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Expression of the Tumour-associated Antigen CA-242 in Transitional Cell Bladder Tumours: a Comparison with CA-50

P. Lipponen, M. Eskelinen and I. Hemmilä

The tissue expression of carbohydrate antigen CA-242 was analysed in formalin-fixed biopsy specimens from 147 transitional cell bladder tumours. The staining was related to established prognostic factors and survival during a mean follow-up of over 12 years and the staining results were also compared to expression of CA-50 antigen. Forty-one percent (60/147) of the tumours were negative for CA-242 and 59% (87/147) were positive. Normal bladder mucosa was positive for CA-242 and the umbrella cells in particular showed intense positive staining. In tumours, the umbrella cells were usually positive (when present) and in tumour tissue, positive cells appeared either as individual positive cells or in groups. None of the tumours was entirely positive for CA-242. The tissue expression of CA-242 could not be significantly related to TNM classification, papillary status, WHO grade or quantitative variables (DNA ploidy, S phase fraction, mitotic frequency, nuclear factors). The tissue expression of CA-242 was significantly weaker than the expression of CA-50. The expression of CA-242 was related to favourable prognosis in survival analysis ($P = 0.04$). The results show that the expression of the novel tumour marker antigen CA-242 as determined in paraffin-embedded material is a weak prognostic factor as compared with established prognostic factors in transitional cell bladder tumours.
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INTRODUCTION

THE EXPRESSION of blood group antigens and other blood group-related carbohydrate antigens has been extensively studied in bladder cancer [1–6]. Previous analyses show that deletion of blood group-related antigens is usually a sign of poor differentiation [1–6], high T-category [1–6], DNA aneuploidy [2, 6] and high invasive potential [2, 3, 6]. Blood group antigen Lewis a has been studied in bladder cancer and it also has prognostic relevance [5]. Lewis^a-related CA-50 shows heterogeneous tissue expression in bladder cancer similar to Lewis^a, and the loss of the expression of CA-50 has been related to several malignant histopathological characteristics and poor outcome [1, 6]. Monoclonal antibody CA-242 was obtained after immunisation of mice with a human colorectal adenocarcinoma cell line COLO 205 [7, 8]. The structure of the antigenic determinant of the CA-242 molecule is not completely defined but it seems to be a sialylated carbohydrate structure related to type I chain. It is related to the antigenic determinant of CA-50 (not identical) as defined by antibody CA-50 [8]. We have recently analysed the tissue expression and prognostic value of the CA-50 antigen in transitional cell bladder tumours [6]. Deletion of the antigenic determinant of CA-50 was related to malignant histopathological characteristics and poor outcome [6]. We have now analysed the same biopsy specimens for the expression of CA-242, since at present there are no data available on the expression of this antigen in transitional cell bladder tumours, and, furthermore, compared the results to CA-50.

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PATIENTS AND METHODS

Patients

The study comprised patients with newly diagnosed transitional cell bladder cancer between 1970 and 1987 and the follow-up analysis was performed in June 1991, the mean (S.E.) observation period being 12.0 (0.3) years. In total there were 147 patients with a mean (S.E.) age of 66.7(0.8) years and the female/male ratio was 26/121. The treatment and follow-up investigations were carried out according to standard practice [9]. The initial staging of tumours was based on results of excretory pyelography, transurethral biopsy, cytological examination of voided urine and bimanual palpation under anaesthesia. Screening for metastasis included chest radiography, laboratory tests, abdominal ultrasonography, and when appropriate, bone scintigraphy and lymphography. Tumour, nodes and metastasis (TNM) classification was done according to UICC 1978 [10] and was based on above mentioned examinations plus the pathologists' reports. Follow-up investigations were at 3-month intervals during the first 2 years and thereafter at 6-month intervals. If a recurrent tumour was observed the follow-up programme was restarted. Treatment of recurrent tumours was based on the same principle as the treatment of primary tumours. The causes of death were verified from patient files, autopsy reports and from the files of the Finnish Cancer Registry.

