Integrin VLA-4 enhances sialyl-Lewis\(x^a\)-negative melanoma adhesion to and extravasation through the endothelium under low flow conditions

Shile Liang and Cheng Dong
Department of Bioengineering, The Pennsylvania State University, University Park, Pennsylvania

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Liang S, Dong C. Integrin VLA-4 enhances sialyl-Lewis\(x^a\)-negative melanoma adhesion to and extravasation through the endothelium under low flow conditions. Am J Physiol Cell Physiol 295: C701–C707, 2008. First published July 16, 2008; doi:10.1152/ajpcell.00245.2008.—During their passage through the circulatory system, tumor cells undergo extensive interactions with various host cells including endothelial cells. The capacity of tumor cells to form metastasis is related to their ability to interact with and extravasate through endothelial cell layers, which involves multiple adhesive interactions between tumor cells and endothelium (EC). Thus, it is essential to identify the adhesive receptors on the endothelial and melanoma surface that mediate those specific adhesive interactions. P-selectin and E-selectin have been reported as adhesion molecules that mediate the cell-to-cell interaction of endothelial cells and melanoma cells. However, not all melanoma cells express ligands for selectins. In this study, we elucidated the molecular constituents involved in the endothelial adhesion and extravasation of sialyl-Lewis\(x^a\)-negative melanoma cell lines under low flow in the absence of polymorphonuclear neutrophils (PMNs). Results show the interactions of \(\alpha_x\beta_\text{i} (\text{VLA-4})\) on sialyl-Lewis\(x^a\)-negative melanoma cells and vascular adhesion molecule (VCAM-1) on inflamed EC supported melanoma adhesion to and subsequent extravasation through the EC in low shear flow. These findings provide clear evidence for a direct role of the VLA-4/VCAM-1 pathway in melanoma cell adhesion to and extravasation through the vascular endothelium in a shear flow. PMNs facilitated melanoma cell extravasation under both low and high shear conditions via the involvement of distinct molecular mechanisms. In the low shear regime, \(\beta_\text{2}\)-integrins were sufficient to enhance melanoma cell extravasation, whereas in the high shear regime, selectin ligands and \(\beta_\text{2}\)-integrins on PMNs were necessary for facilitating the melanoma extravasation process.

...tumor cell extravasation; vascular adhesion molecule-1; leukocytes; inflammation

MALIGNANT MELANOMA has a high propensity for metastatic spread making it the most deadly form of skin cancer. An important step in the metastatic process is the arrest of melanoma cells in the venous or capillary bed of the target organ and subsequent extravasation (3, 5). Therefore, it is important to identify and characterize adhesive receptors on the endothelial and melanoma surfaces that mediate their specific adhesive interactions. P-selectin and E-selectin have been reported as adhesion molecules that mediate the cell-to-cell interaction of endothelial cells (EC) and melanoma cells (35, 36). Members of the selectin family are structurally related, containing a lectin-binding domain that recognizes specific carbohydrates (30). However, not all melanoma cells express ligands for selectins (29, 37), which raised the question as to whether selectin-mediated binding of melanoma cell to the EC is necessary for initiating the adhesion cascade of melanoma migration through the endothelium and whether there are other mechanisms for melanoma cell adhesion to the EC.

Expression of integrin VLA-4 (\(\alpha_x\beta_\text{i}, \text{CD49d/CD29}\)) on the surface of several melanoma cell lines has been reported (22). VLA-4 binds to domains 1 and 4 of vascular adhesion molecule-1 (VCAM-1), an immunoglobulin superfamily member induced by inflammatory mediators on the EC (7, 18). Adhesion mediated by VLA-4 expressed on lymphocytes and VCAM-1 on the inflamed EC is one of the first steps in extravasation of these cells into tissue sites of inflammation (46). Several previous investigations have examined potential binding mechanisms of VLA-4 to purified VCAM-1 molecules under shear conditions in vitro (50, 51). Another in vitro study has also shown that high affinity interaction of VLA-4 and VCAM-1 enhances migration of human melanoma cells across activated endothelial cell layer (26). However, this study was carried out under static conditions without considering any effects of physiological shear flow existing in hematogenous metastasis. In addition, many other in vivo studies have indicated that VLA-4 expression can enhance the ability of intravenously injected tumor cells to extravasate and grow in the lung (12, 39). However, as an end-point assay, in vivo studies aimed at understanding cellular responses under shear conditions have had problematic difficulties because they could not quantitatively define the exact features of the hemodynamic environment. Moreover, it is hard to determine whether a corresponding response is due to shear forces or to other factors associated with the complex in vivo fluid dynamic environment. In other words, a mechanism related to a potential involvement of VLA-4 in melanoma adhesion to and subsequent extravasation through the EC under shear flow conditions is not entirely understood or well characterized.

