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The Roles of Akt and NOSs In Regulation of VLA-4-Mediated Melanoma Cell Adhesion To Endothelial VCAM-1 After UVB-irradiation

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Abstract

UVB-reduced avidity between M624 melanoma and HUVEC cells is dependent on the interaction of VLA-4 with its endothelial ligand VCAM-1. Our previous studies suggested that a spatial organization of $\alpha 4$ integrin, one of the two subunits of VLA-4, on the melanoma cell surface contributed to the changes in avidity for VCAM-1 upon UVB-irradiation. In this study, we demonstrate that Akt plays an important role in regulation of the expression and surface level of $\alpha 4$ integrin on melanoma cells upon UVB-irradiation. While the cell surface level of $\alpha 4$ integrin is not significantly affected by UVB-irradiation or Akt inhibitor alone, it is dynamically altered after UVB-irradiation when Akt is inhibited. Inhibition of Akt also reverses the reduction of avidity of cells after the irradiation. Our data also shows that UVB reduces the level of Akt. The inhibition of Akt activity correlates with a reduced amount of coupled cNOS and reduced amount of iNOS after UVB-irradiation. However the effect of NOSs on melanoma cell adhesion appears due to their roles in regulation of apoptosis after UVB-irradiation. Base on these results, we propose that the UVB-induced reduction of avidity of melanoma cells is coordinatively regulated by NOSs and Akt through two differential mechanisms.

Introduction

The very late antigen-4 integrin (VLA-4, $\alpha 4\beta 1$ integrin) expressed on human melanoma cells can potentially mediate tumor cell metastasis by tethering, rolling, and adhering to vascular cell adhesion molecule-1 (VCAM-1) expressed on endothelial cells, similar to peripheral blood mononuclear cell (PBMC) trafficking to lymphoid organs and to sites of inflammation [1–4]. The ability of melanoma cells to adhere to cytokine-activated endothelium correlates with VLA-4 expression [2,5]. The avidity of VLA-4 to endothelial cells is regulated by several cytoplasmic proteins [6–8]. Phosphorylation and dephosphorylation of integrin $\alpha 4$ alters its binding affinity to paxillin, a cytosolic signaling adaptor protein, and thus regulates migration of cells [9,10]. Protein kinase B (PKB, Akt) is a serine/threonine protein kinase, which has been shown to play important roles in regulation of cell adhesion via different mechanisms [11–15]. However, it has never been shown if Akt mediates adhesion by regulating an adhesion molecule such as $\alpha 4$ integrin upon UVB-irradiation.

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Akt activity is often co-regulated with iNOS and eNOS. Akt can stimulate iNOS expression via the NF- κ B pathway [16]. Akt can also phosphorylate eNOS to increase eNOS stability and coupling [17]. In return, an elevation of iNOS can cause feedback inhibition of Akt [18]. UVB-induced activation of Akt plays an important role in regulation of cell cycle progression and apoptosis [19–21]. However, it is not known if the Ultraviolet B light (UVB)-induced Akt activation can also affect adhesive affinity of the irradiated cells. A recent study indicated that UVB-irradiation leads to a readily observable redistribution of α 4 but not β 1 on the cell surface, resulting in reduced adhesion between M624 melanoma and endothelial cells [22]. Recent studies also indicated that UVB-irradiation dynamically regulates the activities of iNOS and eNOS, including nNOS and eNOS [23,24], which can potentially affect adhesive affinity of cells [25–27]. However, little is known about the relationships among NOS, Akt and melanoma cell adhesion after UVB-irradiation. Using the same M624 melanoma model, this study was to elucidate the relationships of α 4 integrin, Akt, eNOS and iNOS. Their effect on UVB-reduced melanoma cell adhesion to endothelium was also determined under hemogenous shear stress, a critical step for melanoma cells to establish distant metastases. Our results suggest that NOSs and Akt regulate avidity of melanoma cells after UVB-irradiation via independent signaling pathways.

Materials and Methods

Cell Culture and Treatment

The M624 cells (human melanoma cell line) were cultured in 100 mm² cell culture plates in Dulbecco's modification of eagle's medium (DMEM) (Mediatech, Manassas, VA) with 10% (v/v) fetal bovine serum (FBS) (Denville, Metuchen, NJ). Penicillin and streptomycin (Invitrogen, Carlsbad, CA) were added to the culture medium, and the cells were incubated at 37°C and 5% CO₂.

