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QUARTERLY

Differences of $\alpha_3\beta_1$ integrin glycans from different human bladder cell lines✪

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Expression as well as properties of integrins are altered upon transformation. Cell adhesion regulated by integrins is modulated by glycosylation, one of the most frequent biochemical alteration associated with tumorogenesis. Characterisation of carbohydrate moieties of $\alpha_3\beta_1$ integrin on the cultured human bladder carcinoma (T-24, Hu456, HCV 29T) and human normal ureter and bladder epithelium (HCV 29, Hu609) cell lines was carried out after an electrophoresis and blotting, followed by immunochemical identification of α_3 and β_1 integrin chains and analysis of their carbohydrates moieties using highly specific digoxigenin-labelled lectins. In all the studied cell lines $\alpha_3\beta_1$ integrin was glycosylated although in general each subunit differently. Basic structures recognized in β_1 subunit were tri- or tetraantennary complex type glycans in some cases sialylated (T-24, HCV 29, HCV 29T) and fucosylated (Hu609, HCV 29T). Positive reaction with *Phaseolus vulgaris* agglutinin and *Datura stramonium* agglutinin suggesting the presence of β1-6 branched N-linked oligosaccharides was found in cancerous cell lines (T-24, Hu456) as well as in normal bladder epithelium cells (Hu609). High mannose type glycan was found only in β_1 subunit from Hu456 transitional cell cancer line. On the other hand α_3 subunit was much less glycosylated except the invasive cancer cell line T-24 where high mannose as well as sialylated tri- or tetraantennary complex type glycans were detected. This observation suggests that changes in glycosylation profile attributed to invasive phenotype are rather associated with α_3 not β_1 subunit.

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Abbreviations: BSA, bovine serum albumin; mAbs, monoclonal antibodies; TBS, Tris buffered saline. Agglutinins were from: AAA, *Aleuria aurantia*; DSA, *Datura stramonium*; GNA, *Galanthus nivalis*; MAA, *Maackia amurensis*; PHA-L, *Phaseolus vulgaris*; SNA, *Sambucus nigra*.

Integrins are a family of heterodimeric cell adhesion molecules involved in a variety of biological processes. These cell surface receptors mediate cell–extracellular matrix and cell–cell interactions. They consist of noncovalently bound α and β subunits that associate in various combinations forming ligands of different specificity. To date, at least 22 different integrins are known. It has become clear that integrins are not only involved in cell adhesion but in signal transduction as well. It is thought that β subunit intracellular domain participates in association with the cytoskeleton in focal adhesion [1–4].

There are a number of indications that integrins are altered upon transformation [5–9]. One of the most consistent observation is that the polarized distribution of integrins seen in normal epithelia is often lost in carcinoma. In many cases, overall expression of integrins tends to be reduced in tumors [2]. However, there is no clear pattern for the effects of transformation on extracellular matrix receptor expression. In general, the tumor cells have a much greater diversity of integrin expression than their normal cell counterparts, particularly for such receptors as $\alpha_3\beta_1$ and $\alpha_v\beta_1$ that recognize many different ligands and provide versatility for tumor cells populations [8]. However, the loss or the gain of expression of a particular integrin has not been linked directly to malignant transformation, rather the changes in integrin expression seem to be tumor and integrin specific.

Recently, the $\alpha_3\beta_1$ integrin has attracted attention since its function seemed to be versatile depending on the presence of other integrins and bivalent cations [10]. The $\alpha_3\beta_1$ integrin has been shown to be a receptor for laminin [11–13], fibronectin [14] and collagen [10]. Laminin 5 which is expressed by carcinoma tissues of various organs, has been identified as the preferential ligand for the $\alpha_3\beta_1$ integrin [13]. This integrin has been suggested to be involved in cell–cell interactions since it may directly interacts in a homophylic [15] or in a heterophylic mode [16].

