Role of \( \beta \)3 Integrins in Melanoma Cell Adhesion to Activated Platelets under Flow*

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Mechanisms mediating tumor cell attachment to the vessel wall under flow conditions are largely unknown. Therefore, we analyzed the ability of human melanoma cells to adhere to an immobilized matrix during blood flow and determined the role of platelets in this process. In a parallel plate flow chamber, M21 melanoma cells were suspended in human blood and perfused over a collagen I matrix at a wall shear rate of 50 s\(^{-1}\) (2 dynes/cm\(^2\)) to simulate venous flow over a thrombogenic surface. Melanoma cell interaction with the matrix or blood cells and platelets was monitored and quantified by fluorescence and confocal laser microscopy. Despite their ability to adhere to collagen I under static conditions, M21 cells failed to attach directly to this matrix during blood flow. However, they associated with adherent thrombi, and this resulted in stable melanoma cell arrest. Inhibition of platelet activation or platelet integrin \( \alpha \)IIb/\( \beta \)3 function abolished M21 cell attachment. Melanoma cell interaction with thrombi was specific and required \( \beta \)3 integrin expression. M21-L cells which lack integrin \( \alpha \)IIb/\( \beta \)3 failed to associate with thrombi and to arrest during blood flow. Transfection of these cells with the integrin subunits \( \alpha \)v or \( \alpha \)IIb resulted in variants expressing \( \alpha \)v/\( \beta \)3, as in the wild type, or \( \alpha \)IIb/\( \beta \)3. Both variants were able to associate with thrombi and to arrest during blood flow. Therefore, \( \beta \)3 integrin-mediated binding to activated platelets represents an efficient mechanism for melanoma cell arrest under flow, and this may contribute to the role of platelets in hematogenous metastasis.

During metastasis, tumor cells disseminate to distant organs via the lymph or the blood stream (1). The arrest of metastasizing tumor cells within the blood stream is a prerequisite for their extravasation. This step is rate-limiting during hematogenous metastasis. In the blood stream, tumor cells are exposed to flow-dependent shear forces, plasma proteins, blood cells, and platelets, all of which may affect tumor cell survival, arrest, and extravasation. Mechanisms which mediate tumor cell arrest involve adhesive interactions of tumor cells with vascular cells and their matrices. A major limitation of our current knowledge of such adhesive interactions originates from the fact that most experimental models used for their study are based on static conditions that are different from those present in the vasculature. Specifically, effects generated by blood flow and the resulting shear forces may critically affect adhesive cell interactions (2). Therefore, the goal of the present study was to analyze tumor cell arrest under flow conditions. This was achieved by using an in vitro model that mimics flow in the vasculature. The system is based on a parallel plate flow chamber combined with confocal laser microscopy and real-time recording to document and quantify tumor cell adhesive properties under flow.

Tumor cells originating from solid tumors express a variety of adhesion receptors which support their attachment to extracellular matrices present in tissues and the vessel wall, such as collagen, fibrin, fibronectin, laminin, vitronectin, and von Willebrand factor (3). Adhesion receptors also participate in tumor cell interactions with the intact endothelium of the vasculature. This has been demonstrated mostly under static conditions, and many of these adhesion receptors were identified as members of the integrin family including \( \beta \)1 integrins containing the \( \alpha \) subunits \( \alpha \)1, \( \alpha \)2, \( \alpha \)3, \( \alpha \)4, \( \alpha \)5, \( \alpha \)6, \( \alpha \)7, and the \( \alpha \)v integrins \( \alpha \)v\( \beta \)3 and \( \alpha \)v\( \beta \)5 (4). Static conditions are, however, unlikely to occur in the blood stream. It has been suggested that passive entrapment of tumor cells in narrow capillary vessels may favor their attachment to the endothelium or exposed sites of the subendothelial matrix via adhesion receptors genuine to the tumor cells. Yet shear conditions can reach maximal levels in capillary passages (5). Therefore, adhesion mechanisms mediating tumor cell attachment to the vessel wall would have to be designed such that adhesive interactions can be established under flow conditions and continuously withstand shear stress. While mechanisms for flow-resistant adhesion have been identified for platelets as well as for leukocytes and enable them to fulfill their specific functions during hemostasis and inflammation (6, 7), it is yet unknown whether metastasizing tumor cells possess similar mechanisms. It has long been thought that platelets may assist hematogenous dissemination of metastasizing cells (8–10). Perhaps the most convincing evidence is the inhibition of metastasis by experimental thrombocytopenia shown for a variety of tumors (11–14). We therefore hypothesized that tumor cells may interact with platelets and thereby acquire specific mechanisms which mediate platelet anchorage under flow. To test this hypothesis we selected a human melanoma cell model, because melanoma is the most malignant form of skin cancer characterized by a high frequency of metastasis during early stages of the disease. M21 melanoma cells suspended in human blood failed to attach directly to a collagen I matrix under flow, even at a relatively low wall shear rate of 50 s\(^{-1}\) (2 dynes/cm\(^2\)) which corresponds to venous blood flow (5). However, the melanoma cells were able to associate with attached platelets that had become activated and engaged in thrombus formation due to their initial matrix contact. The interaction between melanoma cells and platelets resulted in stable melanoma cell arrest during continued blood flow. The association between mela-