UVB Irradiation

The power of the UVB lamp (UVP Inc., Upland, CA) was determined by a UVX digital radiometer (UVP Inc., Upland, CA) after the lamp was warmed up for 5 min. The cell culture medium was replaced with phosphate buffered saline (PBS, 1 ml/plate) during UVB irradiation (50 mJ/cm²). After the irradiation, the original medium was added to cell culture plates and the cells were returned to incubator for further analysis.

Cell Treatment

N-Nitro-L-Arginine Methyl Ester (L-NAME), N-Acetyl-L-Cysteine (L-NAC), N-([3-(Aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride (1400w), Akt 1/2 kinase inhibitor (Akt I) were purchased from Sigma-Aldrich (St. Louis, MO). The final concentrations for the treatment were 2 mM L-NAME, 25 mM L-NAC, 25 μ M 1400w, 200 nM Akt I. The cells were pre-treated with each chemical for 1 h and then UVB-irradiated. After the irradiation, the cells were incubated for 18 h in the same medium with each inhibitor.

Western Blotting

The antibodies against eNOS, iNOS, Akt 1, α 4 integrin and mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti- β -actin was purchased from Sigma-Aldrich (St. Louis, MO) and anti-rabbit IgG was purchased from Bio-Rad (Hercules, CA). Two different conditions were used for SDS-PAGE. The first one was low-temperature, SDS-resistant SDS-PAGE as previously described [24]. Generally, the protein samples were prepared in Laemmli buffer (0.32 M Tris-HCl, pH 6.8, 0.5 M glycine, 10% SDS, 50% glycerol, and 0.03% bromophenol blue) without boiling and separated on reducing SDS-

PAGE (with 2.5% 2-mercaptoethanol) at 4°C. The second one was a regular SDS-PAGE. The protein samples in Laemmli buffer were boiled and separated on SDS-PAGE at room temperature. The proteins were then transferred onto a nitrocellulose membrane, which was then blocked with 5% (w/v) skim milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h and then incubated with proper antibodies at 4°C overnight. After washing with TBST, the membrane was incubated with HRP-conjugated anti-rabbit antibody for 1 h at room temperature. The membrane was then washed three times in TBST, two times in TBS, and developed in West Pico Supersignal chemiluminescent substrate (Pierce, Rockford, IL).

Flow Cytometry

After treatment, cells were harvested in 15 ml tubes and washed with DPBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ twice. Then each sample with 10^6 cells was suspended in 100 μl DPBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and was incubated with 5 μl FITC-conjugated CD49d antibody (BD sciences, Franklin Lakes, NJ) in the dark for 30 min. After incubation, the cells were washed twice again. The cell pellets were suspended in 400 μl DPBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and ready for the analysis of FACSsort analyzer (BD sciences, Franklin Lakes, NJ). 20,000 cells were counted for each sample.

Parallel Plate Flow Chamber Cell Adhesion Analysis

Dynamic flow adhesion assays were performed using a parallel plate flow chamber in a laminar flow environment as previously described [1,2,28]. 50 $\mu\text{g}/\text{ml}$ of VCAM-1 protein (R&D system, Minneapolis, MN) was 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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ for 2 h at room temperature. M624 melanoma cells ($10^6/\text{ml}$ in DPBS flow buffer with $\text{Ca}^{2+}/\text{Mg}^{2+}$) were perfused through the chamber on immobilized VCAM-1 protein surface at 0.45 dyn/cm^2 . The movement of the cells was monitored and recorded by a video system for 5 min/sample. The total number of the attached cells in 5 min were counted [28].

Results and Discussion

UVB-irradiation reduces the expression of $\alpha 4$ integrin in M624 melanoma cells