Histological methods

Histological samples were preoperative biopsy specimens from primary tumours. The samples were fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin or Van Gieson stains for grading. The samples were graded histologically according to WHO [11] and the papillary status of tumours was recorded.

Quantitative methods

The methods of flow cytometry [12], nuclear morphometry and mitotic frequency analysis [13] have been reported in detail previously in the literature. In brief, the measurements were determined as follows.

The mitotic figures were counted with a consultation microscope by two observers using an objective magnification of 40× (field diameter 490 μm). The mitotic figures were identified and counted from the most cellular areas of the tumour in 10 consecutive microscope fields (diameter 490 μm) and the number of mitotic figures was corrected for the fraction of neoplastic epithelium in each field. Thus, the final mitotic index (volume corrected mitotic index) expresses the number of mitotic figures/mm^2 of neoplastic epithelium.

In nuclear morphometric measurements the IBAS 1 and 2 image analyser was used. The images of the most atypical, well preserved microscopic fields were selected subjectively and focused on a video screen through a video camera attached to the microscope (magnification 40×). A mean of 75 nuclei were traced using a digitiser tablet and a mouse connected to the computer which also automatically calculated the morphometric nuclear parameters. In this analysis mean nuclear area and standard deviation of nuclear area were used.

The samples used for flow cytometry (FCM) were biopsy specimens from the primary tumours, fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin. One to three 50 μm thick sections were cut, deparaffinised and the single cell suspensions were stained with propidium iodide. The DNA was determined by Facs Star flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, California, U.S.A.). A 488 nm argon laser line run at 200 mW was used for fluorescence excitation. As the staining intensity of fixed nuclei varied from one sample to another, no internal standard was included. The lowest peak was given a DNA index (DI) value of 1 and the DI of other peaks were calculated with this as a reference. The DNA ploidy was assessed in a blinded manner without knowledge of the clinicopathological data. Histograms with a symmetrical G0/G1 peak were classified as diploid and if two G0/G1 peaks were present the histogram was classified as aneuploid. In survival analysis, tumours were categorised into two groups according to the DI value 1.05. Tumours with a DI 1.05 were recorded as diploid and tumours with a DI > 1.05 were considered aneuploid. The S phase fraction (SPF) was calculated using a modified rectilinear method. In aneuploid cases SPF was calculated for the aneuploid stemline only. The mean of CV (coefficient of variation) of the measurements was 5.50% (range 4.00–7.50%). The DI was available in 133/147 (90%) of cases and S phase fraction could be analysed in 113/147 (77%) of cases.

Immunohistochemistry

Tissue supernatant containing mouse monoclonal antibody CA-242 (IgG1) (Wallac, Turku, Finland) was used for the CA-242 staining. For immunohistochemical demonstration of CA-242, 5-μm sections from primary transitional cell carcinomas were deparaffinised and rehydrated. Endogenous peroxide was blocked by 3% hydrogen peroxide for 5 min followed by a wash for 5 min with phosphate-buffered saline (PBS). Tissue sections were incubated with the monoclonal mouse anti-human CA-242 [7] diluted at 1:5000 in PBS. Several dilutions were tested to find optimal staining before the entire series was processed and enzymatic pretreatment was also tested, but it did not change immunoreactivity. Sections were then washed twice for 5 min with PBS and incubated for 20 min with horse anti-mouse biotinylated secondary antibody (Vector, California, U.S.A.) diluted at 1:200 in PBS. Slides were washed twice in PBS for 10 min and incubated for 20 min in preformed avidin-biotinylated peroxidase complex (ABC, Vectastain Elite kit, Vector). Sections were washed twice for 5 min with PBS, developed with diaminobenzidine tetrahydrochloride substrate (Sigma, U.K.), slightly counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted. Positive control (adenocarcinoma of colon) was always positive for CA-242 and negative control (adenocarcinoma of colon processed without primary antibody) was always negative for CA-242. The immunostaining in the present cohort for CA-50 was performed using the same staining method and has been described in detail elsewhere [6]. The CA-50 antigen (Pharmacia, Sweden) was diluted at 1:40 in staining procedures [6].

