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QUARTERLY

Adhesion properties of human bladder cell lines with extracellular matrix components: the role of integrins and glycosylation✪

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Integrin subunits present on human bladder cells displayed heterogeneous functional specificity in adhesion to extracellular matrix proteins (ECM). The non-malignant cell line (HCV29) showed significantly higher adhesion efficiency to collagen IV, laminin (LN) and fibronectin (FN) than cancer (T24, Hu456) and v-raf transfected (BC3726) cell lines. Specific antibodies to the α_2, α_5 and β_1 integrin subunits inhibited adhesion of the non-malignant cells, indicating these integrin participation in the adhesion to ECM proteins. In contrast, adhesion of cancer cells was not inhibited by specific antibodies to the β_1 integrin subunit. Antibodies to α_3 integrin increased adhesion of cancer cells to collagen, LN and FN, but also of the HCV29 line with colagen. It seems that α_3 subunit plays a major role in modulation of other integrin receptors especially in cancer cells. Differences in adhesion to ECM proteins between the non-malignant and cancer cell lines in response to Gal and Fuc were not evident, except for the v-raf transfected cell line which showed a distinct about 6-fold increased adhesion to LN on addition of both saccharides. *N*-Acetylneuraminic acid inhibited adhesion of all cell lines to LN and FN irrespective of their malignancy.

Many studies have attributed the altered adhesive behaviour of tumour and transformed cells to changes in the expression pattern and affinity of their integrins (Morini *et al.*, 2000; Schramm *et al.*, 2000). Most integrins are able to bind various ligands with different affinities. The high affinity state of an integrin for its ligand may be a constitutive condition depend-

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Abbreviations: ECM, extracellular matrix; FN, fibronectin; Fuc, fucose; Gal, galactose; LN, laminin; NeuAc, *N*-acetylneuraminic acid.

ing on the cell type, or may be induced by signalling through other cells surface receptors (Ivaska & Heino, 2000). Rapid upregulation of the integrin function is induced by numerous stimuli acting either from outside the cell or from its interior (Humphries, 1996). The interactions of cells with extracellular matrix (ECM) depend on the expression and function of integrin receptors. On the other hand, changes in the expression and structure of carbohydrates may be considered as a universal feature of malignant transformation. It is known that protein–carbohydrate interactions play a crucial role in recognitive events. In particular, galectins that specifically bind β galactose residues have been implicated as modulators of cell adhesion, since they are secreted into the extracellular space and cross-link the glycoconjugates on the cell surface and ECM proteins, most probably laminin (LN) (Zhou & Cummings, 1993) and fibronectin (FN) (Ozeki *et al.*, 1995). In accordance with the proposed function galectins enhance (Kuwabara & Liu, 1996) or inhibit (Hadari *et al.*, 2000) cell matrix interactions. Carbohydrate-based recognition occurring on the cell surface depends also on the presence of sialic acid residues, which potentially can inhibit intermolecular and intercellular interactions due to their negative charge. However, they can also act as ligands recognised by a variety of sialic acid binding lectins (Kim & Varki, 1997). Also sialyl Lewis[a] antigens are implicated in the adhesion of human cancer cells (Ugorski & Laskowska, 2002).

The results of flow cytometry of the same human non-malignant ureter epithelium (HCV29), malignant human bladder carcinoma (T24, HU456) and v-raf transfected HCV29 (BC3726) cell lines (Laidler *et al.*, 2000) indicated significant differences in expression of the β_1 integrin subunit in cell lines irrespective of their malignancy and nonsignificant differences in the expression of the α_2, α_3 and α_5 subunits between malignant and non malignant cell lines. To specify more closely the role of these integrins in the progression of the transitional bladder cancer we have extended our studies to the adhesion efficiency assays of the above cell lines to collagen type IV, LN and FN, using specific anti-integrin antibodies and also three monosaccharides, i.e. fucose (Fuc), galactose (Gal) and *N*-acetylneuraminic acid (NeuAc), affecting cell adhesion.

MATERIALS AND METHODS

Chemicals. PVDF membranes were from Millipore. Collagen IV, fibronectin, laminin, bovine serum albumin (BSA) solution, crystal violet, proteinase inhibitor cocktail, and fucose were from Sigma Chemical Co. Galactose and *N*-acetylneuraminic acid were from Serva. All remaining chemicals were of analytical grade.

