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The structure of the oligosaccharides of N-cadherin from human melanoma cell lines*

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N-cadherin is calcium-dependent cell adhesion molecule that mediates cell-cell adhesion and also modulates cell migration and tumor invasion. N-cadherin is a heavily glycosylated protein. Many studies have demonstrated that malignant transformation of a number of cell types correlates with changes of cell surface N-linked oligosacharides. We have studied the carbohydrate profile of N-cadherin synthesized in human melanoma cell lines and the effect of this protein and complex N-glycans on *in vitro* migration of melanoma cells from the primary tumor site—WM35 and from different metastatic sites WM239 (skin), WM9 (lymph node), and A375 (solid tumor). N-cadherin was immunoprecipitated with anti-human N-cadherin polyclonal antibodies. Characterization of its carbohydrate moieties was carried out by SDS-PAGE electrophoresis and blotting, followed by immunochemical identification of the N-cadherin polypeptides and on-blot deglycosylation using PNGase F for glycan release. N-glycans were separated by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and their structures identified by the computer matching of the resulting masses with those derived from a sequence database. The assay of *in vitro* chemotaxic cell migration was performed using QCM™ Cell Invasion Assay (Chemicon).

N-cadherin from WM35 (primary tumor site) possessed high-mannose and biantennary complex type glycans with α2–6 linked sialic acid. N-cadherin from WM239, WM9, and A375 cell lines possessed mostly tri- or tetra-antennary complex type glycans. In addition, N-cadherin from WM9 (lymph node metastatic site) and A375 (solid tumor metastatic site) contained heavily α-fucosylated complex type chains with α2,3 linked sialic acid. Blocking of N-cadherin-mediated intercellular interaction by N-cadherin-specific antibodies significantly (of about 40%) inhibited migration of melanoma cells. Inhibition of synthesis of complex type N-glycans by swainsonine (mannosidase II inhibitor) led to 50% decrease of cell migration.

The results indicated differences between N-cadherin glycans from primary and metastatic sites and confirmed influence of N-cadherin and complex -type N-glycans on *in vitro* migration of melanoma cells.
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Keywords: **N-cadherin, N-glycans, melanoma, MALDI MS, migration**

Abbreviations: **HexNAc: *N*-acetyl-hexosamine; Fuc: fucose; Sia: sialic acid; MALDI MS: matrix-assisted laser desorption ionisation mass spectrometry; PNGase: F peptide N-glycosidase F.**

Introduction

The pattern of expression of cell adhesion molecules and their properties play a pivotal role in controlling processes of cell division, migration, differentiation and death.

Cadherins are transmembrane glycoproteins, which provide strong intercellular adhesion in a Ca^{2+} dependent manner. Classic cadherins are the transmembrane components of cellular junctions. They are composed of three segments: an large extracellular domain, which mediates homophilic type cell adhesion, a transmembrane domain, and a highly conserved cytoplasmic domain, that interacts with catenins to link cadherins to the actin cytoskeleton. Cadherins play a major role in epithelial cell-cell adhesion [1,2]. Moreover, their role in cell differentiation, transformation, and invasion has been also documented [3–5] with some of these functions most probably depending upon the activaction of intracellular signal transduction cascades [6].

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Cadherins are major cell-cell adhesion molecules involved in the development and maintenance of skin. Normal cultured human melanocytes express both E-cadherin and P- cadherin. E-cadherin is primarily responsible for adhesion of melanocytes to keratinocytes [7–9]. N-cadherin is expressed in highly invasive tumor cell lines that usually lack E-cadherin expression. Progressive loss of E-cadherin and expression of N-cadherin during cancer progression not only releases melanoma cells from control by keratinocytes, but also provide new adhesive properties [10]. N-cadherin plays an essential role in controlling the strength of cell-cell and cell-matrix interactions. N-cadherin expressed in melanoma cells not only mediates cell-cell interactions between adjacent cancer cells and stromal fibroblasts and endothelial cells but also promotes survival and migration of melanoma cells [10]. It is expressed by several human fetal tissues and re-expressed by the corresponding neoplasm, and in this way contributes to the melanoma invasive phenotype [7,11].