Oligosaccharides play a vital role in normal growth and development of living organisms [17]. N-glycosylation of integrin $\alpha_5\beta_1$ is of fundamental importance for assembly of the subunits and maintaining of binding ability [18]. In this paper we have analysed the carbohydrate moieties of $\alpha_3\beta_1$ integrin in five human bladder cell lines, using reaction with specific lectins, the method applied recently by others in study of integrin glycans [19, 20].

MATERIALS AND METHODS

Cell lines and culture conditions. The cell lines of non-malignant transitional epithelial cell of bladder – Hu609, ureter – HCV 29 and cancer lines from transitional cell cancer of urine bladder – Hu456 [21], T-24 (HTB-4, ATCC, [22]) and BC3726 (v-raf transfected HCV 29 line, later named HCV 29T) were used. These lines were obtained from Cell Line Collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland).

All cell lines were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Boehringer), 100 units/ml of pencillin, 100 μg/ml of streptomycin. Cells after reaching confluency were washed twice and then harvested in phosphate buffered saline. The cell pellets were homogenized in 50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and protease inhibitor coctail (Sigma P-2714) by sonification (three times 5 s each) (Bandelin electronic UW 70 Sonopuls Gm 70) extracted for 1 h on ice in the same buffer containing additionally 0.1% Triton X-100 and 0.3% protamine sulphate. Finally cell extracts were clarified by centrifugation at 35 000 $\times$ ***g*** for 1 h (L7-65 ultracentrifuge, Beckman).

Mouse monoclonal antibodies (mAbs) to human integrin subunit β_1 (clone 2A4) from Genosys, and to α_3 (clone P1B5) from DAKO were used. Secondary goat anti-mouse antibodies were purchased from Boehringer.

A Glycan Differentiation Kit, containing the digoxigenin (DIG)-labelled lectins: *Galanthus nivalis* agglutinin (GNA) – specific for terminal Man (α1-2, α1-3 or α1-6) Man units; *Sambucus nigra* agglutinin (SNA) – specific for NeuAc (α2-6) Gal; *Maackia amurensis* agglutinin (MAA) – specific for NeuAc (α2-3) Gal; *Datura stramonium* agglutinin (DSA) – specific for Gal (β1-4) GlcNAc; *Aleuria aurantia* agglutinin (AAA) – specifically binding Fuc (α1-2, α1-3, α1-6) GlcNAc and *Phaseolus vulgaris* agglutinin (PHA-L) – specific for (β1-6) branched N-glycans were purchased from Boehringer. PVDF membranes were from Millipore. Other chemicals were of the highest purity and were purchased from Sigma.

SDS/PAGE and blotting. Cell extract proteins (100 μg) were separated by 8% polyacrylamide gel electrophoresis in the presence of SDS in non reducing condition according to Laemmli [23] and transferred to an PVDF membrane, by electrophoretic blotting [24] for 16 h at constant current 250 mA at 4°C. The efficiency of protein transfer was at least 95% as checked by staining the gel with Coomassie Brilliant Blue R-250. Proteins immobilised on PVDF membrane were stained with Ponceau S followed by destaning in H_2O.

Glycan chain analysis. Glycan chain analysis of α_3 and β_1 subunits was performed with the use of a Glycan Differentiation Kit according to Haselbeck *et al.* [25] as described in details earlier [26].

Immunodetection of α_3 and β_1 subunits. The blots were blocked in TBS/Tween (0.02 M Tris/HCl, pH 7.6, containing 0.15 M NaCl and 0.1% Tween 20), with 1% bovine serum albumin (BSA). Afterwards, membranes were sequentially incubated with specific mAbs diluted in TBS/Tween with 1% BSA (1:200 for β_1 subunit and 3:200 for α_3 subunit) for 18 h, and after triple wash with TBS/Tween, incubated with the alkaline phosphatase coupled goat antimouse Ig (1:500 in TBS/Tween with 1% BSA) for 1 h. The α and β subunits were localized on the sheet with 4-nitroblue tetrazolium as a substrate.

