

## Aberrant Glycosylation of $\alpha_v\beta_3$ Integrin is Associated with Melanoma Progression

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**Abstract.** *N-glycosylation of integrins plays an important role in cancer progression. Increased  $\alpha_v\beta_3$  integrin expression during melanoma progression is well-documented but the role of its glycans in tumorigenesis is still poorly understood. In the present study we used the WM793 primary melanoma cell line and its highly metastatic variant, WM1205Lu, to examine  $\alpha_v\beta_3$  glycosylation. Lectin precipitation, enzyme digestion and the use of swainsonine (SW) showed that  $\alpha_v\beta_3$  integrin glycosylation differs significantly between primary and metastatic melanoma cells. High-mannose structures and complex glycans with bisecting N-acetylglucosamine (GlcNAc) were more abundant in both subunits of primary cells. We also observed a shift in the sialylation of  $\alpha_v\beta_3$  integrin related to reduction of  $\alpha$ 2-6-linked sialic acid expression and an increase of  $\alpha$ 2-3 sialylation of both subunits in melanoma progression. Metastatic melanoma migration on vitronectin (VN) was reduced in the presence of antibody against  $\alpha_v\beta_3$  and the lectins phytohemagglutinin-L (PHA-L), Sambucus nigra agglutinin (SNA) and Maackia amurensis (MAA) in woundhealing assays. Our results show that the acquisition of metastatic competence by melanoma cells is accompanied by alteration of  $\alpha_v\beta_3$  integrin glycosylation and that both  $\alpha_v\beta_3$  and  $\beta$ 1-6-branched sialylated complex-type N-glycans promote metastatic melanoma migration on VN.*

Aberrant glycosylation of integrins is closely associated with the metastatic competence of different cancers (1, 2), including melanoma (3, 4). Integrin  $\alpha_v\beta_3$  is highly N-glycosylated during post-translational processing; the  $\alpha_v$

subunit possesses 13 potential sites of asparagine-linked (N-linked) glycosylation and the  $\beta_3$  subunit possesses 6 of them (5). Integrin  $\alpha_v\beta_3$  is an adhesion protein important in tumor angiogenesis, metastasis and inflammation.  $\alpha_v\beta_3$  is the main vitronectin (VN) receptor but it also binds other extracellular proteins, fibronectin, fibrinogen, laminin, von Willebrand factor and collagens (3, 6). It probably is the most prominent surface receptor up-regulated in different tumor types and it is, thus, considered to be one of the most important prognostic biomarkers of cancer (7). Humanized monoclonal antibodies against  $\alpha_v\beta_3$  have been tested on mice and also in clinical trials as anticancer drugs to inhibit tumor growth, angiogenesis and metastasis (8).

Melanoma is one of the most common and most aggressive human cancers. Acquisition of metastatic competence by melanoma cells growing in dermis as the vertical growth phase (VGP) is generally an irreversible step of melanoma progression. Most therapies are ineffective when melanoma is diagnosed at that stage. The formation of metastatic lesions in distinct organs is the leading cause of death among melanoma patients. The search for effective ways to inhibit propagation of melanoma demands a comprehensive examination of the changes occurring during the transition from VGP to metastatic lesions. Glycosylation is an essential process in the contact of cells with extracellular matrix proteins (ECM). Changes in the N-oligosaccharide composition of integrins can modify the adhesive interactions of tumor cells with the surrounding environment, thus facilitating their spread. Increased  $\alpha_v\beta_3$  integrin expression during melanoma progression is well documented (9-11) but the role of its glycans in tumorigenesis is still poorly understood. In the present study we analyzed  $\alpha_v\beta_3$  integrin glycosylation in the last two stages of melanoma progression. For this, we chose two genetically-related human melanoma cell lines: WM793 primary cells derived from early VGP and WM1205Lu cells from lung metastasis. Our results show that the acquisition of metastatic competence by melanoma cells is accompanied by alteration of  $\alpha_v\beta_3$  integrin N-glycosylation. High-

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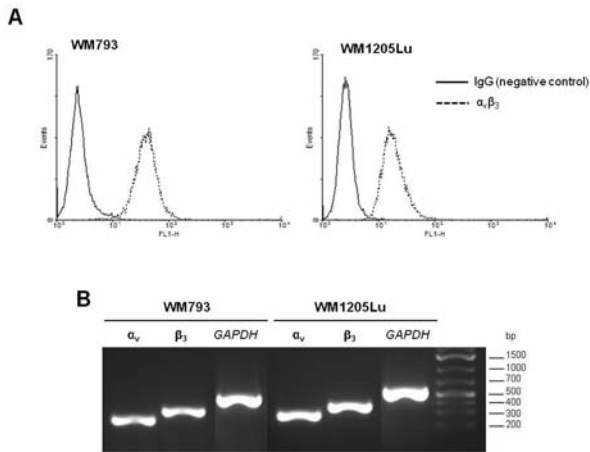


Figure 1. Primary WM793 and metastatic WM1205Lu melanoma cells show similarly high expression of  $\alpha_v\beta_3$  integrin on protein (A) and gene (B) levels. (A) Cells were trypsinized and incubated with anti- $\alpha_v\beta_3$  primary monoclonal antibody (MAB1976), followed by incubation with the secondary antibody conjugated with FITC. Integrin  $\alpha_v\beta_3$  expression was determined by flow cytometry, removing nonspecific fluorescence relative to the IgG control. (B) Total RNA (1  $\mu$ g) was reverse-transcribed and cDNA was amplified by RT-PCR. The PCR products or  $\alpha_v$ ,  $\beta_3$  and GAPDH (endogenous control) were resolved on 2% agarose gel containing ethidium bromide.

mannose oligosaccharides and sialylated complex-type N-glycans with bisected N-acetylglucosamine (GlcNAc) undergo change in WM1205Lu metastatic cells. We also report that both  $\alpha_v\beta_3$  integrin and sialylated complex-type oligosaccharides promote only metastatic and not primary melanoma migration on VN.