Neoplastic transformation in human tumor cells is generally accompanied by structural alteration in cell surface oligosaccharides [12]. The expression of branched and sialylated or fucosylated complex type N-linked oligosacharides in malignant tumor cells appears to be directly associated with their metastatic potential [13,14]. Human cancer of breast, colon, bladder, and melanomas show increased levels of β1-6GlcNAc branched N-glycans of tri- and tetra-antennary type, formed due to the increased activity of N-acetylglucosaminyltransferase V [12,15,16]. The appearance of N-linked glycans with β1-6GlcNAc branches correlates with metastasis and progression of tumor [16,17]. Moreover, sialoglycans on the surface of human colon cancer cell have been implicated in cellular adhesion and metastasis. The common structural motif among adhesion molecules in colon cells is the terminal NeuAcα2,3Gal-R glycosidic epitope. Swainsonine, an inhibitor of Golgi α-mannosidase II, blocks synthesis of complex type N-linked oligosacharide. Inhibition of N-linked processing with the alkaloid swainsonine, blocked tumor cell invasion, thus increasing tumor cell adhesion *in vitro* [12].

Recent analysis of N-glycosylation profile of proteins from various melanoma cells indicated N-cadherins as one of the proteins undergoing changes in oligosaccharide composition in different cancer melanoma cell lines.

Here we present the data, showing the differences between N-cadherin glycans isolated from melanoma primary and various metastatic sites. The results indicated that N-cadherin and complex type N-glycans significantly promote *in vitro* migration of melanoma cells.

Materials and methods

Materials

All standard chemicals of analytical grade were purchased from Sigma, Poznań, Poland.

Antibodies

Mouse mAb against N-cadherin (A-CAM clone GC-4, Sigma, Poznań, Poland) was used in blocking experiments for inhibition of N-cadherin mediated migration. Another anti N-cadherin: pAb rabbit anti-N-cadherin pAb (Takara, Japan) was used for western blotting and immunoprecipitation. Mouse IgG_1 Negative Control (Dako, Denmark) was used in blocking experiments for control inhibition of N-cadherin mediated migration through matrigel.

Cell lines

Melanoma cell lines were obtained from the Department of Cancer Immunology, University School of Medical Sciences at the Great-Poland Cancer Centre (Poznań, Poland). The WM35 line was from the primary radial growth phase tumor site while WM9 was the lymph node metastatic line, WM239, the skin metastatic line (all of them established by Meenhard Herlyn, The Wistar Institute, Philadelphia, USA), and A375 (ATCC-CRL-1619), [18] the solid tumor metastatic line. The cell lines were cultured in the RPMI–1640 medium (Sigma, Poznań, Poland) containing 10% foetal bovine serum (GibcoBRL^{TM}) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml; Polfa, Tarchomin, Poland). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

Homogenisation

Cells were harvested from culture dishes with a rubber policeman, washed with PBS and homogenised on ice by triple sonification, 5 s each (Bandelin Electronic) in 50 mM Tris/HCl pH 7.5 containing 1 mM EDTA and proteinase inhibitor cocktail (Sigma, Poznań, Poland). The homogenate was left on ice for 1 h with 1% Triton X-100 and 0.3% protamine sulphate and centrifuged at 18,000 $\times g$ for 1 h at 4°C. Protein concentration in the supernatants was determined according to Bradford (1976).

Immunoprecipitation

The cleared cell homogenate (15 mg of total protein) was incubated with 200 μg of anti-N-cadherin pAb (Takara, Japan). Immunoprecipitation of N-cadherin was performed using standard procedures, described earlier [19].

Gel electrophoresis

The gel ($85 \times 70 \times 1.5$ mm) was prepared in the presence of SDS in reducing conditions using a discontinuous buffer system according to Laemmli (1970). Immunoprecipitates (300 μg of total protein per lane) of human melanoma cell lines (WM35, WM239, WM9, A375) were separated using 4.5% stacking and 8% separation gel in 4 h run.

Western blotting

Proteins were transferred onto PVDF membrane overnight using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 150 mA in 25 mM Tris, 192 mM glycine, 20% methanol at pH 8.4.

Immunodetection of N-cadherin

Immunodetection of N-cadherin on PVDF membrane was performed using standard procedures, described earlier [19]. The blot was incubated for 1 h with rabbit anti-human N-cadherin (Takara, Japan) diluted 1:1,000 in 2% BSA/TBS/Tween.

Glycan analysis

Individual protein bands corresponding to the N-cadherin were excised from the PVDF membrane and glycan analysis was carried out according to the method described by Kuster *et al.* (1997) as modified by Hoja-Łukowicz *et al.* (2000).

