Platelets in cancer

From basic research to therapeutic implications

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Summary
Platelets are well-known for their major role in primary hemostasis and thrombosis. Cancer patients frequently manifest thrombotic events and present abnormalities in blood coagulation which appear to be linked to altered platelet function and turnover. Moreover, numerous studies indicate an intimate cross-talk between platelets and tumor growth, angiogenesis and metastatic dissemination. Finally, several experimental data and clinical trials suggest possible benefits of anti-platelet drugs on some cancers.
Here, we will review the current state of basic biological research regarding the role of platelets in cancer progression. We also critically review the possible clinical applicability of some anti-platelet therapies to limit tumor growth and prevent metastatic dissemination.

Zusammenfassung
Die wichtige Rolle der Plättchen in der primären Hämostase und Thrombose ist gut bekannt. Krebspatienten weisen häufig thrombotische Ereignisse und Unregelmäßigkeiten der Blutgerinnung auf, die mit Veränderungen der Plättchenfunktion und des Thrombozyten-Turnover verbunden zu sein scheinen. Außerdem wiesen zahlreiche Studien auf eine enge Wechselwirkungen zwischen Plättchen,Tumorwachstum, Angiogenese und Metastasierung hin. Schließlich lassen experimentelle Daten und klinische Studien einen möglichen Nutzen von Thrombozytenaggregationshemmern bei einigen Krebsarten vermuten.
Wir geben einen Überblick zum aktuellen Stand der biologischen Grundlagenforschung bzgl. der Rolle der Plättchen bei der Krebsprogression. Auch die Eignung einiger Thrombozytenaggregationshemmer zur Eindämmung des Tumorwachstums und zur Prophylaxe der metastatischen Tumorausbreitung haben wir kritisch geprüft.

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Cancer is the uncontrolled growth of cells in a given tissue to a cell mass which may spread throughout the body to form metastasis, ultimately leading to death. Cancer comprises several pathologies whose symptoms and disease course differ depending on the cell type initially affected, the multiple cellular and molecular factors involved, the type of tissue affected, genetic predisposition and environmental factors. Characterization of alterations affecting oncogenes and tumor suppressors helped to establish the fundamentals of cancer biology (1). Accordingly, therapeutic anticancer strategies have primarily focused to target tumor cells. Cumulating experimental and preclinical data indicate that carcinogenesis and tumor progression are not cell-autonomous processes, but rather involve complex multiple interactions with tumor microenvironment (2). Tumor microenvironment is the cellular and molecular environment in which tumors grow, including blood vessels, pericytes, fibroblasts, immune cells, bone-marrow derived cells, growth factors, cytokines and extracellular matrix (ECM) molecules. Tumor microenvironment provides a necessary blood supply and a favorable milieu which stimulates their growth and invasion, prevents from immune recognition and promotes survival in circulation, until they extravasate and seed at distant organs. Currently, it is believed that cellular and molecular components of the tumor microenvironment constitute a barrier protecting against certain drugs and therapies and may favor development of resistance against therapeutic approaches (3). These concepts have become widely recognized and increased the relevance for the development of new therapeutic targets to treat human cancers.

The first association between cancer and blood dates back to the Indian surgeon Sushruta, who lived approximately 3000 years ago and who described that tumor entry into the blood stream leads to blood vessel constriction and compression. In 1865, an association between hemostatic abnormalities and cancer was clinically recognized by the French clinician Armand Trousseau. He reported several cases of thrombophlebitis in patients who were later diagnosed with gastric cancer (4). He emphasized the increased formation of platelet-rich thrombi and hypercoagulability

in these patients. Recent studies indicated that the chance of diagnosing cancer is significantly elevated after pulmonary embolism or primary deep venous thromboembolism (5). More evidence for platelet involvement in the tumor process, relies on the link between an elevated platelet count (thrombocytosis) and malignant tumors which was reported by Reiss et al., in 1872 (6). Since then, thrombocytosis has been shown to be correlated with advanced, often metastatic steps of cancer and appears to be a negative prognostic factor for many human cancers (7).

Platelets are small anucleated cellular fragments, derived from megakaryocytes in the bone marrow. One third of the platelets are sequestered in the spleen while two thirds of them circulate freely in the bloodstream at a count comprised between 150 and 400 thousands per microliter, providing a potent biomarker for diagnostic and clinical studies. The principal duty of platelets is to prevent hemorrhages and post-traumatic bleedings (8). Following vascular injury, platelets are recruited to the exposed subendothelial ECM proteins, leading to their activation. Platelet activation triggers the release of a variety of biological active substances including adhesive glycoproteins, growth factors, cytokines, coagulation factors, from the so-called α-granules, and soluble agonists such as adenosine diphosphate (ADP), adenosine triphosphate (ATP) and serotonin from the dense (δ) granules along with the production and release of thromboxane A2 (TxA2). These mediators contribute to the recruitment of circulating platelets by upregulating the affinity of integrin aIIbb3 for its ligands, soluble fibrinogen (FGN) and von Willebrand factor (VWF), thereby leading to the formation of a hemostatic plug. A functional coagulation system is additionally required for effective hemostasis completion. The coagulation cascade is initiated by tissue factor (TF) which is exposed upon vascular injury and amplified by negatively charged phospholipids exposed at the platelet surface, supporting the assembly of the tenase and prothrombinase complexes. This cascade leads to the generation of thrombin, which mediates the conversion of FGN into an insoluble fibrin network stabilizing the clot.

Beyond hemostasis and thrombosis, platelets are critically involved in many biological processes including inflammation, embryonic development, innate and adaptive immunology and tissue regeneration to cite a few (9–11). Their role in cancer is known for long but the molecular mechanisms underlying the interactions between cancer cells and platelets only begin to be unraveled. On the one hand, tumor cell induced platelet activation and aggregation may trigger thrombosis in patients with cancer. On the other hand, platelets recruited by tumor cells may favor tumor growth, angiogenesis and metastasis. Recent reviews have been published which all exhaustively describe the most up-to-date data concerning the involvement of blood platelets in the progression of cancer and metastasis (12–15). What we would like to do in the present review is to illustrate and to critically analyze the available data on the role played by platelets in various aspects of the tumor process including thrombosis, angiogenesis and metastatic dissemination and to explore potential therapeutic implications either using existing antiplatelet drugs or new drug candidates to target new receptors and pathways in order to improve cancer treatment.

