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Effect of $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrin glycosylation on interaction of melanoma cells with vitronectin

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The metastatic transformation of melanocytes is associated with altered expression of adhesion molecules, including $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrins. Integrin $\alpha_v\beta_3$ is a primary vitronectin (VN) receptor, while both integrin types take part in adhesion to VN when they are in complex with uPAR. Although their role in melanoma cell interaction with VN is of great interest, the influence of N-oligosaccharides attached to these glycoproteins is still unappreciated. The present study assesses the role of $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrins and the influence of their glycosylation status on WM9 and WM239 metastatic melanoma cell interactions with VN. Cell adhesion to and migration on VN were selected as the studied cell behaviour parameters. Function-blocking antibodies and swainsonine (SW) treatment were used in these tests. Both cell lines interacted with VN in an integrin-mediated but cell-line-specific manner. In WM9 cells, migration was not completely inhibited by antibodies against $\alpha_3\beta_1$ or $\alpha_v\beta_3$ integrins, suggesting the participation of other VN receptors. In both cell lines in coprecipitation test the formation of an integrins/uPAR complex was shown. In the presence of SW formation of the complex did not occur, suggesting the participation of glycosylation in this proccess. Additionally, the adhesion properties of WM9 cells were changed after SW treatment. Our results suggest that in these two metastatic cell lines integrin-linked N-oligosaccharides influence the VN adhesion receptor activity and function.

Keywords: adhesion, integrin, glycosylation, migration, vitronectin

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INTRODUCTION

In melanoma a number of studies have shown malignant transformation to be associated with a general up-regulation of cell adhesion molecules (Fogel *et al.*, 2003; Haass *et al.*, 2005; Kuphal *et al.*, 2005). A comparative analysis of integrin expression in different stages of melanoma has identified β_3 (particularly $\alpha_v\beta_3$) integrin expression as one of the most specific markers of the transition of melanoma from the radial growth phase (RGP) to the vertical growth phase (VGP) (Natali *et al.*, 1995; Haass *et al.*, 2005; Sousa & Espreafico, 2008). Increased expression of $\alpha_v\beta_3$ integrin — the classic vitronectin (VN) receptor (Horton, 1997) — is closely associated with increased cell invasion and metastasis. Interestingly, the risk factor for melanoma is also associated with increased levels of VN in the skin (McKeown-Longo & Panetti, 1996), which has been seen in advanced melanoma tumours. Integrin $\alpha_v\beta_3$ has a limited distribution in normal tissues, is absent in normal melanocytes or benign nevi, and is overexpressed in invasive melanomas (Eliceiri & Cheresh, 2000; Baroni *et al.*, 2003). Therefore it is suggested that $\alpha_v\beta_3$ integrin is crucial in the malignant transformation of melanoma cells (Seftor *et al.*, 1999; Mitjans *et al.*, 2000; Boukerche *et al.*, 2000; Jin & Varner, 2004).

Another integrin whose increased expression correlates positively with the degree of dermal invasiveness of melanoma cells is $\alpha_3\beta_1$ integrin (Tsuji, 2004). Expression of $\alpha_3\beta_1$ is likely to influence the malignant behaviour of tumour cells by regulating their motility. Migration and invasion of glioma and melanoma cells on either high-affinity or low-affinity ligands were inhibited by antibodies against $\alpha_3\beta_1$ integrin subunits (Melchiori *et al.*, 1995; Tsuji *et al.*, 2002). It has been shown that $\alpha_3\beta_1$ integrin acts as a *trans*-dominant inhibitor of the functions of the other integrins in mouse keratinocytes (Hodivala-Dilke *et al.*, 1998). This integrin has also been shown to interact with other (non-integrin) surface receptors, in a *cis*-acting manner. One of them is a GPI-linked glycoprotein, a member of the plasmin activator system — uPAR, which is suggested to take part in cancer cell invasion and migration (Tsuji, 2004).

Malignant transformation of cells is generally accompanied by changes in their interactions with extracellular matrix (ECM) proteins in a way that facilitates migration and creates conditions for invasion. The molecular basis of the integrin-ligand interaction is not fully understood, but some experimental evidence suggests that posttranslational events are important determining factors. Although integrin-mediated adhesion to ECM proteins is based on the interaction of specific amino-acid residues, the strength of binding may be modulated by various factors including glycosylation of integrins (Zheng & Hakomori, 2000; Nadanaka *et al.*, 2001; Guo *et al.*, 2002; Seales *et al.*, 2003; Pocheć *et al.*, 2003), the glycosylation status of ECM proteins (Zheng & Hakomori, 2000), as well as carbohydrate–carbohydrate interactions (Ono *et al.*, 2000; Wang *et al.*, 2001). In addition to direct bind-

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Abbreviations: BSA, bovine serum albumin; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; MMP, matrix metalloproteinase; PBS, phosphate-bufferd saline; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene fluoride; RGD, Arg-Gly-Asp sequence; RGP, radial growth phase of melanoma; SDS/PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SW, swainsonine; uPAR, urokinase-type plasminogen activator receptor; VGP, vertical growth phase of melanoma; VN, vitronectin.

ing of complementary carbohydrate motifs, bridging to cross-linked carbohydrate structures or lectins is a common binding mode (Kaltner & Stierstorfer, 1998).