Monoclonal antibodies. The following monoclonal antibodies (mAbs) were used: specific to the α_2 (clone P1E6) and α_3 (clone P1B5) integrin subunits (DAKO); specific to the α_5 (clone CDw49e) and β_1 (clone CD29/GPIIa) subunits (Genosys Biotechnologies).

Cell lines and culture conditions. The following cell lines were used: non-malignant transitional epithelial of ureter, HCV29, and transitional cancer of ureter bladder, Hu456 (Vilien *et al.*, 1983), T24 (HTB-4, ATCC (Bubenick *et al.*, 1973)), and v-raf transfected HCV29 line, BC3726. These cell lines were obtained from the Cell Line Collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland). The cell lines were cultured in RPMI 1640 medium (Sigma Chemical Co.) containing 10% fetal bovine serum (FBS) (GibcoBRL[TM] or Boehringer) and antibiotics (penicilin — 100 U/ml, streptomycin — 100 μg/ml, Polfa Tarchomin, Poland). The cells were grown in monolayers in a 95% air/5% CO_2 atmosphere at 37°C in a humidified incubator.

Cell adhesion assay. 96-well culture plates (Costar) coated alternatively with 10 μg/ml collagen IV, LN or FN were blocked for 90 min

at 37°C with 1% heat-denatured BSA. Next the wells were washed twice with phosphate-buffered saline (PBS) and once with RPMI 1640 to remove the excess of BSA prior to the addition of cells. Before the adhesion assay, cells were washed in serum-free medium and starved for 30 min. Cells were trypsinized and reseeded at a density of 5×10^4 cells per well on the different matrix proteins. Cells were allowed to adhere for 30 min at 37°C in a CO_2 incubator. Non-adherent cells were removed by washing three times with PBS. Adherent cells were fixed for 10 min in 96% ethanol, washed three times in PBS, stained with 0.1% crystal violet for 25 min, washed in tap water and air-dried. Stained cells were lysed overnight on a shaker in 0.5% Triton X-100 to release the dye. The absorbance at 600 nm determined using an ELISA-reader (El_x 808_{IV} Ultra Microplate Reader, Bio Tek Instruments) was proportional to cell numbers (Kueng *et al.*, 1989). Each data point was calculated from two separate experiments performed in triplicate and was expressed as the mean ± standard deviation (S.D.). Non-specific cell adhesion as measured on BSA-coated wells was subtracted.

For competition experiments, cells were pre-incubated with monoclonal antibodies against the α_2, α_3, α_5 or β_1 integrin subunits at a concentration of 1.4 μg/ml, 2 μg/ml, 1.2 μg/ml and 1.5 μg/ml, respectively, on a shaker at room temperature for 30 min prior to seeding on matrix protein coated wells. After pre-incubation with mAbs, cell viability was higher than 97%. The antibody remained present during the assay. Control competitions with non-specific IgG had no effect on adhesion.

In order to study the participation of carbohydrate moieties of the cell surface glycoproteins as well as ECM proteins in the adhesion process, we analysed the adhesion efficiency of the cells to LN and FN in the presence of 0.1 M Fuc, Gal or NeuAc solutions in PBS.

Statistics. The significance of the differences between mean values were computed using Dunkan's new multiple range test (*$P < 0.05$ or **$P < 0.01$).

Other methods. Protein was determined by the Comassie Brilliant Blue-binding assay method (Bradford, 1976) using bovine serum albumin as standard.

RESULTS

Cell adhesion

The cell lines examined attached to collagen IV, LN and FN with different efficiencies (Fig. 1). The adhesion efficiency of the normal HCV29 cells to all ECM proteins was significantly higher than that of T24, Hu456 and BC3726 cells, except for Hu456 and BC3726 cells adhesion to collagen IV.

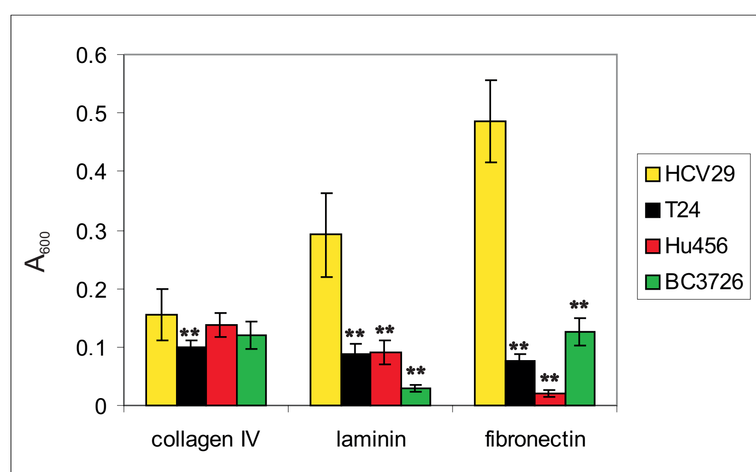

Figure 1. Adhesion of human non-malignant HCV29 and malignant T24, Hu456, and BC3726 cells to collagen IV, laminin and fibronectin.

