cancer cells used and requires an interaction between platelets and cancer cells when the platelets are not artificially preactivated (33). Our results indicate that the cytotoxic effect of platelets on cancer cells is mainly due to the production of microparticles involved in recruiting and activating macrophages that facilitate cell-cycle arrest. Further studies will determine if this pathway is specific to colorectal cancer or is also activated following the interaction of platelets with other types of cancers.

Numerous studies underline the effects of PMPs on the recruitment and activation of monocytes (34, 35). This process classically induces oxidative burst activation of monocytes and the release of proinflammatory cytokines such as TNFα (36, 37). In accordance with these studies, we showed that iMPs (containing RANTES, MIF, CXCL-12, and IFNγ) that were released following platelet–tumor cell interactions increased the attraction of monocytes and that monocyte–platelet–tumor cell interactions induced cell-cycle arrest in vitro and decreased tumor growth in vivo. Proinflammatory cytokines, such as IFNγ and TNFα, are known to induce M1 macrophage polarization with strong tumoricidal activity (38). The tumoricidal activity of macrophages may be due to cytokine-mediated induction of cell death and phagocytosis such as TRAIL, CCL2, and IL8 (39). Alternatively, macrophage-mediated phagocytosis could be an important part of the antiinflammatory immune response. However, the exact mechanisms supporting the tumoricidal activity of the macrophages in vivo remained to be determined.

Recently, the notion of tumor-educated platelets has been highlighted and provides an interesting tool for cancer diagnostics (40–42). The main way a tumor influences platelet RNA profiles is through the uptake of tumor-derived microvesicles (41). Here, we demonstrated that platelets can also educate tumor cells via (i) the release of their alpha-granule contents into the tumor cells, (ii) the microvesicle-dependent transfer of adhesive proteins, such as the β3-integrin subunit, (iii) the fact that platelet transfer could induce the expression or repression of different RNAs in cancer cells. Based on our results, we propose that platelet-educated cancer cells also exist and play an important role in tumor behavior and metastasis formation. Here, we focused on and highlighted the role played by iMPs in these processes. However, the overexpression/repression of mRNA could also be an essential step in the regulation of the tumor and the formation of metastases. These points need to be clarified.

These mechanisms lead to the induction of EMT and are responsible for the enhanced ability to interact with the endothelium. Moreover, the iMPs generated during platelet–tumor cell interactions prime the endothelium and enhance the adhesion of tumor cells to the endothelium. These results are in accordance with the findings of Pasquier and colleagues, who demonstrated an MP-mediated cross-talk between endothelial cells and tumor cells involved in the activation of endothelial cells and the formation of a prometastatic vascular niche (43). Janowska and colleagues demonstrated that PMPs can transfer platelet-derived CD41 in six lung cancer cell lines, which is known to enhance the metastatic potential of cancer cells with an increased ability to adhere to endothelial cells (44, 45). Additionally, it has been recently shown that PMPs can infiltrate solid tumors and suppress tumor growth mainly via the delivery of miR-24 (10). Thus, platelets (as well as iMPs) that are released in the bloodstream during cancer progression may contribute to the metastatic process by facilitating the interactions between tumor cells and the endothelium.

Altogether, our results indicate that iMPs, by being present in the tumor microenvironment and by disseminating information (via cytokines and integrins) in the bloodstream, play an important role in the behavior of a tumor and may represent an interesting target to successfully treat colorectal cancer as well as a pertinent biomarker of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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