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The role of platelet activation in tumor metastasis

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Running title: Platelets during cancer progression

Keywords: platelets, adhesion molecules, metastasis, P-selectin, selectin ligands, tumor emboli, thrombosis, Trousseau syndrome
Abstract

Platelets are highly reactive components of the circulatory system, which exert not only haemostatic activity but also contribute to modulation of various pathological conditions including inflammation, atherosclerosis and cancer metastasis through the release of cytokines, chemokines and the presentation of several adhesion molecules. During cancer metastasis, the formation of platelet-tumor cell aggregates in the circulation facilitates immune-evasion and the microvascular arrest of tumor cells at distant sites. Several adhesion molecules, e.g. integrins and glycoproteins (GPs), were shown to be involved in this process. Recent findings indicate that P-selectin is another main mediator of platelet-tumor cell interactions. Other effects of activated platelets on cancer progression are associated with a release of platelet-derived factors stimulating tumor growth and angiogenesis. Any interference in platelet-tumor cell interactions resulted in attenuation of cancer metastasis. The well recognized albeit not fully characterized function of platelets during cancer progression defines platelets as potential targets for cancer therapy. Specifically, the rapid expression of P-selectin on the cell surface of activated platelets and its strong association with metastasis provide a rationale for P-selectin inhibition as an anti-metastatic treatment.
Introduction

The main challenge for cancer treatment still remains the control of metastasis. Metastasis is a multistep process initiated by tumor cells which escape from a primary tumor and enter the blood circulation or the lymphatic system. While large numbers of tumor cells may enter the blood stream from a primary lesion, only very few survive the host defense mechanisms, arrest in the vasculature of distant organs and finally form metastatic foci. There is increasing evidence that platelet activity may facilitate this process. Platelets have been associated with cancer since 1865, when the French physician Armand Trousseau discovered and described the association of excessive blood coagulation with cancer progression [reviewed in 1]. Although the initial clinical observations of migratory thromboses and platelet-rich microthrombi are mostly associated with gastrointestinal malignancies, the majority of advanced cancer patients develop thrombotic abnormalities and hypercoagulable state [1-4].

The clinical correlation between platelet dysfunction and cancer progression is supported by a large body of evidence obtained from several animal models [e.g. 5,6]. Experimentally induced thrombocytopenia (depletion of platelets) was shown to reduce the development of metastasis both in transplant and syngeneic mouse tumor models [7]. Various human and animal tumor cells were found to be capable of inducing platelet aggregation and activation [e.g. 3,8-11]. Any interference in platelet-tumor cell interactions with anti-platelet agents has consistently demonstrated potent anti-metastatic effects [reviewed in 10,12]. In this article, the mechanisms of platelet contribution to metastasis are reviewed and possible perspectives for therapeutic application are discussed.

Physiological functions of platelets

Platelets are highly reactive cellular effectors of hemostasis and thrombosis in humans and other mammals. Vessel wall injury or thrombus formation stimulates platelets to adhere to subendothelial matrix and to undergo activation leading to aggregation and release of
mediators for further aggregation, angiogenesis and inflammation. Together with a display of many adhesion molecules, platelets mediate the subsequent recruitment of inflammatory cells and enable multidirectional interactions among leukocytes, endothelial cells, subendothelial matrix, and other platelets. However, in part the same processes cause adverse clinical events associated with thrombotic vessel occlusion [13].

Platelets are small anucleate cell fragments derived from megakaryocytes in the bone marrow. The platelet membrane contains a dense layer of glycoproteins (GPs) and integrins, which mediate their adhesive properties leading also to aggregation. Although most of the platelets never become activated or adherent in their life time, their exposure to activating molecules, e.g. von Willenbrand Factor (vWF), collagen etc. causes a rapid activation resulting in adhesion and aggregation [13]. The principal platelet adhesive receptor is GP Ib/IX/V complex which upon binding to vWF both induces platelet adhesion and activation. Platelets bound to vWF undergo activation of the GPIIb/IIIa receptor which facilitates high affinity binding to fibrinogen. Activation of platelets results in surface expression of adhesion molecules responsible for an enhanced and rapid adherence. In addition, activation of platelets induces shape change, which is associated with a rearrangement of cytoskeletal proteins resulting in actin polymerization and extension of filapodia [14]. Adhesive properties of platelets have to be strictly regulated to maintain function during hemostasis.