The changes most commonly observed in tumour cell glycoproteins are these: a shift of N-glycans to the highly β1,6-branched type as a result of increased expression of *N*-acetylglucosaminyltransferase V (GlcNAcT-V); the presence of poly-*N*-acetyllactosamine structures; and hypersialylation of cell surface glycoproteins (Guo *et al.*, 2000; 2002; Hakomori, 2002; Gu & Taniguchi, 2004; Siddiqui *et al.*, 2005; Laidler *et al.*, 2006). It is well documented that β1,6-branched N-oligosaccharides are present on integrin subunits including β_1, α_3 and α_v (Lityńska *et al.*, 2002; Chakraborty & Pawelek, 2003; Ochwat *et al.*, 2004; Przybyło *et al.*, 2007; Kremser *et al.*, 2008) and the number of integrin subunits acting as substrates for GlcNAcT-V is apparently correlated with melanoma development and progression (Ochwat *et al.*, 2004; Przybyło *et al.*, 2007). Our previous work showed that $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrins possess cell-line-specific glycosylation patterns in two metastatic melanoma cell lines, WM9 and WM239 (Kremser *et al.*, 2008). Both subunits of $\alpha_3\beta_1$ integrin in WM9 cells originating from lymph node metastasis showed more diverse glycan types than did WM239 cells originating from skin metastasis. In the case of $\alpha_v\beta_3$ integrin it was just the reverse: the glycan pool from WM239 cells was much more diverse than that from WM9 cells. Nevertheless, both integrins showed the presence of tumour-associated glycans regardless of the differences in glycosylation profiles between these integrins in the two cell lines.

The aim of the present study was to analyse the participation of $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrins in cell adhesion and migration on VN in WM9 and WM239 melanoma cell lines. With the use of swainsonine (SW), a specific inhibitor of α-mannosidase II, we also assessed the influence of glycosylation on $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrin function.

MATERIALS AND METHODS

Chemicals. The Immobilon P membrane, rabbit polyclonal antisera against α_3, α_v integrin subunit, mouse monoclonal anti-$\alpha_v\beta_3$ integrin (clone LM609) antibody and vitronectin from human plasma (for adhesion and wound healing assay) were purchased from Millipore (Bedford, MA, USA). Mouse monoclonal anti-$\alpha_3\beta_1$ integrin antibody (clone IA3) was from Abcam (Cambridge, UK). Mouse IgG_1 — negative control and rabbit polyclonal anti-mouse F(ab')2 FITC-conjugated antibody were from Dako (Carpenteria, CA, USA). RPMI 1640 medium with Glutamax-I (RPMIG), bovine serum albumin (BSA), trypsin/EDTA solution, penicillin/streptomycin solution, PMSF, goat anti-mouse AP-conjugated antibody, swainsonine (from *Metarrhizium anisopliae*), GRGDS peptide, high molecular mass standards and protease inhibitor cocktail were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Mouse monoclonal antibody (no. 3937) against human uPAR (CD87) was from American Diagnostica Inc. Phosphate-buffered saline (PBS) was from GibcoBRL™ (Paisley, UK). Sheep anti-rabbit AP-conjugated immunoglobulin, Immunoprecipitation Kit (Protein G), 4-nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate solution were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Calcein AM was from Biotium (Hayward, CA, USA). All remaining chemicals were of analytical grade, commercially available.

Cell lines and culture conditions. Two human melanoma cell lines WM9 from lymph node metastasis, and WM239 from skin metastasis were obtained from the Department of Cancer Immunology, University School of Medical Science at Great-Poland Cancer Center (Poznań, Poland). The cells were cultured in RPMIG 1640 supplemented with 10% foetal calf serum (FCS) and antibiotics (penicillin/streptomycin) in standard conditions.

Cell adhesion assay. Cell adhesion assay was performed in 96-well culture black solid plates (Costar) coated with 0.7 μg/ml VN and blocked with 1% heat-denatured BSA. For some experiments cells were cultured for 24 h in serum-free RPMIG 1640 containing additionally swainsonine (10 μg/ml). After reaching subconfluency, cells were starved in serum-free medium for 30 min, harvested and reseeded at a density of 5×10^4 cells per well in serum-free medium. Cells were allowed to adhere for 30 min at 37 °C in a humidified atmosphere. Non-adherent cells were removed and adherent ones were stained with calcein AM (2 μM) for 30 min. Next, cells were washed with PBS and centrifuged. The fluorescence was measured at 495/520 nm. Non-specific cell adhesion, as measured on BSA-coated wells, was subtracted. All data are given as percentage of fluorescence of 5×10^4 cells, taken as 100%.