Previous researchers have also investigated roles of polymorphonuclear neutrophils (PMNs) in facilitating melanoma cell adhesion to and subsequent extravasation through the EC. They reported a possible mechanism through the binding of intercellular adhesion molecule (ICAM-1) on melanoma cells and \(\beta_\text{2}\)-integrins (CD11a/CD18 and CD11b/CD18) on PMNs (33, 44). However, those studies used a transfected L-fibroblast that constitutively expresses E-selectin and ICAM-1 as the adhesion substrate to model the EC. A potential role of VCAM-1 on the EC that might bind to VLA-4 on melanoma cells was not considered.

In the present study, the interactions of melanoma cells and the EC in a shear flow were investigated. Results have indi-
cated that in low shear flow, human melanoma cells expressing VLA-4 adhered to and extravasated through the EC. In contrast, adhesion and transendothelial migration of human melanoma cells with low expression of VLA-4 were significantly lower. VLA-4/VCAM-1 binding itself was not strong enough to support melanoma adhesion and extravasation under high shear flow conditions. The presence of PMNs was able to enhance melanoma-EC interactions that resulted in significant increases in melanoma extravasation under flow conditions, which is mediated by the expressions of β3-integrins and selectin ligands on the surface of PMNs. This study provides important insights into how melanoma cells adhere to the EC in the presence of shear forces in the microcirculation. Considering that VCAM-1 expression on the surface of HPMEC, they were treated with soluble chemoattractant type IV collagen (CIV; 100 μg/ml; 3 h) (Sigma). The center 12 wells of the bottom plate were filled with soluble chemoattractant type IV collagen (CIV; 100 μg/ml in RPMI 1640/0.1% BSA) (BD Biosciences). Surrounding control wells were filled with medium (RPMI 1640/0.1% BSA). We have shown that melanoma cells express αβ3-integrin receptors for soluble collagen IV protein and migrate toward collagen IV stimulation, which agrees with previous finding (8). After the bottom of the filter was scraped by using a cell scraper, the apparatus was assembled by placing the filter on the bottom plate followed by addition of a sealing gasket and the top plate. The chamber was primed with 37°C medium to eliminate bubbles in the system. Control experiments using HPMEC monolayer only or PMNs alone perfused over HPMEC monolayer were done to examine whether HPMEC alone or PMNs migrated toward the chemoattractant under the experimental conditions. For melanoma cell migration assay, 5 × 10^5 melanoma cells alone or 5 × 10^5 melanoma cells and 5 × 10^6 PMNs together were perfused into the chamber under various shear flow conditions for 4 h in a 37°C, 5% CO₂ incubator. To quantify migration, migrated cells were stained with Hema3 solution (Fisher Scientific, Pittsburgh, PA) and counted using an inverted microscope (Diaphot 330, Nikon, Japan) with NIH Image software (version 1.60). Around 99% PMNs were still alive after the 4-h flow assay tested by trypan blue.