Scoring of immunoreactivity

One investigator analysed the sections "blind" by using two methods of scoring. The sections were divided into being either negative or positive for CA-242. Secondly, a staining index was calculated. The staining intensity of cells in the sections was scored using arbitrary units 0, 1, 2 and 3 in order of increasing intensity: 0 corresponded to no positive staining, 1 to slight or weak staining, 3 to heavy or marked staining and 2 to intermediate staining between 1 and 3. The fraction (f) of cells of each staining in the whole section was evaluated. The staining index (I) was calculated using the formula

$$I = 0 \times f1 + 1 \times f2 + 2 \times f3 + 3 \times f4$$

where f1–f4 corresponded to the fractions of cells of each staining intensity. The scoring of CA-50 expression was done using the same method [6].

Statistical methods

In basic statistical calculations the SPSS/PC+ program package was used in a Toshiba T3200 computer and the statistical tests used are indicated in connection with the results when appropriate. Univariate survival analysis (log rank analysis) was based on the life-table method with statistics by Lee and Desu [14].

RESULTS

Normal bladder mucosa (n = 4) was positive for CA-242 as shown in Fig. 1(a) and (b) and the umbrella cells in particular were positive for CA-242 (Fig. 1a and b). Forty-one per cent (60/147) of the tumours were totally negative for CA-242 and 59% (87/147) showed positive staining. In CA-242-positive tumours the umbrella cells (when present) were usually positive for CA-242. The expression of CA-242 showed intratumoral heterogeneity and positive cells appeared as individual positive cells (Fig. 1c and d) or as groups of positive cells (Fig. 1e). None of the tumours showed uniform positive staining and the staining index was usually low. In inverted papilloma, only umbrella cells were positive whereas one adenocarcinoma that was analysed showed weak uniform staining as shown in Fig. 1f. In transitional epithelium, the antigen was mainly located on cell membranes and accordingly the staining gave an impression of a honeycomb structure (Fig. 1e). The staining of cytoplasm was always weaker than the staining of the cell membranes.

The staining result was independent of papillary status, WHO grade and TNM classification (Table 1). The staining index

