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Carbohydrate moieties of N-cadherin from human melanoma cell lines✪

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Expression of N-cadherin an adhesion molecule of the cadherin family, in tumor cells is associated with their increased invasive potential. Many studies suggested the role of N-linked oligosaccharides as important factors that contribute to metastasis by influencing tumor cell invasion and adhesion. N-cadherin is a heavily glycosylated protein. We have analysed the carbohydrate profile of this protein synthesized in human melanoma cell lines: WM35 from the primary tumor site and WM239, WM9, and A375 from different metastatic sites. N-cadherin was immunoprecipitated with anti-human N-cadherin polyclonal antibodies. Characterisation of its carbohydrate moieties was carried out by SDS/PAGE electrophoresis and blotting, followed by immunochemical identification of the N-cadherin polypeptides and analysis of their glycans using highly specific digoxigenin or biotin labelled lectins. The positive reaction of N-cadherin from the WM35 cell line with *Galanthus nivalis* agglutinin (GNA), *Datura stramonium* agglutinin (DSA) and *Sambucus nigra* agglutinin (SNA) indicated the presence of high-mannose type glycans and biantennary complex type oligosaccharides with α2–6 linked sialic acid. N-cadherin from WM239, WM9, and A375 cell lines gave a positive reaction with *Phaseolus vulgaris* leukoagglutinin (L-PHA) and lotus *Tetragonolobus purpureas* agglutinin (LTA). This indicated the presence of tri- or tetra-antennary complex type glycans with α-fucose. In addition, N-cadherin from WM9 (lymphomodus metastatic site) and A375 (solid tumor metastatic site) contained

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Abbreviations: DSA, *Datura stramonium* agglutinin; GNA, *Galanthus nivalis* agglutinin; L-PHA, *Phaseolus vulgaris* leukoagglutinin; LTA, lotus *Tetragonolobus purpureas* agglutinin; MAA, *Maackia amurensis* agglutinin; pAb, polyclonal antibody; SNA, *Sambucus nigra* agglutinin.

complex type chains with α2–3 sialic acid (positive reaction with *Maackia amurensis* agglutinin — MAA).

The results demonstrated that N-glycans of N-cadherin are altered in metastatic melanomas in a way characteristic for invasive tumor cells.

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

Cadherins are transmembrane glycoproteins, which provide strong intercellular adhesion in Ca^{2+} dependent manner. Classic cadherins are composed of a large extracellular domain, which mediates homophilic type cell adhesion, a transmembrane domain, and a highly conserved cytoplasmic domain, which interacts with actin cytoskeleton. Many different cadherins are known, the predominant ones being E-cadherin (cell-CAM 120/80, Arc-1, uvomorulin), N-cadherins (A-CAM, N-cal-CAM), and P-cadherin (placental cadherin). Cadherins play a major role in epithelial cell–cell adhesion; moreover, their role in cell differentiation, transformation, and invasion has been also documented (Freemont & Hoyland, 1996; Menger & Vollmar, 1996; Alpin *et al.,* 1999; Hazan *et al.,* 2000). Normal cultured human melanocytes express both E-cadherin and P-cadherin, but it is E-cadherin that is primarily responsible for adhesion of melanocytes to keratinocytes (Tang *et al.,* 1994; Hirohashi, 1998; Hsu *et al.,* 2000). N-cadherin plays an essential role in controlling the strength of cell–cell and cell–matrix interactions. It is expressed by several human fetal tissues and re-expressed by the corresponding neoplasm (Hsu *et al.,* 1996; Sanders *et al.,* 1999).

Changes in cadherin expression in melanoma are characterised by a significant loss of membranous P-cadherin and E-cadherin and *de novo* expression of membranous N-cadherin (Sanders *et al.,* 1999; Johnson *et al.,* 1999; Laidler *et al.,* 2000).