Cell adhesion is presented as absorbance units (A) at 600 nm. Values are expressed as mean ± standard deviation of two independent experiments performed in triplicate. Bars indicate S.D. and *$P < 0.05$ and **$P < 0.01$.

Effect of monoclonal antibodies on cell adhesion

In order to assess whether $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$ or other integrins of the β_1 integrin family were responsible for mediating adhesion to collagen IV, LN or FN, cell adhesion assays were performed in the presence of specific anti-integrin monoclonal antibodies.

As shown in Fig. 2A, mAb P1A6 to the α_2 subunit inhibited Hu456 and BC3726 cells adhesion to collagen IV, increased attachment of T24 cells, but had no effect on HCV29 cells adhesion level. In contrast, mAb P1B5 to α_3 subunit increased adherence of all cells to collagen IV (Fig. 2A). On the other hand, mAb CD29/GPIIa to β_1 subunit had no effect on any cells adhesiveness to collagen IV (Fig. 2A). Similarly, this mAb did not affect the ability of T24, Hu456 or BC3726 cells to adhere to LN, but caused a decline in HCV29 cell adhesion to LN (Fig. 2B).

mAb P1B5 to the α_3 subunit inhibited HCV29 cells attachment to LN and FN (Fig. 2B, C). However, this mAb had an opposite effect on T24 and Hu456 cells adhesion level to LN and FN, failed to affect BC3726 cell adhesion to LN and caused an increase in the attachment to FN (Fig. 2B, C). Similarly, mAb CDw49e to the α_5 subunit inhibited HCV29 and BC3726 cells attachment to FN and increased T24 and Hu456 cells binding (Fig. 2C). Incubation of cells with non-specific IgG did not influence binding to collagen IV, LN nor FN (not shown).

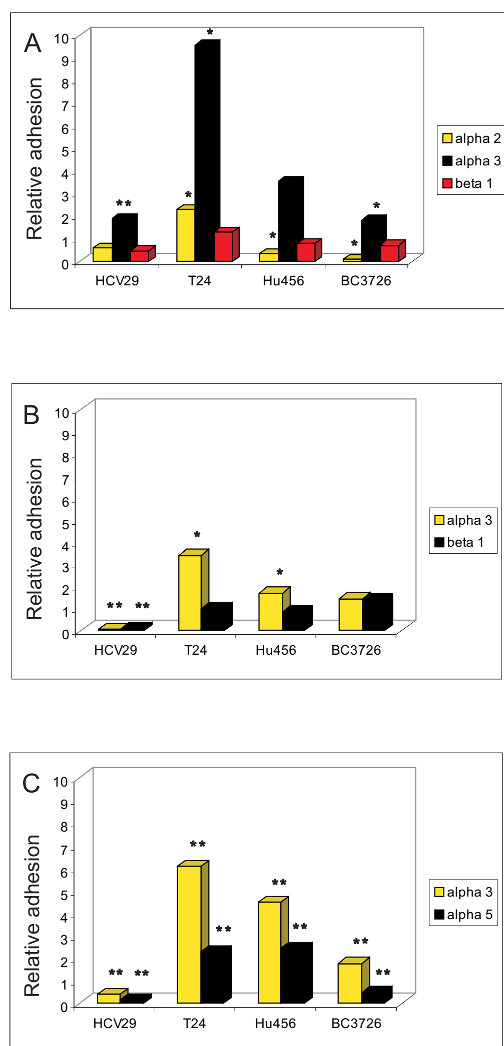

Figure 2. Effect of monoclonal antibodies to α_2, α_3, α_5 and β_1 integrin subunits on adhesion of HCV29, T24, Hu456 and BC3726 cells to collagen IV (A), laminin (B) and fibronectin (C).

Cell adhesion of cells adhering in the presence of the indicated antibodies is presented relatively to control adhesion (cells without additions). Each result is the average of two independent experiments performed in triplicate. Relative to control taken as one. $^*P < 0.05$ and $^{**}P < 0.01$.