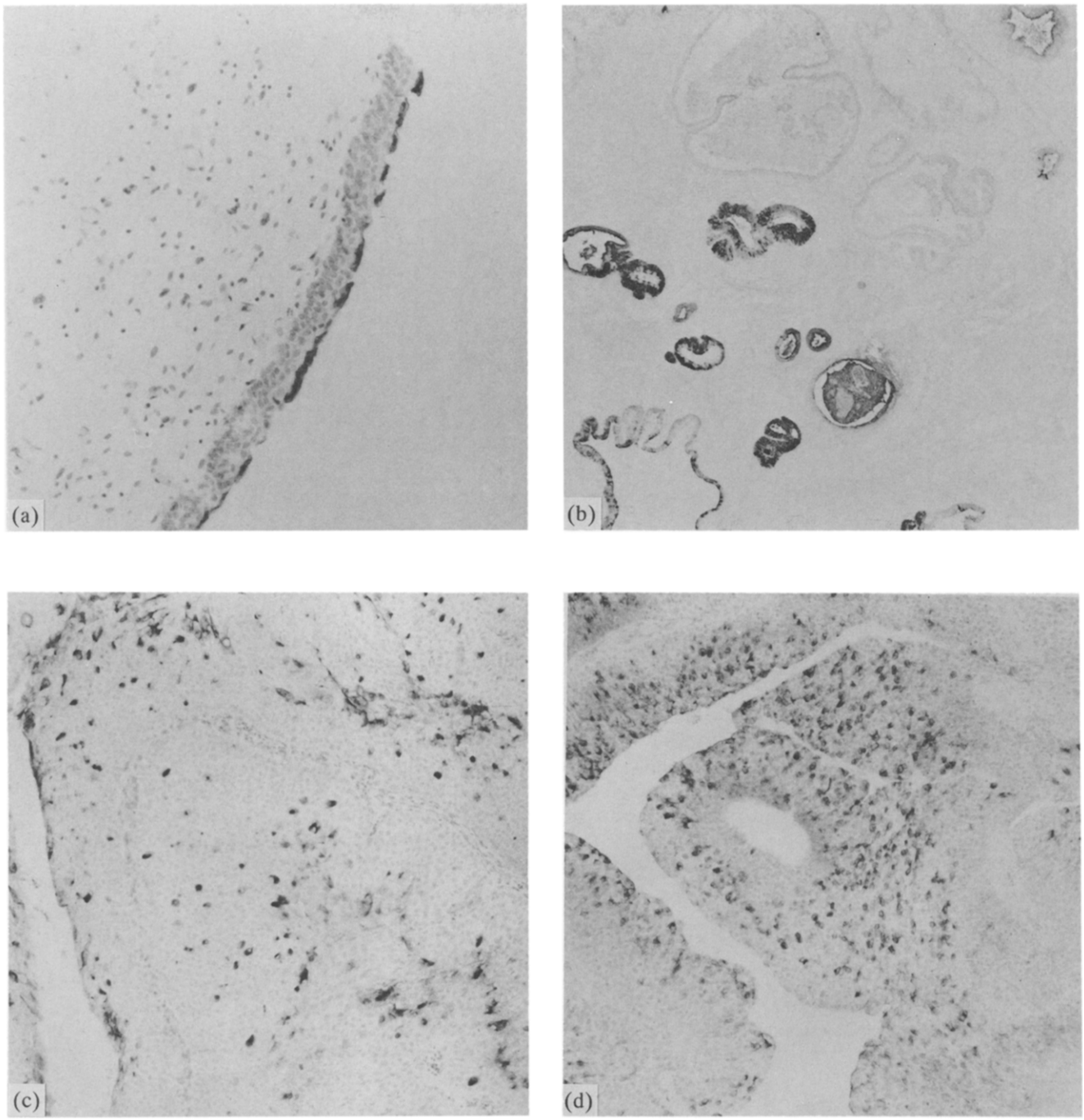

Fig. 1. (a) In normal transitional epithelium, umbrella cells stain positively for CA-242 antigen. Magnification 250×. (b) In prostatic urethra transitional epithelium exhibits variable positivity while the prostatic epithelium (top right) is negative for CA-242. Magnification 100×. (c–d) In transitional cell tumours individual positive cells were present. Magnification 250×.

values did not show significant differences among the different groups either. The staining was independent of morphometric nuclear factors, mitotic frequency and flow cytometric factors (data not shown). The progression of tumours (T, N and M categories) was not related to expression of CA-242.

In survival analysis the positive tumours had a more favourable prognosis than the negative ones (Fig. 2). Papillary tumours could be categorised into prognostic groups by this antibody ($\chi^2 = 3.3, P = 0.066$) whereas in Ta-T1 tumours CA-242 had no prognostic value.

A comparison with CA-50

Sixteen of the tumours were negative and 73 were positive for both of the antibodies. Ten tumours were negative for CA-50 and positive for CA-242. Forty-two tumours were negative for CA-242 and positive for CA-50 ($\chi^2 = 5.4$, $P = 0.019$). The staining indices for CA-242 and CA-50 were positively correlated ($R = 0.293, P < 0.01$). The above shows that the same tumours were significantly more often positive for both of the antigens. Also, the tissue expression of both of the antigens was similar since CA-50 was also more intensely expressed at cell membranes