**Platelets function beyond hemostasis**

The activity of platelets is also controlled by 7-transmembrane receptor family consisting of thrombin, prostaglandin and chemokine receptors [for review see 13,14]. Protease activation receptor-1 (PAR-1) and PAR-4 are specifically activated by thrombin. After stimulation, platelets also release various substances contributing to the local activation and additional recruitment of platelets and leukocytes. Platelets contain two types of distinct secretory vesicles, α-granules and dense granules. Upon activation, these granules fuse with the cell
membrane and release a number of bioactive molecules into the local periphery and simultaneously expose adhesion receptors on platelets surface [15]. α-Granules contain large proteins and adhesion molecules, like P-selectin, GPs, growth factors, and thrombospondin. Dense granules contain small molecules including serotonin, Calcium ions, ADP and ATP, which help recruiting other platelets [16]. Platelets synthesize and release thromboxane A₂ on stimulation with collagen, thrombin or ADP, thereby further promote recruitment and activation of platelets [17].

Platelets, in addition to exerting hemostatic activity can also contribute to immunity and inflammation. Activated platelets release a number of secretory molecules including chemokines, cytokines, growth factors, coagulation factors and metalloproteinases [18,19]. To the most potent chemokines secreted by platelets belong: CCL5 (also known as RANTES), CCL3 (macrophage inflammatory protein -1), and CXCL4 (platelet factor 4, PF4) [18]. Platelet’s CCL5 binds to endothelium and is the primary chemokine responsible for a recruitment of monocytes and T cells [20,21]. The surface expression of CD40 ligand on activated platelets induces rapid activation of endothelial cells associated with secretion of chemokines and expression of adhesion molecules responsible for recruitment of leukocytes [22,23]. Detection of functional protein synthesis in platelets will probably lead to identification of further novel biological activities [24]. The messenger RNA profiling of human platelets, together with a validation of platelet protein synthetic activity can further modulate platelet phenotype and function based on a stimuli and a cellular context [25,26].

**Platelets during cancer progression – clinical evidence**

Hemostatic abnormalities associated with human cancer are common, yet diverse. In some cases, patients with occult malignancy develop deep vein thrombosis or pulmonary embolism [1]. In other cases, clinical findings may relate to disseminated intravascular coagulation (DIC) or to thrombohemorrhagic syndromes, which are commonly referred to as cancer
coagulopathy [2,27]. The pathophysiology of thrombosis in cancer is complex but likely includes all aspects of Virchow’s triad: stasis of blood, vascular trauma and hypercoagulability of blood itself [1,4]. The clinical evidence for higher platelet counts, high platelet turnover, and the presence of activated platelets in the circulation generally indicates poor prognosis in most cancers [3,28-30]. Although substantial clinical, epidemiologic and pathologic evidence validate the cancer associated thrombosis, this phenomenon has been considered an epiphenomenon of cancer progression for a long time. However, there is large body of evidence that platelets not only contribute to coagulation but rather promote cancer development and progression, through directly effecting tumor growth, angiogenesis and metastatic dissemination [1,9,31-34].

**Platelets during metastasis – experimental evidence**

The clinical correlation between platelets dysfunction and cancer progression was further supported by the findings that platelets have a significant role in many animal models of metastasis [reviewed in 10,35]. Similarly to the role of platelets during hemostasis, many platelet activities may contribute to cancer progression (Figure 1). Several mechanisms were associated with the supporting function of platelets during metastasis and include: platelet-tumor cell aggregation; immune-evasion; promotion of tumor cell adhesion and growth; and enhancement of angiogenesis. Clear evidence for the role of platelets was derived from experiments where experimentally induced thrombocytopenia, reduction of circulating platelets, resulted in attenuation of metastasis in several tumor models [5,7,8]. Similarly, injection of tumor cells in platelet-deficient mice, Nf-E2-/- [36], caused marked protection against hematogenous metastasis [37].