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Dulbecco’s modified Eagle’s medium; PGE1, prostaglandin E1.

The system was developed to distinguish melanoma cells from blood cells or phagia, PA) at 50 nM final concentration. PPACK inhibits thrombin function.

Venipuncture from healthy volunteers into PPACK (H-D-Phe-Pro-Arg- chloromethyl ketone hydrochloride, Bachem Bioscience Inc., Philadelphia, PA) at 50 nM final concentration. PPACK and 5 units/ml ADP scavenger (Sigma) was centrifuged at 2500 g for 30 min at 37°C and 100 mmol/L NaCl, 2.7 mM KCl, 0.4 mM NaH2PO4, 10 mM NaHCO3, and 5 mM dextrose) contain 1 unit/ml aprotinin. The resuspended blood cells were centrifuged again at 2250 × g for 10 min. Washing of the blood cells was repeated two more times using HEPES-Tyrode buffer as above but containing only 0.2 unit/ml aprotinin and finally no aprotinin. The final pellet was reconstituted in HEPES-Tyrode buffer, pH 7.4, containing 50 mg/ml bovine serum albumin and 1 mM CaCl2 (20).

Staining of Cells and Blood for Flow Studies—A simple staining system was developed to distinguish melanoma cells from blood cells or platelets and to identify the cellular components of attached heteroaggregates. Melanoma cells were suspended in plain DMEM at 2 × 106 cells/ml and stained with hydroethidine (P3iSciences, Inc., Washington, PA) at 20 μg/ml final concentration for 30 min at 37°C and then washed twice to remove excess dye. Hydroethidine intercalates into the DNA forming red fluorescent labeled chromosomes of the cells. Pre-stained melanoma cells were suspended in human blood containing 10 μM mepacrine (quinacrine dihydrochloride; Sigma) which concentrates in cell cytoplasmic granules and platelet dense granules. Thus all cells and platelets in the suspension acquired a green fluorescence while the melanoma cells could be identified by their unique red fluorescence. Filter settings at 488/515 nm (excitation/emission) for mepacrine and at 534/590 nm for hydroethidine allowed differential detection of melanoma cells in the presence of whole blood. The hydroethidine signal was detected at lower intensity with the mepacrine filter settings; however, the hydroethidine filter settings almost completely excluded the mepacrine signal. Therefore, melanoma cells were identified within heterogeneous cellular structures. Erythrocytes were not visible in the fluorescent field due to the quenching effect of hemoglobin.

Neither of the dyes, hydroethidine or mepacrine, impaired cell adhesive functions as was tested in stationary assays.

Preparation of Collagen I Matrix and Flow Chamber Assembly—Suspensions of fibrillar collagen I (bovine Achilles tendon; Sigma) were prepared as described earlier (21). In brief, 2 mg/ml collagen I from bovine Achilles tendon (Sigma) was suspended in 0.5 M acetic acid, pH 2.8, homogenized at room temperature for 3 h, centrifuged at 1,000 rpm for 10 min, and stored at 4°C. For matrix preparation, glass coverslips (1.24 × 50 mm, Corning Inc., Corning, NY) were coated with 200 μl of collagen suspension in a humid atmosphere for 1 h at room temperature. The coverslips were rinsed three times with DMEM prior to assembly of the flow chamber. The flow chamber consisted of the matrix-coated glass coverslip as the bottom which was placed onto a metal frame. A rectangular silicon gasket (250 μm thick) was placed onto the coverslip before mounting a 37 × 65 × 12-mm acrylic block as the top of the flow chamber. This block was secured to the frame by four screws and contained two 27-mm long and 4-mm wide drainings used as the chamber inlet and outlet, respectively. The inner dimensions of the flow chamber were 14 × 28 × 0.2-mm (22). The chamber was filled with DMEM and connected to a pump system via medium-filled silicon tubing before placing it onto the heated and enclosed stage of the microscope. All flow experiments were carried out at 37°C. Based on the design of the flow chamber, a given flow rate generated a corresponding wall shear rate which was constant throughout the chamber. The relationship between the wall shear rate (μνd/dz) (velocity gradient evaluated at the wall) and shear stress is (μνd/dz) = μv, where μ is the velocity of the fluid, v is the distance to the surface, r is the fluid shear stress, and μ is the fluid viscosity. The shear rate (μνd/dz) has the units of m/s2 (15). This unit was used throughout the present studies to address the flow conditions used in the experiments.

