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UVB-Irradiation Regulates VLA-4-Mediated Melanoma Cell Adhesion To Endothelial VCAM-1 Under Flow Conditions

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Abstract

The major aspect contributing to the mortality of melanoma is its ability to spread, or metastasize. Ultraviolet B light (UVB) is considered an indirect cause of melanoma formation. However, little is known about the potential effects of UVB to melanoma metastasis. Integrins, a large family of cell adhesion molecules expressed on the melanoma cell surface, are important for cell signaling, growth, and migration during metastasis. Most critically, tumor cell tissue invasion is dependent on the initial interaction of tumor cells with vascular endothelium at the target organ, and there is increasing evidence for a prominent role of melanoma very late antigen-4 (VLA-4) integrin binding to its endothelial ligand vascular cell adhesion molecule-1 (VCAM-1) in this process. This research focuses on the quantitative modulation of VLA-4 integrin expression and function on melanoma cells after UVB irradiation. The present data show that at 3, 12, and 18 hours post-UVB irradiation, VLA-4 expression was unchanged relative to untreated cells, but adhesion to VCAM-1 decreased significantly. Immunofluorescence studies implied that the spatial organization of VLA-4 on the melanoma cell surface contributed to the changes in avidity for VCAM-1 upon UVB irradiation. With increased understanding of the molecular mechanisms underlying melanoma-endothelial interactions upon UVB irradiation, clinical advances for melanoma may be developed.

Keywords

Melanoma; VLA-4; VCAM-1; UVB; tumor metastasis; HUVEC

INTRODUCTION

As with other types of cancer, melanoma metastasis is a complex process that consists of a series of distinct steps that transfer tumor cells from a primary site to a distal location [1]. Initially, tumor cells invade surrounding tissue and enter either the lymphatic system or the blood vasculature. The circulating tumor cells eventually roll, adhere, and anchor to vessel endothelium, and then extravasate through to establish secondary colonies in the target organ. These events are highly dependent on the interaction of various cell adhesion molecules (CAMs) expressed on tumor cells and endothelial cells.

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Integrins, a family of CAMs, are heterodimeric adhesion receptors composed of α and β subunits (of an available 19 α subunits and 8 β subunits in mammals). Integrins are widely expressed on tumor cells and play vital roles in cancer metastasis [1,2]. The VLA-4 integrin ($\alpha 4\beta 1$ integrin) expressed on human melanoma cells can potentially mediate tumor cell metastasis by tethering, rolling, and adhering to VCAM-1 expressed on endothelial cells, similar to peripheral blood mononuclear cell (PBMC) trafficking to lymphoid organs and to sites of inflammation [3–6]. VLA-4 has also been shown to enhance transendothelial migration via engagement of VCAM-1 [7]. According to previous reports, the ability of melanoma cells to adhere to cytokine-activated endothelium correlates VLA-4 expression [4,8]. However, an important feature of VLA-4, like other cell adhesion molecules, is that protein expression levels do not necessarily dictate functional capacity. The latter is actually regulated through activation states and/or spatial organization induced by numerous stimuli [9–12].

UVB irradiation is considered a significant risk factor but perhaps indirect cause of melanoma [13]. Overexposure to UVB in neonatal or juvenile mice, but not adult mice, has been linked with a significantly higher risk of melanoma in later life [14]. However, little is known about the potential mechanistic effects of UVB on melanoma metastasis. Thus the present study was undertaken to examine the effects of UVB irradiation on melanoma cell adhesion to endothelium under hematogenous shear stress, a critical step for melanoma cells to establish distant metastases. This work highlights the molecular pathway underlying melanoma-endothelial interactions and reveals the vital role of VLA-4 and VCAM-1, post-UVB irradiation.