A rapid formation of platelet-tumor cell emboli is detected in the microvasculature of lungs upon tail vein injection of tumor cells in mice [5,9,38,39]. While the mechanism of platelet activation *in vivo* has not been fully characterized, there is abundant evidence that tissue
factor (TF)-dependent activation of coagulation cascade and platelets is associated with cancer progression [40-43]. TF is a membrane-associated protein expressed on tumor cells and activated endothelial surfaces. Circulating TF was found to be primarily present in microparticles, which promotes thrombus formation [44]. TF-initiated activation of platelets is mediated by thrombin which stimulates protease-activated receptors (PARs) and thereby platelet aggregation. Addition of thrombin to tumor cells resulted in enhancement of metastasis in many cancer models [38,45]. Accordingly, the down-regulation of TF expression in melanoma cells led to an attenuation of experimental metastasis [46]. The relevance of thrombin-induced platelet activation during hematogenous metastasis was evaluated in Par4-/- deficient mice, in which platelets were irresponsible to thrombin [37]. The absence of PAR4 led to attenuation of metastasis. The ability of hirudin treatment to further reduce metastasis in Par4-/- mice is consistent with the role of thrombin in this process which acts also independent of platelet activation [37]. PAR signaling has also direct effects on tumor cells and increase cell proliferation, invasiveness and metastasis [47].

Besides platelet-activating function, thrombin is implicated in fibrin depositions in primary tumor and during metastasis [38,48]. Fibrinogen deposits are often found in solid tumors in humans [48]. Moreover, Palumbo and colleagues have demonstrated that fibrinogen deficient mice are protected from experimental metastasis of B16-F10 melanoma cells [49]. However, hirudin treatment enhanced the protection against metastasis seen in fibrinogen deficient mice. Taken together, both the thrombin-induced platelet activation and the fibrin formation contribute to metastasis.

Platelets adherence to tumor cells was shown to be mediated by surface expression of P-selectin, GPIIb/IIIa and GP Ib/IX/V [5,39,50-52]. Inhibition of GPIIb/IIIa complex with a specific antibody reduced experimental metastasis of several cancer cells [5,53,54]. Attenuation of metastasis was observed in GPIb/IX deficient mice, thereby implicating GPIb/IX receptor to cancer progression [55]. Similarly, it was shown show that P-selectin
profundely modulates platelet-tumor cell interaction, and its inhibition led to attenuation of metastasis in several models [39,50,56].

**Platelets P-selectin and tumor cell aggregation**

P-selectin is one member of vascular cell adhesion molecules collectively called selectins that is involved in adhesive interactions of leukocytes and platelets within the blood circulation. P-selectin is present in α-granules of platelets and Weibel-Palade bodies of endothelium and upon activation, is rapidly expressed on a cytoplasmic membrane. While cell-surface expression of P-selectin is temporal in nature, the constitutively expressed L-selectin on leukocytes is proteolytically cleaved after the binding, resulting in activation. Selectin ligands belong to the group of sialylated, fucosylated lactosamine oligosaccharide structures containing the terminal tetrasaccharide sialyl Lewis^x^ -sLe^x^, which are carried on glycoproteins like P-selectin glycoprotein ligand-1 and mucins [57,58]. In addition, glycosaminoglycans also serve as physiological ligands for P- and L-selectin. Epithelial cancer cells, carcinomas, commonly express mucins, carriers of selectin ligands. During metastasis, the entry of invasive carcinomas into the blood circulation makes these cells potential candidates for interactions with other blood constituents, including platelets, through selectin-mediated interactions. The contribution of P-selectin to metastasis was demonstrated in several studies using P-selectin deficient mice [39,50,59]. The absence of P-selectin led to attenuation of metastasis. To identify whether P-selectin expression on platelets or endothelium facilitates metastasis, bone marrow reconstitution experiments followed by injection of melanoma cells were evaluated [59]. In addition to platelets, endothelial P-selectin was shown to contribute to metastasis. Furthermore, a temporal inhibition of P-selectin mediated interactions by a single heparin injection led to attenuation of metastasis similar to the one observed in the absence of P-selectin [39]. Enzymatic removal of carcinoma mucins from tumor cells prior to intravenous injection, resulted in similar attenuation of
metastasis as was observed in the absence of P-selectin, further supporting the relevance of P-selectin mediated platelet-tumor cell interactions. Tumor cell induced platelet aggregation was shown to protect tumor cells from immune-mediated elimination of the cells [60]. Inhibition of platelet-tumor cell interaction led to an accelerated NK-cell mediated tumor cell lysis in vitro and in vivo [60-62].