Fluorescence and Confocal Laser Microscopy—The microscopy system was specifically designed to visualize, record, and quantify the dynamics of cell-cell and cell matrix interactions during flow experiments. It consisted of an inverted microscope equipped with fluorescence and confocal laser modes (Axiovert 135, LSM 410, Zeiss, Oberkochem, Germany) and was controlled by a computer using LSM software (Zeiss). For real time analysis, cell adhesive events were monitored and recorded by a video camera using the fluorescence mode. To examine the three-dimensional topography of adherent cellular structures, optical sections of the specimens along the z axis were acquired using the confocal laser mode.

The Acquisition and Quantitation of Cell Adhesion—Images were captured either directly during a flow experiment or from video tapes that had been recorded during the experiment. To classify attached objects, to determine their location along the x and y coordinates, to count objects, and to measure surface area coverage by attached objects, the images were processed using MetaMorph® image processing software (Universal Imaging Corp., West Chester, PA). To quantitate melanoma cell arrest, the hydroethidine-stained cells (1 × 105 in 1 ml of DMEM) were suspended in 3 ml of mepacrine-containing blood and perfused over the collagen I matrix at a wall shear rate of 50 ± 15 s−1. Without interrupting the flow or changing the flow rate, the chamber was then perfused with 3% paraformaldehyde in phosphate-buffered saline and reacted with appropriate fluorescent labeled antibodies which were captured as follows. We designed a computer program which directed the mobile stage of the microscope to 50 predefined x and y positions starting from a reference point. The program further controlled the acquisition of images. At each position, two images were captured using filter settings to detect red fluorescence (melanoma cells), green fluorescence (platelets and leukocytes), and blue fluorescence (erythrocytes). From these images, attached melanoma cells were enumerated based on their unique red fluorescence, and thrombi were identified and counted based on their size and green fluorescence. To differentiate thrombi-associated from directly matrix-attached melanoma cells, the coordinates of the melanoma cells were determined and compared to those of thrombi. During or at the end of experimental runs, numerous attached objects which were identified as melanoma cells containing thrombi using the fluorescence mode were routinely subjected to confocal z-sectioning in order to verify their cellular composition (platelets

The abbreviations used are: mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle’s medium; PGE1, prostaglandin E1.
FIG. 1. Time course of platelet adhesion and M21 melanoma cell arrest on collagen I during blood flow. M21 cells (2.5 x 10⁶/ml final) were suspended in whole human blood containing PPACK as anticoagulant. This suspension was perfused over a glass coverslip coated with collagen I at a constant wall shear rate of 50 s⁻¹. Collagen I was chosen as matrix to ensure platelet adhesion and activation at this low shear rate. The melanoma cells were prelabeled with hydroethidine (red) and washed before their addition to blood which contained mepricrin (green). All cells and platelets in the suspension acquired a green fluorescence (filter setting 488/515 nm) (upper panels), while the melanoma cells could be identified by their unique red fluorescence (filter setting 543/590 nm) (lower panels). At each time point, images were captured with both filter settings at identical x and y coordinates. The bar in the lower right panel represents 10 μm. Locations of tumor cells within thrombi are indicated by (+) in the upper right panel.

and blood cells detected at 488 nm, melanoma cells at 543 nm). The area within which the 50 measuring fields were defined was 4 x 6.3 mm. This area was located 1 mm away from the inlet and 3 mm away from the lateral margins of the flow channel. This location was kept constant and was arbitrarily chosen, acknowledging that platelets and leukocytes attached to, and melanoma cells began to associate with, adherent thrombi already within the immediate vicinity of the inlet and thus became depleted from the suspension which reached the measuring area.