Effect of monosaccharides on cell adhesion to LN

A significant effect was observed in the case of NeuAc, whose presence decreased or completely reduced adhesion to LN of all the cell lines tested (Fig. 3A). Attachment of HCV29 cells was also inhibited in the presence of Gal or Fuc, by about 21% and 52% of control level (Fig. 3A). In the case of the cancer cell lines (T24, Hu456) the presence of Gal or Fuc inhibited or had no effect on adhesion to LN (Fig. 3A). On the other hand, addition of Gal and Fuc caused a significant increase in the attachment of BC3726 cells to LN (Fig. 3A).

Effect of monosaccharides on cell adhesion to FN

Similar as in the case of LN-binding NeuAc, reduced adhesion of all the cell lines tested to

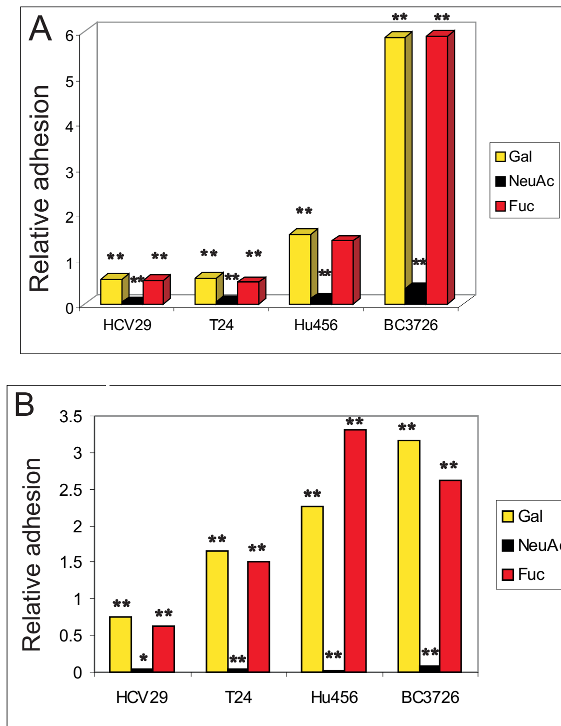

Figure 3. Influence of different monosaccharides on adhesion of HCV29, T24, Hu456 and BC3726 cells to laminin (A) and fibronectin (B).

Cell adhesion was measured in the presence of 0.1 M solutions of Gal, NeuAc or Fuc. Cell adhesion is presented relatively to control adhesion (cells without additions). Each result is the average of two independent experiments performed in triplicate. Relative to control taken as one. *$P < 0.05$ or **$P < 0.01$.

FN to 0–9% of control level (Fig. 3B). Adhesion of HCV29 cells was inhibited or reduced by Gal and Fuc as in the case of LN (Fig. 3B). Surprisingly, the cancer cells in the presence of Gal or Fuc adhered more efficiently to FN (Fig. 3B). The BC3726 cells behaved in the same way as on LN being more adhesive in the presence of both this monosaccharides.

DISCUSSION

Our results show that adherence of human bladder non-malignant HCV29 cells to collagen IV, LN and FN is higher than of cancer cells. It is known that the interactions between cells and ECM proteins largely depend on the expression of functional integrins on the cell surface, mainly of the β_1 integrin family (Curley *et al.*, 1999). Adhesion of non-malignant cells was inhibited by antibodies against the $\beta_1, \alpha_2, \alpha_3$ and α_5 integrin subunits indicating that these integrins are important receptors in non-malignant cells. These results are consistent with those of Heino (2000) and Nykvist *et al.* (2000) that the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins are high-affinity receptors for type IV collagen, while according to Akiyama *et al.* (1995) integrin $\alpha_5\beta_1$ is the major FN receptor for most cells. A lack or even reverse response characteristic for cancer human bladder cells to anti-integrin antibodies requires an explanation.

A single cell type may express multiple integrins with an apparently redundant ligand specificity. The functional significance of this redundancy is not understood but it is clear that different binding sequences are recognised by separate receptors that may function cooperatively and each can lead to different cellular response. Signalling events initiated by the occupancy of one integrin can suppress functions associated with other integrins, a process known as integrin cros-talk, e.g. integrin $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Blyston *et al.*, 1994; 1999; Simon *et al.*, 1997). Positive and negative co-operation as well as trans-dominant inhibition have been described for several integrins (Diaz-Gonzales *et al.*, 1996).

