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Reduced HLA-A,B,C Expression in Tumourigenic v-*raf* Transfected Human Urothelial Cells

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Abstract—*Tumourigenic (TGrIII) human urothelial cells grown* in vitro *have previously been demonstrated to have a markedly decreased expression of* β_2*-microglobulin and HLA-A,B,C antigens as compared to non-tumourigenic (TGrII) human urothelial cell lines. Furthermore, during 'spontaneous'* in vitro *transformation of a non-tumourigenic (TGrII) human urothelial cell line Hu*609 *into a tumourigenic (TGrIII) subline Hu*609*T/LLH, changes in morphology and tumourigenicity have been demonstrated to be accompanied by a decreased HLA-A,B,C expression.*

*After malignant transformation of the non-tumourigenic (TGrII) human urothelial cell line HCV*29 *by DNA transfection with the v-*raf *oncogene, four sublines could be isolated. In this study we have investigated these sublines for their expression of membrane bound HLA-A,B,C antigens and provide further evidence that an inverse relationship exists between tumourigenicity and monomorphic HLA-A,B,C expression. Treatment of the cells with recombinant human interferon* α *for 3 days increased the expression of HLA-A, B, C antigens by* 50–150% *indicating that at least some of the reduced HLA-A,B,C expression could be due to decreased synthesis of HLA-A,B,C antigens. All the transfected cell lines overexpress v-*raf *and c-*myc.

INTRODUCTION

IT IS widely accepted that HLA-A,B,C antigens serve as recognition elements for effector T cells in the immune response against e.g. tumour cells. HLA-A,B,C antigens are membrane bound glycoproteins that are present on virtually all nucleated normal cells. They consist of a 45,000 mol. wt heavy chain non-covalently associated to the 12,000 mol. wt β_2-microglobulin. The external portion of the heavy chain can be divided into an outermost polymorphic part and an innermost monomorphic part. Evidence has been provided that in particular the polymorphic part serves as a recognition structure for the cytotoxic T lymphocytes. Quantitative and/or qualitative changes in HLA-A,B,C expression on tumour cells would therefore probably affect the host immune response [1]. Reduced expression of HLA-A,B,C antigens has been demonstrated in biopsies from melanomas, carcinomas of the breast, lung, kidney and colon/rectum [2–4], but the decreased HLA-A,B,C expression in the latter appears not to influence survival of the patients [5]. In contrast, increased expression of HLA-A,B,C has been demonstrated in biopsies from e.g. human hepatocellular carcinomas [6]. Cell lines established from neuroblastoma, small cell lung carcinoma and choriocarcinoma have been demonstrated to have marked decreased expression of HLA-A,B,C [7–9].

Human urothelial cell lines propagated *in vitro* can be classified according to their *in vitro* characteristics into various grades of transformation (TGr) [10–12]. Malignant (TGrIII) cells differ from pre-malignant (TGrII), slightly transformed (TGrI) and normal (TGr0) cells by their morphology, their invasiveness *in vitro* into co-cultured normal tissue, their tumourigenicity in nude mice, by the glycosolation of membrane glycoproteins, and by their apparent lack of polymorphic HLA-A,B epitopes [13]. We have previously demonstrated that an inverse relationship between monomorphic HLA-A,B,C expression and tumourigenicity exists in these human urothelial cell lines [14]. Furthermore we have demonstrated that during a 'spontaneous' *in vitro* transformation of the pre-malignant (TGrII) urothelial cell line Hu609 into a malignant (TGrIII) subline Hu609T/LLH, changes in morphology and tumourigenicity were accompanied by a decreased expression of HLA-A,B,C antigens [15].

We have recently been able to induce malignant transformation of the immortalized and non-tumourigenic (TGrII) human urothelial cell line HCV29 after introduction of the v-*raf* oncogene by DNA transfection [16]. Three tumourigenic and one non-tumourigenic HCV29 sublines could be isolated.