Image Processing and Classification of Attached Objects—To describe the relationship of thrombus size and melanoma cell association with thrombi, attached objects were classified as single platelets, microthrombi, small thrombi, or large thrombi. For this purpose the images were processed as follows. The background intensity of each image was determined and subtracted in order to standardize images which were acquired during different experimental runs. For binarization, the threshold was set to a level where the binarized image resembled the original image best. This is an arbitrary process which requires that the chosen threshold level is kept constant throughout measurement of all images in order to allow a valid comparison of the measured areas. In each of 10 randomly chosen images, the surface area coverage of 30 single platelets was measured, and average surface areas were determined. Based on these measurements, the objects were classified as single platelets (1–70 pixels), microthrombi (71–250 pixels), small thrombi (251–1000 pixels), or large thrombi (>1000 pixels) wherein the distinction between thrombus classes is arbitrary. The total area of one optical field corresponded to 215,384 pixels.

RESULTS

To test our hypothesis that platelets may assist melanoma cell arrest during blood flow, we initially tested whether the melanoma cells can stimulate and induce platelet aggregation by using the classical stir aggregometer approach. This is important because the activation state of platelets may be critical for platelet-melanoma cell interaction (10). M21 cells (or their variants, as below) were added to human platelet-rich plasma from citrate-treated blood at final concentrations of 5 x 10⁵, 1 x 10⁶, or 5 x 10⁶ cells/ml and in the absence of other stimulants. The melanoma cells failed to induce platelet aggregation under these conditions, whereas 2 μM ADP, as a control, did (data not shown). For this reason, and because we wanted to examine the role of platelets on melanoma cell arrest during blood flow at a low shear rate, we chose collagen type I as an experimental matrix. At a low wall shear rate of 50 s⁻¹, corresponding to a venous shear rate, collagen I promotes platelet adhesion mediated by integrin α2β1 (23) and this results in platelet stimulation (24) and thrombus formation (25).

Time Course of Platelet Adhesion and Melanoma Cell Arrest—At a wall shear rate of 50 s⁻¹, platelets readily attach to the collagen I matrix, initially as single platelets. Within the measuring area, microthrombi consisting of 2–10 platelets formed within the first minute of perfusion. Within the second minute, additional platelets attached to adherent microthrombi resulting in the formation of small thrombi which continued to grow larger in size with continued perfusion. Thrombus formation indicated that the attached platelets had become activated and this promoted platelet cohesion. M21 melanoma cells failed to interact with the matrix during the first 2 min of perfusion. After this initial period, immobilized M21 cells were detected, and this coincided with the appearance of larger size thrombi (Fig. 1). Analyzing the positions of arrested melanoma cells revealed that 93% or more of the attached M21 cells were associated with platelet-containing thrombi. Within the measured surface area, 36–49% of the counted large thrombi contained M21 melanoma cells. M21 cells which did not establish contact to a thrombus largely failed to anchor to the matrix. Analysis of the cellular composition and three-dimensional topography of melanoma cell-containing thrombi by confocal z sectioning under continued flow showed platelets in direct contact with the melanoma cells and revealed the presence of leukocytes in most of the analyzed thrombi. A representative example of a melanoma cell-containing thrombus is shown in Fig. 2. In the majority of cases, the melanoma cells were not in contact with the matrix but attached to underlying platelets, sometimes in the vicinity of leukocytes. The rare individually attached M21 cells which were not associated with thrombi adhered directly to the matrix. These data demonstrate that melanoma cells suspended in blood can interact with platelets and thereby associate with and become incorporated into thrombi under flow conditions. This interaction with platelets greatly promoted melanoma cell arrest on the matrix during blood flow. This result was con-
To investigate the relationship between thrombus size and melanoma cell arrest under flow, we examined the effect of increasing concentrations of anti-\(\alpha_{IIb}\beta_3\) on thrombus size and melanoma cell arrest (Fig. 4). At low anti-\(\alpha_{IIb}\beta_3\) concentrations (20 or 50 \(\mu\)g/ml), the numbers of adherent single platelets or microthrombi were strongly increased, whereas the number of small thrombi was slightly decreased compared to untreated or mAb AV-10-treated controls. Formation of large thrombi was strongly inhibited even at the lowest anti-\(\alpha_{IIb}\beta_3\) concentration tested. At a higher concentration (100 \(\mu\)g/ml), anti-\(\alpha_{IIb}\beta_3\) inhibited any size of platelet thrombi. Like formation of large thrombi, platelet-dependent melanoma cell arrest was strongly inhibited at all antibody concentrations tested (Fig. 4). These results indicate that thrombus size affects melanoma cell arrest under flow. This is supported by the observations that, during the time course of flow experiments, M21 cell attachment coincided with the appearance of larger size thrombi and that, at the end of experimental runs, arrested M21 cells were found associated mostly with large thrombi.