**RESULTS**

**Expression of adhesion molecules.** Surface expression of specific adhesion molecules on cells was characterized using various antibodies. Both WM9 and C8161 expressed ICAM-1 and VLA-4 (Table 1). VLA-4 expression on WM9 was significantly higher than that on C8161, of which the expression was close to the background level. Furthermore, neither WM9 nor
C8161 expressed sialyl-Le^a^ and sialyl-Le^b^, the ligands for selectins. Unstimulated HPMEC constitutively expressed ICAM-1. VCAM-1 and E-selectin expression on HPMEC were slightly above the background level (Table 1). Upregulation of E-selectin and VCAM-1 were detected after TNF-α (300 U/ml) stimulation for 4 h (E-selectin) or 24 h (VCAM-1) on HPMEC, respectively. There was no P-selectin expression with or without TNF-α stimulation on HPMEC.

**VLA-4/VCAM-1 interactions support melanoma adhesion to the EC under low shear conditions.** To undergo transendothelial migration, tumor cells first have to stably adhere to the EC. Because VLA-4 is known to be able to bind purified VCAM-1 molecules, we examined whether VLA-4-expressing melanoma cells would interact with VCAM-1-expressing HPMEC cells under shear flow conditions. Tethering, rolling, and adhesion are three distinct steps of circulatory cells interacting with the EC in a shear flow. Tethering is defined as the transient interaction between a moving cell and the EC in a hydrodynamic flow, which can be measured as the transient stop of the motion of a cell. Rolling is defined as the transient interaction between a cell and the EC substrate in a fluid flow, where the velocity of the cell is significantly lower than the velocity of a noninteracting cell near the surface. Adhesion is the firm attachment of cells to the EC. WM9 cells tethered on nonstimulated HPMEC under low shear stresses at 0.5 and 1 dyn/cm^2^ for 5 min before the detachment assays were initiated by a twofold step increase in the shear stress at every 20-s interval. The number of cells that remained bound was determined under each shear stress. VCAM-1 expression level on the HPMEC affected cell resistance to detachment. Treatment with TNF-α for 24 h upregulated the VCAM-1 expression on the HPMEC; at the same time, it also dramatically increased shear resistance of the adherent melanoma cells and thus increased the strength of adhesion of melanoma cells under flow conditions (Fig. 2). WM9 cell withstood higher shear resistance than C8161 for which VLA-4 expression was significantly lower, suggesting that the expression level of VLA-4 on melanoma cells also contributed to their arrest on the HPMEC (Fig. 2).

**VLA-4/VCAM-1 interactions support melanoma extravasation through the EC under low shear conditions.** To investigate whether increases in the number and strength of arresting melanoma cells on the EC through VLA-4 and VCAM-1 would increase the extravasation under flow conditions, flow migration assays were carried out. Control experiments were allowed to accumulate on the HPMEC under 0.5 dyn/cm^2^ for 5 min before the detachment assays were initiated by a twofold step increase in the shear stress at every 20-s interval. The number of cells that remained bound was determined under each shear stress. VCAM-1 expression level on the HPMEC affected cell resistance to detachment. Treatment with TNF-α for 24 h upregulated the VCAM-1 expression on the HPMEC; at the same time, it also dramatically increased shear resistance of the adherent melanoma cells and thus increased the strength of adhesion of melanoma cells under flow conditions (Fig. 2). WM9 cell withstood higher shear resistance than C8161 for which VLA-4 expression was significantly lower, suggesting that the expression level of VLA-4 on melanoma cells also contributed to their arrest on the HPMEC (Fig. 2).