MATERIALS AND METHODS

Antibodies and Reagents

The following primary monoclonal antibodies (mAb) were purchased from BD Biosciences: anti-human $\beta 7$ integrin (FIB504) mAb, anti-human CD29 (mAb13) mAb, anti-human CD43 (1G10) mAb, anti-human CD49b (12F1-H6) mAb, anti-human CD49f (GoH3) mAb, anti-human VCAM-1 (51-10C9) mAb, anti-human CD162 (KPL-1) mAb, anti-sLe^{x/a} (HECA-452) mAb, and anti-human sLe^x (CSLEX-1) mAb. Isotype control antibodies, and fluorescein isothiocyanate (FITC)-conjugated and tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies were also purchased from BD Biosciences. Anti-human sLe^x (KM93) and sLe^a (KM231) mAbs were from Calbiochem (San Diego, CA). Anti-human CD49d (HP2/1) mAb and anti-human CD49e (SAM-1) mAb were obtained from Beckman Coulter (Miami, FL). Recombinant human VCAM-1 was purchased from R&D Systems (Minneapolis, MN).

Cell Culture

The melanoma cell line M624 was cultured in Dulbecco's Modification of Eagle's Medium supplemented with 10% fetal bovine serum (FBS). The cells (5×10^6) were seeded in 100 mm cell culture dishes one day before experiments. Primary Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD) and used on passage 2 through 6 exclusively. The cells were cultured in M199 medium with supplements as following: 100 μ g/ml Heparin (Sigma, St. Louis, MO), 50 μ g/ml endothelial cell mitogen (Biomedical Technologies Inc., Stoughton, MA), 10% heat inactivated FBS, and 2 mM L-glutamine [15]. For adhesion assays, HUVECs were stimulated for 8 h with 10 ng/ml IL-1 β to induce adhesion molecule expression.

UVB Irradiation

UVB was generated from a 15 W UVB lamp (UVP, Upland, CA). The intensity of UVB was standardized by a UVB meter (UVP) and set at 3 mW/cm². M624 cells were irradiated with 50 mJ/cm² UVB (290–320 nm wavelength), with the medium replaced with DPBS during the irradiation. After UV irradiation, fresh medium was added to each plate, and cells were maintained in regular culture conditions for a designed time period of 3–18 h until analysis.

Parallel Plate Flow Chamber Cell Adhesion Assays

Dynamic flow adhesion assays were performed using a parallel plate flow chamber in a laminar flow environment as described previously [3,4,15]. The flow chamber was placed on the stage of an inverted microscope with an auto-controlled heater to maintain environmental temperature at 37°C, which is optimal for integrin-mediated adhesion [16]. Cell movement was monitored and recorded by a video system. For rolling velocity analysis the still images were captured from the video (QuickTime, Apple, Cupertino, CA 95014), pseudo-colored by iVision-Mac (BioVision Technologies, Inc, Exton, PA) and merged. Attachment assays were performed by perfusing M624 melanoma cells (10⁶/ml flow buffer of 0.1% BSA/DPBS containing Ca⁺⁺/Mg⁺⁺) on immobilized HUVEC cells at the appropriate flow rates to obtain wall shear stresses of 0.5–2.0 dyn/cm², thereby mimicking the fluid mechanical environment of microcirculation and post-capillary venules [17]. The total number of interacting melanoma cells (rolling or adherent on HUVECs in the field of view) was counted [15,18]. To analyze if the adhesive interactions between M624 and HUVECs were VCAM-1-dependent, HUVEC monolayers were pretreated with function-blocking anti-VCAM-1 mAb (51-10C9, 10 µg/ml) for 30 min at 37°C. Rolling velocity was calculated as the distance traveled by an attached cell divided by the period of observation (5 s).

For the study of M624 cells interactions with VCAM-1 proteins, 20 µg/ml of VCAM-1 proteins were placed on a 100 mm petri dish overnight at 4°C followed by blockade of non-specific binding sites with 0.1% bovine serum albumin (BSA) in DPBS containing Ca⁺⁺/Mg⁺⁺ for 2 h at room temperature. Melanoma cells in the flow buffer were pumped through the chamber on immobilized VCAM-1 protein at 0.5 dyn/cm² as described above.