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In the present study we have investigated these sublines for their membrane expression of monomorphic and polymorphic HLA-A,B,C antigens. We present data indicating that the tumourigenic transfected urothelial cells, which have been demonstrated to overexpress v-*raf* as well as c-*myc*, have a markedly reduced expression of monomorphic HLA-A,B,C antigens as compared to the non-tumourigenic parent cells. Treatment of the cells with recombinant human interferon α (rHu-INF-α) increased the expression of monomorphic and polymorphic HLA-A,B,C antigens, suggesting that some of the reduced HLA expression could be due to decreased synthesis. These data therefore provide further evidence that an inverse relationship exists between tumourigenicity and HLA-A,B,C expression in human urothelial cell lines.

MATERIALS AND METHODS

Cells

The non-tumourigenic but immortalized human urothelial cell line HCV29 was originally established by J. Fogh (Memorial Sloan Kettering Institute, U.S.A.) from a biopsy of histologically normal bladder mucosa taken from a patient previously irradiated because of bladder papillomata. This cell line was received in our laboratory in 1977. The cells were propagated *in vitro* in standard medium Fib.41b with 10% foetal bovine serum (FBS) and classified as TGrII according to Christensen *et al.* [10]. The HCV29-T112C1 and HCV29-T112D1 sublines were isolated after transformation of the HCV29 cell line by DNA transfection with the v-*raf* oncogene as described elsewhere [16]. The transfection method used was a modified standard $CaPO_4$–DNA precipitation method. The plasmid pNRraf construct contains the v-*raf* oncogene as well as the *neo*^{r} gene conferring resistance against G418. Expression of v-*raf* mRNA and protein was demonstrated by Northern and Western blot analysis respectively.

Antisera

A murine monoclonal antibody (MoAb W6/32) against the monomorphic part of HLA-A,B,C antigens (Seralab., U.K.) was used to demonstrate the presence of HLA antigens. A panel of six semi-specific human HLA-A,B alloantisera directed against the polymorphic part of HLA-A,B antigens was kindly provided by Dr A. Svejgaard at the Tissue Typing Laboratory of the University Hospital in Copenhagen, and used in a modified standard NIH complement dependent cytotoxicity assay [15]. Normal human AB serum served as a negative control.

HLA typing assay

Cells were placed in the wells of a Terasaki plate and incubated for 24 h at 37°C/5% CO_2. After plating the cells were washed in PBS and incubated with antisera for 60 min at room temperature. After renewed washing, pre-tested rabbit complement was added followed by incubation at 37°C/5% CO_2 for an additional 90 min. The plate was washed and stained with fluorescein diacetate (stains the cytoplasm of living cells green) and ethidium bromide (stains the nuclei of dead cells red).

Cytotoxic index (C.I.) =
(number of dead cells divided by total number of cells) × 100%.

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

Indirect immunofluorescence assay

200,000 cells in 100 μl PBS + 1% FBS (PBS*) were placed in 70 × 11 mm plastic tubes and incubated with MoAb W6/32 for 30 min on ice, washed twice in PBS* and resuspended in FITC conjugated rabbit anti-mouse Ig followed by an additional incubation for 30 min on ice. Cells were tested for fluorescein intensity staining with a Leitz microscope photometer connected to an Epson HX 20 computer. All tests were repeated 2–3 times and at least 100 cells with membrane staining were measured each time. To prevent fading during measurements, *p*-phenylendiamine was added to the final cell suspension [17].

Treatment with recombinant human interferon α (rHu-INF-α)

Cells were treated for 3 days with 500 units of rHu-INF-α (Intron A: activity 1.7×10^8 IU/mg protein—kindly provided by Schering, DK) per ml of standard medium. Cells were then trypsinized lightly with 0.05% trypsin + EDTA and tested for their expression of HLA using the immunofluorescence assay and the HLA typing assay.

RESULTS

DNA transfection of the non-tumourigenic human urothelial cell line HCV29 with the v-*raf* oncogene was accompanied by changes in morphology and tumourigenicity (Table 1). From the tumour produced by T112D1 only outgrowth of morphology type 2 cells could be observed (cell line T112D1expl.). In a renewed tumourigenicity test these cells were no longer able to produce tumours after subcutaneous injection of 5.0×10^7 cells.

