Eur J Cancer Clin Oncol, Vol. 23, No. 7, pp. 991–995, 1987.
Printed in Great Britain

0277-5379/87$3.00+0.00

Changes in HLA A,B,C Expression During "Spontaneous" Transformation of Human Urothelial Cells *In Vitro*

SVEND S. OTTESEN,* JØRGEN KIELER and BRITTA CHRISTENSEN
The Fibiger Institute, Ndr. Frihavnsgade 70, 2100 *Copenhagen Ø, Denmark*

Abstract—*The immortalized but non-tumourigenic and non-invasive human urothelial cell line, Hu* 609, *known to express the appropriate HLA A,B antigens (A* 2,−; *B* 5,−) *has previously been demonstrated to undergo "spontaneous"* in vitro *transformation into an invasive and tumourigenic subline, Hu* 609*T/MV. This subline does not express the polymorphic HLA epitopes.*

In the present investigation we have followed two additional "spontaneous" transformations of the Hu 609 *cell line into malignant sublines. Evidence is presented that the development of morphological changes and tumourigenicity were accompanied by a gradual loss of the expression of polymorphic HLA A,B epitopes and a reduction in the expression of monomorphic HLA A,B,C antigens. Antigens could be detected again after neuraminidase treatment.*

We conclude that the urothelial Hu 609 *cell line after "spontaneous" transformation still possesses the HLA A,B epitopes. The observed quantitative differences in HLA expression between Hu* 609 *and its malignant sublines may be due to masking of the HLA antigens by sialic acid containing tumour-associated highly branched glycoproteins.*

INTRODUCTION

HLA CLASS I (HLA A,B,C) antigens consist of a 45,000 mol. wt membrane-bound glycoprotein associated with the 12,000 mol. wt β_2-microglobulin by non-covalent binding. The extracellular portion of the antigen can be divided into three domains. The two outermost domains (α_1, α_2) constitute the polymorphic part of the antigen, whereas the innermost domain (α_3) constitutes the monomorphic part [1]. HLA A,B,C antigens are present in virtually all nucleated cells, and play a crucial role in the cell-mediated immunological response against virus-infected cells [2] and tumour cells [3]. Quantitative changes in HLA A,B,C expression in tumour cells may therefore influence the ability of the cytotoxic T-lymphocytes to recognize and destroy these cells [4]. Furthermore, an inverse relation between HLA expression in tumour cells and their metastatic potential seems to exist [5].

Various characteristics of human urothelial cell lines propagated *in vitro* permit a classification of these cell lines according to grade of transformation (TGr) [6]. Pre-malignant TGr II and malignant TGr III cells differ from normal TGr 0 and slightly transformed TGr I cells in growth pattern and morphology and by having an infinite life span. Furthermore, TGr III cells differ from TGr II cells by their morphology [6], their tumourigenicity in nude mice [6, 7], their ability to invade normal tissue *in vitro* [6, 8], and in the glycosylation of membrane glycoproteins [9]. Moreover, they also seem to have lost the expression of polymorphic HLA A,B epitopes [10].

The apparently "spontaneous" transformation of two TGr II cell lines (Hu 609 and HCV 29) into TGr III cell lines (Hu 609T and HCV 29T) has previously been described [10]. One of these two cell lines, Hu 609, was derived from the histological normal ureter of a patient operated for a renal carcinoma. During *in vitro* cultivation this cell line has been demonstrated to undergo apparently "spontaneous" transformation at least five times. The HLA type of the donor's leucocytes was HLA A 2,−; B 5,7, and the Hu 609 cells have been demonstrated to express HLA A2 and B5 [10]. However, these polymorphic HLA epitopes could not be detected on a corresponding "spontaneously" transformed TGr III subline (Hu 609T/MV) [10].

Accepted 20 January 1987.
*To whom reprint requests should be sent.