Many studies suggested the role of N-linked oligosaccharides in cancer metastasis by influencing tumor cell adhesion and invasion (Dall'Olio, 1996; Dennis *et al.,* 1999). In particular, malignant transformation was associated with increased size (branching) of oligosaccharide chains, extensive poly-N-acetyllactosaminylation and sialylation of some cell membrane glycoproteins. 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 (Dennis *et al.,* 1999; Prokopishyn *et al.,* 1999; Datti *et al.,* 2000). The appearance of N-linked glycans with β1-6GlcNAc branches in rodent tumor models correlates with metastasis and progression of tumor (Datti *et al.,* 2000). 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 linkage.

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 (Lityńska *et al.,* 2001).

This a novel finding that carbohydrates of N-cadherin precipitated from the cell line from primary melanoma site differ from those of N-cadherin precipitated from cell lines derived from metastatic sites in different organs.

MATERIALS AND METHODS

Materials. All standard chemicals of analytical grade were purchased from Sigma.

Cell lines. Melanoma cell lines were obtained from the Department of Cancer Immunology,

University School of Medical Sciences at 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 (both of them established by Meenhard Herlyn, The Wistar Institute, Philadelphia, U.S.A.), and A375 (ATCC-CRL-1619, Giard *et al.*, 1973), the solid tumor metastatic line. The cell lines were cultured in the RPMI–1640 medium (Sigma) containing 10% foetal bovine serum (GibcoBRL^{TM}) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml from Polfa, Tarchomin, Poland). Cells were incubated 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 phosphate-buffered saline (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). The homogenate was left on ice for 1 h with 1% Triton X-100 and 0.3% protamine sulphate and centrifuged at 18000 × ***g*** for 1 h at 4°C. Protein concentration was determined in the supernatants according to Bradford (1976).

Immunoprecipitation. The cleared cell homogenate (2.1 mg of total protein) was incubated with 10 μg anti-N-cadherin pAb (Takara) overnight at 4°C on an orbital rotator. Afterwards, the sample was mixed with 30 μl of homogeneous protein-A agarose suspension (Boehringer Mannheim) and agitated for 3 h at 4°C on a orbital rotator. Immunoprecipitates were washed: twice with 50 mM Tris/HCl buffer, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and proteinase inhibitor cocktail; twice with 50 mM Tris/HCl buffer, pH 7.5, containing 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate and finally twice with 50 mM Tris/HCl buffer, pH 7.5, supplemented with 0.1% Nonidet P-40 and 0.05% sodium deoxycholate. The immunoprecipitated proteins were eluted by boiling the immunoprecipitates for 5 min in SDS/PAGE sample buffer (125 mM Tris/HCl, pH 6.8, 2% SDS, 2% mercaptoethanol and 10% glycerol) and separated by SDS/PAGE.

Gel electrophoresis. The gel (85 × 70 × 1.5 mm) was prepared in the presence of SDS 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. The PVDF membrane was blocked for 3 h at room temperature in TBS/Tween (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) containing 2% BSA (bovine serum albumin). The blot was incubated for 1 h with rabbit anti-human N-cadherin (Takara) diluted 1:1000 in 2% BSA/TBS/Tween. Afterwards, it was washed three times for 15 min each with TBS/Tween, and incubated for 1 h with goat anti-rabbit IgG-AP conjugate (Sigma at 1:4000 dilution in 1% BSA/TBS/Tween). Subsequently, the blot was washed three times with TBS/Tween, followed by a triple wash with TBS for 5 min each. N-cadherin was detected using NBT/X phosphate solution (NBT, 4-nitro blue tetrazolium chloride, X-phosphate, 5-bromo-4-chloro-3-indolyl phosphate, Boehringer Mannheim) in 100 mM Tris/HCl with 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5.