Requirement of \(\beta_3\) Integrin Expression for Melanoma Cell-Platelet Interaction—Large thrombi that attached to the matrix may locally change the pattern of blood flow and thereby alter shear conditions in their vicinity. We therefore examined whether the observed association between melanoma cells and thrombi was specific or due to passive entrapment at sites of potentially altered flow fields. Specific association between the melanoma cells and platelets likely depends on a receptor-mediated adhesive interaction. We hypothesized that \(\beta_3\) integrins might be involved, using a mechanism similar to that which governs platelet-platelet interaction in thrombus formation. M21 cells express integrin \(\alpha\psi\beta_3\) but not \(\alpha_{IIb}\beta_3\) (Fig. 5). To examine whether \(\alpha\psi\beta_3\) participates in M21 cell association with platelet thrombi during blood flow, we compared M21 cells to M21-L cells which lack \(\alpha\psi\psi\psi\) expression (16). To attribute differences in the adhesive capacities of M21 and M21-L cells to \(\alpha\psi\beta_3\) function, M21-L cells were transfected with \(\alpha\psi\) to reconstitute \(\alpha\psi\beta_3\) expression (M21-L4 cells) (19). M21 wild type and its variants M21-L and M21-L4 expressed comparable levels of collagen receptor, integrin \(\alpha\psi\beta_1\) (Fig. 5). Under static conditions, the cells attached readily and equally well to collagen I (19). In contrast, platelet-dependent melanoma cell arrest to collagen I during blood flow was reduced by 93% when using M21-L cells (\(\alpha\psi\psi\beta_3\)) compared to M21 wild type cells (\(\alpha\psi\psi\beta_3\)). Association with platelet thrombi and arrest under flow was fully restored in M21-L4 cells (\(\alpha\psi\psi\psi\beta_3\)) as shown in Fig. 6. In contrast to platelet-dependent melanoma cell arrest, formation of large thrombi and occasional direct attachment of melanoma cells to the matrix under flow occurred independently of the M21 cell type used (Fig. 6). This indicates that the interaction of M21 cells with platelet containing thrombi during blood flow is specific and depends on integrin \(\alpha\psi\beta_3\) expression by the melanoma cells. The specificity of M21...
Cell association with thrombi was supported by confocal z-sectioning of a large number of melanoma cell-containing thrombi. This analysis revealed that the sites of melanoma cell association at the thrombi were random and included the center, the top, and the sides of the thrombi regardless of their exposure to the direction of blood flow. Therefore, melanoma cell attachment to thrombi appeared to be independent of changes in the flow fields in the vicinity of thrombi.

During platelet aggregation and thrombus formation, interaction of platelets is mediated by integrin αIIbβ3. To investigate whether expression of this integrin by melanoma cells supports their association with platelets, we transfected M21-L cells with αIIb, resulting in αIIbβ3-expressing M21-LI1b cells (Fig. 5). M21-LI1b cells associated with platelet thrombi during blood flow, and this mediated their arrest to the matrix to an extent comparable to that found for the αvβ3-expressing M21 or M21-L4 cells (Fig. 6). Platelet-dependent arrest of both M21-L4 and M21-LI1b cells was blocked by platelet inhibition through treatment with PGE1 or anti-αIIbβ3 mAb LJ-CP8, corresponding to the data shown for M21 wild type cells in Fig. 3. This indicates that β3 integrin expression by melanoma cells supports their interaction with platelets under flow and may suggest that the mechanism involved is related to that of platelet-platelet interaction.

Requirement of Plasma Proteins for Platelet-mediated Melanoma Cell Arrest—To analyze whether plasma proteins are required for platelet-mediated melanoma cell arrest during blood flow, we tested the ability of M21 cells to attach to...
matrix-bound platelets in washed blood preparations. When M21 cells were suspended in human blood and perfused over collagen I as in Fig. 3 in the absence or presence of increasing concentrations of mAbs LJ-CP8 (function blocking anti-\(\alpha_{\text{IIb}}\beta_{3}\)) \((\square)\) or AV-10 (not blocking anti-\(\beta_{3}\)) \((\triangle)\). For each experimental condition, images were captured with filter settings for green (all cells and platelets) or red fluorescence (melanoma cells) at 50 predefined positions. For quantitation of attached objects the images were binarized, and object classifiers were defined based on surface area coverage of individual platelets, micro thrombi containing two to five platelets, small thrombi, or large thrombi (1–70, 71–250, 251–1000, and >1000 pixels, respectively, the total measured area corresponded to 215,384 pixels) using MetaMorph® image processing software. Identification and quantitation of thrombus-associated melanoma cells were done as in Fig. 3. Each data point represents mean numbers ± S.D. of attached objects of each class in 50 images expressed as a percent of control. Controls were done in the absence of antibody at the same day at the beginning and at the end of the experiment using blood from the same donor. Note differences in the scaling of the y axes.