Cancer-thrombosis connection

Cancer patients often suffer from thromboembolic diseases, such as superficial and deep vein thrombosis, pulmonary emboli, as well as arterial thrombosis and embolism. Thromboembolic disease is the second leading cause of death in cancer patients and thromboprophylaxis with low molecular weight heparin significantly reduces the incidence of symptomatic venous thromboembolism (VTE) in ambulatory cancer patients treated with chemotherapy (16). The "Khorana risk assessment model" which includes several clinical variables such as site of cancer, obesity, leukocytosis, anemia and thrombocytosis has been proposed to predict risk of cancer-associated thrombosis. This risk prediction model, which has been validated in several large cohorts of patients in various clinical settings, provided evidence that thrombocytosis is an important biomarker of cancer-associated thrombosis, indicating a role for platelets in this process (7). Cancer-mediated thrombocytosis may be explained by the ability of several tumor cells to produce and regulate thrombopoietin (TPO), (17, 18), a key cytokine which stimulates megakaryocyte formation and platelet production. In a multicenter study involving 619 patients with epithelial ovary cancer, thrombocytosis was found to be associated with high plasma level of TPO and interleukin-6 (IL-6), and linked to an advanced disease and poor survival (17). It has also been reported that circulating IL-6 is a risk marker of thromboembolic manifestations. Interestingly, experiments performed with orthotopic mouse models of ovary cancer, provided evidence and confirmed that tumor cell-derived IL-6 upregulated the production of hepatic TPO (17).

In addition to thrombocytosis, several platelet activation markers were found to be upregulated and could contribute to the prothrombotic state of cancer patients. For example, ovarian cancer patients had elevated number of CD63 positive platelet microparticules reflecting a procoagulant phenotype (19). Moreover, in several studies, key markers of platelet activation, including CD40 ligand, β-thromboglobulin or P-selectin exposed at the platelet surface or soluble in the plasma (13) are increased in cancer patients compared to non-cancer control subjects. Thus, the combination of increased number of circulating platelets along with upregulation of circulating prothrombogenic factors establishes the hypercoagulable state contributing to Trousseau's syndrome.

The concept of cancer cells as inducers of platelet activation and aggregation has been well established in vitro. Tumor cells can trigger changes in platelet activation through several mechanisms. The activation by direct interaction and subsequent aggregation of platelets, termed as TCIPA (tumor cell-induced platelet aggregation) has been shown to occur in vitro with lung, colon, breast, pancreatic and prostate cancer cells (20). One possible mechanism of TCIPA involves sialoglycoprotein Aggrus/podoplanin found in various tumor cell lines, such as glioblastoma, mesothelioma, lung, esophageal squamous cell and

colon carcinoma (21, 22). The C-type lectin-like receptor (CLEC-2) expressed on platelets was identified as one of the counter receptors of podoplanin. Podoplanin binding to CLEC-2 transmits platelet-activation signals through Src family kinases, Syk and phospholipase Cγ2 in platelets (23). Blockade of the podoplanin-CLEC-2 interaction inhibits TCIPA in vitro, as well as metastasis in vivo (24). TCIPA is also induced by tumor derived cathepsin B and matrix metalloproteinases (MMPs) in several tumor cell lines as well as by released ADP (20).

Cancer cells can also trigger indirect platelet activation. They can initiate the coagulation cascade trough their ability to express tissue factor, thereby generating thrombin, the most potent platelet agonist. Their ability to release procoagulant microparticules, can also initiate thrombin generation (25). Finally, platelets can be activated through cancer-induced formation of neutrophil-extracellular DNA traps (NETs), which may result in platelet aggregation and thrombus formation (26) (►Fig. 1).

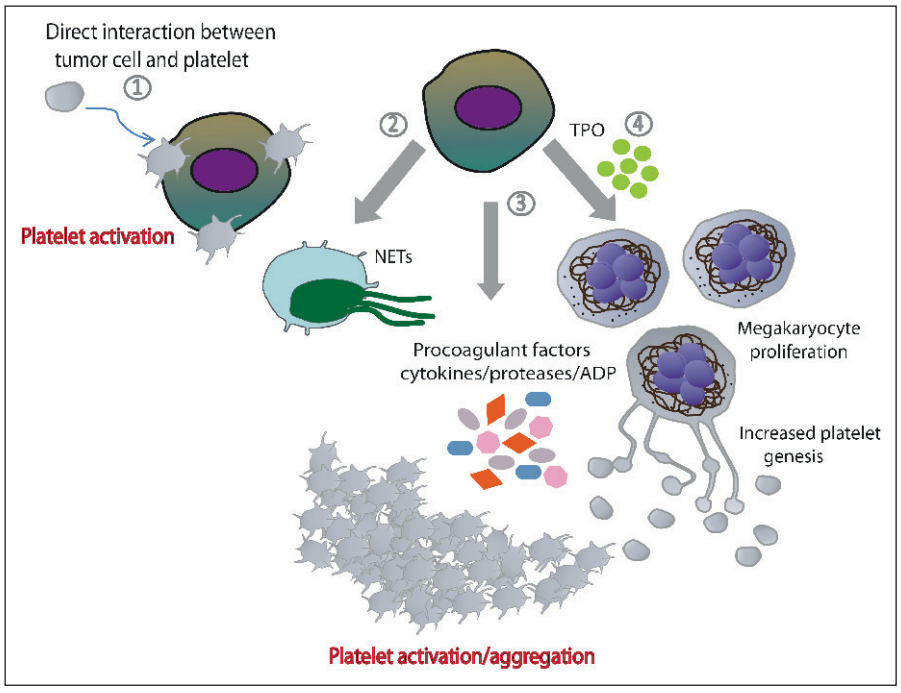


Fig. 1 Mechanisms involved in tumor cell-induced thromboembolic events: Tumor cells through their ability to bind directly platelets (1), to promote NET formation (2), to release pro-activatory and pro-coagulant factors (3) could trigger platelet aggregation and activation. Additionally, tumor cells may regulate TPO levels to stimulate thrombocytosis (4), thereby leading to a prothrombotic phenotype. For the details, see the text.