Here we report a gradual loss of expression of polymorphic HLA A,B and a reduction of expression of monomorphic HLA A,B,C antigens during two additional "spontaneous" transformations of Hu 609 (TGr II) cells into malignant TGr III cells (Hu 609T/LLH and Hu 609T/HJ). Antigens could be detected again after neuraminidase (NANase) treatment. These results suggest that changes in the grade of sialylation of the glycoprotein structure of the membrane may be the cause of the observed alteration of HLA expression.

MATERIALS AND METHODS

Cells

Hu 609, Hu 609T/LLH and HU 609T/HJ cell lines were propagated in a standard Fib.41B medium [6] with 10% inactivated foetal bovine serum (FBS). Two batches of Hu 609 cells (BC 97/102 and BC 118/123) stored in liquid N_2 were analysed in the present investigation.

Antisera

Semi-specific human HLA alloantisera directed against the polymorphic part of HLA A,B antigens were obtained by the courtesy of Dr. A. Svejgaard from pregnant women and tested for specificity at the Tissue Typing Laboratory of the University Hospital in Copenhagen. Normal rabbit serum and nine unrelated semispecific HLA antisera, as well as rabbit antisera against Hu 609 cells, served as negative and positive controls, respectively. One monoclonal antibody (MoAb) W6/32 (Seralab, Sussex, U.K.) which reacts with the monomorphic part of HLA A,B,C antigens [11] was used to demonstrate the presence of HLA antigens.

Cytotoxicity assays

HLA antigens were demonstrated by one of two types of complement-dependent serum-mediated cytotoxicity (CDC) tests. All assays were carried out in duplicate.

1. As a standard CDC assay we used a modification of the procedure as previously described [12]: target cells suspended in standard medium were placed in the wells of a Terasaki plate and incubated for 24–48 hr in 37°C/5% CO_2. After plating the cells were washed with PBS and incubated for 60 min at room temperature with 5 μl of antiserum. After renewed washing, 5 μl of pretested rabbit complement diluted 1 : 3 were added and the plate was re-incubated at 37°C/5% CO_2 for 90 min. The plate was washed and stained with 5 μl fluorescein diacetate (3 μg/ml) for 10 min followed by staining with ethidium bromide (250 μg/ml) for an additional period of 10 min. Fluorescein diacetate stains the cytoplasm of living cells green and ethidium bromide (EtBr) stains the nuclei of dead cells red.

Cytotoxic index (C.I.) = (No. of red cells divided by total No. of cells) × 100%.

Corrections for negative controls were made by the subtraction of the cytotoxic index for cultures treated with normal rabbit serum (specific C.I.).

2. ^{51}Cr release assay: chromium labelling was carried out with ^{51}Cr (Amersham, sp. act. 325 mCi/mg chromium) at a final concentration of 25 μCi/ml medium for 18 hr. After washing, 5000 cells placed in 70 × 11 mm plastic tubes were treated with antiserum for 1 hr at room temperature. Pretested rabbit complement (GlaPo, Århus, Denmark) diluted 1 : 3 was added and incubated at 37°C/5% CO_2 for an additional period of 90 min. Spontaneous ^{51}Cr release was studied in cells kept in culture medium. Maximal release was measured in cells treated with 1% sodium dodecyl hydrogensulphate (SDS).

Specific cytotoxicity (%) = (^{51}Cr release from antibody treated cells minus spontaneous release minus negative control divided by ^{51}Cr release from SDS treated cells minus spontaneous release) × 100%.

Neuraminidase treatment

A total of 1.0×10^6 cells suspended in 1.0 ml PBS were treated for 30 min at 37°C with 10 units of neuraminidase (NANase) (from Vibrio Cholera, 500 units/ml BDH, U.K.). After washing the cells were resuspended in culture medium (1.0×10^5 cells/ml) and used in the ^{51}Cr release assay. The viability of the treated cells was ≥90% as estimated by the trypan blue exclusion test.

