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Correlation between classification of human urothelial cell lines and HLA-A,B,C expression

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Summary. Quantitative changes in major histocompatibility class I antigen expression in tumour cells are believed to affect the host immune response against the tumour. In tumourigenic (TGrIII) human urothelial cell lines the apparent loss of polymorphic HLA-A,B epitopes has previously been demonstrated. In the present study, 3 non-tumourigenic (TGrII) and 6 tumourigenic (TGrIII) human urothelial cell lines have been investigated for their quantitative expression of monomorphic HLA-A,B,C and B_2-microglobulin. Evidence is provided that an inverse correlation exists between tumourigenicity and HLA-A,B,C and B_2-microglobulin expression. Furthermore, treatment of the cells with neuraminidase partly restored the expression of monomorphic HLA-A,B,C suggesting that at least some of the observed quantitative differences could be due to masking of the membrane bound HLA antigens by sialic acid-containing glycoconjugates.

Introduction

Membrane glycoproteins coded for by the major histocompatibility complex (HLA-A,B,C) are important recognition structures for the detection and elimination of virus-infected cells and tumour cells by cytotoxic T-lymphocytes. They consist of a heavy chain non-covalently associated to the light chain, B_2-microglobulin. The extracellular part of the heavy chain can be divided into an outermost polymorphic portion and an innermost monomorphic portion. Alterations in the expression of HLA-A,B,C antigens probably affect the host immune response [3].

In several tumours from mice both quantitative and qualitative alterations in H-2 antigen expression have been demonstrated, and transfection studies have shown that highly tumourigenic H-2 deficient cells could be converted into non-tumourigenic cells after the introduction of a single H-2 antigen [6].

In contrast, little is known about alterations in HLA-A,B,C expression in human tumours [5]. Reduced expression of HLA-A,B,C antigens has been demonstrated in biopsies from melanomas and carcinomas of the breast, lung and colon/rectum [6, 12, 13]. Cell lines established from neuroblastoma [11], small cell lung carcinoma [4] and choriocarcinoma [17] have been demonstrated to have marked decreased expression of HLA-A,B,C.

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Human urothelial cell lines propagated in vitro can be classified according to their in vitro characteristics into various grades of transformation (TGr) [2]. Normal (TGr0) and slightly transformed (TGrI) cells differ from pre-malignant (TGrII) and malignant (TGrIII) cells in their morphology and finite life-span in culture. TGrIII cells differ from TGrII cells in their morphology [2], their tumourigenicity in nude mice [2, 19], in their ability to invade normal tissue in vitro [2, 10], in the glycosylation of membrane glycoproteins [1] and in their apparent lack of polymorphic HLA-A,B epitopes [18]. In the present study we demonstrate an inverse relationship between the TGrII and TGrIII classification of 9 human urothelial cell lines and their expression of the monomorphic part of HLA-A,B,C. Furthermore, it is shown that HLA expression can partly be restored after treatment with neuraminidase (NANase), suggesting that the observed quantitative differences may be due to both a decreased synthesis of HLA-A,B,C antigens and a masking of membrane-bound HLA-A,B,C antigens by sialic acid-containing glycoconjugates.

Materials and methods

Cells. A panel of 9 human urothelial cell lines (see Table 1) classified according to grade of TGr as defined in our in vitro model were propagated in standard medium Fib.41b [2] with 10% heat inactivated fetal bovine serum (FBS). Before test, cells were trypsinized with 0.05% trypsin, washed twice and resuspended in phosphate-buffered saline (PBS) or standard medium.

Antisera. A monoclonal antibody (MoAb) W6/32 (Serotec, Sussex, England) directed against the monomorphic part of HLA-A,B,C antigens [15] and a fluorescein isothiocyanate (FITC) conjugated rabbit anti-human B_2-microglobulin (FITC ra-a-B_2) purchased from Dakopats, Copenhagen, Denmark were used to demonstrate the presence of HLA antigens.