<table>
<thead>
<tr>
<th>Expression of adhesion molecules</th>
<th>WM9</th>
<th>C8161</th>
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<tbody>
<tr>
<td>Control IgG (Control IgM)</td>
<td>5.9±0.3 (10.2±1.0)</td>
<td>6.2±0.4 (11.2±0.8)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>102.2±3.1*</td>
<td>64.2±1.3*</td>
</tr>
<tr>
<td>VLA-4</td>
<td>34.8±2.1*</td>
<td>8.8±0.4*</td>
</tr>
<tr>
<td>sialyl-Le^a^</td>
<td>(10.5±1.3)</td>
<td>(12.1±1.2)</td>
</tr>
<tr>
<td>sialyl-Le^b^</td>
<td>(11.0±1.3)</td>
<td>(11.3±0.6)</td>
</tr>
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Values are mean fluorescence intensity from flow cytometry. Tumor necrosis factor-α (TNF-α) stimulation time was 4 h for E-selectin or 24 h for all other adhesion molecules. ICAM-1, intercellular adhesion molecule-1; sialyl Le^a^, sialyl Lewis^a^; VLA-4, α4β1-integrin; VCAM, vascular cell adhesion molecule. *P < 0.05 compared with respective nonstimulated cases.
showed there were no migrations of HPMEC or PMNs toward the chemoattractant (data not shown). Under low shear stress at 0.5 dyn/cm², 24 h TNF-α treatment on HPMEC significantly increased both WM9 and C8161 extravasation when compared with the nonstimulated control cases (Fig. 3). But because of its low VLA-4 expression level, C8161 extravasation through the TNF-α-treated HPMEC did not even reach the level similar to WM9 through nontreated HPMEC. Functional blocking of VLA-4 on WM9 using anti-α₄ mAb reduced melanoma extravasation significantly, confirming that VLA-4/VCAM-1 interactions played an important role in supporting WM9 extravasation (Fig. 3). However, under a higher shear stress at 2 dyn/cm², melanoma cell extravasation was almost abolished for both TNF-α-stimulated and nonstimulated cases (data not shown). These data correlated with the data of melanoma cell adhesion to the HPMEC, where the adhesion of melanoma cell to the HPMEC was near baseline level when shear stress reached 2 dyn/cm².

VLA-4/VCAM-1 interactions enhance melanoma extravasation through the EC in the presence of PMNs. To investigate whether binding of VLA-4 on melanoma cells and VCAM-1 on the EC would further affect melanoma extravasation in the presence of PMNs, WM9 cells were perfused together with PMNs into the flow migration chamber and their extravasation through 24 h TNF-α-stimulated HPMEC was measured under different shear conditions. In the presence of PMNs, WM9 extravasation increased significantly compared with that in the absence of PMNs (Fig. 4). To further examine roles of PMNs in melanoma extravasation, PMNs were treated with either a function-blocking antibody against β₂-integrins or neuraminidase, an enzyme that cleaves terminal sialic acid residues from cell surfaces. Under low shear stress at 0.6 dyn/cm², blocking β₂-integrins reduced WM9 extravasation significantly (Fig. 4A); however, treating PMNs with neuraminidase did not impair WM9 extravasation in the low shear regime (Fig. 4A). In contrast, at the shear stress level of 2 dyn/cm², both β₂-integrin blockade and neuraminidase treatment of PMNs effectively abolished the PMN-facilitated WM9 extravasation (Fig. 4B). In addition, under both shear stresses at 0.6 and 2 dyn/cm², blocking β₂-integrins resulted in a similar level of WM9 extravasation with or without existence of PMNs.

Antibody interference assays were next used to delineate the role of VLA-4 on WM9 cells in their extravasation in the presence of PMNs (Fig. 4). In the low shear regime, VLA-4 blockade significantly reduced WM9 extravasation in the presence of PMNs (Fig. 4A). In the case where PMNs were treated with antibody against β₂-integrins, blocking VLA-4 further brought down the extent of WM9 extravasation, which was not observed in the case where PMNs were treated with neuraminidase (Fig. 4A). In contrast, under high shear stress, blocking VLA-4 did not interfere with PMN-facilitated WM9 extravasation (Fig. 4B), which correlates with the low levels of melanoma extravasation in the absence of PMNs. Along these lines, blocking VLA-4 did not further inhibit WM9 extravasation in cases where PMNs were treated with either an antibody against β₂-integrins or neuraminidase.