Protein reduction and alkylation

The excised PVDF pieces (8 × 2 mm) were placed into Eppendorf tubes and washed twice with 20 mM $NaHCO_3$, pH 7.0 for 15 min each. The final wash was discarded and replaced by 300 μl of fresh 20 mM $NaHCO_3$ pH 7.0 supplemented with 20 μl of 45 mM dithiothreitol (DDT) and the fixed protein was reduced at 60°C for 30 min. After cooling to room temperature (RT) 20 μl of 100 mM iodoacetamide (IAA) was added and the protein alkylated for 30 min at RT in the dark. The reducing and alkylation reagents, as well as residual SDS were then removed by incubation in 1:1 acetonitrile/fresh 20 mM $NaHCO_3$, pH 7.0 for 60 min. Subsequently the membrane pieces were incubated in the blocking solution (Roche) overnight at 4°C.

In situ digestion

Prior to deglycosylation PVDF pieces were washed three times with 20 mM $NaHCO_3$ pH 7.0 for 15 min each. The washings were discarded and replaced with 3U of PNGase-F in 50 μl of 20 mM $NaHCO_3$, pH 7.0 and incubated at 37°C for 12–16 h.

Sugar extraction

After deglycosylation the incubation buffer was completely dried in a SpeedVac (JW Electronic, Poland) and then dissolved in 10 μl of ultrapure water (Milli-Q Plus Millipore, Bredford, CA, USA), and applied onto a microcolumn clean-up.

Esterification of sialic acid

The glycans were dissolved in 50 μl of ultrapure water and passed through a small column, microcolumn packed with 5 μl Dowex AG-50 (Na^+ form) glycans were eluted with 50 μl of ultrapure water and dried in a SpeedVac. The glycans were resuspended in dry DMSO (5 μl) and iodomethan (5 μl) and allowed to stand for 2 h at room temperature. After 2 h incubation, 5 μl DMSO was added and the sample was completely dried in a SpeedVac.

Microcolumn clean-up of sugar

Prior to MALDI MS, a microcolumn consisting of an Eppendorf GELoader pipette tip [20] packed with 5 μl of AG-3 (OH^- form, bottom) and AG-50 (H^+ form, top) (BioRad) each was used to separate glycans from their accompanying salts. The column was washed with 100 μl water and an aliquot of the sugar sample was applied. Glycans were eluted with 100 μl water and dried in a SpeedVac.

MALDI MS

Matrix-assisted laser description ionisation (MALDI) mass spectra were recorded by using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystem) equipped with a delayed extraction mode. The MALDI matrices were prepared by dissolving 25 mg of 2,5-dihydroxybenzoic acid in 1 μl of acetonitrile/0.2% trifluoroacetic acid (70:30, v/v). Typically 1 μl of the analyte was then added. Mass calibration was performed with the MH^+ ion of insulin set at *m/z* 5734.6 and peptide ion at *m/z* 1209.7. Raw data were analysed using the computer software provided by the manufacturer and are reported as average masses.

Analysis of MALDI MS spectras

The final structures were determined based on the data recorded and the following assumptions:

(a) each *m/z* value found in MALDI-MS spectra represents the protonated form of a glycan MH^+. These forms exist as adducts with either sodium ion(s)—[M + Na^+] or potassium ion(s) [M + K^+] and the intensities of the former adducts are a few fold (ca. 5–6-fold) higher that those of the latter ones.

(b) to determine the glycan structure based on its [M + Na^+] or [M + K^+] value two alternative approaches were used: Database for analysis of MALDI-MS glycan spectra as routine software tool of the mass-spectrometer in Dipartimento di Chimica Organica e Biochemica Complesso Universario Monte S. Angelo, Napoli, Italy—Angela Amoresano. Computer program developed based on the algorithm:

$$\begin{aligned} M/Z = \{[&axM_N + bxM_H + cxM_F + dxM_S \\ &- (a + b + c + d)x18 + wxNa^+ + qxK^+] : z\} \\ &(M_N\text{—}221.2,\ M_H - 180.2,\ M_F - 164.2, \\ &M_S\text{—}\ 309.3,\ Na^+\text{—}22.0,\ K^+\text{—}38.1) \end{aligned}$$

prepared in accordance with commonly accepted rules and order of synthesis of N-glycans in aim to attribute to a given MH^+ ([M + Na^+]/[M + K^+]

value, all the possible N-glycan structures that could be combined from the following building block units: *N*-acetylglucosamine—M_N, mannose—M_H, galactose—M_H, fucose—M_F and sialic acid—M_S. A comparison of the results of interpretation of a given *m/z* value, using both methods was always performed prior to the final determination of each detected N-glycan of N-cadherin.

