Glycans in melanoma screening. Part 2. Towards the understanding of integrin N-glycosylation in melanoma

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Abstract

Although melanoma is one of the most studied malignancies, it still remains challenging for biomedicine. Since aberrant glycosylation has been considered as an important hallmark of cancer for many years, melanoma glycomic studies give a chance of better understanding the biology of the disease. The multistage nature of melanoma development, which is accompanied by changes in the expression of adhesion receptors from the integrin family, provides a chance for searching for neoglycoforms of proteins that can be considered as future sensitive melanoma biomarkers. The β1,6-branching, sialylation and fucosylation seem to be important modifications of integrin N-glycans in the case of malignant melanoma progression.

Introduction

The incidence of malignant melanoma (*melanoma malignum*) is still rising worldwide. The most abundant type of the disease is cutaneous melanoma, which derives from the melanocytes present in the epidermis, but very rarely it can also start in other parts of the body in which melanocytes reside (e.g. uvea, conjunctiva, oral mucosa or digestive tract). Melanoma is a highly metastatic cancer, in which the 5-year survival rate for patient with early primary tumour (Stage I) exceeds 90%, and dramatically drops to 13% when distant metastatic lesions are present (Stage IV) [1,2]. The difference in survival rates between primary and metastatic melanoma potentially reflects the big changes in cancer cell physiology that accompany progression of the disease. Melanoma seems to be a very good model of epithelial neoplasm because its development is realized through five, quite well-distinguished histological stages: common acquired nevus, dysplastic nevus, RGP (radial growth phase) primary melanoma, VGP (vertical growth phase) primary melanoma and metastatic melanoma [3]. Therefore it is not surprising that malignant melanoma is one of the most studied cancers, with approx. 5000 independent cell line strains available for research [4]. The multistage nature of the development and the availability of cells from the different stages of melanoma progression provides a chance for making a quite complex description of molecular events accompanying the gain of metastatic potential of transformed cells.

Glycosylation in cancer studies

In the post-genomic era, studies of glycan structure and function become complementary to proteomic research. This is why the idea of structural and functional glycomics is becoming considered an important part of modern biochemistry. Cell-surface glycoproteins contribute to a variety of interactions between the cell and its surroundings, such as adhesion and migration [5,6], and surprisingly the potential chemical information hidden behind the protein glycans dramatically exceeds the information capacity of proteins and nucleic acids taken together [7]. Therefore it is not surprising that aberrant glycosylation accompanies various diseases, including cancer [8]. Changes in glycosylation occur in essentially all types of human cancers, but the detailed analysis of glycosylation still remains challenging because of the heterogeneity and complexity of oligosaccharides. However, advances in modern analytical techniques used in glycosylation analysis, such as MS and HPLC, have undoubtedly helped us to understand the cancer cell glyco-code [9–11]. The good understanding of differences in glycosylation between malignant and healthy tissues can influence biomedicine in many ways. First, the neoglycoforms of cancer cell proteins which are released from the cell through a secretory pathway or via proteolysis can be treated as potential sensitive and specific cancer biomarkers. Secondly, we can study the exact influence of changed glycosylation on cancer cell biology (at the molecular and cellular level) and, finally, manipulating the glycosylation machinery can be considered as a novel therapeutic strategy [12–16].

There is considerable evidence of aberrant glycosylation during progression of cancer, including melanoma [17]. These changes can concern glycosphingolipids [18], but the most studied glycoconjugates in this field are glycoproteins. There are some well-characterized hallmarks of O- and N-glycosylation associated with malignancy. These include incomplete synthesis of glycans, altered glycosylation of mucins (such as MUC family mucins), changes in the synthesis of histo-blood group antigens (especially from the Lewis family), increase of β1–6 GlcNAc branched N-glycans which can result in the presence of highly branched

Key words: glycan, integrin, melanoma screening, N-glycosylation.
Abbreviations used: CAM, cell-adhesion molecule; ECM, extracellular matrix; MALDI, matrix-assisted laser-desorption ionization; RGP, radial growth phase; VGP, vertical growth phase.
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structures (tri- and tetra-antennary), increased synthesis of polylactosamine chains, and finally changes in sialylation and fucosylation [16,19,20]. Unfortunately, we are still far from a precise description of the functional significance of these alterations in malignancy.