For competition experiments, cells were pre-incubated with monoclonal function-blocking antibodies specific for $\alpha_v\beta_3$ or $\alpha_3\beta_1$ integrin subunits, anti-uPAR (all at a concentration of 0.5 μg/well) as well as RGD peptide (0.5 mg/ml) and EDTA (10 mM) on a shaker at 37 °C for 1 h prior to performing adhesion test. Control competition with non-specific IgG_1 (80 μl/ml) was performed (not shown). Changes in adhesion after antibody treatment were calculated by comparing adhesion of untreated (taken as 100% adhesion) and treated cells. All data are given as percentage of adhesion compared to untreated cells (control).

Scrape-wound healing assay. Scrape-wound healing assay was performed in a 6-well culture plate as described in details by (Lityńska *et al.*, 2006). Briefly, cells were grown to confluence on VN-coated surface (0.7 μg/ml), and after aspiration of the medium, the cell-coated surface was scraped with a 200 μl pipette tip in a single stripe. Then the surface was washed twice with RPMIG 1640, covered with medium supplemented with 10% FCS and the wounds were allowed to heal for 24 h at 37 °C.

In order to investigate the participation of integrins and uPAR in this process, in some experiments the wound healing was performed in culture medium containing anti-$\alpha_3\beta_1$ integrin, anti-$\alpha_v\beta_3$ integrin, anti-uPAR antibodies (5 μg/ml), RGD peptide (0.5 mg/ml), EDTA (10 mM) or non-specific IgG_1 (80 μl/ml). Migration of cells into wounded areas was evaluated with an inverted microscope and photographed. The average extent of wound closure was evaluated by multiple measurements of the width of the wound. Ten measurements of three separate trials were made for all these conditions. Changes in cell migration rate after antibody treatment were calculated by comparing migration of untreated (taken as 100% migration) and treated cells. All data are given as percentage of migration compared to untreated cells (control).

Flow cytometric analysis. The expression of integrin subunits was assessed by flow cytometry as previously described by Laidler *et al.* (2000). Briefly, cells

(1×10^5) were fixed in PBS containing 4% paraformaldehyde for 10 min in 4 °C. Next the cells were incubated for 45 min on ice with antibodies against anti-$\alpha_v\beta_3$ integrin (clone LM609, at a concentration of 20 μg/ml), anti-uPAR antibody (mAb 3937, at a concentration of 25 μg/ml) or normal mouse IgG_1 (80 μl/ml) as a negative control. Cells were then washed in PBS and next incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG $(Fab')_2$ fragments (50 μl/ml) for 45 min on ice and washed again. Finally, cells were assessed for fluorescence in a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). A total of 10^4 cells were analyzed for each immunofluorescence profile using the Macintosh Cell Quest program. The analysis of a gated cell population was based on a comparison of the fluorescence intensity of the control and test samples. The results are presented as mean ± standard deviation of three independent experiments.

Immunoprecipitation and immunoblotting. After reaching early confluency the cells, cultured with or without SW, were pelleted by centrifugation and were homogenized on ice in extraction buffer (50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1 mM PMSF and protease inhibitor cocktail) by sonication (Sonies, Vibra-Cell), followed by incubation in the same buffer containing additionally 3% protamine sulphate for 1 h on ice. Finally, cell extracts were clarified by centrifugation at $30\,000 \times g$ for 30 min. Protein concentration was determined in the supernatants according to Peterson (1977). The clarified cell lysate (300 μg of protein) was incubated with 3 μl of Protein G-agarose. After that it was incubated with 2 μl of anti-uPAR antibody, overnight at 4 °C on an orbital rotator. Then the sample was mixed with 15 μl Protein G-agarose and incubated for another 3 h at 4 °C on an orbital rotator. After that the precipitated material was washed three times with buffers (Immunoprecipitation Kit, Roche Diagnostics GmbH). The precipitated proteins were eluted by boiling the Protein G-bound complexes for 10 min in SDS/PAGE sample buffer (Laemmli, 1970) in reducing or non-reducing conditions.