Absorption experiment

Before absorption, 5.0×10^6 Hu 609T cells suspended in 1.0 ml PBS were treated with 50 units of neuraminidase for 30 min at 37° C. After washing, 15 μl of MoAb W6/32 (diluted 1 : 1000), anti-HLA A2 or anti-HLA B5 were absorbed with 7.5×10^5 cells for 60 min at room temperature. Antisera were tested for remaining activity against non-transformed Hu 609 cells using the standard CDC test.

RESULTS

Transformation of the two batches of pre-malignant (TGr II) Hu 609 cells judged by morphological criteria began in the 42nd and 48th passages, respectively, as can be seen in Table 1. In the course of 1–3 additional passages the sub-cultures showed the uniform type 3 morphology characteristic of malignant TGr III cultures (see footnote to Table 1).

Using both the ^{51}Cr release assay and the standard CDC test the Hu 609 cells were found to

Table 1. HLA expression in "spontaneously" transformed human urothelial cells

Passage No.	Batch Hu 609–BC 118/123 Assay: standard CDC test* Morphological type‡	Tumourigenicity§	HLA A2	HLA B5 Specific C.I.(%)	HLA B7	Batch Hu 609–BC 97/102 Assay: ^{51}Cr release† Morphological type‡	Tumourigenicity§	HLA A2	HLA B5 Specific cytotoxicity(%)	HLA B7
18	2	0/2	21	24	5					
20						2	0/2	62	35	8
42	2/3	nt		nt						
43	3	nt	0	0	0					
44	3	2/2		nt						
45						2	nt	24	37	6
48						2/3	nt		nt	
52						3	nt	16	8	0
53						3	2/2		nt	
54						3	2/2	1	0	0
56						3	2/2	2	3	0

*Standard CDC assay performed as described under Materials and Methods.

†^{51}Cr release assay performed as described under Materials and Methods.

‡Morphological type: 1: Large elongated or polygonal cells with small nuclei. 2: Variable size and shape with small nuclei. 3: Small, round or polygonal cells with large nuclei.

§Formation of progressively growing tumours after s.c.injection of 1–5 × 10^7 human urothelial cells into nude BALB/c mice. No. of takes/total No. of mice.

nt: Not tested.

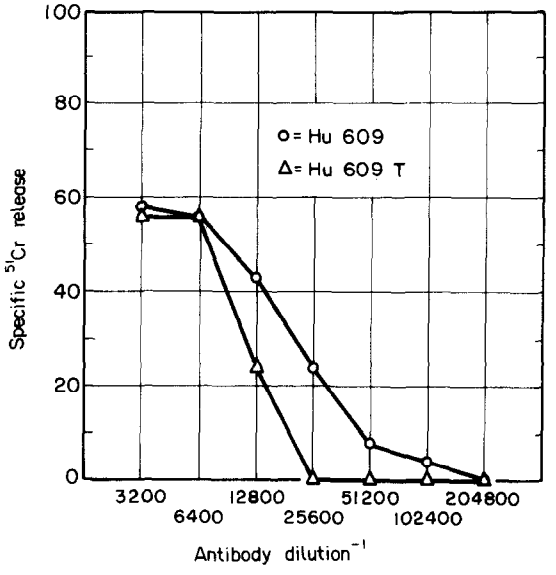

Fig. 1. Demonstration of the monomorphic part of HLA A,B,C antigens on Hu 609/Hu 609T/LLH using MoAb W6/32 and the ^{51}Cr release assay (see footnote to Table 1).

express HLA A2 and B5 before transformation (Table 1). Weak reactions were also found with anti-B7, but these did not exceed the weak cross-reactions seen with unrelated semi-specific HLA antisera. During "spontaneous" morphological transformation, the expression of HLA A2 and B5 gradually decreased and finally disappeared a passage before or after the demonstration of tumourigenic properties.