**Materials and Methods**

**Cell lines and reagents.** VGP WM793 and highly metastatic WM1205Lu human melanoma cell lines were obtained through participation in the ESTDAB project (the European Searchable Tumour Cell-Line Database; IPD-ESTDAB: <http://www.ebi.ac.uk/ipd/estdab/>).

Polyclonal primary antibodies used in western blotting against  $\alpha_v$  (AB1930) and  $\beta_3$  (AB1932) integrin subunits were obtained from Millipore (Chemicon, Temecula, CA, USA). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (7074S) was purchased from Cell Signaling Technology (Danvers, MA, USA). Alkaline phosphatase-conjugated sheep anti-rabbit IgG (AP322A) for Western blotting and mouse anti- $\alpha_v\beta_3$  (MAB1976, clone LM609) used in flow cytometry, wound-healing assays and confocal microscopy were obtained from Millipore (Chemicon). FITC-conjugated rabbit anti-mouse IgG, F(ab')<sub>2</sub> (F0313) and mouse IgG1 (X0931), used as a negative control in flow cytometry, were obtained from Dako (Glostrup, Denmark) and Cy3-conjugated goat anti-mouse (115-166-003) from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Biotinylated lectins (GNA, B-1245; PHA-L, B-1115; PHA-E, B-1125; DSA, B-1185; LEL, B-1175; SNA, B-1305; MAA, B-1265; AAA, B-1395) and agarose-

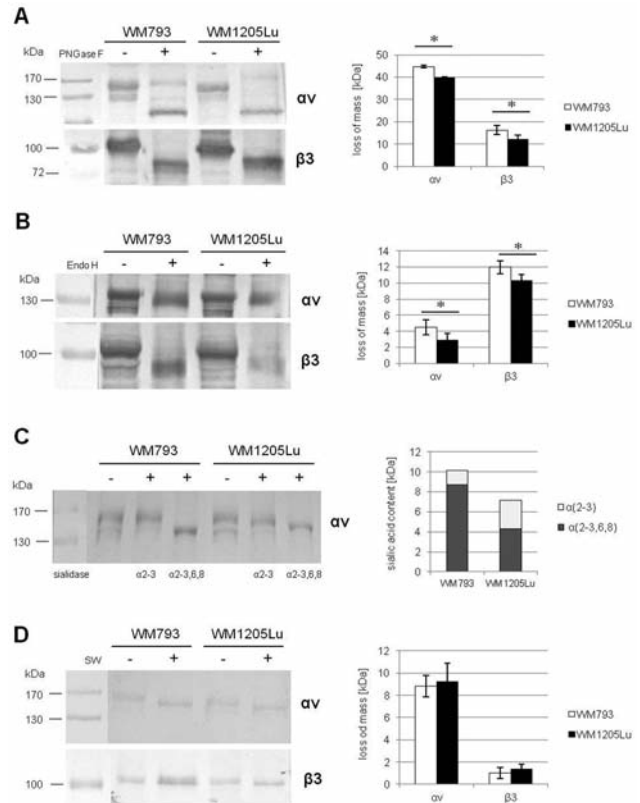


Figure 2. Glycosylation of  $\alpha_v\beta_3$  integrin differs between primary and metastatic melanoma cells in the amount of (A) total N-glycans, (B) high-mannose and hybrid structures and (C) sialic acids but not (D)  $\beta$ 1-6-branched structures. (A) N-glycans were released from glycoproteins using PNGase F from *Flavobacterium meningosepticum*, (B) high-mannose and hybrid type glycans using Endo H from *Streptomyces plicatus*, (C)  $\alpha$ 2-3-linked sialic acids using *Streptococcus pneumoniae* sialidase and  $\alpha$ 2-3,6,8-linked sialic acids using *Arthrobacter ureafaciens* sialidase; (D) synthesis of complex-type glycans, including  $\beta$ 1-6-branched glycans, was blocked using swainsonine (SW) from *Metarrhizium anisopliae*. The amount of glycans in  $\alpha_v$  and  $\beta_3$  integrin subunits was calculated based on loss of molecular mass in SDS-PAGE. Values are means $\pm$ SD of three independent experiments, asterisked values differ significantly between primary and metastatic cells at  $p < 0.05$  by Duncan's test.

bound streptavidin (SA-5010) were obtained from Vector Labs (Burlingame, CA, USA). Vitronectin (CC080) was purchased from Millipore (Chemicon). Swainsonine (SW) isolated from *Metarrhizium anisopliae* (S8195) was purchased from Sigma-Aldrich (St. Louis, MO, USA). N-glycosidase F (PNGase F) isolated from *Flavobacterium meningosepticum* (11 365 181 001) and endo- $\beta$ -N-acetylglucosaminidase H (Endo H) from *Streptomyces plicatus* (11 088 726 001) were obtained from Roche (Mannheim, Germany), *Streptococcus pneumoniae* sialidase (N7271) from Sigma-Aldrich and *Arthrobacter ureafaciens* sialidase (GK80040) from Glyko (Prozyme, Hayward, CA, USA).

**Cell culture conditions and cell lysate preparation.** The cells were grown in RPMI1640 medium (Invitrogen, Paisley, UK) supplemented with 10% FBS (Gibco, Paisley, UK), 100 U/ml