Equal amounts of proteins (1/3 of precipitated material) were separated on 10% SDS/polyacrylamide gels under non-reducing conditions for integrin detection or 12% SDS/polyacrylamide gels under reducing conditions for uPAR detection, according to Laemmli (1970). Following separation, the proteins were electrotransferred to Immobilon P membranes in Tris/Glicyne Buffer (Bio-Rad) containing 20% methanol, for 18 h at constant amperage at 0.1 A with cooling (Bio-Rad). Next, the membranes were blocked in 1% BSA in TBS/Tween (0.02 M Tris/HCl, pH 7.6, containing 150 mM NaCl and 0.1% Tween 20). Afterwards, membranes were incubated for 1 h in 1% BSA in TBS/Tween containing one of the following antibodies, specific for different integrin subunits: α_3, α_v or uPAR (1 : 2000 dilution). After triple wash with TBS/Tween, blots were incubated with alkaline phosphatase-coupled sheep anti-rabbit (1 : 4000 dilution) or goat anti-mouse Ig (1 : 2000 dilution) in 1% BSA in TBS/Tween, for 1 h. The subunits were localised with the use of 4-nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolylophosphate solution. Relative mass was determined using high molecular mass standards.

Statistics. Statistical analysis was performed with the use of Duncan's new multiple range test. A P value less than 0.05 was considered statistically significant.

RESULTS

In the first part of this work we compared adhesion to and migration on VN of two metastatic melanoma cell lines of different origin. The WM239 cells from skin metastasis adhered to VN with a higher efficiency than did WM9 cells from a lymph node. The adhesion levels were 70% for WM239 and 50% for WM9 cells. After 24 h incubation, wound closure was 23% for WM239 and 33% for WM9 cells (Kremser *et al.*, 2008); this seemed consistent with the reverse tendency observed in the adhesion tests.

Cell adhesion and migration assays performed in the presence of EDTA or RGD peptide revealed that interaction of WM9 and WM239 cells with VN was exclusively mediated by integrins in an RGD-dependent manner. The presence of EDTA in the culture medium prevented melanoma cell attachment to VN in the adhesion assay, and caused their detachment from VN-coated surfaces in wound healing tests (not shown). RGD-containing peptide blocked the adhesion and migration completely (not shown). Several lines of evidence have demonstrated that there are a few vitronectin receptors acting in an RGD-dependent manner, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_8\beta_1$ and $\alpha_{IIb}\beta_3$ integrins (Humphries *et al.*, 2008). Flow cytometry data showed $\alpha_v\beta_3$ integrin expression to be cell-line-dependent and high on WM9 (70%) and WM239 (90%) cells (Fig. 2). The level of adhesion seemed related to the level of cell surface expression of this integrin. The antibodies against $\alpha_v\beta_3$ almost completely inhibited the adhesion of both tested cell lines to VN, confirming the role of this integrin as a primary VN adhesion receptor (Fig. 1). At the same time, antibodies against $\alpha_v\beta_3$ integrin did not slow migration completely; it was reduced by 25% (in WM9) and by 70% (in WM239) (Fig. 3), suggesting that $\alpha_v\beta_3$ nevertheless is not the exclusive VN receptor in these cells. Incubation of the melanoma cells with non-specific IgG_1 did not influence the adhesion to or migration on VN (not shown).

It is well known that formation of the uPAR–$\alpha_3\beta_1$ complex leads to adhesion to VN, with uPAR playing a role of VN receptor (Wei *et al.*, 1994; 2001). Our observations suggested that the participation of uPAR in WM9

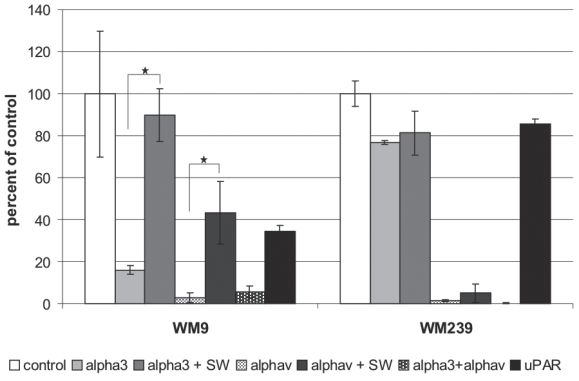

Figure 1. Effect of antibodies against $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrins and uPAR on melanoma cell adhesion to vitronectin (VN)
WM9 and WM239 cells were cultured in the presence or absence of swainsonine (SW) and pretreated with specific antibodies. Then they were allowed to adhere to VN (details in Materials and Methods). Changes in adhesion rate after antibody treatment were estimated by comparing adhesion of untreated (taken as 100% adhesion) and treated cells. All data are given as percentage of adhesion *versus* that of untreated cells (control). Values are means ± standard deviation of three replicates. Asterisk indicates $P < 0.05$.