Lectin analysis. The PVDF membrane was incubated overnight in blocking reagents (Boehringer Mannheim) at 4°C. The blot was washed twice with TBS and once with lectin buffer 1 (TBS, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5). Subsequently, the blot was incubated for 1 h with digoxigenin labelled lectins, GNA, SNA, DSA (1 μg/ml dilution in buffer 1) and MAA (5 μg/ml dilution in buffer 1), or biotin labelled lectin L-PHA, LTA

(5 μg/ml dilution in buffer 1). The membrane was washed three times with TBS and incubated with anti-digoxigenin–AP conjugate diluted 1:1000 in TBS or Extr Avidin-AP conjugate (Sigma) diluted 1:1000 in TBS. The lectins were detected using NBT/X phosphate in 0.1 M Tris/HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, at pH 9.5.

RESULTS

N-cadherin was immunoprecipitated from four melanoma cell lines: WM35 (from the primary tumor radial phase), WM239, WM9, and A375 (from metastatic sites).

N-cadherin immunoprecipitated from various melanoma cell lines was identified with rabbit anti human N-cadherin antibody as a 133 kDa polypeptide (Fig. 1), which corresponded well to the values reported for this cell adhesion molecule in various human cells (Dufour *et al.,* 1999; Hazan *et al.,* 2000; Laidler *et al.,* 2000).

Glycan chain analysis performed with the use of digoxigenin labelled lectins, GNA, SNA, MAA and DSA and biotin labelled lectins, L-PHA and LTA, allowed for characterisation of the oligosaccharide component of this cell adhesion molecule. The carbohydrate moieties of N-cadherin from all studied human melanoma cell lines are presented in Table 1.

The positive response of N-cadherin from the primary tumor, the WM35 cell line, with GNA, DSA, and SNA indicated the presence of high-mannose type glycan and complex biantennary type ones with α2–6 linked sialic acid (Fig. 2A, B, C). Additionally, N-cadherin from WM239, WM9, and A375 cell lines that underwent metastasis gave a positive response with L-PHA and LTA (Fig. 2E, F). The positive reaction with L-PHA suggested the presence of β1,6 GlcNAc branched tri- or tetra-antennary complex type glycan (Fig. 2E). The presence of α-fucose was demonstrated by a positive reaction with LTA lectin (Fig. 2F). N-cadherin from WM9 (lymphomodus metastatic site) and A375 (solid tumor metastatic site) contained also complex type chains with α2,3-linked sialic acid that was shown to result in their positive reaction with MAA (Fig. 2D).

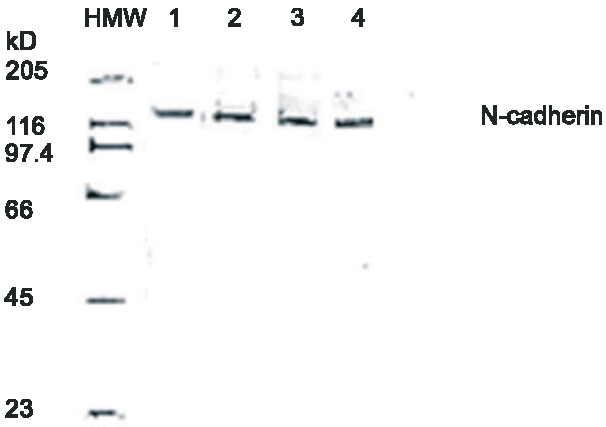

Figure 1. Western blot analysis of immunoprecipitates of N-cadherin from human melanoma cell lines.

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 mass standards stained with Amino-black.

DISCUSSION

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 (Laidler *et al.,* 2000). Parallel studies have been carried out on the profile of N-glycosylation of proteins from the same melanoma cell lines. The results suggested that N-cadherin might belong to the proteins undergoing changes in glycosylation due to metastasis (Lityńska *et al.,* 2001).

In normal epidermis, E-cadherin serves as an intercellular glue between melanocytes

and keratinocytes, enabling gap junctional communication. During melanoma development and progression, loss of E-cadherin and upregulation of N-cadherin expression results in a switch of communication partners to N-cadherin expressing melanoma cells and adjacent dermal fibroblasts (Hsu *et al.,* 1996; 2000). A promoter role for N-cadherin in invasion of various human cancers, including breast (Hazan *et al.,* 2000), prostate (Bussemakers *et al.,* 2000), stomach, and melanoma (Sanders *et al.,* 1999; Herlyn *et al.,* 2000) has been implicated. A cell line with a low invasion rate was converted to a highly invasive one by transfection and expression of N-cadherin (Nieman *et al.,* 1999).