Other methods

Protein content was determined by the dye-binding assay method [27] using bovine serum albumin as a standard. Gels were calibrated for molecular weight determination using the Sigma standard Kit for electrophoresis in SDS.

RESULTS

In all cell lines the presence of α_3 and β_1 subunits was revealed by the reaction with specific monoclonal antibodies in cell extracts as well as by flow cytometry. The expression was comparable in all cell lines and ranged from 61–87% for β_1 and 71–93% for α_3 subunits (data not shown). The apparent molecular weights of α_3 and β_1 subunits ranged from 124 000 to 138 000 and from 112 000 to 120 000, respectively.

The identification of integrin subunits and reaction with a given lectin are presented in Figs. 1 and 2. Only one of five examined human bladder cell lines, invasive bladder cancer cell line T-24 expressed α_3 subunit that reacted with every used lectin except MAA. The positive reaction of α_3 subunit from this line with DSA, SNA, GNA and PHA-L indicated the presence of high-mannose type glycans as well as tri- and/or tertaantennary complex type glycans with sialic acid. In two lines, Hu456 and HCV 29 α_3 subunits reacted only with high-mannose recognising lectin – GNA, suggesting that only this type of oligosaccharide was present. No reaction of α_3 polypeptide from HCV 29T and Hu609 lines with any of the lectins was observed under condition used in this experiment. In all five examined cell lines β_1 subunit was glycosylated as indicated by the positive reaction with the lectins used. Reaction with PHA-L suggested

the presence of tri- and/or tetraantennary complex type glycans in all lines except β_1 integrin chain from HCV 29. The presence of sialic acid in β_1 subunit of integrin from T-24, HCV 29 and HCV 29T lines was indicated by

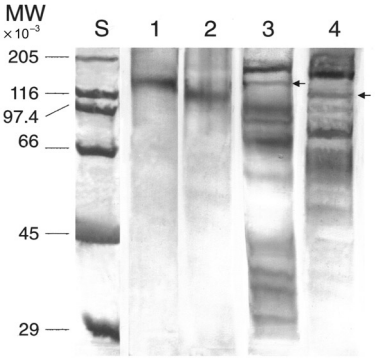

Figure 1. Characterisation of glycan types of α_3 and β_1 subunits from HCV cell line probing with: 1, anti α_3 antibody; 2, anti β_1 antibody; 3, *Galanthus nivalis* agglutinin; 4, *Maackia amurensis* agglutinin.

the reaction with MAA and/or SNA, the lectins staining sialic acid α (2–3) and α (2–6) linked to galactose, respectively. Additionally, β_1 subunits from HCV 29T, Hu456 and Hu609 possessed core fucose. Only β_1 subunit from Hu456 line reacted with GNA suggesting presence of high mannose type glycans.

DISCUSSION

Different studies have indicated that the role of carbohydrates as components of glycoproteins includes stabilisation of protein conformation, modulation of physicochemical properties and biological function. They provide ligands for specific recognition and binding events mediating protein targeting, cell–matrix or cell–cell interactions [17]. One of the most frequent biochemical changes associated with tumorogenesis and metastasis is the altered expression and structure of cell membrane oligosaccharides [28–33]. The only changes observed up to now during the malignant transformation of bladder tumor was the loss of AB0 antigens from the cell surface [34] due to the allelic loss of the AB0 glycosyltransferase-encoding genes [35]. Here we described the preliminary characterisation of oligosaccharides of $\alpha_3\beta_1$ integrin from human bladder cell lines. In all the studied cell lines this protein was glycosylated although in general each subunit differently irrespective of the correlation between glycosylation state of α_3 and β_1 subunits.