Cytotoxicity assay. Chromium labelling was carried out with ^{51}Cr (Amersham, UK, sp. act. 325 mCi/mg chromium) at a final concentration of 25 μCi/ml medium for 18 h. After trypsinization and washing, 5000 cells resuspended in standard medium were treated with MoAb W6/32 in serial dilutions for 60 min at room temperature. Pre-tested rabbit complement (GlaPo, Århus, Denmark) diluted 1:3 was added and incubated for an additional pe-

riod of 90 min. Spontaneous ^{51}Cr release was studied in cells kept in standard medium. Maximal release was measured in cells treated with 1% sodium dodecyl sulphate. Ab_{50} = Antibody dilution^{-1} which gave 50% of maximal cytotoxicity in cells treated with MoAb W6/32.

NANase treatment. Cells (1.5×10^6) suspended in 1.0 ml of PBS were treated for 30 min at 37° C with NANase (5 u/ml, from *Vibrio cholerae*, BDH, Pook, England). After washing cells were resuspended in PBS + 1% FBS and used in the immunofluorescence assay. The viability of the treated cells was ≥90% as estimated by the trypan blue exclusion test.

Immunofluorescence assay. Cell suspensions (2.5×10^6/ml 100 µl) were placed in 70 × 11 mm plastic tubes and treated for 30 min at 4° C with 50 µl undiluted MoAb W6/32 or FITC ra-a-B_2 (1:3). After washing twice in PBS + 1% FBS, cells treated with FITC ra-a-B_2 were resuspended in 25 µl glycerol + *p*-phenylenediamine [9] and tested for fluorescence staining. The MoAb W6/32-treated cells were resuspended in 100 µl affinity purified FITC conjugated rabbit anti-mouse immunoglobulins (Dakopats, Copenhagen, Denmark) and incubated for an additional period of 30 min at 4° C. Cells were washed twice and resuspended in 25 µl glycerol with *p*-phenylenediamine, mounted on glass slides and tested for membrane fluorescence staining in a Leitz fluorescence microscope equipped with a high-voltage Xenon lamp. The primary antibody replaced with PBS + 1% FBS or FITC conjugated rabbit anti-mouse immunoglobulins served as negative controls respectively. Quantitative immunofluorescence membrane staining was tested using a Leitz microscope photometer connected to an Epson HX 20 computer. All tests were repeated 3–4 times and at least 100 cells with membrane staining were measured each time. Fluorescence intensity is presented as the difference between the fluorescence intensity of the experimental cells and the mean of the negative control cells.

Results

Using ^{51}Cr-labelled target cells in the complement-dependent cytotoxicity test, all human urothelial cell lines investigated reacted with the MoAb W6/32 against the monomorphic part of HLA-A,B,C antigens (Table 1). Furthermore, the non-tumourigenic TGrII cell lines apparently expressed a higher concentration of HLA-A,B,C as compared to the tumourigenic TGrIII cells. Unexpectedly, the T24 cell line used in this study, expressed high amounts of HLA.

Table 1. Correlation between transformation grade (TGr) and HLA-A,B,C expression on human urothelial cell lines

Cell line	TGr	Monomorophic HLA-A,B,C (Ab_{50})[a]
Hu1734	II	23.500
HCV29	II	20.500
Hu609	II	20.000
HCV29T	III	12.000
Hu609T/LLH	III	8.500
Hu961a	III	8.500
Hu456	III	12.000
Hu1703He	III	nt.
T24	III	41.000

nt. = not tested
[a] As demonstrated by the ^{51}Cr release assay
AB_{50} = Monoclonal antibody (MoAb) W6/32 dilution^{-1} which gave 50% of maximum cytotoxicity

Table 2. Quantitative HLA-A,B,C expression on human urothelial cell lines as demonstrated by an indirect immunofluorescence test

Cell line	% Positive cells Intensity ± SD		Increase (%)
	Untreated	+ Neuraminidase (NANase)	
Hu1734	100 22.2 ± 3.6	100 22.7 ± 2.8	2*
HCV29	100 26.0 ± 8.9	100 24.0 ± 4.5	−8*
Hu609	100 22.0 ± 5.0	100 33.5 ± 7.6	52
HCV29T	100 4.1 ± 0.8	100 6.5 ± 1.3	59
Hu609T/LLH	100 4.5 ± 1.0	100 6.9 ± 1.9	53
Hu961a	100 3.8 ± 1.0	100 4.3 ± 1.2	13*
Hu456	80 2.8 ± 1.1	100 5.9 ± 1.6	111
Hu1703He	85 3.1 ± 0.2	92 3.4 ± 0.5	10*
T24	100 16.6 ± 1.5	100 22.7 ± 0.9	37