**DISCUSSION**

This study was designed to analyze the ability of human melanoma cells to adhere to an immobilized matrix during blood flow and to determine the role of platelets in this process. This is pertinent to tumor metastasis because tumor cell arrest within the vasculature is required for extravasation and thus limits the metastatic capacity of tumor cells that disseminate via the blood stream. In this report we provide evidence that efficient melanoma cell arrest to a collagen I matrix during blood flow depends on the interaction of the melanoma cells with platelets which have already established matrix contact. Despite their ability to adhere to collagen I under static conditions in an integrin \(\alpha_{2}\beta_{1}\)-dependent manner (19), M21 human melanoma cells largely failed to attach directly to this matrix during blood flow. They were, however, able to associate with platelets, and this interaction resulted in M21 cell arrest. The flow-resistant interaction of M21 cells and platelets depended on platelet activation and thrombus formation. Moreover, the interaction was found to be specific and required \(\beta_{3}\) integrin function on both the melanoma cells and the platelets.

The ability of platelets to support tumor cell arrest during
...blood flow may contribute to the role of platelets in hematogenous metastasis. A number of reports indicate that interference with platelet function in vivo reduced tumor metastasis in animal models. Moreover, reduction of platelet counts inhibited metastasis of a variety of tumor cells including melanomas, carcinomas, and sarcomas (11–14). In these cases, metastasis was reduced regardless of the ability of the tumor cells to induce platelet aggregation in vitro (26–28). Tumor cells can induce platelet aggregation by releasing ADP (29–33), generating tissue factor (34), or by other mechanisms leading to thrombin formation (35–37). It is conceivable that two distinct mechanisms are involved in platelet-assisted tumor cell arrest in the vasculature: first, an induction of platelet activation and aggregation by tumor cells or their released factors which is possibly accompanied by passive entrapment of tumor cells in platelet aggregates; and second, a specific adhesive interaction between platelets and tumor cells. Both types of interaction may allow tumor cells to utilize indirectly the platelet-specific mechanisms for successful adhesion during blood flow. Since M21 melanoma cells failed to induce platelet activation in vitro, we sought to define conditions for their local exposure to stimulated platelets. Therefore, we chose collagen I as the matrix because it represents a thrombogenic surface and promotes platelet adhesion and thrombus formation at low shear rates, corresponding to venous blood flow (23–25). Although collagen I is present in the vessel wall, it becomes exposed to blood only in deep vessel injury or upon rupture of atherosclerotic plugs (38, 39). Metastasizing cells are unlikely to encounter this matrix in the vasculature. Therefore, collagen I was used in this study solely as a model for a thrombogenic surface. The collagen I matrix was perfused with M21 melanoma cells suspended in whole human blood. It has to be considered that plasma contains collagen-binding proteins, such as fibronectin, von Willebrand factor, or vitronectin (40), which potentially modified the matrix during the experiments. However, it has been reported that a function-blocking antibody to the α2 subunit of the platelet collagen receptor α2β1 fully inhibited platelet adhesion to collagen I during perfusion with whole blood at wall shear rates up to 1600 s⁻¹ (41). Other investigators showed that at wall shear rates of 1500 s⁻¹ or higher, von Willebrand factor immobilized to the collagen I matrix during blood flow becomes the relevant adhesive ligand for platelets, since a recombinant fragment of von Willebrand factor, von Willebrand factor 445–733 containing the binding site for a2 integrin expression for melanoma cell association with platelets and arrest during blood flow. M21 melanoma cells were suspended in either plasma depleted, washed blood (open bars), washed blood containing 2 mg/ml human fibrinogen (light gray bars), or untreated whole blood (gray bars). The suspensions were perfused over a collagen I matrix at a wall shear rate of 50 s⁻¹. Melanoma cell attachment and their association with thrombi was measured as in Fig. 3. Surface area coverage by platelets or thrombi of various sizes was determined as in Fig. 4. Each bar represents the mean numbers of pixels (± S.D., n = 3 runs using the same blood and washed blood preparation) for the surface coverage of attached objects as classified in Fig. 4 or the numbers of arrested tumor cells at 50 predefined positions. Washed blood was prepared as detailed under “Materials and Methods.”
flow over collagen I at 50 s⁻¹ in our experiments were not significantly affected by the presence of mAb L1b-1 which blocks GPIb function (data not shown). Together, this suggests that collagen remained the major matrix component for platelet adhesion at the low shear rate used in our experiments. A possible modification of the collagen matrix did not improve platelet-independent melanoma cell attachment even after prolonged periods of blood flow.