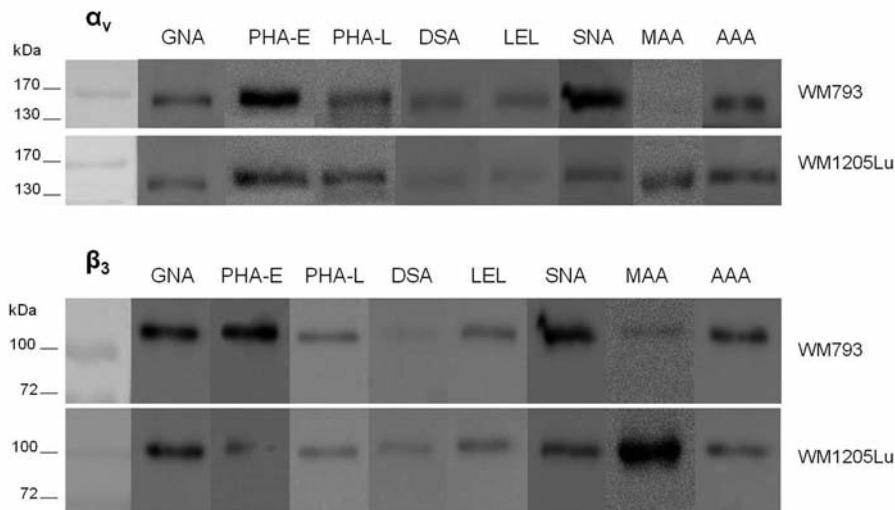


Figure 3. The content of high-mannose glycans and sialylated complex-type structures with bisecting GlcNAc in  $\alpha_v\beta_3$  integrin changes during acquisition of the metastatic phenotype. Cell lysate glycoproteins (100  $\mu$ g) were incubated with biotinylated lectins GNA, PHA-L, PHA-E, DSA, LEL, SNA, MAA and AAA, follow by precipitation using agarose-bound streptavidin. Glycoproteins released by boiling in Laemmli sample buffer were destined for  $\alpha_v$  and  $\beta_3$  integrin subunit immunodetection.

penicillin and 100  $\mu$ g/ml streptomycin (Sigma-Aldrich) at 37°C in a CO<sub>2</sub> incubator (Lab Line Instruments, Melrose Park, IL, USA). Cells were harvested upon 70-80% confluency using 0.25% trypsin solution containing 0.02% EDTA (T3924; Sigma-Aldrich). For some experiments the cells were cultured in the presence of swainsonine (10  $\mu$ g per 1 ml medium) for 24 h.

The cells were lysed in RIPA buffer (89900; Thermo Scientific, Rockford, IL, USA) containing protease inhibitor cocktail (P2714; Sigma-Aldrich). The mixture was centrifuged (15,000 rpm) for 30 min at 4°C. The protein concentration was quantified in the collected supernatant using the Total Protein Kit, Micro Lowry, Peterson's Modification (TP0300; Sigma-Aldrich) to enable comparison of equal total protein amounts between samples of the two cell lines.

**Reverse transcription-polymerase chain reaction (RT-PCR) for  $\alpha_v\beta_3$  integrin expression.** Semiquantitative RT-PCR was used to assess  $\alpha_v$  (ITGAV) and  $\beta_3$  (ITGB3) gene expression. RNA was extracted from WM793 and WM1205Lu cells using the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) and reverse transcription was carried out using the Omniscript RT kit (Qiagen) according to the manufacturer's instructions. PCR was performed using Taq PCR Master Mix (Qiagen) in a DNA Engine PTC-200 thermocycler (MJ Research, Watertown, MA, USA). Each reaction consisted of 35 cycles of denaturation (1 min at 94°C), primer hybridization (1 min at 59°C for both  $\alpha_v$  and  $\beta_3$  and 59.6°C for *GAPDH*) and elongation (2 min at 72°C). The reaction began from an initial denaturation (3 min at 94°C) and the final step was elongation (10 min at 72°C). The expression of  $\alpha_v$  and  $\beta_3$  integrin subunits was normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) used as housekeeping gene. The primer sequences were as follows: GACTGTGTGGAAGACAATGTCTGTAAACCC ( $\alpha_v$  forward), CCAGCTAAGAGTTGAGTTCAGCC ( $\alpha_v$  reverse), CTGGTGTTTACCACTGATGCCAAG ( $\beta_3$  forward), TGTTGA GGCAGGTGGCATTGAAGG ( $\beta_3$  reverse), 5'-CCACCCATGGC

AAATTCATGGCA-3' (*GAPDH* forward), and 5'-TCTAGACGGC AGGTCAGGTCCACC-3' (*GAPDH* reverse).

The PCR amplification products were resolved on 2% agarose gel containing ethidium bromide and were visualized in a transilluminator (UVItec, Cambridge, UK). The amplification product lengths were 305 bp for  $\alpha_v$ , 393 bp for  $\beta_3$  and 599 bp for *GAPDH*.

**Flow cytometry.** The cells were harvested by trypsinization and washed in cold PBS. Then the cells were incubated 45 min with mouse antibody against the  $\alpha_v\beta_3$  complex (1:100), followed by incubation with polyclonal rabbit anti-mouse antibody conjugated with FITC (1:10) for 45 min. After both incubations, the cells were centrifuged (1,100 rpm, 5 min, 4°C) and washed with cold PBS. Stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) using the CellQuest software (BD Biosciences, San Diego, CA, USA). The expression level of  $\alpha_v\beta_3$  integrin was determined relative to nonspecific binding of mouse IgG1 (negative control).

**Enzymatic digestion of glycans.** The N-oligosaccharides were released from the glycoproteins with peptide-N-glycosidase F from *Flavobacterium meningosepticum* (11 365 181 001; Roche). The cell extract proteins were denatured 3 min in 50 mM sodium phosphate buffer, pH 7.5, containing 5% SDS. After cooling to RT, SDS was neutralized with 10% nonidet P-40 (NP-40). Glycans were digested overnight at 37°C using 0.5 mU PNGase F per 15  $\mu$ g glycoproteins.

The content of high-mannose and hybrid glycans in integrin subunits was determined using Endo H from *Streptomyces plicatus*. The cell glycoproteins were suspended in sodium phosphate buffer, pH 5.5, to obtain the 50 mM final buffer concentration. Protein denaturation was carried out in the presence of 2% SDS for 3 min. After cooling to RT, 25 mU Endo H per 15  $\mu$ g glycoproteins was added and the samples were incubated overnight at 37°C.

The cell extract proteins (15 µg) were treated with *Streptococcus pneumoniae* α2-3 sialidase and *Arthrobacter ureafaciens* sialidase, which specifically removes α2-3,6,8-linked sialic acids. Glycoproteins were digested in the incubation buffers (50 mM sodium phosphate buffer, pH 6.0, for α2-3 sialidase; 50 mM sodium acetate, pH 5.0, for α2-3,6,8 sialidase) at 37°C overnight.