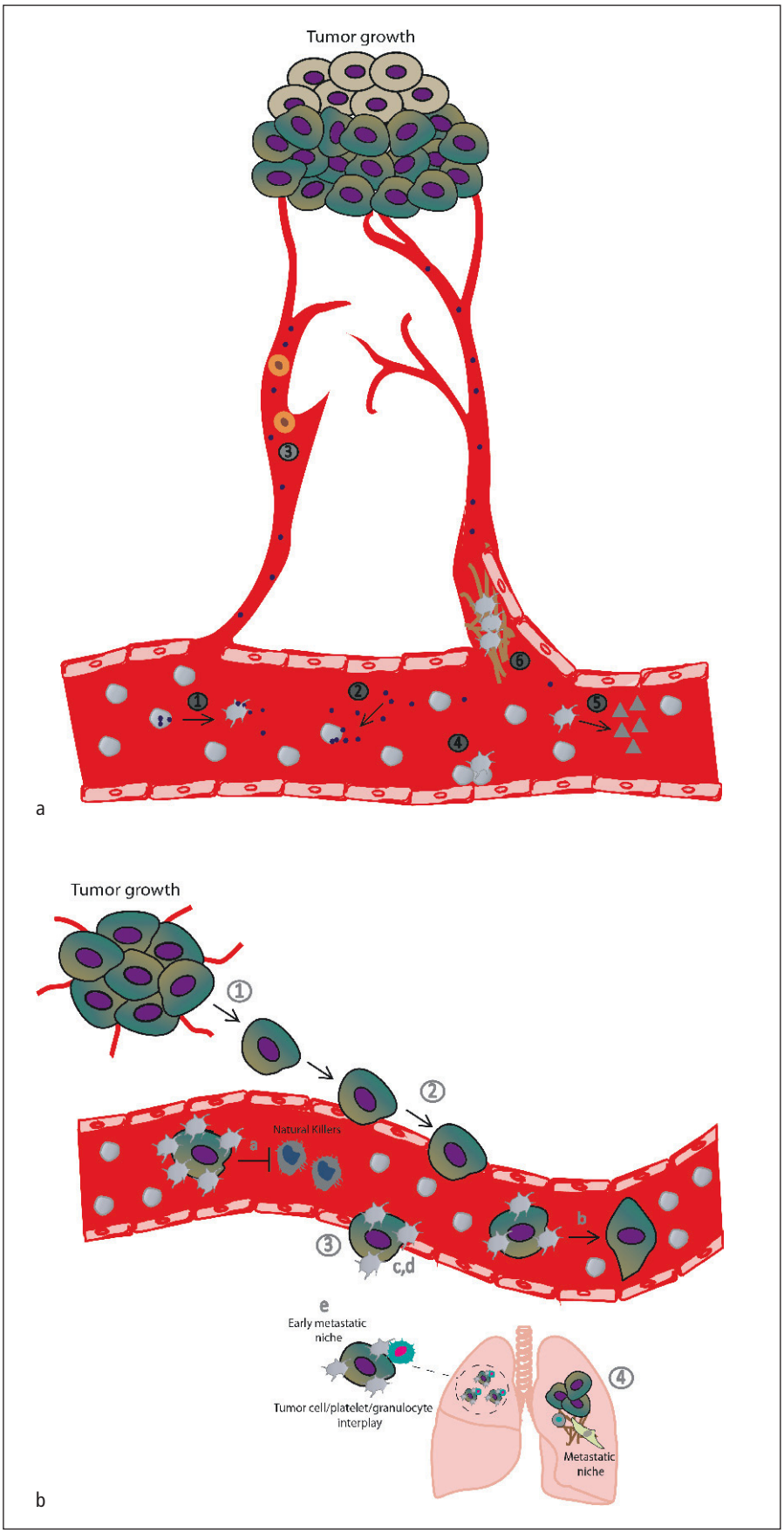
To what extent all these events occur in patients is difficult to assess and tools lack at the moment to properly interfere with them. We will discuss later the impact of antiplatelet therapy on platelet/cancer cell interaction.

Platelet-mediated tumor angiogenesis

Beyond a certain size (>1–2 mm^3), tumors initiate angiogenesis, the formation of new capillaries from preexisting blood vessels, to provide the oxygen and nutrients essential for their growth (27). There is experimental evidence that the role of platelets in angiogenesis may contribute to tumor growth and survival (►Fig. 2a).

Platelets contain both pro- and anti-angiogenic factors, which can be released upon platelet activation. Examples of positive regulators of angiogenesis in platelets are vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), lysophosphatidic acid (LPA), angiopoietin (Ang), and MMP-1, –2, and –9, while negative regulators comprise platelet factor-4 (PF-4), plasminogen activator inhibitor type-1 (PAI-1), thrombospondin, tissue inhibitor of MMPs and endostatin. Several studies suggested that release of pro- and anti-angiogenic factors is a tightly regulated process. It has been proposed that pro- and anti-angiogenic factors are stored in distinct α-granules in the same platelet and that their release is regulated by a selective stimulation of the thrombin proteinase-activated receptors (PAR) –1 and PAR-4 (28), while tumor cell derived ADP would promote the release of the pro-angiogenic factor VEGF through the activation of the $P2Y_{12}$ receptor, without affecting the release of the anti-angiogenic factor endostatin (29, 30). In contrast, TxA2, another important soluble agonist, has been proposed to promote the release of endostatin but not VEGF (29). Such a tightly regulated mechanism of differential secretion with selective release of pro or anti angiogenic factors is somewhat difficult to reconcile with the common knowledge that platelet activation results from simultaneous stimulation of multiple pathways. In addition, the concept of functional co-clustering of proteins in distinct granules was recently challenged in experimental settings using quantitative immunofluorescence microscopy techniques and micro ELISA arrays (31, 32). Thus, it will probably be very challenging to determine under which in vivo conditions the selective stimulation of various platelet receptors, such as PAR-1, PAR-4, $P2Y_{12}$ and thromboxane receptors may occur.

Platelets have been reported to take up and sequester pro-angiogenic mediators, which could indirectly regulate angiogenesis. It has been shown that a small amount of VEGF secreted from microscopic subcutaneous tumors resulted in elevated levels of platelet VEGF (33). In ischemic hind limb and tumor xenograft hypoxia models, platelets promoted mobilization and recruitment of bone marrow-derived cells to neovascularized hypoxic tissues to favor angiogenesis, and this homing was dependent not only on release of the α-granule content but also on the sequestration of growth factors and cytokines by platelets



(34). In agreement with these results, Kuznetsov et al., showed that human platelets adsorb cytokines released by luminal breast cancer cells in order to deliver them to indolent disseminated tumors ensuring their outgrowth through vessel formation (35).