The association of M21 melanoma cells with platelets under flow depended on platelet activation and thrombus formation since PGE₂ or anti-αIIbβ3 abolished M21 cell interaction with platelets. In the presence of these inhibitors, attachment of individual platelets and micro thrombi was strongly enhanced; however, they failed to support M21 cell adhesion. The increase in single platelet and micro thrombus attachment in the presence of platelet inhibitors may be explained by the fact that platelets were not depleted from the streaming blood due to incorporation into large thrombi as observed in the absence of platelet inhibitors. Association of M21 cells with thrombi coincided with the occurrence of large size thrombi. Detailed analyses of mural thrombogenesis revealed that the blood flow pattern in the vicinity of growing thrombi is characterized by standing vortices that develop upstream and downstream of the thrombi. This leads to considerably reduced velocities in and near the recirculating regions which in turn permit the local accumulation of platelet-activating agents that are released or induced by the aggregating platelets within the thrombi (43). It is therefore plausible that tumor cells may be passively trapped close to or between nearby growing thrombi. We have ruled out that the M21 melanoma cell interaction with thrombi during blood flow was due to on-specific entrapment. We found melanoma cells associated with thrombi at random sites, indicating that changes in flow did not affect the interaction. Moreover, we demonstrated that the M21 cell association with thrombi was a specific, receptor-mediated process. Failure of M21 cells to bind to adhered platelets in which the integrin αIIbβ3 function had been blocked suggests that αIIbβ3 participates in the interaction between platelets and melanoma cells. This concept is supported by previous reports (44, 45) and is substantiated by the finding that platelets from Glanzmann’s thrombasthenic patients, which lack αIIbβ3 expression, failed to interact with tumor cells in vitro (46). In order to identify a potential counter receptor for platelet integrin αIIbβ3 expressed by the melanoma cells, we reasoned that αIIbβ3 may bind to a related receptor expressed by the melanoma cell using a mechanism similar to that which governs platelet-platelet interaction in thrombus formation. Evidence suggests that an αIIbβ3-related integrin expressed by tumor cells might be involved in their interaction with platelets (46, 47). This αIIbβ3-related molecule may represent tumor cell integrin αvβ3, since these receptors share characteristic homologies and a ligand recognition repertoire. In order to examine the role of melanoma cell integrin αvβ3, we utilized an M21 cell variant that lacks αvβ3 expression (M21-L) (16) in comparison to a variant in which αvβ3 expression was reconstituted upon transfection (M21-L4) (19). Using this system, we demonstrated that αvβ3 expression was required for M21 cell association with platelets, and that this resulted in efficient M21 cell arrest on a collagen I matrix during blood flow.

In human melanoma, expression of αvβ3 was found to be restricted to the metastatic phenotype (48) and to commence with the onset of vertical growth within the primary lesion (49). Furthermore, we have evidence that αvβ3 contributes to the tumorigenicity of human melanoma cells (19) and to adhesion interactions with fibrinogen and its breakdown products which occur in the tumor stroma (50). Thus, melanoma cell αvβ3 seems to be involved in early stages of tumor development. We now provide evidence suggesting that this adhesion receptor may also be involved in later stages of melanoma progression by contributing to melanoma cell-platelet binding and arrest during blood flow.