In each of the above cases, the reaction was stopped by heating at 100°C for 10 min after overnight incubation at 37°C. Both digested and nondigested samples were boiled in Laemmli sample buffer (LSB, 161-0737; Bio-Rad, Hercules, CA, USA) prior to SDS-PAGE on 10% polyacrylamide gels. Glycan amounts were calculated based on loss of molecular mass in UVImap Image Quantification software (UVItec).

**Lectin precipitation.** Cell lysate proteins (100 µg) were incubated with 4 µl biotinylated lectins (GNA, PHA-L, PHA-E, DSA, LEL, SNA, MAA, AAA) in HEPES buffer (10 mM HEPES, pH 7.5, containing 150 mM NaCl, 0.1 mM CaCl<sub>2</sub> and 0.01 mM MnCl<sub>2</sub>). After overnight incubation at 4°C, the glycoprotein-lectin complexes were precipitated using 20 µl agarose-bound streptavidin for 3 h at 4°C. After centrifugation (14,500 rpm, 5 min, RT), the supernatants from lectin-treated lysates were removed. The precipitates were washed three times in HEPES buffer and once in PBS and glycoproteins were released from the glycoprotein-lectin-agarose complexes by boiling in LSB at 100°C for 10 min. After final centrifugation, the supernatants were collected and destined for integrin subunit immunodetection.

**Western blotting.** Glycosylation of integrin subunits was analyzed by Western blotting. Equal amounts of proteins (15 µg) in LSB (for the β<sub>3</sub> subunit, 5% β-mercaptoethanol was added) were separated on 10% SDS-PAGE gel, transferred to a PVDF membrane and detected using the appropriate antibodies. After blocking in 1% BSA in TBST, the membranes were incubated for 1 h at RT with polyclonal rabbit anti-α<sub>v</sub> and anti-β<sub>3</sub> antibodies diluted 1:1000 in 1% BSA in TBST. Then, goat anti-rabbit IgG was applied in a 1:4000 dilution, followed by incubation at RT for 1 h. The secondary anti-rabbit antibodies were conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP) and the integrin subunits were visualized by colorimetric reaction and chemiluminescence, respectively. Colorimetric visualization was done by incubating the membranes with BCIP and NBT substrates for AP (Roche) and chemiluminescence using Western blotting luminol chemiluminescence reagent (Santa Cruz Biotech.) in the Gnome System (Syngen, Cambridge, UK).

**Cell adhesion assay.** Cell adhesion abilities were tested as described previously by Janik *et al.* (12) with minor modifications. A 96-well plate was coated with vitronectin (VN, 0.1 µg/ml PBS) and left overnight at 4°C. Control cells and cells treated with swainsonine (10 µg/ml) 24 h before the assay were starved in serum-free RPMI 30 min before collecting by trypsinization. The cells (5×10<sup>4</sup>) were seeded into wells coated with VN and allowed to adhere for 1 h at 37°C. After removal of nonadhering cells, adhering cells were stained with calcein for 20 min. Fluorescence was measured at 495/520 nm with a microtiter plate reader (Infinite F200 Pro; Tecan, Grödig, Austria). Nonspecific binding of cells to BSA-coated wells was subtracted.

**Confocal microscopy.** Cells were seeded on VN-coated (0.1 µg/ml PBS) or uncoated coverslips overnight at 4°C. Before staining, the cells were washed three times with PBS, fixed in 2% PFA for 20 min

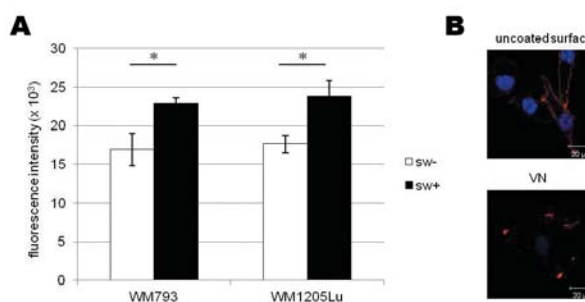


Figure 4. Melanoma cells bind strongly to vitronectin (VN). (A) Both WM793 primary and WM1205Lu metastatic cell adhesion to VN is affected by β1-6-branched N-glycans as determined with the use of swainsonine (SW). Values are means±SD of three repetitions, and differ significantly between treated and untreated cells at p<0.05 by Duncan's test. (B) Integrin α<sub>v</sub>β<sub>3</sub> clusters in focal adhesions of melanoma cells adhering to VN, unlike dispersed expression on the whole membrane of cells growing on an uncoated surface.

at RT and incubated with monoclonal antibodies against α<sub>v</sub>β<sub>3</sub> integrin (1:10 in 2% BSA in PBS) for 45 min, followed by Cy3-conjugated goat anti-mouse antibody (1:100 in 2% BSA in PBS) for 45 min at RT. The slides were mounted with Vectashield media with DAPI, the coverslips were sealed and were kept at 4°C. Cell fluorescence was visualized using a Zeiss confocal microscope (Jena, Germany) and analyzed in an LSM Image Browser (Zeiss, Jena, Germany).

**Wound-healing assay.** Cells were seeded onto a 6-well plate precoated overnight at 4°C with VN. Three scratches were made in the cell monolayer of each well with a yellow pipette tip. Migrating cells were incubated 24 h in the presence of antibodies against α<sub>v</sub>β<sub>3</sub>, lectins PHA-L, PHA-E, SNA and MAA, as well as 1-deoxymannojirimycin (dMM), an inhibitor of N-glycan synthesis that induces accumulation of oligomannose-type chains. The wound sites were photographed in 10 random fields using the Zeiss AxioVision Rel.4.8 image analysis software. The average extent of wound closure was determined by measuring the width of the wound at the starting point and after 24 h.

**Statistical analyses.** The results are given as means of three measurements±standard deviation (SD). The Duncan's test was used to compare mean values. p-Values<0.05 were considered significant.