Beside the release, storage and delivery of soluble mediators, platelets have also been proposed to participate in angiogenesis by directly or indirectly regulating endothelial cell function. Human platelets were able to promote tube formation of human umbilical vein endothelial cells in a matrigel assay (36). This effect was independent of platelet activation or granule release, suggesting that direct platelet/endothelial interactions can enhance angiogenesis in malignancy. Conversely, the coagulation end-product fibrin, which is fre-

Fig. 2 Schematic illustrations

a) multiple ways of platelet-mediated tumor angiogenesis: Platelets may stimulate tumor angiogenesis through the secretion of pro-angiogenic growth factors (1), the take-up and sequestration of pro-angiogenic mediators from tumor and tumor microenvironment (2), and the recruitment of bone-marrow-derived cells to neovascularized tumor sites (3). Direct interactions between platelets and endothelial cells (4) may also stimulate tumor angiogenesis. Finally, angiogenic sprouting may be supported by shedding of microparticules (5) and endothelial cell migration on platelet-enriched fibrin clots (6). For the details, see the text.

b) main steps of metastatic dissemination: Metastatic cascade is a multi-step process which includes: local invasion enabling detachment of tumor cells from primary tumor site (1), tumor cell attachment to the vascular wall and entry into the circulation (intravasation), (2), exit of tumor cells from the bloodstream (extravasation), (3) and finally tumor cell survival and proliferation at secondary organs (4), such as lung to form metastases, through involvement of a local microenvironment (metastatic niche). Within the bloodstream, platelets escort tumor cells through several steps of metastasis and may support this pathological process by: protecting tumor cells from immune system surveillance (a), conferring a mesenchymal phenotype to epithelial tumor cells (b), reinforcing tumor cell/blood vessel interplay (c), promoting tumor cell extravasation (d), and establishing early metastatic niches (e) within the vasculature of distant organs to promote tumor cell survival and proliferation. For the details, see the text.

quently found deposited in tumors, constitutes a provisional matrix that supports endothelial cell adhesion, survival and migration to promote angiogenesis (37).

Platelets have also been shown to stimulate angiogenesis following the shedding of microparticules. Platelet-derived microparticules (PMP) promote proliferation, migration and angiogenic sprouting of endothelial cells in vitro (38). They increase the mRNA levels of pro-angiogenic factors, such as VEGF, hepatocyte growth factor and MMP-9 in tumor cells, which may subsequently support tumor angiogenesis and growth (39). Brill et al., reported that PMP are capable to induce angiogenic sprouting in vitro and in vivo to a similar extent as whole platelets (40). An association between PMP and tumor progression has been shown in patients with prostate and gastric cancer (41). In addition, the levels of PMP in patients with gastric cancer were strongly correlated with the levels of angiogenic factors, such as VEGF, IL-6 and RANTES (Regulated on Activation Normal T Cell Expressed), further supporting a possible role of PMP in tumor angiogenesis (42).

Therapies targeting VEGF signaling are often unsuccessful in cancer patients. Approval to use Bevacizumab, a monoclonal antibody against VEGF-A, has been recently revoked by the United States Food and Drug Administration in women with breast cancer (43). Therefore, more appropriate anti-angiogenic strategies aiming to limit cancer progression are needed. Whether targeting the pathways of platelet-mediated angiogenesis could represent a potential anti-cancer strategy is rather speculative but remains to be addressed. Finally, whether angiogenic profiling of platelet α-granules in patients with cancer may constitute a predictive marker of the risk of disease progression and overall prognosis is a question for future stimulating studies.

Mechanisms of platelet-mediated metastasis

Metastasis is the major cause of mortality in patients suffering from cancer, and therapeutic interventions directed against this pathological process are limited since the underlying mechanisms are not fully understood. To metastasize, tumor cells must undergo successive several steps of cancer progression: detachment from the primary tumor, intravasation into the vascular system directly or via the lymph nodes, survival in the circulation, attachment to the endothelium and extravasation, and finally survival and proliferation in distant organs. Survival and proliferation of disseminated tumor cells at distant sites need a supportive specialized micro-environment. This concept known as seed and soil theory, was formulated by Paget in 1889, suggesting that microenvironment niches (soil) need to be compatible to tumor cells (seed) (44).

The first evidence for a role for platelets in this phenomenon came from studies reported by Gasic et al., in which thrombocytopenia was closely associated with reduced metastasis (45). Moreover, injection of platelets in thrombocytopenic mice restored the capacity to form metastases (46). Interfering with platelet production by disturbing megakaryocyte maturation also resulted in inhibition of metastasis in an experimental mouse model (47). Several mechanisms have been proposed to explain the role played by platelets (▶Fig. 2b). They can contribute to metastasis by shielding tumor cells from immune host system, by triggering epithelial-mesenchymal transition, by mediating tumor/vascular wall interaction and by various mechanisms helping extravasation of tumor cells from host vasculature. They are also able to mediate tumor cell survival and growth at distant sites by guiding establishment of metastatic niches.

Platelet-mediated protection from immune system surveillance

Cytotoxic natural killer (NK) lymphocytes, which induce tumor cell lysis, represent the major threat towards tumor cells in the blood circulation. Platelets are likely the first blood cells to interact with tumor cells (48) and have been proposed to serve as physical guards to protect tumors cells from immune system surveillance (49).