DISCUSSION

The interaction of blood-borne tumor cells with vascular EC is a key component of the hematogenous spread. In this study, we have demonstrated that under low shear stress, VLA-4 on melanoma cells alone interacted with VCAM-1 on the EC to tether melanoma cells in a hydrodynamic flow and mediated melanoma cell adhesion to the EC. Two melanoma cell lines that express different levels of VLA-4 on the surface were chosen to investigate the roles of VLA-4 in facilitating melanoma adhesion. Studies have shown VLA-4 expression in melanoma correlates with tumor metastasis (1, 13). Although there were no studies comparing the in vivo metastatic properties of WM9 and C8161 side by side, individual studies have shown either WM9 (19) or C8161 (15) can form metastasis in vivo. A previously published paper (10) has compared the WM9 and C8161 in vitro in terms of invasiveness, chemotactic
shear stress 2 dyn/cm², there was no extravasation of WM9 cells by themselves. PMNs both reduced WM9 extravasation significantly. Blocking of VLA-4 on melanoma cells was functionally blocked using antibodies against its α4-subunit, and the extravasation of WM9 cells was measured afterwards in the presence of PMNs. A: under low shear stress 0.6 dyn/cm², WM9 cell alone could resist shear flow and migrate through HPMEC monolayer. The presence of PMNs increased WM9 extravasation significantly. Blocking β2-integrins reduced WM9 extravasation significantly; however, the treatment of neuraminidase on PMNs did not significantly affect WM9 extravasation. VLA-4 blocking reduced WM9 extravasation significantly even in the presence of PMNs. In the case where PMNs were treated with antibody against β2-integrins, blocking VLA-4 further brought down WM9 extravasation, which did not happen in the case where PMNs were treated with neuraminidase. Values were means ± SE for N ≥ 3. B: under high shear stress 2 dyn/cm², there was no extravasation of WM9 cells by themselves through the EC. In the presence of PMN, WM9 cell migration dramatically increased. Blocking of VLA-4 on WM9 did not affect WM9 cell extravasation mediated by PMNs. β2-Integrins blocking and neuraminidase treatment on PMNs both reduced WM9 extravasation significantly.

Previous studies have shown the binding of VLA-4 and VCAM-1 can mediate T lymphocytes (4, 23) and monocytes (28) rolling and subsequent firm adhesion to the EC in a shear flow. Similar to these findings, our results indicated that interactions between VLA-4 and VCAM-1 enhanced melanoma cell tethering and adhesion to the EC. However, our results showed that most melanoma cells adhered to the EC immediately without entering a rolling phase. Giavazzi et al. (14) similarly found that VLA-4/VCAM-1 interactions mediated direct adhesion of melanoma cells to the EC without a rolling step. The difference could be due to the distinction between conformational states of the VLA-4 molecules expressed in different cell types. The conformational states may be responsible for multiple roles of VLA-4 in cell rolling and firm adhesion, respectively (6, 51). Other tumor cell types seem to use different mechanisms to adhere to the EC in the presence of a shear flow, which could be attributed to the expressions of adhesion molecules on the surface of tumor cells. For example, studies done on colon carcinoma adhesion indicated that rolling is a necessary step for the firm adhesion of such tumor cells to the EC (41, 47). Previous studies have shown colon carcinoma cells express carbohydrate antigens such as sialyl-Le⁰ and sialyl-Leᵃ that can bind to the selectins on the EC (20, 21). More recent studies showed colon carcinoma cells also express CD44 variant isoforms (CD44v), which may mediate their adhesion to the EC via binding to selectins expressed on the EC (38). The melanoma cells used in our study did not express sialyl-Le⁰ or sialyl-Leᵃ. In addition, they only express CD44 standard isoforms (CD44s) from our recent experiment (data not shown), which has been shown to bind E- and P-selectins with low affinity (16).