## Results

**Expression of α<sub>v</sub>β<sub>3</sub> integrin on gene and protein levels.** In this study we evaluated α<sub>v</sub>β<sub>3</sub> integrin expression on gene and protein levels. The expression of α<sub>v</sub> (*ITGAV*) and β<sub>3</sub> (*ITGB3*) genes was normalized to *GAPDH* housekeeping gene expression in semiquantitative RT-PCR. We did not observe differences in α<sub>v</sub> and β<sub>3</sub> subunit expression between primary WM793 and metastatic WM1205Lu cells (Figure 1B). Monoclonal antibody against the α<sub>v</sub>β<sub>3</sub> complex was used to detect its cell surface expression. Flow cytometry analysis showed comparatively high expression of integrin in both





by  $\alpha$ 2-3, respectively. We also estimated melanoma sialylation using bacterial sialidases that catalyze the hydrolysis of terminal sialic acids linked by  $\alpha$ 2-3 and  $\alpha$ 2-3,6,8. Lectin precipitation and neuraminidase digestion showed differences in the expression of sialic acids between primary and metastatic melanoma cells. Treatment of cell lysate proteins with  $\alpha$ 2-3 sialidase prior to SDS-PAGE caused different mobility shifts of the  $\alpha$ <sub>v</sub> subunit in the melanoma cell lines due to differences in their amounts of sialic acids (Figure 2C). Subunit  $\alpha$ <sub>v</sub> expressed in WM1205Lu metastatic cells possessed more  $\alpha$ 2-3-linked sialic acids than in WM793 primary melanoma cells but total sialylation was significantly higher in the  $\alpha$ <sub>v</sub> integrin subunit in WM793 primary cells than in the WM1205Lu metastatic cell line. It was not possible to determine  $\alpha$ 2-3-linked sialic acid in the  $\beta$ <sub>3</sub> subunit by this method because the shift in this subunit's mobility was too small.

Immunodetection of both subunits in MAA precipitates showed that  $\alpha$ 2-3-linked sialic acids recognized by MAA were much more abundant in  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> integrin from metastatic than from primary cells. Sialic acids occupying terminal  $\alpha$ 2-6 linkages detected by SNA lectin were observed mainly in  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> integrin from WM793 primary cells (Figure 3). The results of MAA lectin precipitation correspond to *S. pneumoniae* sialidase-induced  $\alpha$ 2-3 desialylation of the  $\alpha$ <sub>v</sub> subunit. Both methods showed higher content of  $\alpha$ 2-3-bound sialic acids in WM1205Lu metastatic cells than in WM793 primary cells. We could not determine sialic acid content in the  $\beta$ <sub>3</sub> subunit by neuraminidase digestion because the differences in electrophoretic mobility between digested and nondigested proteins were too small.

**Bisecting GlcNAc and  $\beta$ 1-6-branched N-glycans.** We analyzed the expression of the bisecting GlcNAc (N-acetylglucosamine) residue  $\beta$ 1-4-linked to  $\beta$  Man of the N-glycan core in  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> integrin by precipitation with *Phaseolus vulgaris* erythroagglutinin (PHA-E). Both subunits of primary melanoma were precipitated in higher amounts than in the metastatic line (Figure 3).

The Golgi  $\alpha$ -mannosidase II inhibitor swainsonine (SW) was used to assess the amount of complex-type glycans, among them  $\beta$ 1-6-branched GlcNAc in  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> integrin subunits. Immunodetection of both subunits did not show differences in motility shifts as calculated by subtracting the molecular mass of SW-treated and untreated proteins (Figure 2D). This result was verified by precipitation with phytohemagglutinin-L (PHA-L) lectin, which specifically recognizes  $\beta$ 1-6-branched complex-type oligosaccharides. The values for PHA-L precipitates in both subunits confirm the results obtained with the use of SW (Figure 3). The intensity of the bands for the  $\alpha$ <sub>v</sub> and  $\beta$ <sub>3</sub> subunits did not differ in intensity between primary and metastatic cells.

**Fucosylation and polylactosamine structures.** Precipitation with *Aleuria aurantia* lectin (AAA), specific for  $\alpha$ 1-6 Fuc, did not show differences in the content of this sugar residue in  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> integrin between the two melanoma cell lines. Binding of integrin subunits by AAA was similar in WM793 and WM1205Lu cells (Figure 3).

To detect glycans with lactosamine repeats GlcNAc $\beta$ (1-4)Gal in integrin subunits, for precipitation we used two lectins with different specificity, *Datura stramonium* lectin (DSA) and *Lycopersicon esculentum* lectin (LEL), followed by immunodetection to estimate the amount of 2-3 and  $\geq$ 3 lactosamine units, respectively. The two cell lines showed similar intensity of the bands corresponding to precipitated  $\alpha$ <sub>v</sub> and  $\beta$ <sub>3</sub> subunits (Figure 3).

**Melanoma cell adhesion to VN.** In functional assays we used VN, the main ECM ligand for the  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> receptor (13); their interaction is implicated in melanoma migration (12). To determine whether  $\beta$ 1-6-branched complex-type N-glycans participate in melanoma cell adhesion to VN, we performed adhesion assays for cells treated with SW. Swainsonine's ability to block synthesis of complex-type N-glycans, among them  $\beta$ 1-6-linked structures, facilitates the study of their function. In the two melanoma cell lines, we observed similar increases of adhesion to VN after SW treatment (Figure 4A). We conclude that  $\beta$ 1-6-linked complex-type glycans affect melanoma cell adhesion to VN.

Confocal microscopy of immunostaining showed that  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> is distributed over the whole surface of melanoma cells cultured on an uncoated surface; in the presence of VN,  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> was clustered in focal adhesion structures (Figure 4B). Figure 4B shows only the results for WM1205Lu cells; the same effect was observed in both melanoma cell lines.

**Role of  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> integrin and glycosylation in melanoma migration on VN.** We used monoclonal antibodies against  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> integrin, unlabeled lectins and glycosylation inhibitor in assays of melanoma wound healing on VN to examine the effects of integrins and the effects of glycosylation on melanoma migration. Antibodies against  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub> significantly reduced metastatic cell migration on VN (ca. 50% of control) but did not influence primary cell motility (Figure 5). Lectin PHA-L and both lectins with sialic acid specificity (SNA, MAA) blocked WM1205Lu cell motility but not WM793 cell motility (Figure 5) indicating that sialylated complex-type glycans with  $\beta$ 1-6 branches are involved in metastatic melanoma migration. Lectin PHA-E and dMM inhibitor had no effect on the migration of melanoma cells on VN.