Figure 1 illustrates an experiment carried out with MoAb W6/32 that reacts with the monomorphic part of HLA A,B,C antigens [11]. The monomorphic HLA epitope was expressed in Hu 609/Hu 609T cells both before and after "spontaneous" transformation from TGr II to TGr III. However, transformation was accompanied by a reduction in the reaction with MoAb W6/32 as measured by the ^{51}Cr release assay.

Treatment with 10 units of NANase per 10^6 cells suspended in 1.0 ml of PBS caused a considerable increase in the B5 and B7 expression and a moderate increase in the expression of the monomorphic part of HLA as can be seen in Table 2. On the other hand, the expression of A2 was not influenced by the NANase treatment. Attempts to use higher concentrations of NANase resulted in an increased sensitivity of the cells at the unspecific toxic effect of the complement (data not shown).

Indirect evidence that A2 was also present in Hu 609T/LLH cells in a masked form was provided by an experiment demonstrating that absorption of MoAb W6/32, HLA A2 and B5 antisera with Hu 609T/LLH cells treated with 2 units of NANase per 10^5 cells suspended in 1 ml of PBS was able to remove the reaction of these antisera with Hu 609 cells (Fig. 2).

DISCUSSION

The present data address, for the first time, the mechanism involved in alteration of HLA A,B,C on human epithelial cells during "spontaneous" transformation from a premalignant into a malignant state *in vitro*. The non-tumourigenic Hu 609

Table 2. Effect of neuraminidase treatment on HLA expression in Hu 609 T/LLH (TGr III) cells

Passage No.		Specific ^{51}Cr release (%)* Non-diluted semispecific HLA antisera A2	B5	B7	AB_{ur}	MoAbW6/32 diluted 1 : 25600 Monomorphic HLA
70	Control	0	0	1	—	0
	NANase†	0	21	23	—	14
72	Control	0	0	1	3	4
	NANase	2	16	27	3	14

*See footnote to Table 1.

†Treatment for 30 min at 37° C with 1 unit of NANase per 10^5 cells suspended in 1 ml PBS. After washing the cells were resuspended in culture medium (1.0×10^5 cells/ml) and used in the ^{51}Cr release assay. Viability of the treated cells ≥ 90% as estimated by the trypan blue exclusion test.

AB_{ur} = Mean value of reactions with three unrelated HLA A,B antisera (range 1–5).

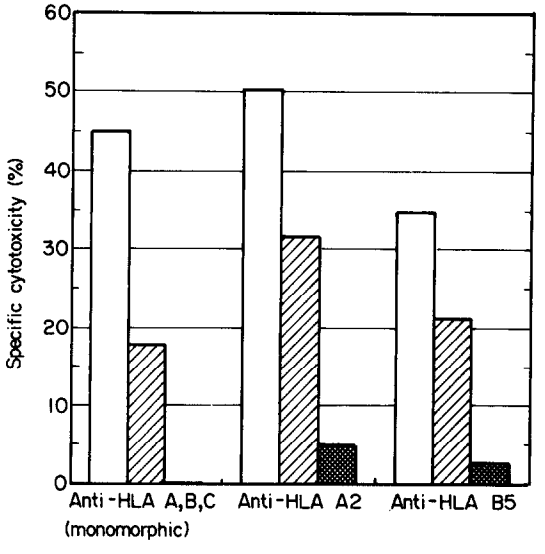

Fig. 2. Absorption of MoAb W6/32 (diluted 1 : 1000), anti-HLA A2 and anti-HLA-B5 with untreated Hu 609T cells (▨), and Hu 609T/LLH cells treated for 30 min at 37° C with 2 units of NANase per 10^5 cells suspended in 1 ml PBS (■). Antisera were absorbed with 3×10^6 cells (1 : 1 v/v) for 60 min at room temperature and tested for remaining activity against nontransformed Hu 609 cells using the standard CDC test (see footnote to Table 1). □ = Unabsorbed antisera.

cells expressed the appropriate polymorphic HLA A,B epitopes, but during the "spontaneous" malignant transformation changes in morphology and tumourigenicity was accompanied by a gradual loss in the expression of polymorphic HLA. The expression of the monomorphic part of HLA, however, was only influenced to a lesser extent, suggesting that the polymorphic HLA epitopes recognized by the semi-specific HLA antisera are still present on Hu 609T cells, but in a masked form.