Integrin $\alpha 4\beta 1$ plays an important role in regulation of the avidity of melanoma to endothelial cells. Our recent report indicated that the surface levels of $\alpha 4$ and $\beta 1$ integrins were not significantly changed after UVB-irradiation, while the spatial organization of $\alpha 4$ integrin was changed [29]. We now determined the effect of UVB on cellular level of $\alpha 4$ integrin in M624 melanoma cells. Western blot analysis showed that $\alpha 4$ integrin was reduced at 18 h post-irradiation (Fig. 1, Lane 2 vs. 1). Since UVB inhibits translation via NOS activation and oxidative stress signaling pathway [30], we determined whether the reduction of $\alpha 4$ integrin was regulated through NOS/oxidative stress-mediated translational inhibition. We used L-NAC to reduce oxidative stress, L-NAME to inhibit cNOS and 1400w to inhibit iNOS. Our data showed that treating each drug did not affect the expression of $\alpha 4$ integrin (Fig. 1, Lanes 3, 5, 7 vs. 1). However, while L-NAC did not have an impact on UVB-reduced $\alpha 4$ integrin expression (Fig. 1, Lane 4 vs. 2), inhibition of NOSs further reduced the $\alpha 4$ integrin level post-UVB (Fig. 1, Lanes 6, 8 vs. 2). Since L-NAC inhibits the UVB-induced phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) and reverses the translation inhibition [30], the results suggest that the UVB-reduced $\alpha 4$ integrin expression is not due to the translational inhibition of the protein synthesis.

The further decrease of $\alpha 4$ integrin by NOS inhibitors suggests that NOS activity partially protects the UVB-induced reduction of $\alpha 4$ integrin. Since iNOS and eNOS are involved in

up- and down- stream signaling of Akt pathway, which can phosphorylate $\alpha 4$ integrin [9,10] and has been shown to play a role in regulation of cell adhesion [13,14], we also determined the effect of Akt inhibitor (Akt I) on UVB-induced reduction of $\alpha 4$ integrin. Unlike the other chemicals, treating the cells with Akt I significantly reduced $\alpha 4$ integrin without or with UVB-irradiation (Fig. 1, Lanes 9, 10 vs. 1, 2). The results suggest that Akt signaling pathway might play a critical role in regulation of $\alpha 4$ integrin expression upon UVB-irradiation.

Akt activity mediates eNOS coupling and iNOS expression after UVB-irradiation

To further determine the role of NOSs and Akt in regulation of melanoma adhesion, we analyzed the expression of NOSs and Akt. While both iNOS and Akt levels are reduced (Fig. 2A, Lane 2 vs. 1), nNOS is not detected (data not shown) and eNOS levels are not significantly changed after UVB-irradiation (Fig. 2B, Lane 2 vs. 1). It was a surprise to detect such a high level of iNOS in non-irradiated cells and it was even more surprising to see the level of iNOS was reduced after UVB-irradiation, which was opposite from what we and others reported for iNOS expression in none melanoma cells [24,31–33]. Since elevated nitric oxide (NO^{*}) produced by iNOS can inhibit UVB-induced apoptosis [24], our observation might explain the survival advantage of melanoma after UVB-irradiation.

The UVB-induced reduction of iNOS and Akt was slightly reversed by treating the cells with L-NAC and L-NAME, which protects eIF2 α from UVB-induced phosphorylation [30]. This suggested that UVB-induced translational inhibition might play a role in regulation of iNOS and Akt expression. Inhibition of Akt further decreased the expressions of iNOS and Akt post-UVB. Since Akt phosphorylates and activates eNOS [17], which plays a role in regulation of cell adhesion [34,35], we analyzed the role of Akt in regulation of eNOS coupling after UVB-irradiation using a SDS-resistant reducing gel [24]. Our data showed that eNOS coupling was increased in an Akt-dependent manner after UVB-irradiation (Fig. 2B, Lane 10 vs. 2). Reduction of free radicals by L-NAC or inhibition of NOSs by L-NAME and 1400w did not significantly affect the UVB-induced eNOS coupling. These results suggest that Akt activity has a protective effect on UVB-reduced expressions of certain gene.

Regulation of surface $\alpha 4$ integrin on melanoma cells after UVB-irradiation