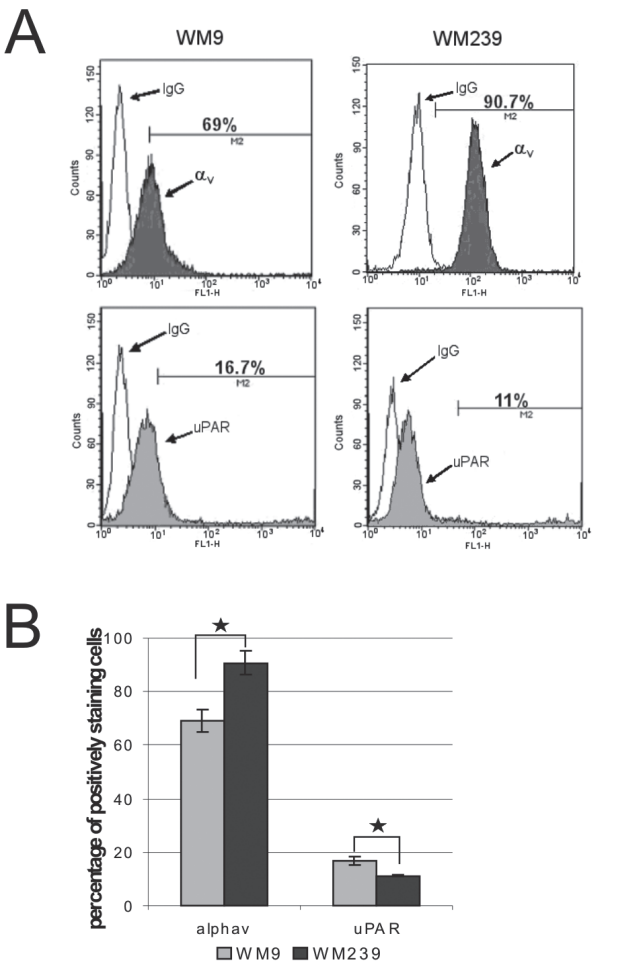

Figure 2. Expression of $\alpha_v\beta_3$ integrin and uPAR on WM9 and WM239 cells

Melanoma cells were examined by flow cytometry for an expression of $\alpha_v\beta_3$ integrin and uPAR, and the data were compared to the results for cells incubated with normal mouse IgG. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG $(Fab')_2$ fragments were used for detection. Fluorescence signals of 10,000 cells were counted and histograms of cell number *versus* log fluorescence were generated. **(A)** Histogram of fluorescence intensity. Grey areas indicate the fluorescence profile of cells after indirect fluorescence staining with anti-$\alpha_v\beta_3$ integrin or anti-uPAR antibodies. Open histograms represent background fluorescence. Relative fluorescence is shown as a logarithmic scale of 4 log cycles on the x-axis, and cell number as a linear scale on the y-axis. **(B)** Quantification of data from flow cytometric analyses. Data from one of three replicates are presented. The negative control for each line is different because the experiments were not run on the same occasion.

and WM239 cell adhesion to VN was negligible in view of the results obtained in the presence of EDTA or RGD peptide. Additionally, the expression level of uPAR on WM9 and WM239 cells was low (16% *vs.* 11%) but differed significantly (Fig. 2). Then we asked whether there was any role for the uPAR–$\alpha_3\beta_1$ or uPAR–$\alpha_v\beta_3$ complex in WM9 and WM239 cell interaction with VN. To probe this we used, instead of function-blocking anti-uPAR antibodies, antibodies directed to the D2-D3 domains of uPAR, where the region interacting with integrins has been described (Degryse *et al.*, 2005; Chaurasia *et al.*, 2006). As an effect, we observed 65% (WM9) and 25% (WM239) reduction of adhesion (Fig. 1), and 65% (WM9) and 15% (WM239) slowing of migration (Fig. 3). Moreover, treatment of the melanoma cells with a function-blocking anti-$\alpha_3\beta_1$ antibody reduced the adhesion efficiency by 85% (WM9) and 25% (WM239) (Fig. 1), and migration speed by 25% (WM9) and 55% (WM239) (Fig. 3). The expression of $\alpha_3\beta_1$ integrin has been estimated previously (Laidler *et al.*, 2000). Both cell lines were found to have similar high levels of this integrin (about 90%). Our results showed that the uPAR–$\alpha_3\beta_1$ as well as the uPAR–$\alpha_v\beta_3$ complex participated in the WM9 and WM239 melanoma cell interaction with VN, but the observed effect was cell-line-specific.

The molecular basis of the receptor-ligand interaction during adhesion is not fully understood. The physical interaction of uPAR with integrins is not constitutive, and has been shown to be strongly promoted by integrin-activating agents as well as urokinase (Wei *et al.*, 2001). Integrin glycosylation represents a modification by which their specificity and affinity is modulated.