The absence of HLA expression has previously been proposed as being a marker of invasive potential [8], and an inverse relation between HLA A,B,C and the degree of differentiation in colon cancer have recently been reported [13]. Biochemical analyses have demonstrated a relatively higher amount of highly branched glycoproteins on TGr III cells than on TGr II cells, and furthermore the urothelial TGr III cells bind more wheatgerm agglutinin and peanut lectin (PNA) than TGr II cells [14]. The latter observation with PNA was only made after pretreatment of the cells with neuraminidase. The combination of these observations with the demonstration of an inverse relation between sialic acid content of the cell membranes and metastatic potential [15] incited us to study the effect of NANase in Hu 609T cells.

The effect of NANase treatment of Hu 609T/LLH cells on the reaction with the MoAb W6/32 and semispecific HLA alloantisera against B5 and B7 suggest that the monomorphic part of the HLA antigens was partially and the polymorphic part completely masked by tumour-associated sialic acid containing highly branched glycoproteins. The reason that the expression of A2 was not influenced by NANase treatment is not clear, but it is conceivable that the concentrations of NANase were too low. This may also explain why NANase did not restore the expression of the monomorphic part of HLA A,B,C completely in Hu 609T/LLH (Table 2) as compared to Hu 609 (Fig. 1). However, higher concentrations of NANase could not be used because the sensitivity of Hu 609T cells to the unspecific toxic effect of complement increased with the higher concentrations of NANase used. This effect of NANase may be due to alterations in the activation of the alternative pathway of complement [16]. Changes in the antigenicity of the HLA A2 antigen is another possible explanation for the failure to demonstrate A2 on Hu 609T/LLH. However, this explanation is less likely since HLA A,B,C antigens have been demonstrated to retain their antigenicity after deglycosylation [17].

Differences in capacity between untreated and NANase treated Hu 609T cells to absorb MoAb W6/32, anti-HLA A2 and anti-HLA B5 presented indirect evidence that the A2 epitope was present on Hu 609T and that the HLA antigens were masked by sialic acid containing tumour-associated glycoconjugates. The effect of NANase treatment on Hu 609T/LLH cells on the expression of HLA A2 and B5 was reproduced in two additional absorption experiments. The effect on the expression of the monomorphic part was confirmed by a quantitative indirect immunofluorescence assay in which NANase treatment was found to increase the membrane staining intensity on Hu 609T/LLH cells by 75% (manuscript in preparation).

Finally, since the HLA alloantisera potentially contain antibodies reactive to antigens other than HLA, it cannot be excluded that NANase treatment exposed such antigens and not the HLA antigens. Against this hypothesis speaks the failure of NANase treatment to increase the sensitivity of the Hu 609T/LLH cells to other unrelated semispecific HLA A,B alloantisera (Table 2).

The 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 antigen serves as the recognition structure for the cytotoxic T-lymphocytes [18]. Masking the HLA antigens, and especially the polymorphic epitopes, could therefore be one way for the tumour cells to escape immune-surveillance. This possibility is being investigated.

Acknowledgements—This work was supported by grants from the Neye Foundation and the JPC foundation. The Fibiger Institute is sponsored by the Danish Cancer Society. Acknowledgement is made of the skillful assistance of Vibeke Ahrenkiel and the advice offered by Dr. Mogens Vilien.