While there seems to be no doubt with respect to the crucial role of N-cadherin expression in melanoma progression, little is know about its carbohydrate moiety. Studies from many laboratories demonstrated the importance of oligosaccharide component of glycoproteins in stabilisation of protein conformation, as well as in modulation of their physicochemical properties and biological function (Dall'Olio, 1996; Laidler & Lityńska, 1997; Dennis, 1999a). Altered glycosylation of glycoproteins is one of the many molecular changes that accompany tumorogenesis and malignant transformation. 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 (Pierce *et al.,* 1997; Dimitroff *et al.,* 1999; Dall'Olio *et al.,* 2000, Petretti *et al.,* 2000).

The results reported here on lectin 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, WM9, and A375). Tri- or tetra-antennary complex type sialylated and fucosylated glycans are specifically present in N-cadherin from all metastases, while absent in this protein from primary radial phase (WM35).

Table 1. Carbohydrate components of N-cadherin from melanoma cell lines

Lectin	Specificity	Cell line			
		WM35	WM239	WM9	A375
GNA	Manα1-(2Man / 3Man / 6Man)Man	+	+	+	+
DSA	Galβ1-4GlcNAc	+	+	+	+
SNA	NeuAcα2–6Gal	+	+	+	+
MAA	NeuAcα2–3Gal	–	–	+	+
L-PHA	β1-6GlcNAc branching	–	+	+	+
LTA	α-Fuc	–	+	+	+

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. However, the fact that N-cadherin, expressed in primary radial phase melanoma cells (WM35), bears no highly branched and less α2,3-sialylated and fucosylated glycans when compared to all metastatic melanomas (WM9, WM 239, A375) suggests a possible association of the oligosaccharide component with cancer progression. Perhaps one of the critical steps in creation of invasive phenotype is the increased