* not significant
2 $P > 0.05$

In an indirect immunofluorescence test with MoAb W6/32 no significant differences could be demonstrated in the amount of stained cells between TGrII and TGrIII cells (Table 2), but there was a 5–9 times higher concentration of HLA-A,B,C on TGrII cells as compared to TGrIII cells as demonstrated by measurements of the intensity of the fluorescence staining. These data were confirmed in a direct immunofluorescence test using a polyclonal antibody against the B_2-microglobulin (Table 3). The apparently higher concentration of HLA-A,B,C on T24 observed in the complement-dependent cytotoxicity test was confirmed in both the immunofluorescence tests. In a subsequent tumourigenicity test the T24 cell line was found to be non-tumourigenic (data not shown).

From Fig. 1 it can be seen that the distribution of the membrane staining intensity between the TGrII cell line Hu609 and its spontaneously transformed TGrIII subline Hu609T/LLH was significantly different, the latter being much more homogeneous as compared to the former. The T24 cell line resembled Hu609 in the distribution of intensity (Fig. 2).

Treatment of the cell lines with 5 u NANase/ml increased the reaction with MoAb W6/32 by 52%–111% in one out of three TGrII and four out of six TGrIII cell lines (Table 2). No effect could be demonstrated on the reaction with the anti-B_2-microglobulin except in the Hu609 cell line, in which the observed increase was of borderline significance ($2P > 0.1$, Table 3).

Table 3. Quantitative B_2-microglobulin expression on human urothelial cell lines as demonstrated by a direct immunofluorescence test

Cell line	% Positive cells Intensity ± SD		Increase (%)
	Untreated	+ NANase	
Hu1734	100 9.6±3.9	100 9.8±3.4	2*
HCV29	100 14.3±2.2	100 13.5±2.9	−6*
Hu609	100 14.4±2.0	100 18.2±2.6	26*
HCV29T	100 1.6±0.5	100 2.0±1.1	25*
Hu609T/LLH	100 2.1±1.1	100 1.9±1.1	−20*
Hu961a	100 1.3±0.8	100 0.9±0.7	−30*
Hu456	94 2.9±1.1	95 3.1±1.3	7*
Hu1703He	77 1.1±0.6	77 0.9±0.2	−18*
T24	100 6.4±0.9	100 7.3±0.4	14*

* not significant
2 $P > 0.05$

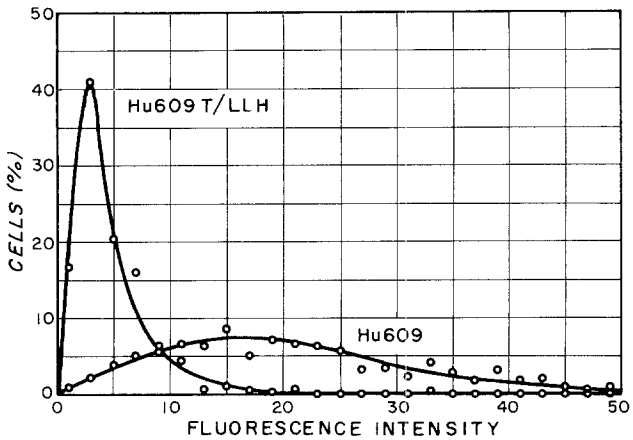

Fig. 1. Relative cellular distribution of membrane fluorescence intensity on Hu609 and Hu609T/LLH cells treated with MoAb W6/32