The faster wound closing by WM793 primary cells than by WM1205Lu metastatic cells (Figure 5) was not the effect of higher migration ability but rather the effect of the much larger area occupied by individual primary cells after scratching. We have also observed this effect in studies of WM793 migration

on an uncoated surface and on fibronectin; an explanation for it has been given in previous publications (14, 15).

## Discussion

While it is clear that the progression of many tumors is accompanied by increased  $\alpha_v\beta_3$  integrin expression (10, 16), no fixed rules have been established on how  $\alpha_v\beta_3$  glycosylation is altered in tumorigenesis (3). In the transition from the radial growth phase (RGP) to the VGP, rapid up-regulation of  $\alpha_v\beta_3$  integrin expression has been shown to be required for melanoma cells to cross the basement membrane (BM) separating the epidermis from the dermis (11, 17-19). In our study, flow cytometry revealed high expression of the  $\alpha_v\beta_3$  complex in both melanoma cell lines (*ca.* 99% of cells) (Figure 1). This is not surprising, considering that the WM793 and WM1205Lu lines represent the last two stages of melanoma progression; however, the transition from the primary to the metastatic stage requires changes allowing cells to cross into blood vessels, remain in circulation and form new lesions in distinct organs after leaving the circulation. It is known that the glycosylation of cell surface proteins must undergo alteration(s) in order for cancer cells to interact with new cell partners and adapt to new environments (20, 21). Rearrangement of glycans accompanies all steps of carcinogenesis, from transformation of normal cells (22, 23) to metastasis (15). To determine the glycosylation profile in the final stages of melanoma progression, we chose  $\alpha_v\beta_3$  integrin, considered a biomarker of melanoma progression. PNGase digestion showed intensive N-glycosylation of  $\alpha_v\beta_3$  integrin, especially the  $\alpha_v$  subunit in which nearly a third of the molecule is built of N-glycans (Figure 2A). We investigated the glycosylation pattern of  $\alpha_v\beta_3$  by immunoblotting the  $\alpha_v$  and  $\beta_3$  subunits in lectin precipitates and by determining molecular mass loss after specific enzyme cleaving and in the presence of SW inhibitor. In both integrin subunits, we found significant differences between primary and metastatic melanoma cells in the content of high-mannose structures and sialylated complex-type glycans with bisected GlcNAc.

Oligomannose and hybrid-type N-glycans are immature structures; they are an intermediate step on the N-glycan synthesis pathway leading to the formation of complex-type structures. The content of high-mannose and hybrid type N-glycans in both  $\alpha_v\beta_3$  integrin subunits was higher for WM793 primary cells than for WM1205Lu metastatic cells (Figures 2B, 3). These incompletely processed glycans are not frequently associated with cancer (24) but changes in their level during tumor progression have been shown in other studies (25-27). Up to now, however, there is no consensus as to the direction of these changes. De Leoz *et al.* (25) observed elevation of high-mannose-type glycans in mouse and human sera proteins during breast cancer

progression. Among other alterations found by Ozcan *et al.* (26) in analyses of structural changes in serum glycoproteins, levels of oligomannose glycans in patients with gastric cancers were lower than in the control group. Kinoshita *et al.* (27) showed reduction of high-mannose glycans in melanoma cell lines during tumor progression; they found slightly lower expression of these structures in the WM266-4 metastatic cell line than in WM115 primary cells from the same patient. Glycosylation is a very sensitive process that depends on many factors, mainly the tissue-specific expression and activity of glycosyltransferases and glycosidases (28); different cancer-dependent effects on the glycosylation profile, including high-mannose expression, are possible. So far, it is not clear whether these structures play a role in cell interaction (29). We did not observe differences in melanoma motility on VN after applying dMM, an inhibitor of N-glycan processing that blocks mannosidase I and leads to accumulation of high-mannose glycans (Figure 5). Zhang *et al.*'s work (30) did not disclose the functional significance of oligomannose structures but, based on comparable binding of dMM-treated H7721 human hepatocarcinoma cells and control cells to human umbilical vein epithelial cells (HUVEC), concluded that high-mannose N-glycans do not mediate cell-cell interaction. Schraen-Maschke and Zanetta (31) determined that oligomannose and hybrid-type N-glycans are involved in homotypic aggregation and proliferation of C6 rat glioblastoma. Liwosz *et al.* (32) showed that the high-mannose/hybrid type N-glycans present on E-cadherin destabilize epithelial cell-cell interactions. A more detailed study is needed to find out whether the decrease of oligomannose glycans in  $\alpha_v\beta_3$  integrin we observed during melanoma progression has a functional significance in melanoma behavior besides the behavior of migration on VN.

It is interesting that high-mannose and hybrid-type structures dominate in the  $\beta_3$  subunits (Figures 2B, 3) representing 73% and 83% of the total N-glycan pool in  $\beta_3$  from primary and metastatic cells, respectively, while, in the  $\alpha_v$  subunit, they account for only 36% in primary and 32% in metastatic cells. Other work also indicates  $\beta$  subunits as the main carriers of oligomannose structures, especially  $\beta_1$  glycoprotein (33). Kawano *et al.* (34) showed the presence of high-mannose structures on  $\beta_1$  subunit in mouse metastatic melanoma B16F1. Veiga *et al.* (35) found that oligomannose glycans are expressed only in the premature isoform of  $\beta_1$ , of lower molecular weight, in B16F10 cells. The authors of the study showed that such glycosylated  $\beta_1$  precursors do not occur on the cell surface; however, other reports demonstrated the presence of such glycosylated  $\beta_1$  forms also in the cell membrane (36).