Matrigel invasion chamber assay

The assay of chemotaxic cell migration was performed using QCM™ Cell Invasion Assay (Chemicon) (insert contains an 8 μm pore size membrane coated with thin layer of ECMatrix™). The migration of melanoma WM35, WM239, WM9 and A375 cells: (i) cultured with swainsonine (inhibition of synthesis of complex type N-glycans) and (ii) treated with anti N-cadherins mAb, was compared with that of untreated, control melanoma cell lines.

(i) For inhibition of complex N-glycans synthesis, cells were cultured with swainsonine (ICN) 10 μg/ml [21] RPMI 1640 medium containing 10% foetal bovine serum for 24 h. (ii) For N-cadherins blocking of function experiments, cells were incubated with monoclonal antibody A-CAM clone GC-4 (Sigma, Poznań, Poland) (40 μg/ml RPMI 1640 medium) or negative control antibodies IgG_1 (Dako, Denmark) at 37°C for 30 min with constant shaking [10].

The melanoma cell lines were loaded into a chamber coated with ECMatrix™ at the density of 5×10^5 cells/ml in a volume of 0.1 ml serum-free RPMI 1640. The plate with cells were incubated for 16 h at 37°C in a CO_2 incubator. Migrating cells on the bottom of the insert were incubated with 150 μl of prewarmed Cell Detachment Buffer for 30 min, dissociated from the membrane and detected by CyQuant GR dye. The fluorescence plate was read using 480/520 nm filter set.

Statistical analysis

Analyses were performed on mean values from replicate experiments, as indicated, using the Student's *t*-test. Result were considered significant at $P < 0.05$.

Results

N-cadherins glycan moiety from melanoma cell lines

N-cadherin immunoprecipitated from four melanoma cell lines: WM35 (primary tumor, radial phase), WM239, WM9, and A375 (metastatic sites) was identified with rabbit anti-human N-cadherin antibody as a 133 kDa polypeptide (Figure 1), which corresponded well to the values reported for this cell adhesion molecule in various human cells [22–24].

Glycan chain analysis performed by MALDI MS, detected oligosacharides as $[M + Na^+]$ or $[M + K^+]$ ions in the positive ion mass spectrum and the spectra acquired (by MALDI MS) for the glycans released from melanoma cell lines were presented in Figures 2A–D.

The carbohydrate moieties of N-cadherin from all studied human melanoma cell lines identified based on MALDI MS spectra are listed in Table 1.

The N-cadherin from the primary tumor, the WM35 cell line, possessed high-mannose type glycan and complex biantennary type ones with α2,6 linked sialic acid (Figure 2A). The signal at *m/z* 1257.6, 1422.3, 1584.3 and 1747.2 corresponded to high-mannose type glycans and the signal at 1826.7 corresponded to hybride type glycan. The *m/z* values of 1341.3, 1503.9, 1666.2 and 1706.7 represented biantennary structures without fucose and sialic acid residues, while the signals at *m/z* 1648.9, 1809.4 concerned the structure with fucose residue and the signals at *m/z* 2117.7, 2523.7 with fucose and sialic acid. The signals at *m/z* of 1852.4, 2175.7, 2217.9 could correspond to triantennary glycans or alternatively to a biantennary oligosaccharides without sialic acid and the *m/z* values of 1970.6, 2158.3, 2335.4 and 2481.5 were attributed to the same structure carrying sialic acid molecules.

The N-cadherin from the skin metastatic tumor site, the WM239 cell line, possessed hybride type glycans and complex type glycans (Figure 2B). The *m/z* value of 1486.3, corresponded to biantennary structures with fucose and the signal at *m/z* 1882.4 indicated biantennary structures with fucose and sialic acid residues. The signal at *m/z* of 1719.3 corresponded to triantennary glycans and the signal at *m/z* 1995.4 corresponded to triantennary glycans with fucose and sialic acid. The tetraantennary type glycans were represented by the signal at 2108.5