Discussion

We have previously demonstrated that during spontaneous transformation of the non-tumourigenic urothelial cell line Hu609 changes in morphology and tumourigenicity were accompanied by a gradual loss in polymorphic HLA-A,B expression and that the HLA antigens could be detected again after NANase treatment [14]. Here we provide further evidence that a significant quantitative difference in HLA-A,B,C expression exists between non-tumourigenic and tumourigenic urothelial cells as demonstrated by two different immunological methods using two different antibodies against the HLA-A,B,C antigen. The data presented here have been confirmed in an immunoperoxidase test (data not shown) and they strongly indicate that an inverse relationship exists between tumourigenicity and HLA-A,B,C expression on human urothelial cells. The distribution of membrane staining intensity showed that the tumourigenic TGrIII cell population was much more homogeneous as compared to the non-tumourigenic TGrII cells, suggesting that cells with tumourigenic potential are characterized by having markedly reduced expression of HLA-A,B,C antigens which may possibly serve as a diagnostic tumour marker. A very recent observation that the expression of B_2-microglobulin in biopsies from bladder mucosa of normal, benign and malignant origin inversely correlated with invasiveness, supports this hypothesis [20].

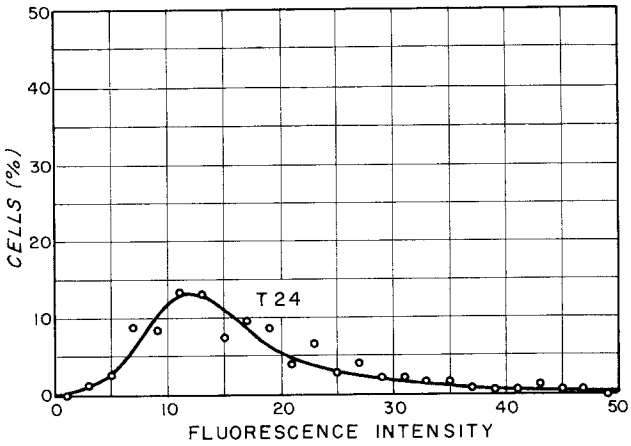

Fig. 2. Relative cellular distribution of membrane fluorescence intensity on T24 cells treated with MoAb W6/32

In agreement with our observation of a relatively high expression of HLA-A,B,C antigens in the T24 cell line used in the present investigation, the distribution of the staining intensity in the fluorescence test was found to resemble that of the Hu609 (TGrII) cell line. Since it is known that non-tumourigenic sublines of T24 exist and that these sublines may differ by several other characteristics (pers communication by J. Bubenik 1986) a re-classification of the T24 cell line propagated in our laboratory was desirable. In a renewed tumourigenicity test it was found that 1.0×10^7 cells of the present cell line were unable to produce tumours in nude mice after s.c. injection. However, this subline was still able to invade normal chick embryonic heart tissue in vitro. These studies will be described in greater detail elsewhere.

None of the cell lines investigated expressed HLA-DR antigens either before or after treatment with NANase (data not shown). These data indicate that the increased reaction with MoAb W6/32 in NANase-treated cells was specific and not due to non-specific reactions.

Debray et al. [1] have previously demonstrated that TGrIII cells have a relatively higher amount of tri- and tetra-antennary glycoproteins on the cell surface as compared to TGrII cells. Treatment with NANase increased the reaction with MoAb W6/32 in one out of three TGrII and four out of six TGrIII cell lines indicating that up to 50% of the membrane-bound HLA-A,B,C antigens can be masked by sialic acid-containing tumour-associated glycoconjugates. In this context it should be mentioned that

comparative studies of normal and malignant bladder mucosa have revealed changes in the sialic acid containing glycocalyx [7]. Previously it has been demonstrated that an inverse relationship exists between sialic acid content of tumour cells and their metastatic potential [21]. Our studies of TGrII and TGrIII cell lines suggest that quantitative and/or qualitative alterations in the distribution of sialic acid on human urothelial cells may also affect their tumourigenicity and invasiveness.

At present there is no satisfactory explanation why the reaction with anti-B_2-microglobulin was not increased in NANase-treated cells. However, steric hindrance as a consequence of the proposed three-dimensional structure of the HLA-A,B,C antigen is a possibility that might be considered.

Cytotoxic T-lymphocytes require both HLA-A,B,C antigens and tumour-associated antigens before they can be activated to kill the tumour cells [3]. Furthermore, observations suggest that the polymorphic portion of the HLA antigens serves as the recognition structure for the cytotoxic T-lymphocytes [16]. Masking the HLA antigens, and especially the polymorphic region, combined with a decreased synthesis of HLA antigens, could be one way for the tumour cells to escape immune surveillance.

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