A number of studies have made clear the importance of sialylation of adhesion proteins in intercellular and cell-ECM contact and shown that any alteration of the sialic acid



composition on the cell surface will change the cell's interactions (37, 38). We analyzed terminal sialic acids linked by  $\alpha$ 2-6 or  $\alpha$ 2-3 to complex-type N-oligosaccharides using the lectins SNA and MAA, respectively. Sialylation of  $\alpha$ 2-6 was more abundant in both subunits of primary cells and sialylation of  $\alpha$ 2-3 in metastatic cells (Figure 3). Neuraminidase digestion confirmed the higher content of  $\alpha$ 2-3-linked sialic acids in  $\alpha_v\beta_3$  integrin from metastatic cells (Figure 2C). The role of  $\alpha$ 2-6 and a 2-3 sialylation in tumor progression has been examined previously (38). Cui *et al.* (39) found that high expression of  $\alpha$ 2-3-linked sialic acid was associated with the metastatic competence of breast cancer. Ranjan and Kalraiya (40) demonstrated that sialic acids linked by  $\alpha$ 2-6 to  $\beta$ 1-6-branched complex-type glycans contribute to the invasiveness of melanoma cells by modulating B16F10 cell adhesion to ECM and BM. Some studies have assessed sialylation of integrin and the functional role of sialic acid alteration. Seals *et al.* (33) found that expression of the ras oncogene in HD3 colon epithelial cells raised  $\alpha$ 2-6 sialylation in  $\beta$ 1 integrins but not in  $\beta_3$  and  $\beta_5$ ; it resulted in altered adhesion to collagen I. Previously, we demonstrated both types of sialic acids in  $\alpha_v\beta_3$  integrin from metastatic melanoma cells (41). Citing Voura *et al.*'s (42) finding that the L1 molecule interacts with  $\alpha_v\beta_3$  integrin on WM239 cells during their transendothelial migration, we suggested that the  $\alpha$ 2-3 sialic acids present on  $\alpha_v\beta_3$  integrin in WM239 cells act as a ligand for the L1 molecule and that these interactions are crucial in transendothelial migration of melanoma cells. In our present model, WM1205Lu metastatic cells able to pass through the vascular endothelium showed elevated  $\alpha$ 2-3-linked sialic acids in  $\alpha_v\beta_3$ . We speculate that the role of these integrin structures in cancer cell extravasation, which we proposed in Kremser *et al.* (41), may also apply to WM1205Lu cells. Numerous studies suggest that sialic acid alterations are carcinoma-specific. Here we showed that  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acid expression on  $\alpha_v\beta_3$  integrin changes during melanoma development.

Wound-healing assays showed the effect of  $\alpha_v\beta_3$  integrin and  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acids only in WM1205Lu metastatic cell migration on VN. Inhibition of the migration of metastatic cells in the presence of SNA and MAA lectins was comparable, despite differences in  $\alpha$ 2-6 and  $\alpha$ 2-3 sialylation of  $\alpha_v\beta_3$ . This may mean that other  $\alpha$ 2-6-sialylated receptors are involved in the contact of metastatic melanoma cells with VN. Janik *et al.* (12) observed reduction of cutaneous melanoma migration on VN in the presence of both anti- $\alpha_v\beta_3$  and anti- $\alpha_v\beta_5$ . Subunit  $\beta_5$  is also an N-glycosylated protein but its glycosylation is poorly known.

The  $\beta$ 1-6 branching of both the  $\alpha_v$  and the  $\beta_3$  subunits, unlike sialylation, did not differ between primary and melanoma cell lines (Figures 2D, 3). The  $\beta$ 1-6 branching of  $\alpha_v\beta_3$  integrin remained unchanged despite an increase of  $\beta$

N-acetylglucosaminyltransferase V (GnT-V) enzyme expression during the transition from VGP to the metastatic phenotype observed in the same cell line pair in our previous study (15). This may mean that  $\alpha_v\beta_3$  integrin is a substrate for GnT-V in an early stage of melanoma progression and that further change in the enzyme level during acquisition of the metastatic phenotype does not influence the  $\beta$ 1-6 branching of this protein. Some studies strongly suggest that synthesis of GlcNAc  $\beta$ 1-6-branched glycans begins in an early stage of cell transformation. In nude mice, Demetriou *et al.* (43) showed that normal lung epithelial cells transfected with GnT-V cDNA demonstrate a cancer-like phenotype due to decreased adhesion to fibronectin (FN) and collagen IV, loss of contact inhibition and increased tumorigenicity. Ito *et al.* (44) compared GnT-V expression between human hepatocellular carcinoma tissues and normal liver cells and also concluded that up-regulation of this enzyme appears in an early phase of carcinogenesis.

Interestingly, lectin precipitation and SW treatment revealed that  $\alpha_v$  chains possess much higher content of  $\beta$ 1-6-branched structures than  $\beta_3$  glycoproteins in both analyzed cell lines (Figures 2D, 3). Demetriou *et al.* (43) showed that the  $\alpha_v$  subunit, not  $\beta_3$ , was the substrate for GnT-V in Mv1Lu lung epithelial cells transfected with this enzyme. We found that  $\beta_3$  subunits are more abundant in high-mannose N-glycans than  $\alpha_v$  in both melanoma lines (Figures 2B, 3).

In cancer development, an increase of the number of antennae in complex-type N-glycans was observed resulting in synthesis of N-glycans of higher molecular weight (45). The data from our work do not confirm this observation. PNGase digestion showed a lower amount of N-glycans in both  $\alpha_v\beta_3$  subunits from metastatic cells than from primary cells (Figure 2A). In both melanoma cell lines, the higher pool of N-glycans in  $\alpha_v\beta_3$  from primary cells was the result of higher amounts of oligomannose structures (Figures 2B, 3) and, generally, more abundant sialylation (Figure 2C) but not the result of differences in branching of complex-type glycans (Figures 2D, 3).