There were no differences in the percentage of monomorphic HLA-A,B,C positive cells between

Table 1. Characteristics of the cell lines included in the present study

Cell line*	Morphological type†	Tumourigenicity in nude mice‡	v-*raf* mRNA and protein§	c-*myc*mRNA‖
HCV29	2	−	−	−
HCV29neo	2	−	−	−
T112C1	3	+	+	+
T112C1expl.	3	+	+	+
T112D1	3	+	+	+
T112D1expl.	2	−	+	+

*The HCV29neo subline was isolated after DNA transfection with neo^r gene alone. T112C1 and T112D1 were isolated after transfection with the pNRraf plasmid. T112C1expl. was isolated after the 2nd *in vivo* passage of the tumour produced by T112C1. T112D1expl. was established from the 1st *in vivo* passage of the tumour produced by T112D1.
†*Morphology*. Type 1: Large epithelial cells with small round nuclei. Type 2: Cells of variable size and shape with small round nuclei. Type 3: Small polygonal or round cells with large nuclei.
‡Tumorigenicity in nude mice was tested by the s.c. injection of 5×10^7 cells.
§Expression of v-*raf* mRNA and protein was demonstrated by Northern and Western blot analysis, respectively.
‖Expression of c-*myc* mRNA was demonstrated by Northern blot analysis.

Table 2. Quantitative expression of HLA-A,B,C antigens as demonstrated by an indirect immunofluorescence test with MoAb W6/32

Cell line	Percentage cells Intensity ± S.D. Untreated	+500 units INF-α/ml	Increase (%)
	100	100	
HCV29	22.6 ± 7.7	32.8 ± 9.8	50*
	100	100	
HCV29neo	22.6 ± 7.5	35.6 ± 11.7	60*
	100	100	
T112C1	5.9 ± 2.7	14.8 ± 5.6	150*
	100	100	
T112C1expl.	9.1 ± 3.3	15.0 ± 6.3	70*
	100	100	
T112D1	7.5 ± 3.8	16.9 ± 5.8	130*
	100	100	
T112D1expl.	19.5 ± 7.5	29.2 ± 12.9	50*

*$P < 0.005$.

the cell lines tested as demonstrated by an indirect immunofluourescence test with the monoclonal antibody MoAb W6/32 (Table 2), but measurements of the membrane staining intensity revealed that the concentration of monomorphic HLA-A,B, C antigens in the tumourigenic cell lines is 2.5–3.8-fold lower than that in the non-tumourigenic cells. These data have been confirmed in a complement dependent cytotoxicity test using ^{51}Cr-labelled target cells and MoAb W6/32 (data not shown). From Figs. 1 and 2 it can be seen that a considerable difference exists in the distribution of membrane

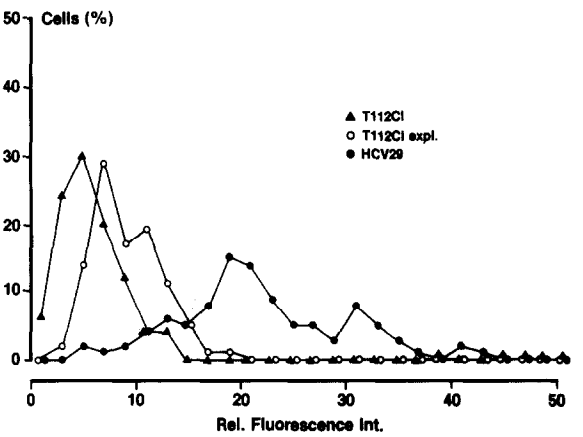

Fig. 1. Relative cellular distribution of membrane fluorescence intensity in HCV29, T112C1 and T112C1expl. treated with the MoAb W6/32.