Several NK sensitive tumor cell lines exhibited decreased metastatic potential after platelet depletion. The proposed underlying mechanism relies on platelet adhesion to the tumor surface, thereby providing a shield and protecting them from NK-cell induced cytotoxic effects. Moreover, platelets were shown to transfer MHC (major histocompatibility complex) class I molecules onto tumor cells to provide a self-signal to NK-cells suppressing their killing activities in vitro (50). It seems that intact platelet activation is required for efficient metastasis in the presence of NK-cells since mice deficient for Gaq, a G protein crucial for platelet activation, exhibited decreased tumor cell survival and metastasis, an effect that was reversed by immunologic or genetic depletion of NK (51). Others had previously shown that platelets inhibit NK-cell cytotoxic activity through soluble factors (52). This has notably been evidenced by the fact that supernatants of activated platelets decreased NK-cell dependent lysis of human leukemia cells in vitro (52). Kopp et al., suggested that platelet-derived TGF-β down-regulates the cytokine NKG2D (Natural Killer Group 2, member D) on NK-cell surface, resulting in decreased NK-cell cytotoxicity in vitro (53). Furthermore, neutralization of TGF-β (transforming growth factor-β) in platelet release reversed this effect and restored normal NK-cell function. Interestingly, down-modulation of NKG2D has been associated with elevated TGF-β levels in plasma of patients with colorectal and lung cancer (54). Studies on modulation of NK-cell cytotoxicity by platelet TGF-β shed a new light on future clinical research. Several inhibitors of TGF-β signaling are currently under evaluation in preclinical models and early clinical trials, including soluble protein receptors, TGF-β antibodies, small-molecule kinase inhibitors, oligonucleotides and peptide aptamers. However, recent in vivo kinetic studies of lung and liver metastasis casted doubt (55) on the concept of the inhibitory effects of platelets on NK cell anti-metastatic activity (49). The authors reported that platelets exert their pro-metastatic effects within the first 1 hour following intravasation of tumor cells into circulation, whereas anti-metastatic effects of NK occurred between 1 and 6 hours after tumor cell inoculation in mice in a platelet-independent manner. Future studies are

required to determine under which in *vivo* conditions and pathological context platelet TGF-β may modulate NK-cell cytotoxicity.

Role of platelets in epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) represents a major developmental regulatory program, which can be reactivated during the progression of some cancers, conferring mesenchymal cell properties to epithelial cells. Tumor cells undergoing EMT lose their adhesive properties and acquire migratory and proteolytic activities, helping them to support the metastatic process. Within the same tumor, the loss of epithelial and the gain of mesenchymal markers have been described to correlate with tumor progression, metastasis and bad prognosis (56). Platelets have been proposed to promote EMT through several signaling pathways upon release of PDGF and TGF-β (57, 58). Here again, TGF-β released by activated platelets plays a key role, in addition to its effect on NK cells. Indeed, TGF-β activates TGF-β/Smad signaling in tumor cells (58). In addition, a direct interaction between tumor cells and platelets appear sufficient to induce Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κb) signaling, which in synergy with TGF-β signaling promotes EMT and efficient metastatic seeding (58). Because platelet TGF-β enhances EMT, it may represent an attractive target to interfere with progression of tumor process. Thus, the rationale of targeting TGF-β deserves further studies to unravel the mechanisms and signaling pathways including immune system and anti-tumor effects. An alternative option to inhibit the switch to pro-metastatic EMT phenotype induced by platelet TGF-β may be by targeting NF-κb activation in tumor cells. However, no drug is currently available to specifically block NF-κb activation.

Platelets in tumor cell/blood vessel interactions

Platelets were proposed to facilitate tumor cell adhesion to endothelial cells, thereby enhancing their extravasation from the circulatory system into the tissues to establish metastasis. Platelet/tumor cell aggregates travel inside the circulation and roll along activated endothelium. P-selectin and CD44 appear to support this process in colon cancer cells (59) and probably in other cancer cells. In agreement with this, P-selectin knock-out mice exhibited decreased tumor growth and metastasis of melanoma and colon cancer cells (60). Similar results were obtained in mice treated by mutated heparin lacking anticoagulant activity, but with P- and L-selectin inhibiting effects (61).

Platelet integrin αIIbβ3 has been proposed to mediate transition from selectin-dependent rolling of tumor cells along the endothelium to stationary adhesion (62). In experimental models, inhibition of this integrin or genetic deficiency of β3 integrin in mice decreased tumor cell colonization in lungs and the number of bone metastases (63–65).

Tumor cells also express integrins, such as αVβ3 integrin, which was shown to support tumor cell interaction with platelets allowing adhesion to the vasculature and metastasis (66). This integrin colocalizes with nectin-like molecule 5 (NECL5) at the invasive edge of tumor cells (67). It has been reported that NECL5 on colon cancer cells enhances experimental metastasis. This effect was shown to be mediated by the interaction of NECL5 with its counter-receptor on platelets, probably CD226 enabling tumor cell attachment to the endothelium, ultimately enhancing metastasis (67). Recently, αVβ3 integrin expressed by breast cancer cells has been shown to interact with platelet-derived autotaxin, thereby leading to early bone colonization by these cells and progression of skeletal metastases in mice (68).

Tumor cell/platelet interactions resulting in platelet activation, the release of soluble mediators may activate endothelial cells. Exposure of VWF on activated endothelial cells could support the recruitment of platelet/tumor cell aggregates through its binding to platelet GPIbα. Jain et al, reported that the absence of GPIbα decreases metastasis of melanoma cells (69), but whether this relies on a loss of tumor cell adhesion to the endothelium remains to be established. In contrast, mice treated with Fab fragments directed against GPIba binding to VWF exhibited enhanced metastasis of the same melanoma cell type (70). Additional studies are required to explain this discrepancy and provide potential mechanisms by which GPIbα may affect metastasis.

Platelet-mediated tumor cell extravasation

After attachment to endothelial cells, tumor cells spread and actively transmigrate through the endothelial barrier. Understanding of the mechanisms of this critical step, known as tumor cell extravasation is essential for development of targeted therapies to prevent metastasis. Various mechanisms by which platelets enhance tumor cell extravasation have been proposed. Overall, a wide range of mediators released by platelets could be involved as shown in mouse models deficient either in granules content or in the secretion machinery (71, 72). More specifically, first the release of adenine nucleotides stored in the δ-granules has been shown to play a key role. his process appears to be mediated by the endothelial receptor $P2Y_2$, since mice deficient for this receptor, exhibited a reduced migration of tumor cells through the endothelial barrier (71). MMPs stored in the α-granules may also be involved in tumor cell extravasation through their ability to degrade the vascular basement membrane and the subendothelial ECM.

Platelet/tumor cell aggregates arrested in the vasculature may cause endothelial cell retraction and exposure of subendothelial collagen thereby facilitating platelet/tumor cell extravasation. Mice lacking the platelet-restricted collagen receptor GPVI exhibited decreased metastasis of Lewis lung carcinoma and melanoma cells (73). Interestingly, this important platelet receptor in thrombosis, is not critical for normal hemostasis (74), which makes it a potentially interesting target devoid of bleeding complications.