We previously showed that UVB-reduced avidity of melanoma to endothelial cells was not dependant on the surface level, but localization of $\alpha 4$ integrin [29]. Now we showed that the total expression of $\alpha 4$ integrin was reduced after UVB-irradiation, especially after treating the cells with NOS and Akt inhibitors (Fig. 1). To determine whether the significant reduction of total $\alpha 4$ integrin resulted in the decrease in surface level of the protein, we measured the surface $\alpha 4$ integrin on M624 melanoma cells using immunofluorescent staining followed by flow cytometry. Our data showed that the intensity of $\alpha 4$ Integrin on the cell surface was not significantly changed but the number of cells that contained surface $\alpha 4$ integrin was decreased after UVB-irradiation (Fig. 3A), which agreed with our previous report [29]. The reduction of surface $\alpha 4$ integrin could be due to apoptotic cell death, which has been shown to cause integrin endocytosis [36]. Treating the cell with L-NAC (free radical scavenger) or 1400w (iNOS inhibitor) reduced the numbers of cells that contained surface $\alpha 4$ integrin without or with UVB-irradiation (Fig. 3B and 3D). Reduction of oxidative stress also reduced the intensity of surface $\alpha 4$ integrin (Fig. 3B). These results suggested that keeping a certain level of oxidative stress could be important for maintaining surface levels of $\alpha 4$ integrin and protecting cells from apoptosis. Treating the cell with L-NAME did not affect the intensity of surface $\alpha 4$ integrin but slightly reversed UVB-induced reduction of the numbers of cells that contained surface $\alpha 4$ integrin (Fig. 3C). This result agreed with our previous report that eNOS promotes UVB-induced apoptosis [23].

While treating the cells with Akt I alone did not affect surface $\alpha 4$ integrin, the expression of $\alpha 4$ integrin changed dramatically after UVB-irradiation. The number of cells that contained surface $\alpha 4$ integrin was significantly decreased, but the intensity of surface $\alpha 4$ integrin was significantly increased (Fig. 3E). The data indicated that inhibition of Akt increased the surface $\alpha 4$ integrin after UVB-irradiation. These results suggest that while Akt protects cells from UVB-induced apoptosis, Akt played an inhibitory role in the surface delivery of $\alpha 4$ integrin. These results also indicated that the surface level of $\alpha 4$ integrin was not always correlated with the total expression level of the integrin (Fig. 3 vs 1).

Two potential mechanisms for regulation of avidity of melanoma cells after UVB-irradiation

The interaction of $\alpha 4\beta 1$ integrin (VLA-4) and VCAM-1 was crucial for the adhesion of M624 melanoma to endothelial cells [29]. We previously showed that UVB-irradiation changed the surface distribution of $\alpha 4$ integrin and thus reduced the adhesive affinity of melanoma to endothelial cells [29]. To determine whether a change of surface $\alpha 4$ level can affect the avidity of melanoma cells, we analyzed the adhesion between melanoma and VCAM-1 protein using parallel plate flow chamber. Our data showed that the avidity of the cells decreased 68% after UVB-irradiation (Fig. 4), which agreed with our previous report [29]. Treating the cells with L-NAC or 1400w alone reduced the avidity of the cells by 71% or 57% respectively (Fig. 4), which correlated with the reduction of surface $\alpha 4$ integrin containing cells as observed (Fig. 3B and 3D). The avidity of the L-NAC or 1400w treated cells was further reduced after UVB-irradiation (Fig. 4), which was not correlated to the unchanged surface $\alpha 4$ integrin after UVB-irradiation (Fig. 3B and 3D). These results suggested that the reduced avidity of the melanoma cells after L-NAC or 1400w treatment might be mainly due to apoptosis. However, the reduced surface level of $\alpha 4$ integrin on L-NAC treated cells (Fig. 3B) also contributed to the reduction of avidity of the cells without or with UVB-irradiation (Fig. 4).

Treating the cells with L-NAME or Akt inhibitor alone did not statistically significantly affect the avidity of the cells (Fig. 4), which correlated with the $\alpha 4$ integrin profile (Fig. 3C and 3E). Inhibition of eNOS reversed the UVB-induced decrease of adhesion between melanoma cells and VCAM-1 by 19% (Fig. 4), which correlated with increased numbers of $\alpha 4$ integrin containing cells after the same treatment (Fig. 3C). This result suggested that while the basal eNOS activity did not play a role in regulation of melanoma cell adhesion, the UVB-induced activation of eNOS led to the endocytosis of $\alpha 4$ integrin via promotion of apoptosis as we previously reported [23,24]. Inhibition of Akt significantly increased avidity of the cells by 48% after UVB-irradiation (Fig. 4, Akt I vs. No Treat). This result indicated that the increased surface level of $\alpha 4$ integrin, but not the number of cells containing the integrin, contributed significantly to the adhesive affinity of melanoma cells.