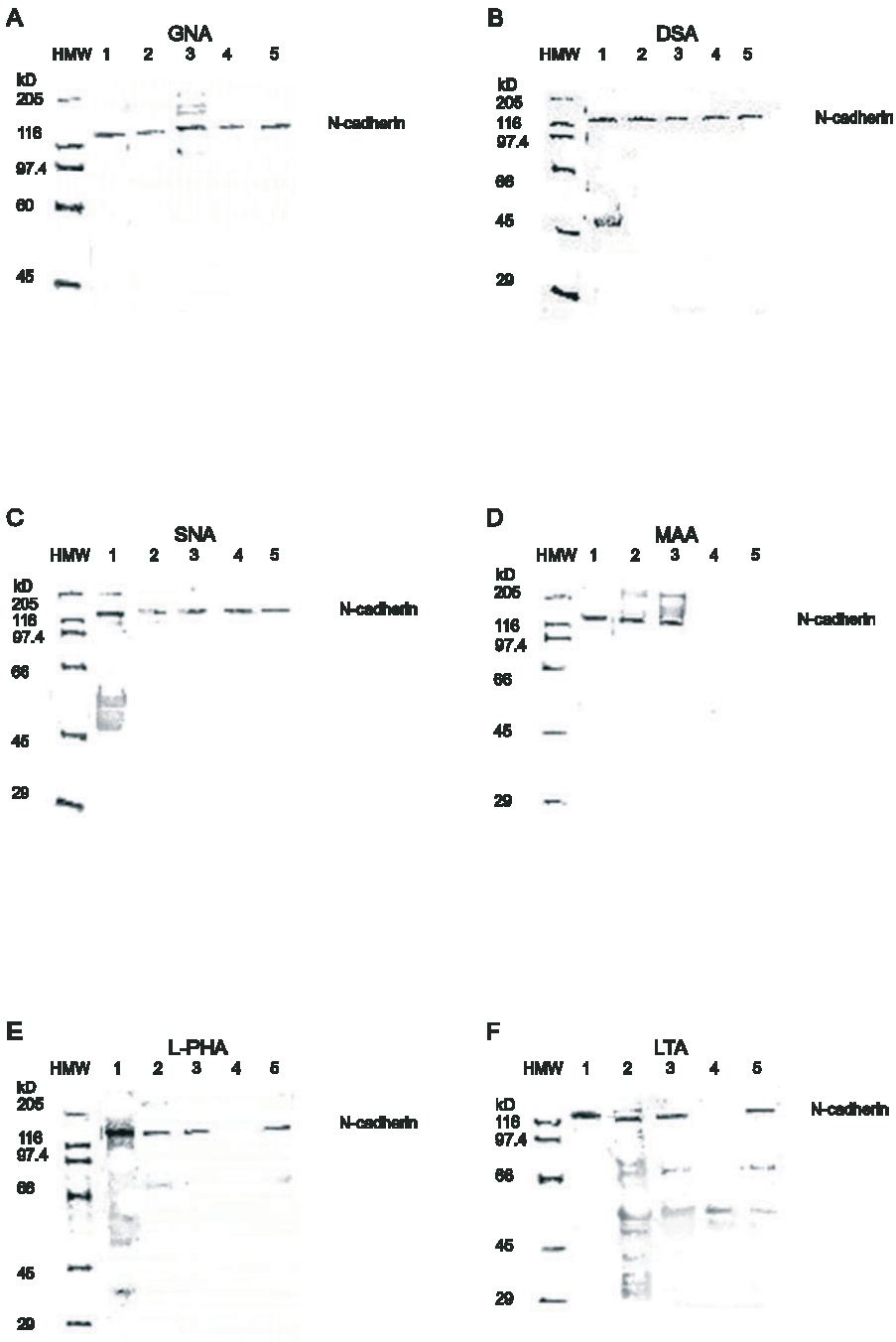

Figure 2. Analysis of N-glycans of N-cadherin from human melanoma cell lines with lectins: A, GNA; B, DSA; C, SNA; D, MAA; E, L-PHA; F, LTA.

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% acrylamide gel, transfered onto a PVDF membrane and immunodetected with anti-N-cadherin pAb (lane 1) or stained with labelled and immunoanalysed lectins (lanes 2–5): 2, A375 cell line; 3, WM9 cell line; 4, WM35 cell line; 5, WM239 cell line; HMW, high molecular mass standards stained with Amino-black.

expression of respective glycosyltransferases: α2,3- and α2,6-sialyltransferases, fucosyltransferase, and GlcNAc-TV accompanying enhanced expression of N-cadherin.

The only observed difference in glycans of N-cadherin between metastatic melanoma lines was the lack of α2,3-sialylation metastatic melanocytes from skin. However, generally it

appears that α2,3-sialylation is important in determination of metastasis. About 90% of colon cancers of I and II grade show an increased activity of α2,6-sialyltransferase and give a positive response with SNA lectin, specific for α2,6-linked sialic acid (Dall'Olio *et al.,* 2000). Loss of α2,6-sialylation in mutant of B16 melanoma is associated with a loss of metastatic potential (Dennis *et al.,* 1999). While there is no question on the role of α2,6-sialylation and increased expression of respective sialyltransferase accompanying cancer progression (Dall'Olio *et al.,* 2000), few reports indicate a specific role of α2,3-sialylation (Pousset *et al.,* 1997). According to Dimitroff *et al.* (1999) in colon cancer cells the α2,3-linked sialic acid bearing proteins are essential in mediating intercellular adhesion. In the melanoma cell lines, except for that derived from skin, investigated in the present and previous reports (Dimitroff *et al.,* 1999), α2,3-sialylation is found to be an important factor in determining the course of metastasis.

The presented results characterised the oligosaccharide component of N-cadherin from melanoma cell lines from different organs 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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