Activated platelets release the endothelial agonist S1P (sphingosine-1-phosphate), a potent inhibitor of vascular leakage. In contrast, LPA stored in α-granules induce permeability of brain endothelial cells (75, 76). These two factors may affect vascular

integrity during tumor cell extravasation. Serotonin is also released from activated platelets and can modulate the vascular tone by inducing vasoconstriction or vasodilation (77). Circulating tumor cells increase serotonin plasma levels and blockade of serotonin receptors or calcium channels have been shown to inhibit experimental liver metastasis (78). However, a direct role of platelet derived serotonin in tumor cell extravasation has not been demonstrated. Another potent vasoactive factor released by platelets is histamine, which enhances vascular permeability and increases leukocyte extravasation (79, 80). Tumor cell incorporation to platelet-leukocyte aggregates arrested at vasculature may generate a favorable milieu stimulating tumor cell extravasation. It remains to be addressed, whether histamine may impact tumor cell extravasation by this mechanism.

NETs have been reported to sequester tumor cells, thereby facilitating tumor cell extravasation in the context of systemic infection (81). Platelets are known to promote formation of NETs in sepsis through platelet-derived Toll-like receptor 4 (TLR4). Recently, platelet derived TLR4 was shown to promote metastasis by interaction with tumor cell-released high-mobility group box1 protein (82). Whether platelet TLR4 may also play a role in tumor cell extravasation or in other steps of cancer progression through mechanisms involving NETs needs to be addressed.

Thus again, a wide range of mediators and various mechanisms have been shown or are suspected to play a role in tumor cell extravasation. Future studies are needed to evaluate at which metastasis step α- and/or δ-granule content could have a functional role. No doubt also that other components of the platelet releasate will appear to play specific roles in these complex processes, including those affecting endothelial permeability, vascular tone and specific cellular mechanisms.

Platelets and metastatic niches

During metastasis, host cells are recruited by disseminated tumor cells to form specialized microenvironment niches. Recently, Labelle et al. demonstrated that tumor cell-activated platelets release CXC chemokine ligand (CXCL) 5 and CXCL-7, which bind to CXCR2 (CXC chemokine receptor 2) at the surface of granulocytes favoring their recruitment to platelet-tumor cell aggregates. This process establishes an early metastatic niche within 2 hours of tumor cell initial arrest in the lung vasculature. The recruitment of granulocytes to the early metastatic niche was strictly dependent on platelet activation (83).

Early metastatic niche differs from Paget's seed and soil theory, which requires a favorable microenvironment (premetastatic niche) that may evolve allowing tumor cell engraftment (metastatic niche) and proliferation at secondary sites. An interesting question is whether platelets affect metastasis by participating in establishment of metastatic niches, and/or through their interplay with molecular and cellular components of metastatic niches, such as fibronectin, collagen, tenascins, bone marrow derived cells or fibroblasts which provide a permissive milieu for the arrival and growth of tumor cells. Tenascin-C (TNC) has been shown to contribute to the generation of stem like niches supporting cancer and thereby initiating survival and proliferation at newly colonized metastatic sites in breast cancer (84). Moreover, cancer associated fibroblasts producing TNC and VEGF have been reported to provide the permissive "soil" for metastatic colonization (85). TNC is also able to recruit flowing platelets directly through integrin α2β1 and indirectly, through integrin αIIbβ3 and the GPIb-IX complex which bind the plasma VWF adsorbed onto TNC (86). Moreover, TNC induces accumulation of fibrin through down regulation of tissue plasminogen activator, which is also known to support flowing platelet recruitment (87). Whether the platelet/TNC interplay influences metastasis remains also to be evaluated.

Therapeutic implications

Current therapeutic options targeting platelets to improve cancer treatment are scarce. Available anti-platelet drugs are aspirin, $P2Y_{12}$ receptor–targeting drugs and integrin $\alpha_{IIb}\beta_3$ blockers which interfere with platelet activation and aggregation (88). As antithrombotic drugs, they are frequently combined to provide a better clinical outcome, notably aspirin and clopidogrel, which represents the current standard of care in the treatment and secondary prevention of coronary artery disease. Considering the role played by platelets in several steps of the tumor process it may appear obvious to use antiplatelet drugs in order to improve cancer patient outcomes in preventive and therapeutic settings. On the other hand, the mechanisms involved in platelet/tumor cells interactions and in the various steps of tumor progression may not be impaired by existing drugs and may require new pharmacological approaches, more specific of these interactions.

Effects of aspirin on cancer: Do platelets play a role?

Aspirin (acetylsalicylic acid) irreversibly inactivates the cyclooxygenase activity of COX-1 and COX-2. As a result formation of prostanoids is prevented, including PGD_2, PGE_2, $PGF_2\alpha$, PGI_2 and TxA2. COX-1 is the only isoform in platelets, while COX-2 is expressed in a large variety of cells, including epithelial cells, endothelial cells and monocytes. Aspirin is known to inhibit more potently COX-1 (>50–100-fold) in anucleated platelets than COX-2 in other cells, firstly because COX-2 is less sensitive to aspirin and secondly because nucleated cells continuously synthesize this enzyme. An inhibitory role of aspirin in cancer was initially reported by Gasic et al., in 1973, who observed metastatic inhibition of MCA6 ascites sarcoma cells in aspirin treated mice (89). Later, additional studies confirmed this effect of aspirin which prevented tumor growth and metastasis in mice (90). A protumorigenic role was initially attributed to the COX-2 isoform, but more recent studies showed that COX-1 also participates in tumorigenesis. Genetic disruption of either COX-1 or COX-2 was shown to reduce intestinal polyp formation in mice (91). COX-1 and COX-2 pathways operate sequentially in intestinal tumorigenesis. Some studies hypothesized that activated platelets may promote tumorigenesis by

triggering COX-2 expression in stromal cells via release of IL-β, PDGF and TGF-β, which lead to tumor progression (92).