The initial studies on melanoma glycosylation suggested a possible link between tumour-associated N-glycosylation epitopes and cell-adhesion properties. Our studies of over 100 melanoma cell lines (uveal and cutaneous) from the ESTDAB (European Searchable Tumour Line Database) Melanoma Cell Bank (Tubingen, Germany) were designed to characterize glycan composition related to tumour progression along with the analysis of cell adhesion to ECM (extracellular matrix) proteins such as fibronectin, laminin and collagen [21]. Our results suggested that cutaneous metastatic melanoma cells revealed more diverse adhesion properties (from not adherent to highly adherent) to ECM proteins in comparison with primary melanoma cells. This may suggest that these cells differ in the assortment of adhesion proteins on their surface, or that the difference in cell behaviour might be due to some changes in the glycosylation of these cell receptors. The lectin screening of cell extracts showed also that proteins of almost all cell lines reacted with GNA (*Galanthus nivalis*; a lectin specific for high-mannose type glycans), AAA (*Aleuria aurantia*; a lectin specific for fucose), SNA (*Sambucus nigra*; a lectin specific for α2,6-linked sialic acids), MAA (*Maackia amurensis*; a lectin specific for α2,3-linked sialic acids) and PHA-L (*Phaseolus vulgaris*; a lectin specific for β1,6 GlcNAc-branched complex type N-glycans) agglutinins. Finally, RT (reverse transcription)–PCR analysis of the expression of glycosyltransferase mRNAs revealed that almost all cell lines expressed sialyltransferases (*SIAT4C* and *SIAT3*) and N-acetylglucosaminyltransferase V (*MGAT5*), the enzyme responsible for the formation of β1–6 GlcNAc-branched glycans. This general type of comparative analysis did not allow proposition of the exact relationships between melanoma glycosylation and its impact on cell physiology, but suggested that cellular adhesion mechanisms indeed could be an interesting candidate for further studies.

Adhesion proteins: the hallmark of melanoma

One of the typical hallmarks of melanoma progression towards a more invasive phenotype includes the changes in adhesion molecule expression [22,23]. One of the most characteristic features is the so called 'cadherin switch' (the loss of E-cadherins found on normal melanocytes, and a shift to N-cadherin on melanoma cells). This change, which is also characteristic of the EMT (epithelial–mesenchymal transition) phenomenon [24], allows melanoma cells to escape from control through keratinocytes and consequently couple to fibroblasts and endothelial cells within the dermis [25]. Our MALDI (matrix-assisted laser-desorption ionization)–MS analyses of melanoma cell glycosylation showed that N-cadherin from primary tumour cells possessed high-mannose and biantennary complex type N-glycans with α2–6-linked sialic acid, whereas N-cadherin from metastatic cells had mostly tri- and tetra-antennary complex type N-oligosaccharides [26].

Besides cadherin changes, melanoma cells show an increase in expression of cell–cell adhesion receptors of the immunoglobulin gene superfamily of CAMs (cell-adhesion molecules), such as MCAM (melanoma CAM), L1-CAM, ALCAM (activated leucocyte CAM), VCAM-1 (vascular CAM-1) and ICAM-1 (intercellular adhesion molecule 1) and changes in the expression of integrins [22]. Integrins are a family of transmembrane adhesion molecules created by non-covalently associated heterodimers of α and β subunits. The integrin headpiece, made by interacting N-terminal domains of both subunits, contains the ECM-binding site, whereas the C-terminal cytoplasmic tail mediates interactions with the cytoskeleton and with intracellular signalling agents [27]. Integrin action during adhesion events consists not only of physical binding to the extracellular ligand, but also involves the induction of the intracellular signalling cascade, thus regulating gene expression, cell growth, cell differentiation and survival from apoptosis [28]. Moreover, integrin activation occurs through the process known as 'inside–out' signalling, in which signals for receptor clustering and conformational changes are delivered from other receptors on the same cell. Therefore integrins can mediate information flux across the plasma membrane in both directions [27].

Expression of integrins in melanoma seems to be of special interest since these receptors play an important role at almost each step of the process of metastasis. In comparison with normal melanocytes, melanoma cells show the loss of integrin $\alpha6\beta1$ and overexpression of receptors such as $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$ and $\alpha v\beta3$ [29,30] and, what is important, some of these changes are characteristic of a particular stage of melanoma progression. Integrin $\alpha v\beta3$ is considered a marker of RGP-to-VGP transition and, consequently, as the one related with the gain of metastatic potential [29]. Integrins $\alpha1\beta1$ and $\alpha2\beta1$ seem to be characteristic of metastatic cells only, whereas integrin $\alpha3\beta1$ (an important laminin receptor) is present on primary and metastatic cells [30,31]. This is why some of these antigens can be considered prognostic for melanoma progression.

There may be various consequences of changes in integrin expression on melanoma cells. The down-regulation of laminin receptor, integrin $\alpha6\beta1$, can result in loss of contact of primary tumour cells with the basement membrane, loosening tumor mass, and therefore it can promote invasion into the dermis. The presence of other integrins such as $\alpha3\beta1$, $\alpha5\beta1$ and $\alpha v\beta3$ can modulate melanoma cell invasiveness, adhesion and migration through interactions with their ECM ligands. Finally, since the integrins can also take part in heterotypic interactions with adhesion receptors from the immunoglobulin gene superfamily, their expression provides a chance for interactions with other cell types during the process of metastasis (e.g. $\alpha v\beta3$–L1-CAM-mediated

adhesion to endothelial cells which can promote cancer cell extravasation) [32]. Such a broad spectrum of potential integrin functions in melanoma progression provides an area for further glycosylation studies.