We performed competition experiments on melanoma cells cultured in medium additionally containing swainsonine, a specific inhibitor of glycosylation preventing the formation of complex type N-glycan, including β1,6-branched oligosaccharides. We used it in order to estimate whether this type of glycosylation of $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrins influences their interaction with VN or their ability to complex with uPAR. After SW treatment, anti-$\alpha_3\beta_1$ antibody reduced WM9 cell adhesion by only 10% in comparison with 65% without SW, and anti-$\alpha_v\beta_3$ integrin antibody by 55%, while without SW the blockage was complete. In WM239 cells, the SW treatment did not influence the blocking effect of any of these antibodies (Fig. 1). We used immunoprecipitation to examine the influence of SW treatment on formation of the complex between uPAR and integrins. Clarified lysates of WM9 and WM239 cells cultured with or without SW were precipitated with antibodies against uPAR. Next, the proteins recovered after precipitation were electrophoresed under reducing or non-reducing conditions, blotted onto a PVDF membrane and probed with antibodies against uPAR, α_3 and α_v integrin subunits. Immunodetection clearly demonstrated that the presence of SW in culture medium prevented the formation of uPAR–α_v or –α_3 integrin complexes in both analysed melanoma cell lines (Fig. 4). That may suggest the participation of glycosylation in this process. Our previous study showed different patterns of glycosylation of both analysed integrins in these cell lines. This may explain the observed differences in the formation of the integrin–uPAR complexes and their role in adhesion and migration processes in the cell lines analysed.

DISCUSSION

There is extensive evidence indicating that changes in the N-glycosylation profile of integrins are related to malignant transformation of cancer cells. In particular, extensive β1,6-branching of N-glycans is a well-accepted hallmark of tumour malignancy. The carbohydrate moieties of cell surface glycoproteins are known to play an important role in cell adhesion and migration (Zhang *et al.*, 2004), while aberrant N-glycosylation has been associated with tumour cells' altered adhesiveness to ECM proteins and increased cell motility/invasiveness (Bellis, 2004).

Multimeric vitronectin is one of the glycoproteins that play key roles in the cellular adhesion and migration of a variety of cell types. Melanoma cells can deposit VN into the ECM. It is well established that VN is recognized by several cell surface receptors including integrins, uPAR and proteoglycans (Hall, 1999). During malignant transformation, melanoma cells start to overexpress $\alpha_v\beta_3$ integrin; this may be their