In summary, our results suggested that the reduction of avidity of melanoma cells is regulated through two different mechanisms after UVB-irradiation. The first mechanism is through the internalization of $\alpha 4$ integrin, which is most likely due to cell apoptosis. Therefore these cells will not survive and contribute to melanoma cell metastasis. The second mechanism is regulated by an Akt-mediated inhibition of surface delivery of $\alpha 4$ integrin. Since Akt also suppressed apoptosis [20], the Akt pathway has potential in being significant in regulation of melanoma avidity and thus distance metastasis after UVB-irradiation.

Research Highlights

- Akt activity is required for UVB-induced eNOS coupling.
- Akt regulates iNOS expression in melanoma cells after UVB-irradiation.

- Akt mediates expression and cell surface delivery of $\alpha 4$ integrin after UVB-irradiation.
- Akt regulates avidity of melanoma to endothelial cells after UVB-irradiation.

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Reference

1. Giavazzi R, Foppolo M, Dossi R, Remuzzi A. *J Clin Invest.* 1993; 92:3038–3044. [PubMed: 7504697]
2. Liang S, Dong C. *Am J Physiol Cell Physiol.* 2008; 295:C701–C707. [PubMed: 18632734]
3. DiVietro JA, Brown DC, Sklar LA, Larson RS, Lawrence MB. *J Immunol.* 2007; 178:3903–3911. [PubMed: 17339490]
4. Springer TA. *Cell.* 1994; 76:301–314. [PubMed: 7507411]
5. Martin-Padura I, Mortarini R, Lauri D, Bernasconi S, Sanchez-Madrid F, Parmiani G, Mantovani A, Anichini A, Dejana E. *Cancer Res.* 1991; 51:2239–2241. [PubMed: 2009542]
6. Binsky I, Lantner F, Grabovsky V, Harpaz N, Shvidel L, Berrebi A, Goldenberg DM, Leng L, Bucala R, Alon R, Haran M, Shachar I. *J Immunol.* 184:4761–4769. [PubMed: 20357260]
7. Sasaki K, Pardee AD, Okada H, Storkus WJ. *Eur J Immunol.* 2008; 38:2865–2873. [PubMed: 18958887]
8. Goldfinger LE, Han J, Kiosses WB, Howe AK, Ginsberg MH. *J Cell Biol.* 2003; 162:731–741. [PubMed: 12913113]
9. Han J, Rose DM, Woodside DG, Goldfinger LE, Ginsberg MH. *J Biol Chem.* 2003; 278:34845–34853. [PubMed: 12837751]
10. Han J, Liu S, Rose DM, Schlaepfer DD, McDonald H, Ginsberg MH. *J Biol Chem.* 2001; 276:40903–40909. [PubMed: 11533025]
11. Wang S, Basson MD. *Exp Cell Res.* 2008; 314:286–296. [PubMed: 17825284]
12. Fayard E, Tintignac LA, Baudry A, Hemmings BA. *J Cell Sci.* 2005; 118:5675–5678. [PubMed: 16339964]
13. Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, Natesan S, Brugge JS. *J Cell Biol.* 2005; 171:1023–1034. [PubMed: 16365168]
14. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. *Mol Cell.* 2005; 20:539–550. [PubMed: 16307918]
15. Dillon RL, Muller WJ. *Cancer Res.* 2010; 70:4260–4264. [PubMed: 20424120]
16. Kao SJ, Lei HC, Kuo CT, Chang MS, Chen BC, Chang YC, Chiu WT, Lin CH. *Immunology.* 2005; 115:366–374. [PubMed: 15946254]
17. Li CJ, Elsasser TH, Kahl S. *J Mol Signal.* 2009; 4:1–13. [PubMed: 19320971]
18. Chan ED, Riches DW. *Am J Physiol Cell Physiol.* 2001; 280:C441–C450. [PubMed: 11171562]
19. Han W, He YY. *Photochem Photobiol.* 2009; 85:997–1003. [PubMed: 19222789]
20. Ibuki Y, Goto R. *Biochem Biophys Res Commun.* 2000; 279:872–878. [PubMed: 11162442]
21. Mildner M, Eckhart L, Lengauer B, Tschachler E. *J Biol Chem.* 2002; 277:14146–14152. [PubMed: 11821397]
22. Wang L, Tang Z, Sun H. *Zhonghua Zhong Liu Za Zhi.* 2000; 22:301–303. [PubMed: 11778555]
23. Wu S, Wang L, Jacoby AM, Jasinski K, Kubant R, Malinski T. *Photochem Photobiol.* 2010; 86:389–396. [PubMed: 20074088]
24. Liu W, Wu S. *Nitric Oxide.* 2010; 23:199–205. [PubMed: 20547234]
25. Conran N, Ferreira HH, Lorand-Metze I, Thomazzi SM, Antunes E, de Nucci G. *Br J Pharmacol.* 2001; 134:632–638. [PubMed: 11588118]