The importance of integrin glycans in melanoma

To perform detailed analyses of the impact of glycosylation on glycoprotein function, the structural analysis of the glycan moiety is an essential step of the research. Glycans can regulate protein function in many ways and on many levels, and nowadays they are treated as one of four basic components of cells. Vertebrates, and especially mammals, have evolved a highly complex glycan repertoire and their heterogeneity still remains challenging for structural analysis. At the molecular level, glycans can shield the protein surface and prevent non-specific protein–protein interactions, protect from proteolysis and increase glycoprotein stability and solubility. Some glycans have a great influence on the conformation of the cellular receptor protein, modulating their ligand-binding activity [33]. At the cellular level, glycans influence cell–cell interactions, as well as interactions of cells with ECM proteins during adhesion, migration and invasion [34].

Integrins are heavily glycosylated proteins possessing multiple potential N-glycosylation sites. There is much evidence showing that glycosylation affects integrin biological activity [5,35]. Glycosylation can be important in integrin heterodimerization, ligand binding, complex forming with other membrane proteins, such as growth factor receptors or tetraspanins, and finally in intracellular signal transduction. It seems, however, that only N-oligosaccharides located on certain protein motifs regulate integrin conformation and biological function [36]. The most studied integrin receptors in the case of their N-glycosylation are integrins $\alpha3\beta1$ and $\alpha5\beta1$ [35]. It was found for instance that N-oligosaccharides present on the $\alpha5$ subunit β-propeller domain are essential for integrin $\alpha5\beta1$ heterodimerization, cell-surface expression and biological function, such as cell spreading and actin cytoskeletal formation, as well as for the proper folding of the $\alpha5$ subunit [37,38]. In the case of $\beta1$ and $\beta3$ subunits, the I-like domains seem to be the most important protein parts for the regulation of functions by N-glycans [39,40].

In the case of melanoma studies, the structural analysis of integrins $\alpha3\beta1$ and $\alpha v\beta3$ glycosylation isolated from cells of different origin was carried out. MALDI–MS characterization of carbohydrate moieties of $\alpha3$ and $\beta1$ subunits from non-metastatic (WM35) and metastatic (A375) human melanoma cell lines showed that these cells differ in glycosylation profiles [41]. The $\beta1$ integrin subunit from both cell lines displayed tri- and tetra-antennary complex-type glycans, but only the A375 cell line displayed the presence of sialylated tetra-antennary complex-type glycan. In contrast, only the $\alpha3$ subunit from metastatic cells possessed $\beta1$–6-branched structures. The presence of this cancer-associated glycan modification on both integrin subunits in the case of more aggressive cells suggests their role in modulating tumour cell adhesion by affecting the ligand-binding properties of $\alpha3\beta1$ integrin. In direct ligand-binding assays, enzymatic removal of sialic acid residues from purified integrin $\alpha3\beta1$ stimulates its adhesion to ECM proteins, such as fibronectin, laminin and collagen IV. Other studies on two metastatic cutaneous melanoma cell lines (WM9 and WM239) indicated that both integrins examined ($\alpha3\beta1$ and $\alpha v\beta3$) possessed heavily sialylated and fucosylated glycans, with $\beta1$,6-branches and short polylactosamine chains [42]. Functional studies revealed that the N-oligosaccharide component of the integrins tested influenced cellular migration on vitronectin and $\alpha3\beta1$ integrin binding to laminin-5.

Our results concerning melanoma cells suggest that $\beta1$,6-branches, as well as sialic acid and fucosylation, can be the most important players in the case of regulation of integrin function by N-glycans. This seems to be in agreement with general observations of N-glycosylation changes during cancer progression. However, the exact function of each particular glycan moiety of integrin receptors still remains a great challenge for researchers. The biology of integrin glycans is unfortunately not easy to study, partly because of the difficulties in isolation of functional heterodimers and their redundancy in the case of ligand binding.

Conclusions

It seems that changes in adhesion molecule expression during melanoma progression act together with the changed glycosylation profile of important adhesion receptors. This 'co-operation' can help cancer cells to metastasize, but also creates the possibility to look for novel mechanisms of regulation of the process, and consequently for new therapeutic targets. It is still not clear whether the differences in integrin glycosylation between primary and metastatic melanoma cell lines result from the changed activity of glycosylation machinery at the level of primary tumour formation and are specific for the prognostic factor of each case of the disease, or are a dynamic process accompanying each stage of the melanoma progression. Further research, using different melanoma models, such as tissue sections and cell lines from different stages of melanoma progression derived from the same patient, is needed. However, integrins seem to be promising candidates for searching for neoglycoforms of proteins, which can be considered as future sensitive melanoma biomarkers.

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