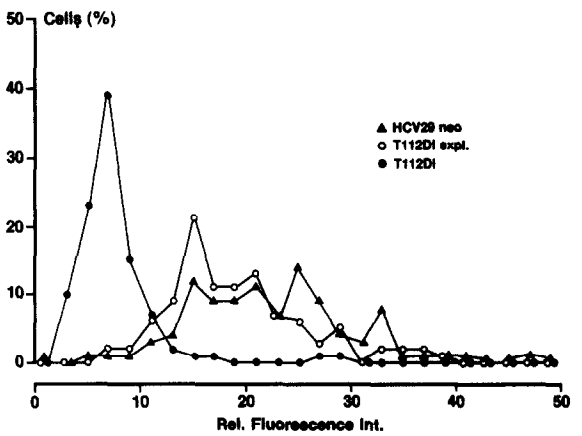

Fig. 2. Relative cellular distribution of membrane fluorescence intensity in HCV29neo, T112D1 and T112D1expl. treated with the MoAb W6/32.

staining intensity of tumourigenic cells as compared to non-tumourigenic cells. Unexpectedly, the transfected but non-tumourigenic subline, T112D1expl. expressed high amounts of HLA-A,B,C.

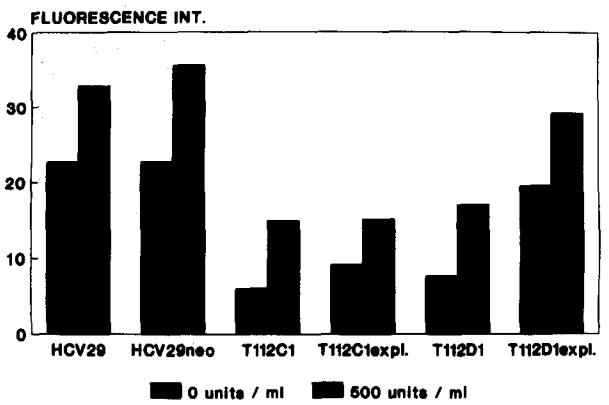

Fig. 3. Effect of treatment with rHu-INF-α on monomorphic HLA-A,B,C antigen expression.

Table 3. Demonstration of polymorphic HLA-A,B,C epitopes by a modified standard complement dependent cytotoxicity test

Cell line	INF-α*	HLA-A,B,C (% spec. cytotox.) Monomorphic	A2	B14	x(unrel.)
HCV29	−	100	100	69	0
	+	100	100	66	0
HCV29neo	−	73	74	35	0
	+	85	88	40	0
T112C1	−	60	57	10	0
	+	79	79	36	0
T112C1expl.	−	77	85	17	0
	+	91	91	43	0
T112D1	−	63	69	33	0
	+	68	72	47	0
T112D1expl.	−	69	65	30	0
	+	74	75	46	0

x(rel): Mean value of two unrelated semispecific HLA-A,B antisera.
*Cells pre-incubated for three days without (−) or with (+) addition of 500 units of INF-α/ml medium

Treatment of the cells with 500 u rHu-INF-α/ml standard medium for 3 days increased the staining intensity with MoAb W6/32 by 50–150% (Table 2 and Fig. 3). The two sublines T112C1 and T112D1 increased the expression of monomorphic HLA-A, B,C considerably more as compared to the other cell lines included.

All the cell lines tested expressed HLA A2;B14–the HLA type of the HCV29 cell line (Table 3)—confirming the origin of the transfected cells. The HCV29-T112C1 and HCV29-T112C1 expl. both express low amount of HLA-B14 as compared to the other cell lines. Pre-treatment of the cells with rHu-INF-α for 3 days increased the expression of appropriate polymorphic HLA-A,B antigens.

DISCUSSION

We have previously demonstrated that tumourigenic human urothelial cells grown *in vitro* express lower amounts of monomorphic HLA-A,B,C antigens and β_2-microglobulin as compared to non-tumourigenic urothelial cells [14]. Furthermore, we have demonstrated that during a 'spontaneous' transformation *in vitro* of the non-tumourigenic urothelial cell line Hu609 into a tumourigenic subline, Hu609T/LLH, changes in morphology were accompanied by a decreased expression of monomorphic and polymorphic HLA-A,B,C antigens [15]. In agreement with out results, the expression of β_2-microglobulin in biopsies from patients with carcinoma of the bladder has been demonstrated to be inversely correlated with invasiveness [18].