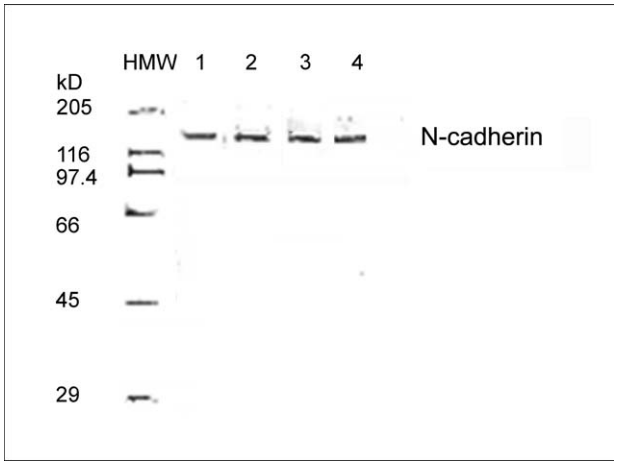
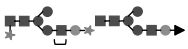
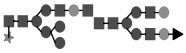
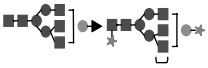
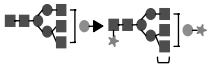


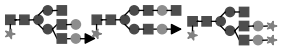
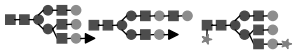
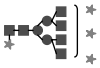
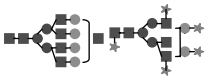
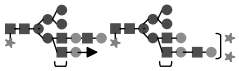
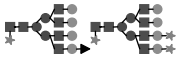
Figure 1. Western blot analysis of immunoprecipitates of N-cadherin from human melanoma cell lines. The cell extracts (300 μg of total protein) of the lines: A375 (lane 1), WM9 (lane 2), WM35 (lane 3), and WM239 (lane 4) were immunoprecipitated with anti N-cadherin pAb as described in Materials and Methods. After precipitation, the samples were analysed by SDS-PAGE using 8% gel, transferred onto a PVDF membrane and immunodetected with anti-N-cadherin pAb. HMW—high molecular weight standards stained with Amino-black.

Table 1. Hypothetical oligosacharide structures observed in MALDI MS spectra of Ncadherin from WM35 (esterified sialic acid), WM239, WM9 and A375 cell line. (■) N-acetyloglucosamina, (●) mannose, (●) galactose, (★) fucose and (▲) sialic acid.

M/z	*WM35*	*WM239*	*WM9*	*A375*
1257.6				
1341.3				
1422.3				
1461.4				
1486.3				
1503.9				
1584.3				
1590.5				
1648.9				
1666.2				
1706.7				
1719.3				
1747.2				
1809.4				
1826.7				
1852.4				
1882.4				
1892.6				
1908.6				
1970.6				
1995.4				
2108.5				
2117.7				
2158.3				
2175.7				
2217.9				
2255.7				

(*Continued on next page.*)

Table 1. (*Continued*).

M/z	*WM35*	*WM239*	*WM9*	*A375*
2280.9				
2304.2				
2320.9				
2328.3				
2335.4				
2481.5				
2523.7				
2598.9				
2613.9				
2631.8				
2793.5				
2818.1				
2834.7				

and the signal at 2255.7 *m/z* indicated tetraantennary structures with fucose residue (Figure 2B).

The analysis of the glycans from WM9 (lymph node) metastatic site, showed some structures that were identical to a few structures found in WM239 cell line that were represented by the signals at *m/z* 1719.3, 1882.4, 1995.4, 2108.5 and 2255.7. The *m/z* value of 1590.5, corresponded to biantennary structures with fucose and sialic acid, the signal at *m/z* 1461.4 indicated the presence of hybride type glycan. The signal at *m/z* of 2328.3 and 2598.9 corresponded to tetraantennary glycans with fucose residues (Figure 2C).

Additionally, N-cadherin from A375 cell lines (solid tumor metastatic site) possessed β1,6 GlcNAc branched tri- or tetraantennary complex type glycans heavily fucosylated and with α2,3- linked sialic acid represented by the values of 2304.2, 2320.9, 2613.9, 2818.1 and 2834.7, and hybride type glycans that corresponded to the signal at *m/z* 2280.9, 2631.8, 2793.5 (Figure 2D).

Migration of melanoma cell lines

The effect of N-cadherin blocking antibody and swainsonine on the invasive capacity of the melanoma cell lines was examined using the ECMatrix^{TM} invasion chamber assay shown in Figure 3. The cells from primary melanoma cell line (WM35) did not migrate through matrigel.