Our results allow us to conclude that in the human bladder, multiple receptors contribute to cell adhesion to ECM proteins and each cell line uses different repertoire of these receptors, as manifested distinctly in cancer cells. This would imply that adhesion of tumour cells is mediated by other integrins, possibly also from a non-β_1 family, e.g. by $\alpha_v\beta_3$ which like $\alpha_5\beta_1$ can serve as an FN receptor.

At least fifteen different molecules have been described as either LN receptor or LN-binding protein, including several members of the integrin family (Belkin & Stepp, 2000). The specificity of integrin $\alpha_3\beta_1$ for its ligands depends on the presence of other integrins that may display a higher activity

for the same ligands (Kreidberg, 2000; Laplantine *et al.,* 2000). There is growing amount of evidence that integrin $\alpha_3\beta_1$ transdominantly regulates integrin $\alpha_6\beta_1$ in mouse keratinocytes (Hodivala-Dilke *et al.*, 1998; Laplantine *et al.*, 2000) or regulates negatively integrin $\alpha_2\beta_1$ in human breast carcinoma cell lines (Lichtner *et al.,* 1998). Our results confirmed the major role for the $\alpha_3\beta_1$ integrin in modulating other adhesion receptors especially in the case of cancer cell lines like T24 and Hu456. However, the mechanism of the postulated shift or cooperation in integrins interaction in malignant cells remains unknown.

Besides, although integrin-mediated adhesion to FN and LN is based on interaction of specific amino-acid residues, the strength of the binding may be modulated by various factors including glycosylation of integrins (Chammas *et al.,* 1993; Zheng *et al.,* 1994), glycosylation status of ECM proteins (Zheng & Hakomori, 2000) as well as carbohydrate–carbohydrate interactions (Wang *et al.*, 2001).

We observed uniform decrease of adhesion of all the cell lines to LN and FN in the presence of NeuAc. These results suggest the participation of NeuAc-bearing glycans in the adhesion to LN and FN regardless of cell type. The high sensitivity of all cell lines to NeuAc does not allow one to consider inhibition of adhesion by NeuAc as a factor discriminating non-malignant from malignant cell lines. According to Morgenthaler *et al.* (1990) and Dennis (1991) NeuAc affects attachment to ECM proteins either by their masking or as a recognition determinant (Morgenthaler *et al.,* 1990; Dennis, 1991). Also in the case of FN it can not be excluded that sialic acid competes with polysialic acid, a very important component of α_5 integrin oligosacccharides participating in adhesion to this ECM protein as has recently been shown (Nadanaka *et al.*, 2001).

The alterations in the adhesion efficiencies of the cell lines in response to Gal and Fuc were cell- and ligand-dependent. The presence of Gal or Fuc decreased adhesion efficiency to LN in the non-malignant HCV29 and T24 malignant cells, while it was increased in BC3726 cells. LN is a heavily glycosylated molecule with only N-linked complex-type oligosaccharides containing poly-*N*-acetyllactosamine units. Due to its unique glycosylation pattern LN can be also recognised by cell surface β 1-4 galactosyltransferase and several members of the galactoside-binding lectin family (Chammas *et al.*, 1994). On the other hand, LN may function as a lectin, recognising carbohydrate moieties of the receptor as it has been shown by Chammas *et al.* (1991) in metastatic murine melanoma.

Ono *et al.* (2000) have found that LN or FN-dependent adhesion in Krieger's D14 cells was enhanced significantly in the presence of Gal. Tunicamycin significantly inhibited adhesion to LN but only slightly affected cell adhesion to collagen IV of metastatic melanoma cell lines (Bironaite *et al.*, 2000). On the other hand, mouse myoblasts did not distinguish between glycosylated and unglycosylated LN substrates indicating that LN carbohydrates are not implicated in those cellular responses (Kostrominova & Tanzer, 1995). These facts although controversial, suggest that glycosylation of LN as well as of surface proteins are important in adhesion events, but as it was shown in our study it takes place in a cell-type dependent manner.

The distinctly different adhesion of BC3726 cells to LN and FN in the presence of monosaccharides compared with the other cell lines may suggest significant changes in properties of the cellular membrane proteins due to v-raf transfection.

From all these results we can conclude that the behaviour of non-malignant bladder cells differs significantly from that of cancer ones with respect to the integrins used as well as the participation of saccharides in adhesion. The cancer cells showed a more veriable pattern of cell adhesion.

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