In this study we have provided further evidence that an inverse relationship exists between HLA-A,B,C expression and tumourigenicity in human urothelial cells. After introduction of the v-*raf* oncogene into the immortalized but non-tumourigenic (TGrII) human urothelial cell line HCV29 by DNA transfection, changes in morphology and tumourigenicity were accompanied by a markedly reduced expression of monomorphic HLA-A,B,C antigens. One of the transfected sublines, HCV29-T112D1 expl., did not retain the low expression of HLA-A, B,C after re-explantation and cultivation and was no longer tumourigenic in a renewed tumourigenicity test. The identity of the transfected cells was confirmed by HLA typing.

Evidence has been presented [19] that inteferon-α enhances the expression of membrane-bound HLA-A,B,C by increasing the synthesis of HLA antigens. Treatment of the transfected cells with rHu-INF-α increased the expression of monomorphic as well as polymorphic HLA-A,B,C antigens indicating that the reduced expression of HLA could be due to a decreased synthesis of these antigens. HLA typing of other TGrIII urothelial cells using the complement dependent cytotoxicity assay is not possible unless the cells are pre-treated with rHu-INF-α (manuscript in preparation).

It has been suggested that the v-*raf* oncoprotein regulates the expression of the c-*myc* oncogene [20]. This oncogene has previously been demonstrated to be overexpressed in tumourigenic (TGrIII) human urothelial cell lines [21], and it is therefore noteworthy that all the v-*raf* transfected urothelial cells included in this study overexpress c-*myc* as compared to the level in the HCV29 cell line. This observation supports the hypothesis that the malignant transformation process involves the activation of functionally different oncogenes [22] and the present observations suggest a similar molecular mechanism to be involved in the alterations in HLA-A,B,C expression in malignant human urothelial cells.

The relationship between the expression of cell surface antigens and proto-oncogenes has been studied by other authors [23–26] with contradictory

results. Thus, introduction of the c-*myc* oncogene into e.g. human melanoma cell lines by DNA transfection has been demonstrated to be accompanied by a decreased expression of HLA-A,B,C [23]. Treatment of these cells with gamma-interferon increased the expression of HLA-A,B,C with a concommittant transient reduction in the expression of c-*myc*. Furthermore, c-*myc* has been demonstrated to be overexpressed in e.g. human small cell lung carcinoma cell lines [8] which has a decreased expression of HLA-A,B,C. Others have found a correlation between the expression of c-*fos* and H-2K antigens [24]. Treatment of highly metastatic clones of the T10 mouse sarcoma cell line with gamma-interferon increased the expression of H-2K antigens and was preceeded by a transient induction of c-*fos* transcripts. *In vitro* transformation of rat thyroid epithelial cell lines by DNA transfection with the v-ras^{ki} oncogene increased the expression of major histocompatibility complex (MHC) class I antigens [25]. No conclusive evidence for a correlation between v-*raf* and HLA-A, B,C has been presented [26]. Further experiments are needed to elucidate the role of oncogene products in the control of MHC antigen expression.

At present we have no satisfactory explanation for the high expression of HLA-A,B,C in the v-*raf* transfected but non-tumourigenic HCV29-T112D1 expl. cell line although this cell line overexpress v-*raf* and c-*myc*.

Quantitative or qualitative changes in the expression of HLA-A,B,C antigens would probably affect the host immune response against the tumour cell [1]. The cytotoxic T lymphocytes require HLA-A,B,C antigens in order to recognize and destroy a tumour cell, and a quantitative decrease in the expression of HLA antigens could be one way for the tumour cells to escape the cytotoxic T lymphocytes. In contrast to T lymphocytes, natural killer (NK) cells are highly active in nude mice and have been suggested to recognize tumour cells which express low levels of MHC class I antigens [27]. Our data apparently are not in agreement with this hypothesis, but further cytotoxicity experiments involving autologous and/or allogeneic NK cells are needed to elucidate this in greater detail.

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