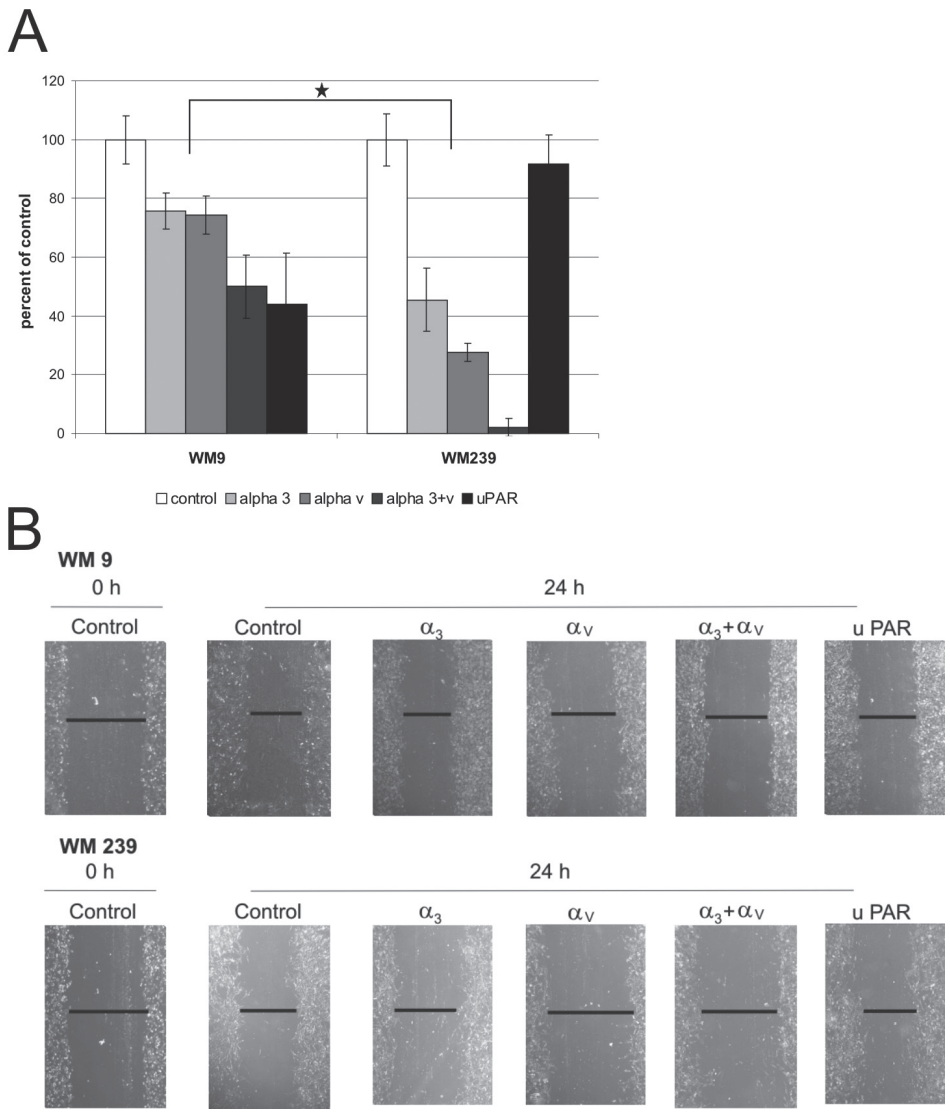

Figure 3. Effect of antibodies against $\alpha_3\beta_1$, $\alpha_v\beta_3$ integrins and uPAR on repair of scratch wounds in monolayers of WM9 and WM239 cells

A line was scratched with a plastic pipette tip through confluent monolayer of cells maintained on VN-coated surface. The scratch-wounded cultures were allowed to heal for 24 h in the presence of specific antibodies. **(A)** Extent of wound closure, quantified by measuring the width of the wound. Width was measured at several locations of the wound, and the mean value was compared with the width of the original wound (0 h). Changes in migration rate after antibody treatment were calculated by comparing the migration of untreated (taken as 100% migration) and treated cells. All data are given as percentage of migration *versus* that of untreated cells (control). Values are means ± standard deviation of three replicates. Asterisk indicates $P < 0.05$. **(B)** Panels show migration of cells into the wounded area in the presence of specific antibodies after 24 h.

mechanism for escaping apoptosis. Ligation of $\alpha_v\beta_3$ with VN triggers signals which help them remain alive during migration steps as well as in an environment of newly colonized organs. Earlier it was demonstrated that WM9 and WM239 cells differ in their interactions with fibronectin (Lityńska *et al.*, 2006). We were interested in their interactions with VN, since both cell lines showed a high level of $\alpha_v\beta_3$ expression as determined by flow cytometry. In agreement with the previous observations, the participation of $\alpha_v\beta_3$ in WM9 and WM239 melanoma cell attachment to and migration on VN was confirmed. The anti-$\alpha_v\beta_3$ antibody almost completely inhibited the adhesion of WM9 and WM239 cells to VN. This antibody also affected the migration of both cell lines on VN, but the effect was more pronounced in WM239 cells. Moreover, experiments with RGD peptide showed complete blockage of cell adhesion to and migration on VN, confirming that this is an integrin-dependent interaction.

A large body of data on cell migration on VN indicates that in most instances cell motility is dependent on the action of $\alpha_v\beta_3$ (Felding-Habermann *et al.*, 2002). Interestingly, work by Aznavoorian *et al.* (1996) showed haptotactic and chemotactic responses to VN to be transmitted by distinct $\alpha_v\beta_3$ integrin-dependent signaling pathways in the human melanoma cell line A2058. A blocking antibody to $\alpha_v\beta_3$ integrin completely inhibited VN-stimulated chemotaxis, while haptotaxis was inhibited by only 50%. In our present study, migration of both cell lines on VN was not completely blocked by the anti-$\alpha_v\beta_3$ antibody. That finding implicates other receptors in this process. We showed that one of them was $\alpha_3\beta_1$ integrin in complex with uPAR. The functional blocking anti-$\alpha_3\beta_1$ antibody affected the tested cells' adhesion to and migration on VN, but the effect was cell-line-specific, indicating that this receptor works *via* different mechanisms in these cells. In adhesion experiments

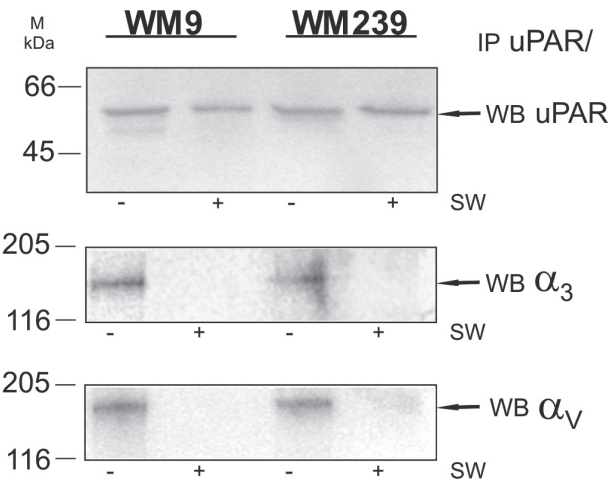

Figure 4. Coimmunoprecipitation of uPAR and integrins in WM9 and WM239 cell extracts cultured in the presence or absence of SW

Upper panel shows material immunoprecipitated with anti-uPAR antibody, separated by SDS/PAGE under reducing conditions and blotted for uPAR using a specific anti-uPAR antibody. Lower panels show immunodetection of α_3 and α_v integrin subunits in material obtained after precipitation with antibodies against uPAR.

the effect of inhibition by anti-$\alpha_3\beta_1$ antibody was more pronounced in WM9 cells, suggesting that in this cell line the binding specificity of this integrin was broadened.