REFERENCES

1. Ploegh HL, Orr HT, Strominger JL. Major histocompatibility antigens: the human (HLA A,B,C) and murine (H-2K,H-2D) Class I molecules. *Cell* 1981, **24**, 287–299.
2. Doherty PC, Blanden RV, Zinkernagel RM. Specificity of virus-immune effector T cells for H-2K or H-2D compatible intractions: implications for H-antigen diversity. *Transplant Rev* 1976, **29**, 89.
3. Hui K, Grosveld F, Festenstein H. Rejection of transplantable AKR leukaemia cells following MHC DNA-mediated cell transformation *Nature* 1984, **311**, 750–752.
4. Kuppers RC, Ballas ZK, Green WR, Henney CS. Quantitative appraisal of H-2 products in t cell-mediated lysis by allogenic and syngenic effector cells. *J Immunol* 1981, **127**, 500–504.
5. Tanaka K, Isselbacher KJ, Khoury G, Jay G. Reversal of oncogenesis by the expression of a major histocompatibility complex Class I genes. *Science* 1985, **228**, 26–30.
6. Christensen B, Kieler J, Vilien M, Don M, Wang CY, Wolf H. A classification of human urothelial cells propagated *in vitro*. *Anticancer Res* 1984, **4**, 319–338.
7. Vilien M, Christensen B, Wolf H, Rasmussen F, Hou-Jensen C, Povlsen CO. Comparative studies of normal, "spontaneously" transformed and malignant urothelium cells *in vitro*. *Eur J Cancer Cin Oncol* 1983, **19**, 775–789.
8. Kieler J. Invasiveness of transformed bladder epithelial cells. *Cancer Metast Rev* 1984, **3**, 265–296.
9. Debray H, Zhu Q, Delannoy P *et al.* Altered glycosylation of membrane glycoproteins in human uroepithelial cell lines. *Int J Cancer* 1986, **37**. 607–612.
10. Vilien M, Wolf H, Rasmussen F. Immunological characterization of cell lines established from malignant and normal human urothelium. *Eur J Cancer Clin Oncol* 1981, **17**, 321–327.
11. Parham P, Barnstable CJ, Bodmer WF. Use of a monoclonal antibody (W6/32) in structural studies of HLA A,B,C antigens. *J Immunol* 1979, **113**, 342–349.
12. Pollack MS, Heagney SD, Livingston PD, Fogh J. HLA A,B,C and DR alloantigen expression on forty-six cultured human tumor cell lines. *JNCI* 1981, **66**, 1003–1012.
13. Momburg F, Degener T, Bacchus E, Moldenhauer G, Hämmerling GJ, Möller P. Loss of HLA A,B,C and *de novo* expression of HLA D in colorectal cancer. *Int J Cancer* 1986, **37**, 179–184.
14. Dus D, Radzikowski C, Debray H, Montreuil J, Christensen B, Kieler J. In: Bøg-Hansen TC, Breborowitcz J, eds. *Lectins—Biology, Biochemistry, Clinical Biochemistry*, Vol. 4, *Proc. VI Int. Lectin Meeting, Posnan, Poland 1984*. Berlin, de Gruyter, 1985, 65–74.
15. Yogeeswaran G, Salk PL. Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. *Science* 1981, **212**, 1514–1516.
16. Kazatchkine MD, Fearon DT, Austen KF. Human alternative complement pathway: membrane-associative sialic acid regulates the competiton between B and 1H for cell-bound C3b. *J Immunol* 1979, **112**, 75–81.
17. Miyazaki J, Appella E, Zhao H, Forman J, Ozato K. Expression and function of a nonglycosylated major histo-compatibility Class I antigen. *J Exp Med* 1986, **163**, 856–871.
18. Stroynowski I, Clark S, Henderson LA, Hood L, McMillan M, Forman J. Interaction of o1 with o2 region in Class I MHC proteins contributes determinants recognized by antibodies and cytotoxic T cells. *J Immunol* 1985, **135**, 2160–2166.