In humans, observational studies indicated that regular use of aspirin was associated with a reduced risk of melanoma, lung, liver, prostate, skin, esophageal and colorectal cancers (90). Moreover, prospective clinical trials have shown that daily aspirin intake as recommended for the prevention of cardiovascular disease reduces the incidence of colorectal cancer after 8–10 years and mortality (93). In more recent analysis of randomized trials, daily treatment with aspirin (75 mg) also decreased the risk of all cancer with metastases (94). Of course, effects of anti-platelet drugs may be dependent on cancer type, stage of tumor progression and cancer risks factors. Aspirin was reported to be more efficient in patients with colorectal cancer than other cancers (94). Reimers et al., reported that aspirin use after diagnosis improves survival of older adults with colon cancer (95). Recently in a meta-analysis of nine observational studies of patients with Barrett's esophagus, aspirin was associated with reduced risk of esophageal adenocarcinoma or high grade dysplasia (96). Conversely, a consensus panel concluded in 2011 that aspirin has only minimal effects on prevention of breast cancer (97).

These data collectively support preventive and therapeutic effects of aspirin on several types of cancer. The key remaining question is whether aspirin exerts its role on cancer through platelet dependent and/or independent mechanisms. The efficacy of the so-called "antiplatelet" lowest dose of 75 mg/day is so far the strongest argument for a role of platelets in cancer-related events.

However, COX-independent mechanisms of aspirin have also been proposed to contribute to its preventive effects in colorectal cancer. Aspirin induces degradation of IkBα, leading to the nuclear translocation of NF-κb, thereby resulting in cell apoptosis. This effect has been demonstrated in vitro and in murine models of colorectal cancer (98). In addition, aspirin was shown to dose-dependently inhibit Wnt/β-catenin, which is a major oncogenic pathway in several cancers (99). Aspirin could also trigger autophagy in colon cancer cells by inhibiting mammalian Target of Rapamycin (mTOR) signaling effectors S6K1 (S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E binding protein-1) through both AMPK (adenosine monophosphate-activated protein kinase)-dependent and independent mechanisms (100). Of note, these pro-apoptotic and anti-proliferative effects triggered by COX-independent action of aspirin have been evidenced in vitro and usually require very high concentrations of aspirin, in the millimolar range, which likely discounts the occurrence of such effects with anti-platelet doses of 75 to 100 mg/day in humans.

Potential limitations of anti-platelet drugs may include an elevated risk of bleeding, especially in patients who are already thrombocytopenic due to chemotherapy or radiotherapy. However, the use of aspirin showed benefit in patients with advanced stage of colorectal cancer, while no major bleeding complications were observed (101). In contrast, other studies suggested caution in the use of aspirin, because a high risk of gastrointestinal bleedings and hemorrhagic strokes were observed in virtually all studies (94, 102–104). Therefore, identification of relevant biomarkers of response to aspirin is urgently needed.

Large randomized clinical trials in patients with active malignancy are required to establish benefits of aspirin. Thus, so far the popular hypothesis that the chemopreventive and chemotherapeutic effects of aspirin could be due to its anti-platelet effects is still speculative and needs to be clearly established. Ongoing randomized clinical trials in patients with different stages of colorectal, non-small cell lung and breast cancers should answer this question in the near future. In one proposed study the investigators have notably focused on platelet dependent mechanisms of aspirin in patients with colorectal cancer (ClinicalTrials.gov NCT02125409).

Evidence of anti-tumoral effects of $P2Y_{12}$-targeting drugs

The $P2Y_{12}$ receptor is a purinergic $G\alpha i_2$-coupled ADP receptor expressed notably on platelets and known to play a critical role in thrombus stability in vivo (105). Two different classes of drugs target this receptor. These are the thienopyridine compounds ticlopidine, clopidogrel and prasugrel which are prodrugs. Their active metabolites irreversibly block the binding of ADP to the receptor, resulting in decreased platelet activation and aggregation, due in large part to a reduced inside-out activation of platelet integrin αIIbβ3 (106). Direct reversible $P2Y_{12}$ receptor antagonists also exist, namely ticagrelor and cangrelor which display similar inhibitory properties. Clopidogrel is widely used clinically to treat coronary artery, cerebrovascular and peripheral vascular diseases (107).

In contrast to aspirin, there is no large scale clinical evidence for any beneficial effect of clopidogrel or any $P2Y_{12}$ targeting drug in cancer patients. As an example, cancer mortality among the CHARISMA trial patients was not influenced by clopidogrel (108).

However, in vitro data indicate that inhibition of the $P2Y_{12}$ receptor results in decreased TCIPA, which is not surprising if one remembers the importance of ADP in tumor cells interactions with platelets (109). Recently, in animal models, clopidogrel was shown to reduce cancer progression. Indeed, in syngeneic orthotopic mice models of pancreatic cancer, clopidogrel inhibited tumor development, metastasis and the extent of thrombosis associated with cancer, at a dose of 8 mg/kg which is 4–8 fold the chronic dose in patients and probably induces complete inhibition of ADP-induced platelet aggregation (110). Similarly, ticagrelor has been found to inhibit lung metastasis in mice (111), whereas genetic deficiency of $P2Y_{12}$, has also been shown to inhibit lung colonization by Lewis lung carcinoma and melanoma cells (112).

Do $P2Y_{12}$ targeting drugs also affect tumor angiogenesis? Earlier work has shown that ticlopidine displayed anti-angiogenic properties in a rat model of subcutaneous fibrin gel chambers (113). However, the thienopyridine SR 25989 R which is the inactive stereoisomer of clopidogrel and completely devoid of any anti-platelet action also appeared to inhibit angiogenesis in both in vitro and in vivo conditions and exhibits an inhibitory effect in an ex-

perimental model of metastasis (114, 115) clearly indicating an off-target effect of thienopyridine compounds on angiogenesis.

Another aspect to consider is the fact that the $P2Y_{12}$ receptor is expressed in cells other than platelets (116), such as on osteoclasts (117). Su et al., reported that mice treated with clopidogrel were protected from pathologic osteolysis and bone loss associated with tumor growth (117). The benefits of $P2Y_{12}$ antagonists in preventing pathological osteolysis and metastasis have been shown in mice, but their effects remain to be studied in humans with malignancy.