There is considerable evidence linking uPAR expression with increased aggressiveness in diverse human tumour types (Blasi, 1999), including melanoma (Besch *et al.*, 2007). Our present study showed uPAR expression to be low in both analysed cell lines. The relatively low expression of uPAR in WM9 and WM239 cells may have been the result of downregulation of uPAR transcription by high expression of $\alpha_v\beta_3$ integrin on these cells, as Hapke *et al.* (2001) observed. Several lines of evidence have confirmed that $\alpha_v\beta_3$ and uPAR influence each other's expression and function in VN adhesion and migration (Tarui *et al.*, 2001; Khatib *et al.*, 2001), although a physical interaction in the cell membrane was reported mainly between uPAR and $\alpha_3\beta_1$ (Wei *et al.*, 2001). Their functional association was confirmed by the observation that an antibody against $\alpha_3\beta_1$ integrin blocked uPAR-mediated adhesion to VN (Zhang *et al.*, 2003). Wei *et al.* (1994) showed that human embryonic kidney cell line 293 adhered to VN in a uPAR-dependent manner: this interaction was not blocked by EDTA or RGD peptides (Wei *et al.*, 1994). Pre-incubation of those cells with anti-$\alpha_3\beta_1$ antibody strongly inhibited their adhesion to VN. In our study we showed that in both cell lines the cell–VN interaction was integrin-dependent. We also confirmed the role of uPAR in this interaction, but rather as a partner of both analysed integrins than the VN receptor itself. Interestingly, however, WM9 cells, which expressed a slightly higher level of uPAR, migrated on VN faster than WM239 cells in the wound healing assay. The same tendency was found in wound healing assays on other surfaces — plastic and fibronectin-coated plates — where the motility of WM9 cells was about twice that of WM239 cells (Lityńska *et al.*, 2006). The invasiveness of WM9 cells has also been determined to be about twice that of WM239 cells by a Matrigel invasion chamber assay (Ciołczyk-Wierzbicka *et al.*, 2004). WM9 cells also showed higher matrix metalloproteinase and uPA activity (not shown). In the present study the use of specific anti-$\alpha_3\beta_1$ and anti-uPAR antibodies resulted in an inhibition of adhesion and migration on VN suggesting that $\alpha_v\beta_3$ is not the exclusive VN receptor in these cells. The stronger inhibition observed in WM9 cells comparing to WM239 cells indicates a more important role of uPAR–integrins complex formation in the interaction of WM9 cells with VN.

In our previous study we showed that $\alpha_3\beta_1$ integrin carries more diverse glycans in WM9 than in WM239 cells, and that these cells have a better efficiency of migration on VN; this migration was delayed in the presence of PHA-L, confirming the role of β1,6-branched N-oligosaccharides in migration processes. At the same time, the WM239 cell migration was not affected by the presence of lectins (SNA, MAA, PHA-L) even though the primary VN receptor — $\alpha_v\beta_3$ integrin — was more variously glycosylated in this cell line than $\alpha_3\beta_1$ was (Kremser *et al.*, 2008). To further analyse the influence of glycosylation on integrin function we used swainsonine, a specific inhibitor of α-mannosidase II leading to the synthesis of shorter and less branched oligosaccharides (Dorling *et al.*, 1980; Tulsiani *et al.*, 1982). We analyzed the contribution of glycans in cocapping of uPAR and integrin $\alpha_3\beta_1$ and $\alpha_v\beta_3$ by immunoprecipitation with anti-uPAR antibody in cell lysates after SW treatment. In both cell lines SW prevented coimmunoprecipitation of both $\alpha_3\beta_1$ and $\alpha_v\beta_3$ with uPAR. Our results are in agreement with those of Gellert *et al.* (2004). Investigating the cocapping of α_v integrin and uPAR, they demonstrated that the interaction on the cell surface was inhibited by *N*-acetyl-D-glucosamine. This showed the formation of this complex to be dependent on glycosylation of both proteins.

Additionaly, WM9 cells pretreated with SW showed less inhibition of adhesion by anti-$\alpha_3\beta_1$ as well anti-$\alpha_v\beta_3$ antibodies while in WM239 cells the effect was not observed.

In conclusion, concerning the different glycosylation patterns observed in both analysed cell lines, and in view of our present observations on adhesion as well as the immunoprecipitation experiments, it is very likely that glycosylation of $\alpha_3\beta_1$ as well $\alpha_v\beta_3$ is also important for their cocapping with uPAR and therefore may influence their interaction with VN.

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