Fluorocytometric Analysis

Surface protein expression on M624 cells was detected using immunofluorescence and flow cytometry. Specific monoclonal antibodies against molecules of interest were first titrated to saturating concentrations. For each sample, cells were suspended in 50 µl saturating concentration of primary mAbs, or the appropriately matched isotype control antibodies, for 30 min on ice, washed twice, and incubated with 50 µl of FITC-conjugated secondary antibodies and kept on ice for 30 min. Cells were washed twice, then resuspended in DPBS with Ca⁺⁺/Mg⁺⁺ for analysis on a FACSsort analyzer (BD Biosciences).

To estimate cell death, M624 cells were double stained with propidium iodide (PI) and ANNEXIN-V using the Apoptosis Detection Kit from BD Biosciences, followed by analysis on the FACSsort according to the manufacturer's instructions.

Immunofluorescence and Deconvolution Microscopy—Deconvolution microscopy was used to analyze the surface localization of α4/CD49d and β1/CD29 integrins (i.e., VLA-4 subunits) on untreated and UVB-treated M624 cells. Briefly, suspended cells were labeled with anti-human α4 mAb (HP2/1) or with anti-human β1 mAb (mAb 13), washed twice, and incubated with corresponding AlexaFluor 568 or AlexaFluor 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA), respectively. After two washes, cells were

fixed for 30 min on ice with 4% methanol-free paraformaldehyde in DPBS. Cells were mounted on glass slides in ProLong Gold Anti-fade Reagent (Invitrogen). Fluorescent images at 0.5 μm increments along the z-axis were acquired with a Leica DMI 6000 inverted microscope through a 40 $\times$ objective and the appropriate filter cubes (Leica Microsystems, Germany), using Simple PCI software (Hamamatsu Corporation, Sewickley, PA). Image stacks were subjected to a 3D-blind deconvolution algorithm using AutoQuant X software (Media Cybernetics, Bethesda, MD) to reduce out-of-focus light in order to obtain reconstructed 3D image projections.

Statistical Analysis

Data are expressed as the mean $\pm$ SD or mean $\pm$ SEM as indicated. Statistical significance of differences between means was determined by one-way ANOVA. If means were shown to be significantly different, multiple comparisons were performed post-hoc by the Tukey t-test. Statistical significance was defined as $p < 0.05$.

RESULTS

Endothelial VCAM-1 supports VLA-4-mediated M624 melanoma cell adhesion under flow conditions

To characterize the relative ability of M624 cells to adhere to vascular endothelium, adhesive interactions were quantified over a range of physiologically relevant shear stresses. Cytokine-stimulated human umbilical vein endothelial cells (HUVECs, 8h with 10 ng/ml IL-1 β) were not capable of supporting M624 tethering in flow at 2 dyn/cm² wall shear stress (data not shown). Shear stress was systematically reduced until stable M624 tethering was achieved at 0.5 dyn/cm² wall shear stress, at which point 37 ± 2 M624 cells adhered to HUVECs (Fig. 1A). The binding was nearly eliminated when melanoma cells were perfused over HUVECs that were pretreated with anti-VCAM-1 monoclonal antibody (Fig. 1A), thus indicating specific adhesion mediated by melanoma VCAM-1 counter-receptors and endothelial VCAM-1.

Fluorocytometric analysis of major cell surface proteins used by leukocytes and epithelial cell carcinomas to adhere to vascular endothelium [2,6,19] was performed to corroborate endothelial adhesion data. It was found that M624 cells expressed high levels $\alpha 4$ (CD49d) integrin (Fig. 2; Table 1), which can exist in a heterodimer with either $\beta 1$ (CD29) or $\beta 7$ integrin subunits. The $\beta 1$ integrin was highly expressed (Fig. 2; Table 1), but the $\beta 7$ integrin was not detected (Table 1), implicating $\alpha 4\beta 1$ (or VLA-4) and not $\alpha 4\beta 7$ as the melanoma ligand for endothelial VCAM-1-mediated adhesion. Melanoma cells failed to express selectin ligands such as PSGL-1 and CD43, or the purported selectin binding epitopes sialyl Lewis X or sialyl Lewis A (Table 1), consistent with the data showing VCAM-1 as the major mediator of melanoma cell attachment.