26. Faruqi TR, Erzurum SC, Kaneko FT, DiCorleto PE. *Am J Physiol.* 1997; 273:H2490–H2497. [PubMed: 9374789]
27. Lahiri M, Martin JH. *Oncol Rep.* 2009; 21:275–281. [PubMed: 19148496]
28. Burdick MM, Chu JT, Godar S, Sackstein R. *J Biol Chem.* 2006; 281:13899–13905. [PubMed: 16565092]
29. Wang L, Venkatesh SS, Burdick MM, Wu S. *Mol. Carcinogenesis.* 2010 In Press.
30. Lu W, Laszlo CF, Miao Z, Chen H, Wu S. *The Journal Of Biological Chemistry.* 2009; 284:24281–24288. [PubMed: 19586904]
31. Sasaki M, Yamaoka J, Miyachi Y. *Exp Dermatol.* 2000; 9:417–422. [PubMed: 11099109]
32. Seo SJ, Choi HG, Chung HJ, Hong CK. *Br J Dermatol.* 2002; 147:655–662. [PubMed: 12366409]
33. Kuhn A, Fehsel K, Lehmann P, Krutmann J, Ruzicka T, Kolb-Bachofen V. *J Invest Dermatol.* 1998; 111:149–153. [PubMed: 9665402]
34. Pandolfi A, Di Pietro N, Siroli V, Giardinelli A, Di Silvestre S, Amoroso L, Di Tomo P, Capani F, Consoli A, Bonomini M. *J Cell Physiol.* 2007; 213:699–709. [PubMed: 17516566]
35. Kaminski A, Ma N, Donndorf P, Lindenblatt N, Feldmeier G, Ong LL, Furlani D, Skrabal CA, Liebold A, Vollmar B, Steinhoff G. *Lab Invest.* 2008; 88:58–69. [PubMed: 18040270]
36. Tsai WH, Chang CW, Lin YS, Chuang WJ, Wu JJ, Liu CC, Tsai PJ, Lin MT. *Infect Immun.* 2008; 76:1349–1357. [PubMed: 18227168]

Fig. 1. The expression of $\alpha 4$ integrin

M624 melanoma cells were treated with L-NAC, L-NAME, 1400w or Akt I in the presence or absence of UVB (50 mJ/cm^2) as indicated. The total cellular levels of Akt and β -actin were determined by western blot analysis using corresponding antibodies.

Fig. 2. The expression of iNOS, eNOS and Akt, and coupling of eNOS

M624 melanoma cells were treated with L-NAC, L-NAME, 1400w or Akt I in the presence or absence of UVB (50 mJ/cm²) as indicated. Panel A: The expression of iNOS and Akt was determined by western blot analysis using corresponding antibodies. Panel B: The monomers and dimers of eNOSs were first separated on a low-temperature SDS-resistant SDS-PAGE and then detected by western blot analysis using anti-eNOS antibody. The levels of β -actin were also probed as a loading control.

Fig. 3. The cell surface level of $\alpha 4$ integrin

M624 melanoma cells were treated with L-NAC, L-NAME, 1400w or Akt I in the presence or absence of UVB (50 mJ/cm^2) as indicated. FITC-conjugated mouse anti-human $\alpha 4$ integrin antibody was added in each sample and the intensity of cell surface $\alpha 4$ integrin was determined by flow cytometry. 2×10^4 cells were counted for each sample. The data represents three independent experiments.

Fig. 4. The adhesive affinity of melanoma cells

M624 melanoma cells were treated with L-NAC, L-NAME, 1400w or Akt I and then untreated or UVB irradiated. The cells were harvested for use in the flow adhesion assay at 18 h post-UVB. The cells were perfused over immobilized soluble VCAM-1 in the parallel plate flow chamber, at a physiologic shear stress of 0.45 dyn/cm^2 . The number of adhesive interactions in a single field of view was counted over a 5 min perfusion period. The experiments were repeated three times. The bars represented as mean $\pm$ SD. The student *t*-test was used to compare the control with UVB irradiation with the inhibitor treatment after UVB irradiation. *: $p < 0.05$.