Elevated  $\beta$ 1-6 N-glycosylation is closely related to tumorigenesis and metastasis (46-48). In our study, PHA-L reduced WM1205Lu motility but not WM793 motility on VN indicating that  $\beta$ 1-6 glycans acted only in metastatic melanoma migration on VN (Figure 5). Binding of both cell lines to VN was similarly stronger after culture in the presence of SW (Figure 4A), likely the result of having similar amounts of  $\beta$ 1-6 glycans in  $\alpha_v\beta_3$  integrin. It is well documented that malignant cells have altered adhesion, which facilitates cell migration to other sites in the body where they establish new tumors. Increased  $\beta$ 1-6 branching is one of the best-characterized N-glycan alterations associated with malignant transformation and progression (49, 50). Our previous work showed that inhibition of  $\beta$ 1-6 branching synthesis in the Golgi apparatus by SW (51, 52) or



blocking of these structures on the cell surface by PHA-L lectin (41, 53) boosted cell adhesion to extracellular matrix proteins and impaired migration on ECM components.

In view of the similarity of  $\beta$ 1-6 glycosylation of  $\alpha_v\beta_3$  in primary and metastatic melanoma, why did we observe the involvement of  $\beta$ 1-6 glycans only in metastatic cells migrating on VN? It might be due to the different glycosylation profile of  $\alpha_v\beta_3$  integrin, which is involved in migration of metastatic but not of primary cells on VN (Figure 5). PHA-E precipitation clearly showed that complex-type N-glycans in both subunits of  $\alpha_v\beta_3$  integrin from WM793 primary cells are modified by bisecting GlcNAc more strongly than in the WM1205Lu metastatic line (Figure 3). The significantly higher content of structures with bisecting GlcNAc in  $\alpha_v\beta_3$  integrin from primary cells may be the reason why WM793 migration was suppressed, despite the high amount of  $\beta$ 1-6 glycans in this integrin. N-oligosaccharides with bisecting GlcNAc have been shown to suppress cancer development (48, 54). An increase of bisecting GlcNAc structures has been found to be associated with loss of metastatic potential (21, 55). In a demonstration of antagonism between bisecting GlcNAc and  $\beta$ 1-6-branched glycans, Zhao *et al.* (56) showed that GnT-V and GnT-III enzymes competitively modified  $\alpha_3\beta_1$  integrin, thus influencing  $\alpha_3\beta_1$ -mediated cell migration. A recent work by Bubka *et al.* (57) did not exclude the presence of GnT-III and GnT-V products on the same glycoprotein in melanoma cells: over-expression of GnT-III leading to higher synthesis of bisecting GlcNAc was accompanied by higher  $\beta$ 1-6 branching in cell glycoproteins, among them the melanoma cell adhesion molecule (MCAM). Taken together, these results suggest that the absence of WM793 primary melanoma migration on VN can be explained by the specifics of  $\alpha_v\beta_3$  integrin glycosylation.

Previously, we established that inhibition of  $\beta$ 1-6 branching synthesis by SW impaired both WM793 primary and WM1205Lu metastatic cell migration on FN (15). In the present experiments, blocking of  $\beta$ 1-6-branched glycans by PHA-L lectin only reduced the migration of metastatic cells on VN. Apparently, the effect of  $\beta$ 1-6-branched glycans on melanoma motility depends on ECM components that require interactions with other integrin receptors. Moreover, it is clear that the interactions of cancer cells with ECM proteins change during cancer progression and cell motility on VN *via*  $\alpha_v\beta_3$  integrin seems to be most important during metastasis (6). Adhesive contact of cells with ECM proteins is needed to trigger the cell signaling pathways that control the metastatic phenotype. We observed clustering of  $\alpha_v\beta_3$  integrin in focal adhesions of melanoma cells adhering to VN, differing from the dispersed expression seen on the whole membrane of cells growing on uncoated surface (Figure 4B). The ability of  $\alpha_v\beta_3$  to cluster in focal adhesions on VN, unlike  $\alpha_v\beta_5$  integrin, was described more than a decade ago (58). Clustering of  $\alpha_v\beta_3$  in focal adhesion

structures in the presence of VN points to the important role of  $\alpha_3\beta_1$ -VN interactions in cancer cells.

Movement of cancer cells through the ECM requires the activity of matrix metalloproteinases (MMPs). Gil *et al.* (59) observed differences in MMP profiles between WM793 primary and WM1205Lu metastatic cells. In zymography tests, they found MMP-2 and MMP-9 activity in metastatic melanoma and only MMP-2 activity in primary cells. We showed high expression of  $\alpha_v\beta_3$  in both of these cell lines but only the metastatic cells responded to  $\alpha_v\beta_3$  monoclonal IgG, which blocked their motility on VN. It is well documented that  $\alpha_v\beta_3$  integrin can regulate the expression and activity of MMPs in cancer cells (60-62) and that acquisition of the melanoma metastatic phenotype requires both elevated  $\alpha_v\beta_3$  and the activity of MMPs (3). Here we also showed that, in melanoma, the malignancy process is accompanied by alteration of  $\alpha_v\beta_3$  glycosylation, which also contributes to metastatic behavior.

It is worth noting that we observed reduction of melanoma cell migration on VN in the presence of the same clone LM609 of mouse antibody against  $\alpha_v\beta_3$  that has been used in preclinical tests of the therapeutic drug Avastin. Several humanized, improved versions of this antibody have been tested in clinical trials as agents to block tumor angiogenesis and metastasis. It is thought that applying a dual therapy directed against  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ , two integrins involved in tumor angiogenesis, will give better therapeutic results (8). The finding that  $\alpha_v\beta_3$  glycosylation is significantly altered during melanoma progression may prove useful in the effort to develop more effective anti-cancer therapies.

Many studies on altered glycosylation in various cancers have examined the total pool of sera or cell glycoproteins, thus yielding information about general tendencies in tumorigenesis. Herein, we focused on one specific cell surface protein. We demonstrated, for the first time, that the pool of high-mannose and complex-type glycans with bisecting GlcNAc on  $\alpha_v\beta_3$  integrin decreases during acquisition of the metastatic phenotype. We also observed a shift of  $\alpha_v\beta_3$  sialylation from  $\alpha$ 2-6 in the primary line to  $\alpha$ 2-3 in metastatic melanoma cells. Future work has to focus on findings that may help in developing targeted therapies aimed at stopping the progression of melanoma, such as increased levels of  $\alpha_v\beta_3$  integrin and alteration of  $\alpha_v\beta_3$  N-glycosylation during acquisition of metastatic competence.

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