Percentages represent mean levels of invasion in 6 probes. N-cadherin blocking antibody (statistical significant, $P < 0.05$) inhibited migration of melanoma cell line by 39.9% in A375 cell line, 35.3% in WM9 and 37.5% in WM239. Swainsonine (mannosidase II inhibitor, that inhibited the synthesis of complex N-glycans, reduced migration by 52.8% in

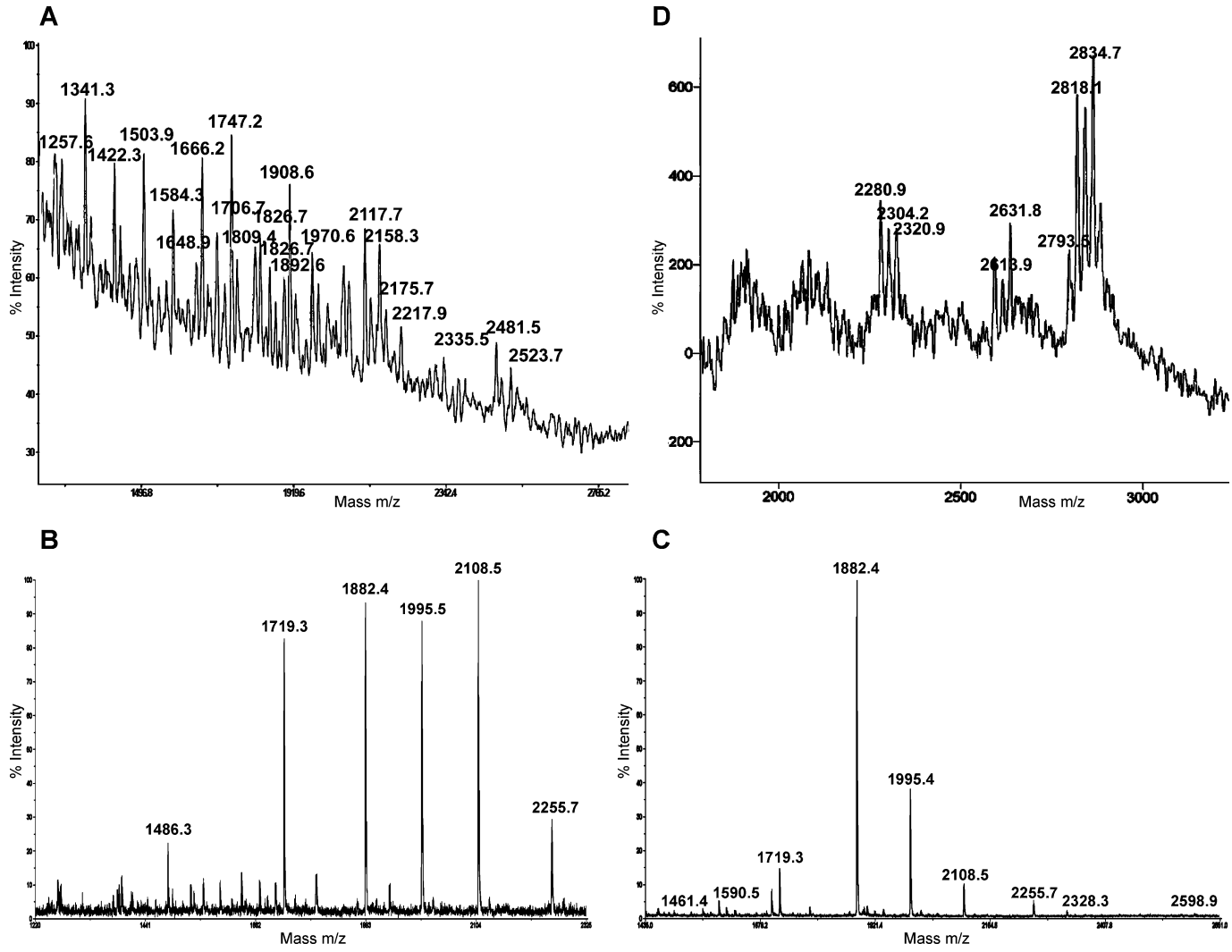


Figure 2. Positive MALDI MS mass spectra of oligosacharides obtained by PNGase-F digestion of N-cadherin from melanoma cell lines: (A) WM35 (estrified sialic acid) *Applied Biosystems Voyager System 6196*, (B) WM239 *Applied Biosystems Voyager System*, (C) WM9 *Applied Biosystems Voyager System* and (D) A375 *Applied PerSeptive Biosystems Cisesma*. The cell extracts (15 mg of total protein) were immunoprecipitated with anti N-cadherin pAb as described in Materials and Methods. After precipitation, the samples were analysed by SDS-PAGE using 8% gel, transferred onto a PVDF membrane and on-blot deglycosylated using PNGase F for glycan release. Profiles of N-glycans were obtained by MALDI MS.