Our knowledge of the structure and function of oligosaccharides of integrins is still fragmentary. Some previous studies showed that N-glycosylation of integrin $\alpha_5\beta_1$ was essential for proper assemble of subunits, acquisition of biological activity and maintaining receptor-fibronectin binding function [36, 37]. Nakagawa *et al.* [18] using three dimensional mapping technique identified 35 kinds of oligosaccharides on human $\alpha_5\beta_1$ integrin, but the most predominant was diantennary di-α(2,3) sialyl/fucosyl structure. Recently, Prokopishyn *et al.* [19] has found that $\alpha_3\beta_1$ integrin expressed by human colon carcinoma cells is a major carrier of oncodevelopmental carbohydrate epitopes whose presence may modulate tumor cell adhesion, migration and invasion. Studies on carbohydrate structure of this integrin performed with glycosidase digestion and lectin blotting, indicated that this was a sialoglycoprotein and that both subunits contained β1-6 branched Asn-linked oligosaccharides. However, more intense staining (reaction with PHA-L) was associated with the β_1 subunit [19].

It should be emphasised that our results confirmed the results of other studies on glycosylation of integrin α and β chains carried out until now. Different glycosylation of each integrin subunit of $\alpha_6\beta_1$ integrin from B16-F10 murine melanoma was shown by Chammas *et al.* [38]. These studies showed that although both chains of this integrin were highly sialylated but only β_1 subunit possessed PHA-L reactive oligosaccharides.

Our data suggest that in all studied cell lines β_1 subunit was more glycosylated than α_3 subunit despite the fact that similar number of

potential glycosylation sites – thirteen for α_3 and twelve for β_1 was predicted [39]. Akiyama *et al.* [36] using endoglycosidase-H for deglycosylation showed that only six potential observed in T-24 line for both subunits and in HCV 29 and HCV 29T lines for the β_1 subunit only. The two other lines Hu456 and Hu609 did not shown any reaction with MAA or SNA.