VLA-4 Mediated M624 Adhesion to VCAM-1 on Endothelial Cells is Sensitive to UVB Irradiation

The effect of UVB on melanoma cell adhesion to endothelium was next assessed, given the possible role of UVB on melanoma metastasis. Relative to untreated cells, UVB-irradiation reduced approximately 70% of M624 cell binding to HUVECs (Fig. 1A). Consistent with untreated cells, essentially all attachment of UVB-treated cells was dependent on endothelial VCAM-1 (Fig. 1A). In addition, the rolling velocity of melanoma cells on HUVECs was significantly increased from $23.8 \pm 1.8 \mu\text{m/s}$ to $42.6 \pm 6.1 \mu\text{m/s}$ after UVB-irradiation (Fig. 1B), demonstrating a decreased adhesive potential in agreement with the reduction in overall attachment of UVB-treated cells (Fig. 1A). Taken together these data indicate that UVB plays an important role in modulating melanoma cell adhesion to VCAM-1. The extent of

the UVB effects was subsequently examined by monitoring the adhesive interactions between melanoma cells and purified VCAM-1 (in the form of dimeric VCAM-1 fused to human Fc portion of IgG) at different time points post-irradiation. The cumulative time-course adhesion data show that the UVB-treated melanoma cells' capacity to bind VCAM-1 decreased in a time-dependant manner. At 3 h post-UVB irradiation, 29 ± 3 cells/mm² adhered to the VCAM-1 over the perfusion period, compared to 58 ± 2 cells/mm² for the non-UVB treated melanoma cells (Fig. 3). The adhesion ability of melanoma cells to VCAM-1 at 12 h post-UVB irradiation was further reduced to 13 ± 1 cells/mm², or 4.6 fold decreased relative to the untreated cells (Fig. 3). The adhesion ability of melanoma cells to VCAM-1 at 18 h post-UVB irradiation was not significantly different from 12 h post-UVB (Fig. 3). No cell binding was observed to negative control substrates, as expected. The results demonstrate that UVB specifically attenuates the avidity of VLA-4 for its ligand VCAM-1.

The Spatial Organization, Not Expression Level, of VLA-4 on the Melanoma Cell Membrane Contributes to the Changes in Avidity for VCAM-1

To explore the mechanism underlying the UVB-induced decrease of melanoma VLA-4, the cell-surface expression of the $\alpha 4$ and $\beta 1$ integrins of untreated or irradiated M624 cells were compared using immunofluorescence and flow cytometry. Neither $\alpha 4$ nor $\beta 1$ integrin expression levels changed significantly in UVB irradiated cells at different recovery time points as evidenced by fluorescence histograms (Fig. 2A–D) and mean fluorescence intensities (Fig. 2E), indicating that the avidity changes on melanoma cells to VCAM-1 were not caused by changes in protein expression level. Importantly, cell death pathways were not controlling adhesion either, as there were no significant differences in percent of apoptotic cells among untreated and UVB treated cells (at any time point post-treatment) as determined by propidium iodide and ANNEXIN-V double staining and flow cytometry analysis (data not shown).

Since expression levels of VLA-4 appeared unchanged by UVB-treatment, another mechanism must govern the observed changes molecular avidity. Thus, the spatial distribution of VLA-4 on the melanoma cell surface was inspected by fluorescently labeling its two subunits, $\alpha 4$ and $\beta 1$, separately. The images obtained by deconvolution microscopy showed a clear increased clustering of the $\alpha 4$ subunit into more distinct groupings (Fig. 4, bottom left panel) after UVB irradiation, compared to untreated cells displaying less punctate staining (Fig. 4, top left panel). However, the $\beta 1$ subunit distribution generally remained diffuse with some localization in both untreated and UVB treated cells, implying $\beta 1$ organization was not as affected by UVB exposure (Fig. 4, right panels). These data imply that UVB-induced $\alpha 4$ integrin relocalization might be responsible for the observed reduction of melanoma cell adhesion to VCAM-1.