A375 cell line, 64.6% in WM9 and 50.09% in WM239 cell line ($P < 0.05$).

Conclusions

We have recently reported that the expression of N-cadherin in various melanoma cell lines was associated with the absence of E-cadherin, commonly viewed as a tumor suppressor cell adhesion molecule [24]. The results of the parallel studies carried out on the profile of N-glycosylation of proteins from the same melanoma cell lines, suggested that N-cadherin might belong to the proteins undergoing changes in glycosylation due to cancer progression [2].

Melanocytes in the normal skin reside at the basement membrane and they adhere to keratinocytes through E-cadherin which allows the formation of gap junctions between the cells. During melanoma progression E-cadherin is replaced by N-cadherin that forms gap junctions with fibroblasts [9,11]. A promoter role of N-cadherin in invasion of various human cancers, including breast [4], prostate [25], stomach, and melanoma [7,10,26] has been implicated. A breast carcinoma cell line BT-20 with a low invasion rate was converted to a highly invasive one by transfection and expression of N-cadherin [27]. In this study we confirmed, that N-cadherin promotes the migration of *in vitro* melanoma cell lines. Blocking of N-cadherin-mediated intercellular interaction by N-cadherin-specific antibodies, significantly (about 35–40%) inhibited

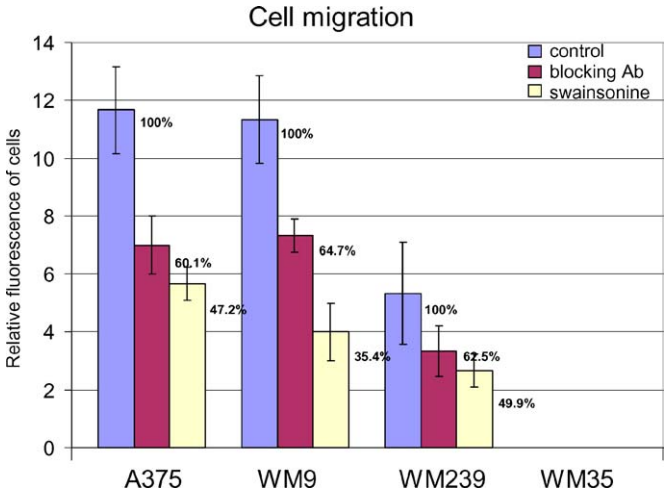


Figure 3. Effects of N-cadherins and complex N-glycans on the invasive capacity of melanoma cell line. Cell migration was performed using QCM™ Cell Invasion Assay (Chemicon). The melanoma cells were loaded into chamber coated with ECMatrix™ at the density of 5×10^5 cells/ml in a volume of 0.1 ml serum-free RPMI 1640. The plate with cells were incubated for 16 h at 37°C in a CO_2 incubator. For the inhibition of synthesis of complex N-glycans, cells were cultured with swainsonine (ICN) 10 μg/ml RPMI 1640 medium containing 10% foetal bovine serum for 24 h. For blocking of N-cadherin experiments, cells were incubated with monoclonal antibody A-CAM clone GC-4 (Sigma, Poznań, Poland) (40 μg/ml RPMI 1640 medium) or negative control antibodies IgG_1 (Dako, Denmark) at 37°C for 30 min with constant shaking. Invading cells on the bottom of the insert were detected by CyQuant GR dye. Statistical significance of the differences *vs* control value are indicated by $P < 0.05$, compared with control.

migration in investigated melanoma cell lines from metastatic sites. Cell migration depends on a delicate balance of cell adhesion and detachment. Under different physiological conditions and microenvironments, cell adhesion molecules can either promote or inhibit migration [28]. Li (2001) showed that N-cadherin mediates migration of melanoma cells on fibroblasts, and that forced expression of N-cadherin in otherwise E-cadherin-positive, N-cadherin-negative melanocytes promoted their migration to fibroblasts. N-cadherin has been postulated to promote both stable and labile cellular interactions [10].