The important role of von Willebrand factor (vWF) in platelet adhesion suggests potential involvement in pathological situation like cancer [63]. To investigate the participation of vWF in metastasis, vWF deficient mice were intravenously injected with melanomas [64]. A significant increase in metastasis was observed in vWF deficient mice, which could be corrected by restoring vWF plasma levels. These findings suggest a surprising protective role of vWF against tumor cell dissemination, while the molecular mechanism remains to be investigated [64].

**Platelet aggregation and Trousseau’s syndrome**

Originally, Trousseau’s syndrome describes the formation of venous and arterial platelet-rich microthrombi with secondary microangiopathic hemolytic anemia, and a frequent association with mucinous carcinomas [2]. Nowadays, Trousseau’s syndrome applies to thrombotic events preceding the detection of an occult cancer. Based on this broader definition of Trousseau’s syndrome, several molecular mechanisms regulating tumor cell induced platelet aggregation were described [65-67]. Since thrombosis occurs in the setting of occult carcinomas, it is reasonable to expect that a factor produced by tumor cells is being released into the bloodstream, which is responsible for thrombotic propensity [2]. There were two main studies investigating Trousseau’s syndrome in animal models [6,68]. In the first model, purified carcinoma mucins, free of any TF, were shown to induce platelet-rich microthrombi formation in vivo [68]. Furthermore, these microthrombi in a mouse model were dependent on the presence of both P-and L-selectin, strongly indicating that a cross-talk between leukocytes
and platelets is required for this process. Platelet aggregates, formed on the carcinoma mucin backbone, were shown to be thrombin independent. Thus thrombin generation is not a proximal step leading to formation of platelet-rich microthrombi, but rather an event dependent on selectin-mediated interactions between platelets and leukocytes. These observations are in agreement with the carcinoma mucin mediated activation of leukocytes through L-selectin signaling, which leads to activation of platelets by a yet unidentified mediator [68]. Finally, the positive effect of heparin treatment in Trousseau’s syndrome patients may simply reflect the capacity of heparin to efficiently block P- and L-selectin mediated interactions. In the second model, targeting of activated human MET oncogene to the mouse liver resulted in slowly progressing hepatocarcinogenesis [6]. Prior to detection of dysplastic nodules all animals developed thrombohemorrhagic syndrome, similar to the symptoms observed in Trousseau’s syndrome.

**Platelet-mediated angiogenesis and tumor growth**

Platelets can exert direct stimulatory action on tumor cells and promote angiogenesis. Secretory granules of platelets are a rich source of many proangiogenic factors including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and others [e.g. 18]. Higher serum levels of VEGF per platelet count were positively correlated with cancer progression and poor prognosis for patients with gastric, renal, colorectal and breast tumors [69-71]. In cancer patients the serum concentration of VEGF corresponds well with the activation status of platelets and neutrophils rather than with the tumor burden [72]. Analysis of platelets from breast cancer patients revealed a markedly higher secretion of VEGF upon activation when compared to platelets from healthy donors [73]. Large numbers of activated platelets were detected in soft tissue tumors, which were associated with abundant expression of VEGF, and ongoing angiogenesis [74]. It is conceivable that VEGF secreted by platelets stimulates angiogenesis by the local activation of
endothelium in vivo, since platelets were shown to stimulate endothelial proliferation in vitro [72]. The contribution of platelets to tumor cell-induced angiogenesis has been thoroughly reviewed elsewhere [34,75].

Recently, platelet-derived bioactive lipid - lysophosphatidic acid (LPA) was found to have a growth-factor-like signaling properties [76]. LPA is a water soluble biolipid consisting of a phosphoglycerol backbone with only one fatty acid chain [77]. Upon generation, LPA exerts its activity either in autocrine or paracrine fashion, on G protein-coupled receptors family of LPA receptors [78]. Activation of downstream signaling pathways leads to cell proliferation, increased survival and enhanced migration. Boucharaba and colleagues have identified platelet-derived LPA as an enhancer of bone metastasis by breast and ovarian cancer cells [76].