DISCUSSION

Progression of melanoma correlates with VLA-4-mediated adhesion to and transmigration through endothelial cell monolayers [3,4,7,20]. More specifically, VLA-4 expressed by melanoma cells mediates binding to VCAM-1 expressed by cytokine-stimulated endothelium [3,4,7,8], similar to VLA-4 expressed by lymphocytes [5,6]. However, little is known regarding the factors controlling VLA-4 expression and function, such as UVB irradiation. In this study, we used the parallel plate flow chamber to quantitatively analyze the adhesive ability of M624 melanoma cells to IL-1 β -stimulated HUVECs. We demonstrated that the adhesion between the melanoma cells and HUVECs is mainly dependent on the interaction between VLA-4 and VCAM-1. When VCAM-1 was blocked with antibody, the amount of melanoma cells bound to HUVECs was reduced by more than 80%, with or without UVB treatment (Fig. 1). The remaining adhesion is not likely due to an interaction between $\alpha 4\beta 7$ and VCAM-1, since $\beta 7$ integrin expression was not detected on

the cell surface. Although flow cytometry screening implied that $\alpha 4$ integrin was incorporated into VLA-4 only, $\beta 1$ integrin could still potentially heterodimerize with $\alpha 2$, or $\alpha 6$ integrins, both of which are expressed on M624 cells (Table 1), to form VLA-2 or VLA-6, respectively. However, they are not likely to have contributed significantly in mediating melanoma cell avidity for HUVECs, as mAb blockade of VCAM-1 abolished cell-cell adhesion (Fig. 1) and none of these molecules are VCAM-1 ligands.

VLA-4 avidity is not only determined by its surface expression levels, but also by its activation states. VLA-4 has a continuum of activation states, at least four of which are distinguishable, enabling it to engage VCAM-1 with varying degrees of affinity [9,21]. Furthermore, it has been shown that VLA-4 avidity is dependent on localization within lipid rafts [22], indicating the importance of cell membrane localization on the function of VLA-4. To further elucidate the mechanism for VLA-4-mediated adhesion upon UVB irradiation, the cell surface expression and membrane distributions of $\alpha 4$ and $\beta 1$ integrins were analyzed before and after UVB treatment. As shown in Fig. 2, the VLA-4 protein expression levels displayed no significant change, indicating that factor(s) other than protein expression level govern the observed changes in VLA-4 avidity for VCAM-1. Deconvolution microscopy images showed that $\alpha 4$ integrin became more clustered after UVB irradiation while $\beta 1$ did not (Fig. 4). Based on these results, a hypothesized model of UVB-induced regulation of VLA-4 integrin function was proposed (Fig. 5), whereby UVB irradiation of melanoma cells causes clustering of $\alpha 4$ integrin that in turn diminishes the ability of the melanoma cells to bind to endothelial VCAM-1. It is possible that the observed clustering reflects the generation of $\alpha 4$ integrin homo-oligomers. The existence of integrin homomers has been proposed through chemical and mathematical modeling [23,24], and several groups have experimentally isolated these species [24–27], most notably the subunits of the $\alpha \text{IIb}\beta 3$ integrin [26,27]. The existence of these homomers is likely to be restricted by integrin activation status, as it has been shown that αIIb homomers appear in the resting, low affinity state [27]. We therefore suggest that the clustering of single integrin chains, particularly the $\alpha 4$ integrin, warrants further study and is currently underway in our laboratories.

In summary, we conclude that VLA-4 is the major contributor for the avidity of melanoma cells to HUVECs. UVB irradiation leads to a readily observable redistribution of $\alpha 4$ but not $\beta 1$ on the cell surface, resulting in reduced adhesion between the two cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UVB	ultraviolet B light
VLA-4	very late antigen-4
VCAM-1	vascular cell adhesion molecule-1
CAMs	cell adhesion molecules

PBMC	peripheral blood mononuclear cell
FITC	fluorescein isothiocyanate
HUVECs	human umbilical vein endothelial cells
LSCM	laser scanning confocal microscopy

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A. Numbers of Adherent Cells **B. Velocity of M624 Cells Rolling On HUVECs** **Figure 1. UVB irradiation reduces M624 melanoma cell adhesion to VCAM-1 expressed on endothelial cells**

Untreated or 3 h post-UVB irradiated M624 melanoma cells were perfused over IL-1 β stimulated HUVEC monolayers in the parallel plate flow chamber at a physiologic shear stress of 0.5 dyn/cm². After 4 min of perfusion, (A) the number of interacting melanoma cells (including all rolling and firmly adherent cells) was counted; and (B) the velocity of rolling cells was measured as described in Materials and Methods. Data in (A) are expressed as the mean $\pm$ SD from three independent experiments, and data in (B) are expressed as mean rolling velocity $\pm$ SD of eleven cells. * p <0.05 versus non-UVB treated control group.