Many studies suggested the critical role of N-cadherin expression in melanoma progression, but little is know about its carbohydrate moiety and possible role in the process. Studies from many laboratories demonstrated the carbohydrate phenotype of tumor cells functions as a biological marker of the malignant potential of a tumor [12,13,29,30]. Aberrant glycosylation is a hallmark of the malignant phenotype. In this study we confirmed, that complex N-glycans promote migration of *in vitro* melanoma cell lines. Swainsonine (mannosidase II inhibitor) inhibited *in vitro* migration of melanoma cells by about 50–60%. The structural characterization of these altered glycoforms has identified carbohydrate motifs associated with tumor tissue. Generally, the most frequently observed cancer related changes in the pattern of glycosylation include the synthesis of highly branched, *N*-acetyllactosaminylated and heavily sialylated N-linked glycans [15,17,31–34].

The results reported here by MALDI MS analysis of immunoprecipitated N-cadherin clearly pointed out the differences in glycosylation pattern between the N-cadherin from primary tumor (WM35) and N-cadherin from metastases: WM239 (skin), WM9 (lymph node), and A375 (solid tumor). The MALDI MS analysis of glycans from melanoma cells lines confirmed the preliminary lectins characterization of N-cadherin glycans [19].

The study showed that N-cadherin from primary melanoma cell lines (WM35) possessed a number of bi- and 2,4-branched triantennary complex type glycans, some of which were α2,6-sialylated [19]. Tri- or tetra-antennary complex type sialylated and fucosylated glycans are specifically present in N-cadherin from all metastases, while this protein is absent in primary radial phase melanoma cell line (WM35). The MALDI MS analysis of glycans from melanoma cell lines confirmed the preliminary lectins characterization of N-cadherin glycans [19].

At present there is no evidence that the observed changes in N-glycosylation of N-cadherin in melanoma cell lines are directly related to melanoma progression and increasing invasiveness. In a previous study [19] we showed that N-cadherin from WM35 cell line less α2,3-sialylated glycans negative reaction with MAA lectin. However, the fact that N-cadherin, expressed in primary radial phase melanoma cells (WM35), bears no highly branched and fewer α2,3-sialylated glycans when compared to all metastatic melanomas (WM9, WM 239, A375) suggests a possible association of the specific oligosacharide component with cancer progression. Perhaps one of the critical steps in creation of an invasive phenotype is the increased expression of respective glycosyltransferases: α2,3- and α2,6-sialyltransferases, fucosyltransferase, and GlcNAc-TV accompanying enhanced expression of N-cadherin.

About 90% of grade I and II colon cancers show an increased activity of α2,6-sialyltransferase and give a positive response with SNA lectin, specific for α2,6-linked sialic acid [32]. Loss of α2,6-sialylation in mutant of B16 melanoma is associated with a loss of metastatic potential [12]. While there is no question on the role of α2,6-sialylation and increased expression of respective sialyltransferase accompanying cancer progression [32], a few reports indicated a specific role of α2,3-sialylation [35]. The observed difference in glycans of N-cadherin between metastatic melanoma lines was the lack of α2,3-sialylation in metastatic melanocytes from skin. However, generally it appears that α2,3-sialylation is important in the determination of a site of metastasis. According to Dimitroff *et al.* (1999) in colon cancer cells the α2,3-linked sialic acid bearing proteins are essential in mediating intercellular adhesion to HUVEC (endothelial cells). These specific carbohydrate-mediated intercellular adhesive events may play

an important role in tumor angiogenesis, metastasis and growth control. Other studies show that elevated levels of cell surface sialic acid and $\alpha 2, 3-$sialyltransferase activity are associated with hepatic metastatic potential of colon cancers [23,36,37]. In the melanoma cell lines from skin, investigated in the present and previous reports [31], $\alpha 2,3$-sialylation was found to be an important factor in determining the course of metastasis.

Interestingly N-cadherin from metastatic site A375 (solid tumor) possessed heavily fucosylated tri or tetrantennary complex type glycans. The presence of terminal fucosylated sequences was demonstrated by immunological methods in many structures of animal and human tumours [30,38]. Higher activity of $\alpha 1,3$-fucosyltransferase was indicated in tumours of colon, stomach, lung [37,39]. Expression of $\alpha 1,2$-fucosyltransferase correlated well with colon, gastric [33,37], prostate cancers [40]. Perhaps, heavily fucosylated tri- or tetrantennary complex type glycans are associated with metastatic potential of melanoma cells.

The presented results characterized the oligosaccharide component of N-cadherin from different melanoma cell lines and demonstrated that N-glycans of N-cadherin are altered in metastatic melanoma cell lines in a way typical for invasive tumor cell N-glycans.

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