Finally, an intriguing observation has been reported concerning prasugrel, the potent third generation thienopyridine. In the TRITON-TIMI 38 trial which compared clopidogrel to prasugrel in the setting of percutaneous coronary intervention, a higher incidence of solid tumors and cancer death was recorded in the prasugrel arm – reviewed by Nanau et al. (118). A possible occurrence in this trial of a chance effect should be taken into consideration. Of note, no tumorigenic effects of prasugrel was reported in mouse xenograft models of prostate, lung and colon cancers and genotoxicity, carcinogenicity assays (119). If the tumor causing effects of prasugrel is confirmed in humans, it would be important to establish whether it is due to an off-target effect or to a more aggressive antiplatelet regimen as compared to clopidogrel.

Effect of integrin αIIbβ3 blockade on cancer

Several αIIbβ3 antagonists, which are exclusively used in acute phases of cardiovascular diseases (120), notably integrilin, have shown to provide some benefits in experimental metastasis (65). It remains to be confirmed that the effect of integrilin is limited to its action on platelets since it has been reported that some cancer cell lines also express αIIbβ3 (121). Zhang et al., proposed an anti-metastatic approach based on the use of a humanized single chain antibody directed against integrin β3 (122). One drawback in targeting platelet αIIbβ3 is the associated bleeding risk which precludes chronic use of such drugs in patients (120). A potential approach to limit the risk of bleedings is to specifically target this integrin under its active conformation. This has been proposed by Stoll et al., demonstrating that a single chain antibody directed against activated αIIbβ3 provide an antithrombotic effect without increasing the bleeding risk (123). Future studies should be conducted to evaluate the effect of such a tool in animal models of metastasis.

Other pharmacological approaches

Platelet-mimicry of tumor cells – Co-targeting anti-platelet strategies?

Therapeutic strategies disrupting the multiple interactions between platelets and tumor cells at an earlier stage may provide advantages to limit tumor progression and metastasis. However, identification of patients with early disease is rather challenging. Tumor cells may express several megakaryocytic genes, such as integrin αIIbβ3, PECAM-1 (Platelet-Endothelial Cell Adhesion Molecule-1)/CD31, thrombin receptors, platelet-type 12-lipoxygenase and acquire a geno-phenotype mimicking platelets (121). This epiphenomenon of platelet mimicry and shared receptors may enable platelet antagonist and inhibitors to simultaneously target tumor cells and platelets and dissipate tumor and platelet cross-talks with tumor microenvironment. Systematic analysis of the markers of platelet mimicry in various cancers and clinical trials investigating the efficacy of shared molecular targets may provide new therapeutic modalities in management of active malignancy.

Platelets as biocompatible drug delivery system?

As mentioned below, targeting of platelet/tumor cell interactions may represent an attractive therapeutic approach. Interestingly, alternative approaches taking advantage of tumor cells preference in interacting with platelets have also been proposed. Indeed, the attributes of platelets, namely, their tendency to uptake a variety of compounds and release them when activated, have been suggested as an efficient drug delivery system (124). Future studies are needed to address whether this strategy might be more effective in killing cancer cells than normal cells while preserving normal physiology.

Experimental limitations

Existing research has provided compelling biological evidence in support of attempting to disrupt physical and functional platelet/tumor interactions and platelet/tumor microenvironment cross-talks to attenuate or inhibit tumor growth, invasion, angiogenesis and tumor metastasis. In mouse models, anti-platelet drugs were shown to reduce tumor growth, angiogenesis and metastasis. However, some data should be interpreted with caution, since evidence has been accumulated for species specific differences in terms of platelet and tumor cell receptors. For example, human platelets are primarily activated by thrombin through PAR-1 and PAR-4, whereas mouse platelets do not express PAR-1 and are predominantly activated through PAR-3 and PAR-4. In several studies, experimental approaches used to evaluate involvement of platelets to induce thrombocytopenia were essentially based on the use of neuraminidase or anti-platelet antibodies. The major drawback of neuraminidase is that sialic acid removal occurs not only on platelets but also on other vascular cells, potentially affecting several biological functions. Thrombocytopenia inducing antibodies present also disadvantages, since they can trigger platelet activation leading to the release of factors potentially affecting molecular pathways in cancer or other circulating cells. Another important point to revisit is that many mouse models used to evaluate mediators released by platelets or platelet receptors in metastasis dismiss the early stages of the metastatic cascade. For example, the frequently used injection of a large number of tumor cells into the circulatory system does not accurately mimic the disease progression as it occurs in patients. Syngeneic orthotopic and genetic mouse models of human cancers are more relevant since they recapitulate progressive

stages of cancer starting from primary tumor development, to tumor angiogenesis and establishment of metastases. Thus, well-designed pre-clinical models with defined alterations in tumors cells, platelets or in other host cells successfully mimicking human diseases are mandatory to dissect the cellular and molecular mechanisms, and to assess the potential and selectivity of anti-platelet agents.

Studies aiming to understand the role of platelets in different steps of metastasis have been mainly conducted in vitro, notably the interactions of platelets between with tumor cells, endothelial cells, leukocytes. This allowed dissection of many mechanisms involved and modulation of signaling pathways. In vivo rabbit and mouse ear chambers have provided some hints on the dynamics of tumor cell intravasation, migration and arrest along the vasculature and platelet-thrombi formation. However conditions are different from native environment, which may not recapitulate more relevantly different steps of tumor progression and metastatic cascade. Tumor cell-endothelial interactions have been extensively studied by microscopy techniques in mice, and not so far in real time. There are also difficulties in tracking platelet-tumor cell interactions within the vasculature. Recently, multiphoton microscopy techniques have been developed to track platelet-tumor cell interactions in mouse liver sinusoid vessels (125). Highly resolutive real time imaging techniques are needed, to analyze more profoundly behavior of platelet/tumor/vascular cell interactions in animal models, thereby leading to more fine elucidation of mechanisms.

Conclusion

Literature describing the contribution of platelets to cancer is significantly growing. It has revealed a highly complex role and the involvement of platelets in many bi-directional interactions with tumor cells and tumor microenvironment. Platelets appear to influence many functions of tumor cells and escort them through different stages of tumor progression. In turn, tumor cells may use their ability to hijack important biological functions of platelets to increase their survival and proliferation capacity promoting the pathogenesis of cancer. In addition, clinical data have led to speculate that anti-platelet medication may provide anti-cancer effects by disturbing platelet-tumor cell cross-talks. However, further fundamental, translational and clinical studies are needed before these drugs can be introduced in clinical cancer care.

Conflict of interest

The authors declare that they have no conflict of interest.

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