Some recent studies have indicated that under dynamic flow conditions there is virtually no melanoma cell adhesion to or extravasation through the endothelial substrate in the absence of PMNs (33, 43). In the presence of PMNs, the interactions of β2-integrins on PMNs and ICAM-1 on melanoma cells can enhance melanoma cell adhesion and subsequent extravasation. However, those studies used transfected L-fibroblast cells that constitutively express only E-selectin and ICAM-1 as the adhesion substrate to model the EC. A potential role of VCAM-1 on the EC that might bind to VLA-4 on melanoma cells in this process was not considered. As endothelial cells express high levels of VCAM-1 on their surface primarily in response to inflammatory stimuli, it is speculated that VLA-4 expressing melanoma cells may preferentially extravasate through the EC at sites of inflammation, as has been known for leukocytes (2, 49). In this study, a human pulmonary microvascular endothelial cell line, which expresses VCAM-1 after stimulation (27), was used to form an EC monolayer. A report (17) has confirmed the ability of tumor cell transendothelial migration after adhesion to the EC by showing the time lapse images of this process. Our results indicated that the adhesion of melanoma cells on the EC through VLA-4/VCAM-1 resulted in tumor cell extravasation under low shear stress. When the shear stress reached 2 dyn/cm², the adhesion of melanoma cells to the EC was abolished, and no extravasation through the EC was detected. Shear stress dictates the forces applied to cells individually (i.e., increase the extent of cell deformation) or on the intermolecular bonds between cells. A previous study (9) has shown that drag force that disrupts individual adhesive bonds between cells reduces when vessel size increases under a constant shear stress. Therefore, when melanoma cells travel with the bloodstream through capillaries, the shear stress would have different effects on the adhesion interaction of melanoma cell with EC.

The presence of PMNs significantly increased the extravasation of melanoma cells. Earlier studies (31, 33) from our
group shows melanoma cells and PMNs form aggregation via ICAM-1/β2-integrins binding under flow conditions. The presence of PMNs did not further increase the transendothelial migration of melanoma cells under static conditions (44). Therefore, the adhesion of PMNs may not be able to induce a change to the endothelial cells that allows easier passage of melanoma cells. Another study (40) indicates that the coculture between melanoma cells and PMNs increases interleukin 8 (IL-8) productions within the tumor microenvironment. IL-8 has been reported to stimulate β2-integrins expression on PMNs (42), which may enhance the adhesion of melanoma cells to the EC via ICAM-1/β2-integrins binding under flow conditions. Similar to a previous study (33), our results confirmed that β2-integrins on PMNs are critical for PMN-facilitated melanoma extravasation under both low and high shear conditions. Previous studies have shown the binding between selectins and their ligands is essential to initiate the rolling of leukocytes on the EC under flow conditions (6, 45). The binding of PMN to selectin activates integrins on PMNs (34) and thus mediates the firm adhesion of PMNs to the EC via adhesion of β2-integrins on PMNs and ICAM-1 on EC (45). After cleaving selectin ligands on PMNs, β2-integrins on PMNs may not be stimulated by interactions of PMN to selectins on the EC, interrupting the firm adhesion of PMNs on the EC. Therefore, the extravasation of melanoma cells through the EC should be reduced since adhesion of PMNs to the EC was found to be necessary for PMN-facilitated melanoma extravasation (33). Very interestingly, our results show the roles of selectin ligands in enhancing melanoma extravasation only become effective under high shear stresses. Although cleaving selectin ligands on PMNs interrupted β2-integrins expression on PMNs, chemokines within the tumor microenvironment such as IL-8 can upregulate β2-integrins expression on PMNs (32, 40), which would mediate the firm adhesion of PMNs to the EC under low shear conditions (25). In contrast, under high shear stress, β2-integrins on PMNs alone were not able to mediate PMNs adhesion; therefore, PMNs adhesion was significantly compromised after cleaving selectin ligands, causing reduced melanoma extravasation. In support of this explanation, previous studies have shown leukocyte rolling and attachment via selectins occurs at around tenfold higher wall shear stresses than adhesions mediated by β2-integrins (48).

In conclusion, this study showed VLA-4 expression on melanoma cells leads to an increase in melanoma cell adhesion to and extravasation across inflamed EC that expressed upregulated VCAM-1, especially under low shear flow conditions. In addition, PMNs could enhance melanoma extravasation under flow conditions, which is mediated by the expressions of β2-integrins and selectin ligands on the surface of PMNs. Considering roles of inflammatory cytokines-chemokines in regulating cell adhesion molecule expressions, the findings from the present study are likely to be important for in vivo investigations, especially the interruption of VLA-4 (on melanoma cells) may be a potential therapeutic intervention to prevent melanoma from metastasis.

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GRANTS

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