It is yet unknown whether platelet binding to the melanoma cells is based on a direct interaction between the two cell types or whether bridging ligands are involved. We hypothesize that platelet integrin αIIbβ3 and melanoma cell integrin αvβ3 interact via divalent or multivalent RGD-containing plasma proteins, such as fibrinogen, von Willebrand factor, fibronectin, or thrombospondin. This concept is supported by a number of reported inhibition studies. Antibodies directed to either platelet αIIbβ3 or to its ligands, as well as RGD-containing peptides and snake venoms, significantly reduced the interaction between tumor cells and platelets in vitro and metastasis in vivo (14, 44, 51–57). The antimetastatic activity of RGD peptides was associated with an accelerated disappearance of isotope-labeled tumor cells from the lungs of treated mice. This indicates that specific adhesion processes were involved in stable tumor cell arrest in the lung capillaries. To address the question whether plasma proteins, envisaged as containing adhesive ligands for the β3 integrins, were required for platelet-mediated melanoma cell arrest during blood flow, we depleted the plasma proteins from blood by repeated washes. As expected, platelet adhesion to the collagen I matrix was not impaired; however, the formation of large size thrombi was drastically reduced, and consequently, platelet-mediated melanoma cell adhesion was also inhibited. Thrombus formation and melanoma cell arrest were partially restored in the presence of added fibrinogen. The lack of a full recovery of thrombus formation and melanoma cell arrest could be due to one or both of the following reasons. First, platelets as well as leukocytes become refractory during the blood-washing procedure, and second, other plasma proteins, such as fibronectin, von Willebrand factor, vitronectin, or thrombospondin, may be involved in addition to fibrinogen. Together, our results indicate that plasma protein(s) are required for platelet-mediated melanoma cell arrest under our experimental conditions. Logically, the mechanism of melanoma cell-platelet heteroaggregate formation needs to be addressed in greater detail, employing experimental procedures which involve minimal disturbance of platelet responsiveness.

Integrin αIIbβ3 mediates platelet cohesion via fibrinogen and/or von Willebrand factor as major bridging ligands in thrombus formation. Therefore, we sought to compare β3 integrins αvβ3 and αIIbβ3 expressed by the same type of melanoma cell for their potential to mediate melanoma cell interaction with platelets. To accomplish this, we transfected M21-L cells with αIIb by a method similar to that described by Kieffer et al. (58), resulting in the M21-L1b variant which expresses αIIb but not αvβ3. Several reports suggest that integrin αIIbβ3 may be expressed by certain tumor cells (59). M21-L1b cells were able to associate stably with platelet-containing thrombi and utilized this mechanism for efficient arrest during blood flow. The extent of M21-L1b cell interaction with platelets was comparable to that found using αvβ3-expressing M21 or M21-L4 cells. This indicates that β3 integrins in general can function as receptors on melanoma cells during their interaction with platelets. This finding may lend further support to the concept that the mechanism involved in melanoma cell-platelet interaction is related to that which governs platelet cohesion in thrombus formation.

We propose that β3 integrin-mediated tumor cell-platelet interaction may represent one possible mechanism to facilitate hematogenous dissemination of tumor cells. It is yet unknown...
whether platelets promote tumor cell arrest on intact endothelium, a process which may involve more than one receptor. These may include other integrins and selectins, the latter of which could contribute to the initial binding events (60–65). Among the integrins, α4β1 represents an example for supporting leukocyte rolling on and attachment to endothelial cells under flow (66, 67). α4β1 is expressed by a variety of tumor cells including certain melanoma cells, and it may be involved in the melanoma cell interaction with the vascular endothelium. αvβ3 and αvβ5 integrins were shown to support tumor cell arrest under flow to fibronectin or vitronectin substrates (68). Stabilization of the initial binding events seemed to depend on the activity of transglutaminase expressed by the tumor cells (69). Under our experimental conditions, one likely candidate to contribute to melanoma cell interaction with thrombi is P-selectin. P-selectin is expressed by activated platelets and was shown to mediate tumor cell-platelet binding under stationary conditions (70). Under flow conditions, P-selectin has been reported to support leukocyte rolling. In our flow experiments, we recorded adhesive events in real time and found no evidence of melanoma cell rolling. A monochototic antibody directed to human P-selectin, known to block P-selectin-mediated adhesion, such as adherence of activated platelets to neutrophils (71) or histamine-induced rolling of leukocytes in postcapillary venules (72), failed to interfere with platelet-mediated melanoma cell arrest under our experimental conditions (data not shown). A more detailed analysis of the initial binding events between activated platelets and melanoma cells during blood flow will reveal a potential contribution of P-selectin. In vivo, association of platelets with tumor cells attached to vascular endothelium has been reported (73) and suggests that platelets may stabilize and protect attached tumor cells during blood flow. In vivo studies will help to clarify whether αβ3 integrin-mediated tumor cell-platelet interaction contributes to the complex process of tumor cell arrest in the microcirculation.

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