Platelets modulate degradation and further remodeling of basement membrane and ECM components, which can serve angiogenesis and tumor cell invasion [79]. Activated platelets secrete gelatinase A [80], heparanase [81], and induce the release of gelatinase and urokinase (u-PA) by endothelial cells and tumor cells in vitro [82]. The role of platelet-derived microparticles (PMV), which are circular fragments shed by activated platelets, was analyzed for its potential to contribute to metastasis [83,84]. PMV were shown to adhere to tumor cells and initiate phosphorylation of mitogen-activated protein kinase p42/44 and expression of membrane type 1- MMP [83]. Addition of PMV to breast cancer cells induced expression of MMP-9 and thereby increased tumor cell transmigration in vitro [84]. In another breast cancer model, platelets promoted invasion of MCF7 cells, which was correlated with an increase of MMP-9 level produced by tumor cells [85].

**Platelets as therapeutic targets for anti-cancer therapies**

Based on the understanding of platelet-tumor cell aggregate formation, potential inhibitors of cancer progression are being explored [reviewed in [10,12]. Platelets adhesion molecules are
involved in mediation of platelet-tumor cell interactions. Since GPIIb/IIIa complex was convincingly shown to mediate this process, platelets antagonist based on its inhibition are being tested [5]. The antagonists of GPIIb/IIIa can be divided into RGD-mimicking nanopeptides and monoclonal antibodies binding this complex. Intravenous injection of monoclonal antibody (e.g. Abciximab) reduced tumor cell induced platelet aggregations and demonstrated antineoplastic effects [86]. Similarly, small molecule oral antagonist of GPIIb/IIIa was shown to efficiently reduce platelet aggregation and metastasis [87].

Although P-selectin mediated platelet-tumor cell interactions are well recognized, the specific inhibition of P-selectin in cancer has not been explored [88,89]. Heparin is a highly sulfated glycosaminoglycan that has been in clinical use as an anticoagulant for many years. Besides its known anti-coagulant activity, heparin can also efficiently bind to P- and L-selectin and was shown to abrogate cancer cell interactions with platelets, thereby attenuating metastasis [39,56,59,90,91]. Furthermore, we could show that heparin derivatives without any anticoagulant activity, but containing P-selectin binding activity attenuate metastasis as efficiently as native heparin [91]. This finding strongly indicates that inhibition of P-selectin is one of the main cancer inhibitory activities of heparin [91]. Based on the encouraging observations with heparin treatment evaluated in retrospective studies, several recent prospective clinical trials have been performed to study this phenomenon [92-95]. The promising results from the clinical studies, primarily in earlier stage patients together with the experimental evidence suggests that heparin treatment directly affect the metastasis rather than by simple inhibition of coagulation[35]. While targeting platelet contribution to cancer progression is actively being pursued, there is at present no clinical trial in cancer patients. A major concern in clinical trials of platelet inhibitors in cancer is the possibility that normal platelet function will be limited leading to bleeding complications.

**Expert opinion**
The current evidence unquestionably links platelets to cancer progression. The clinical observations of abnormal platelet counts as well as their activated state in circulation reflect the host response to factors produced by tumor cells. The potential of platelets to facilitate interactions among tumor cells, endothelium and leukocytes, together with a plethora of available growth factors, chemokines and cytokines defines their central role, both in primary tumor growth as well as in metastasis. But the multifunctional presence of platelets in a number of processes associated with homeostasis rather “complicates” the exploration of platelets as a therapeutic target. From a therapeutic point of view, the function of platelets during metastasis seems to be more important than their role in primary tumor growth. Targeting platelets during primary tumor growth could certainly be therapeutically beneficial yet the feasibility of prolonged treatment in cancer patients remains to be determined. In addition, focusing on the metastasis might be a more feasible way to explore platelets as therapeutic targets. The primary function of platelets during metastasis is related to the rapid platelet aggregation on tumor cells, which might promote several steps of hematogenous metastasis, while the tumor cells are in circulation [12,35,96-98]. It was shown that temporal inhibition of platelet-tumor cell interaction during the hematogenous phase of metastasis is sufficient for significant attenuation of this process [39,56,90,91,99,100]. Thus, platelets could be inhibited in a time-restricted manner, and thereby potentially deleterious side effects associated with continuous targeting of platelets may be reduced. The success of such an approach will largely depend on a finding of the right window for efficient therapeutic intervention. Previously, a similar approach has been proposed for the use of heparin as a potential antimetastatic therapy [96]. The therapeutic window is limited by the time of cancer diagnosis, removal of the primary tumor and a few days afterwards. During this time frame chances are very high that tumor cells are in circulation, thus reduction of platelet-tumor cell interactions could lead to tumor cell elimination. Targeting platelet-tumor cell interactions represent a promising approach to prevent blood-borne metastasis, while taking the current
understanding of the process into account. Since there is no available therapy for treatment of metastasis, any approach towards a better control of this process would be of great benefit.