Figure 2. The expression of VLA 4 on M624 melanoma cells does not change upon UVB treatment

The expression levels of $\alpha 4$ (A, C) and $\beta 1$ (B, D) integrin subunits on untreated or 50 mJ/cm² UVB-irradiated M624 melanoma cells were analyzed by flow cytometry. UVB-irradiated cells were harvested for analysis at 3 (A, B), 12 or 18 h (C, D) after treatment. Specific antibody reactivity is indicated by purple profiles for untreated cells. Open dotted lines represent untreated cell reactivity with negative control isotype-matched antibody. In both A and B, green profiles represent cells 3 h post-UVB treatment. In both C and D, green profiles are for cells 12 h post-UVB treatment, and pink profiles are for cells 18 h post-UVB treatment. The figures are representative of five independent experiments. (E) The mean fluorescent intensities of $\alpha 4$ and $\beta 1$ integrins among untreated and UVB treated cells are not statistically different. Data are expressed as mean $\pm$ SEM of five independent experiments.

Figure 3. UVB reduces avidity of M624 melanoma cell VLA-4 for VCAM-1

Untreated or 50 mJ/cm² UVB irradiated M624 melanoma cells were perfused over immobilized soluble VCAM-1 in the parallel plate flow chamber, at a physiologic shear stress of 0.5 dyn/cm². Melanoma cells were harvested for use in the flow adhesion assay at the indicated time-point post-UVB irradiation. The number of adhesive interactions in a single field of view was counted over a 4 min perfusion period. Data are expressed as the mean±SD from three independent experiments. **p*<0.05 versus non-UVB treated control group.

Figure 4. UVB induces the reorganization of $\alpha 4$ integrin on the surface of M624 melanoma cells
The $\alpha 4$ and $\beta 1$ integrin subunits on untreated or 3 h post-UVB-irradiated M624 melanoma cells were stained by fluorescently-labeled antibodies and then analyzed by deconvolution microscopy. Red AlexaFluor 568-labeled samples show $\alpha 4$ integrin becomes more clustered after UVB treatment, while green AlexaFluor-488 labeled samples show $\beta 1$ integrin with similar staining on untreated and UVB irradiated cells. Scale bars = 1.0 μm .

Figure 5. The hypothesized model for UVB-reduced adhesion between melanoma and endothelial cells

The $\alpha 4\beta 1$ integrin is normally expressed on the M624 melanoma cell surface, thus enabling adhesion to VCAM-1 on endothelial cells under hemodynamic flow conditions. After UVB-irradiation the $\alpha 4$ subunits cluster together independently of the $\beta 1$ chains, leading to a reduced capacity to bind VCAM-1 (i.e., less melanoma cell adhesion to endothelium).

TABLE I

Detection of cell surface molecules on M624 melanoma cells by immunofluorescence and flow cytometry. The specific mAb clones and isotype control antibodies used are described in Materials and Methods. Note the expression of $\alpha 4$ and $\beta 1$ integrin (i.e., VLA-4), but lack of $\beta 7$ (i.e., absence of $\alpha 4\beta 7$), implicating VLA-4 as the mediator of melanoma cell adhesion to endothelial VCAM-1. Data are representative of $n = 2$ independent experiments.

Expression	Family	Name
+	Integrins	CD29 ($\beta 1$), CD49b ($\alpha 2$), CD49d ($\alpha 4$), CD49f ($\alpha 6$)
–	Integrins	CD49e ($\alpha 5$), $\beta 7$
	Sialofucosylated antigens	sLe ^x , sLe ^a , HECA-452
	Other selectin ligands	CD43, CD162