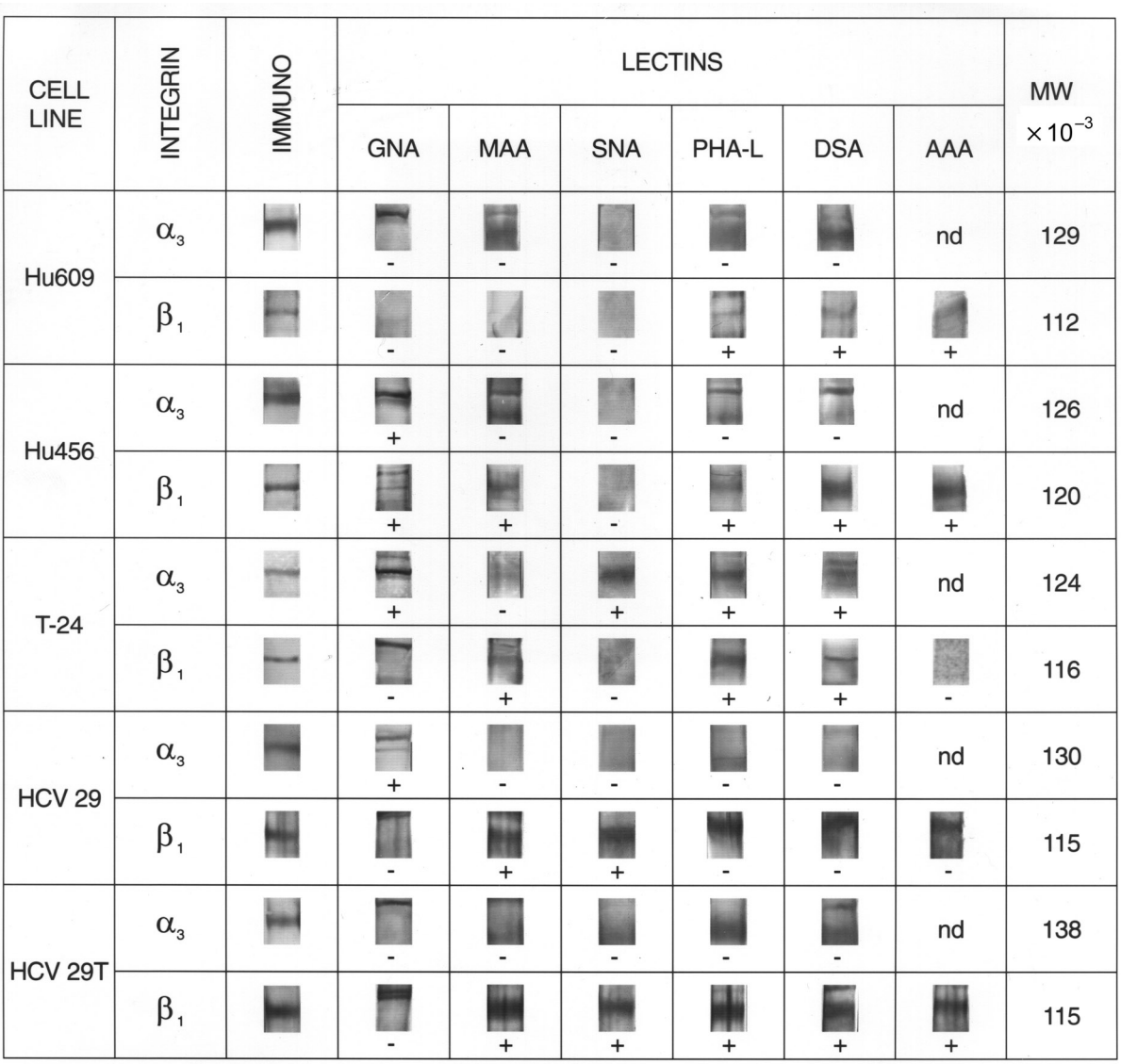

Figure 2. Analysis of $\alpha_3\beta_1$ integrin glycans with specific lectins.

100 μg of cell lysate was run on 8% PAGE/SDS and blotted onto PCVF membrane and probed with lectins: *Galanthus nivalis* agglutinin, *Maackia amurensis* agglutinin, *Sambucus nigra* agglutinin, *Phaseolus vulgaris* agglutinin, *Datura stramonium* agglutinin and *Aleuria aurantia* agglutinin. Immuno – α_3 and β_1 subunit probed with mAbs. (+) Positive reaction with lectin, (–) negative reaction. MW, molecular weight of a given subunit.

N-glycosylation sites of β_1 subunit were glycosylated in $\alpha_6\beta_1$ integrin. Digestion of the soluble $\alpha_3\beta_1$ integrin with N-glycosidase F suggested that in case of β_1 subunit only two or three, and in α_3 subunit only two of all potential N-glycosylation sites were occupied [20]. In our studies the presence of sialic acid was

We did not find clear cut cancer-associated changes in glycosylation profile of β_1 subunit. Among altered patterns, the expression of tri- and tetraantennary oligosaccharides recognised by PHA-L have been associated with malignant transformation of the cells. In case of β_1 subunit positive reaction with PHA-L and

DSA suggesting the presence of β1-6-branched Asn-linked oligosaccharides was observed in cancerous cell lines (T-24, Hu456) as well as in normal cell line (Hu609). On the contrary, the only α_3 subunit showing reaction with PHA-L was that from T-24 line. This observation allowed us to suggest that perhaps changes in glycosylation of α_3 subunit (branching and sialylation) of T-24 cancer cells might be characteristic of invasive phenotype. The presence of this type oligosaccharides was indicated to play an important role in tumor progression by changing cell–cell and cell–extracellular matrix interactions [19, 28, 40, 41] and in case of $\alpha_3\beta_1$ integrin might correlate with specific glycosylation of α_3 subunit.

To solve this problem further more detailed studies using very sensitive methods of glycan analysis are carried out.

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