**Five-year view – future directions**

The complexity of molecular mechanisms that mediate platelet-tumor cell interactions makes a pharmacological inhibition of these interactions rather challenging. In particular, the lack of selectivity poses a major problem in targeting platelets. Although specific inhibition of platelet functions during cancer thrombosis is likely not possible, accumulated evidence from experimental models suggests possible future directions. A first step is to find a cancer type, where cancer progression is shown to be dependent on platelet activation. Platelet microthrombi formation associated with Trousseau’s syndrome is often found in mucin-producing carcinomas, yet the underlying mechanism remains unclear [2]. The current evidence that carcinoma mucins can induce platelet aggregation similar to Trousseau’ syndrome [68] indicates that platelet activation plays an important role in mucin-carrying carcinomas. The formation of platelet aggregates was shown to be dependent on P-selectin, which effectively binds to soluble mucins or mucin-carrying carcinoma cells. These observations make P-selectin a possible candidate for cancer specific therapy. Another issue is to limit the inhibition to affect mostly, if not exclusively, cancer associated mechanisms. This problem will likely be a common one to be encountered by any inhibitor of molecules presented by activated platelets. The main question is whether prolonged inhibition of P-selectin will be associated with deleterious side effects in cancer patients. Intriguingly, there is clinical experience with prolonged inhibition of P-selectin. Therapeutic concentrations of unfractionated heparin used in clinical application were found to be sufficient to effectively inhibit P-selectin [101]. A prolonged application of unfractionated heparin for anticoagulation, commonly used also in cancer patients, has inadvertently achieved significant P-selectin inhibition. Thus P-selectin represents a promising candidate for therapeutic
targeting of cancer progression. This proposition is supported by the known mechanism of action of P-selectin as well as the clinical experience with heparin treatment.

To date there are no anti-platelet agents in clinical trials in the context of cancer. Other platelet inhibitors, including anti-GPIIib/IIIa and anti-GPIb antibodies, effectively attenuate metastasis in animal models and are currently being further developed [53,54]. In addition to adhesion molecules, several platelet activation pathways, including arachidonic acid metabolism (COX/LOX enzymes), ADP, and PAR signaling are being investigated as potential targets for anti-cancer therapy. Finally, the determination of a therapeutic regimen, including dose and time of treatment, will greatly affect the future of an antiplatelet drug as a potential anti-metastatic therapy.

Key issues /Outline

1. Introduction
3. Platelets function beyond hemostasis. The multifaceted role of platelets in immunity and inflammation.
6. Platelets P-selectin and tumor cell aggregation. P-selectin as an important mediator of platelet-tumor cell aggregation.
9. Platelets as therapeutic targets for anti-cancer therapy. Potential approaches to target platelets function during cancer progression.
10. Expert opinion
Grant support

This work was in part supported by Swiss National Science Foundation grant #3100A0-116295 to L.B.

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**This work provide evidence for inhibition of pulmonary metastasis by induction of thrombocytopenia, which can be reconstituted by infusion of platelets.


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**This article documents the key role of platelets (platelet deficient mice) in hematogenous metastasis. PAR-4 signaling on platelets is required for metastasis progression.**


**This work provides evidence for P-selectin as the main mediator of platelet-tumor cell interactions, which promotes metastasis.**


**This article documents the critical role of fibrinogen for the metastatic potential of circulating tumor cells.**


*This work provides evidence for the GPIb contribution to metastasis, through mediation of platelet-tumor cell adhesion.*


*This work documents the contribution of platelets aggregation to tumor cell protection from lysis by NK cells.*


*This work documents the potential of circulating carcinoma mucins to induce platelet aggregations.*


**Figure legend**

**Figure 1. Possible interactions of platelets within the metastatic microenvironment.**

While tumor cells can induce the initiation of this process, activated platelets can further stimulate leukocytes, endothelium and tumor cells. The ability of platelets to affect various processes including platelet-tumor cell emboli formation, immune-evasion; promotion of
tumor cell adhesion and growth; and enhancement of angiogenesis, makes platelets a “versatile” facilitator of metastasis. The functional and the temporal role of platelets during metastasis remain to be characterized.