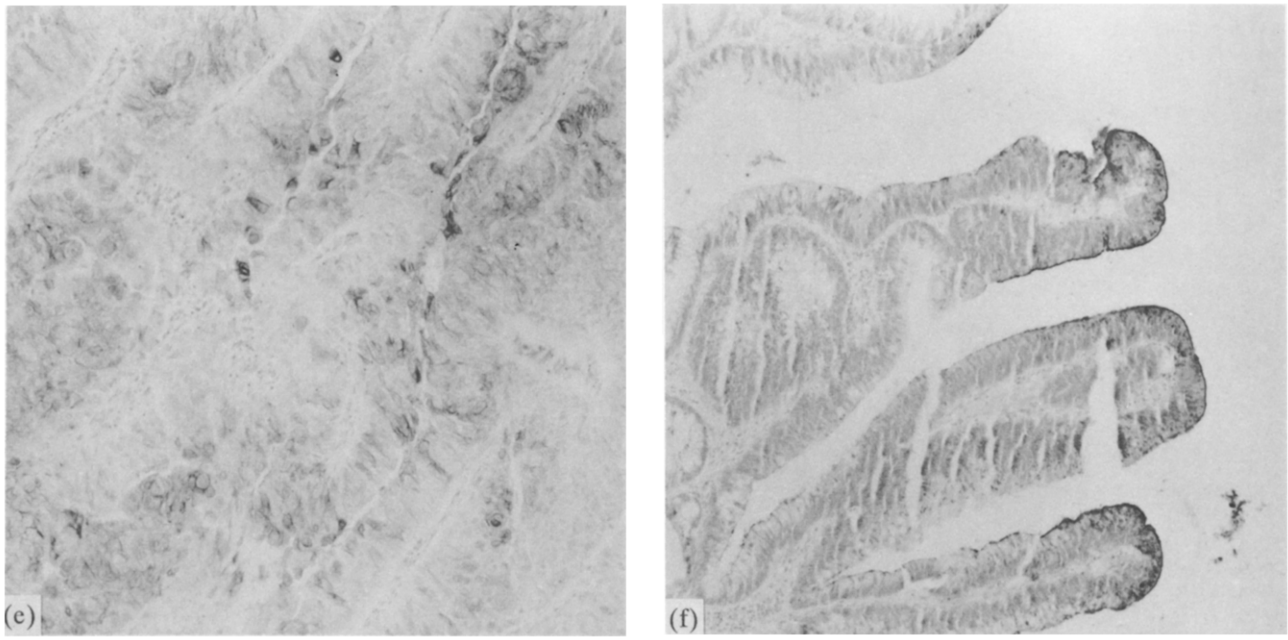

Fig. 1. (e) The positivity for CA-242 was mainly located on cell membranes. The positive cells were arranged as groups. Magnification 400×. (f) Adenocarcinoma of the bladder showed weak uniform staining and the apical border was more intensively stained. Magnification 100×.

than in the cytoplasm. The results also show that the number of CA-242-positive cells in a given tumour was significantly lower than the fraction of CA-50-positive cells. The fraction of positive tumours and the mean values of the staining index in various subgroups of bladder tumours are shown in Table 1. The table shows that CA-242 is randomly expressed in relation to various clinical and histological features, whereas CA-50 expression often shows a significant relationship. In survival analysis, CA-50 has prognostic value in the entire cohort ($\chi^2 = 11.2$, $P = 0.0008$), in papillary tumours ($\chi^2 = 5.8$, $P = 0.0154$) and in Ta-T1 tumours ($\chi^2 = 6.5$, $P = 0.0106$). The tissue expression and prognostic value of CA-50 in these same tumours has been recently reported in more detail elsewhere [6].

DISCUSSION

Tumour-associated antigen CA-242 has been analysed previously in serum samples of patients with pancreatic diseases and elevated concentrations were related to malignant conditions [15]. An immunocytochemical analysis of CA-242 in pancreatic tissue showed that in normal pancreas, CA-242 was expressed in the apical border of glandular epithelium. In well or moderately differentiated carcinomas the majority of carcinomas also expressed this antigen whereas poorly differentiated carcinomas were mainly negative for CA-242 [16].

In the present cohort normal bladder mucosa was positive for CA-242 and umbrella cells in particular were positive which is in line with the results in pancreatic tissue [16]. However, no

Table 1. The fraction of CA-242 and CA-50 positive and negative transitional cell bladder tumours subdivided according to TNM classification, papillary status and WHO grade. The mean (S.E.) of staining index (I) in each category is also shown

Category (*n*)	Positive CA-242/CA-50	Negative CA-242/CA-50	*P**	Mean (S.E.) CA-242(I)	*P*†	Mean (S.E.) CA-50(I)	*P**
TA (28)	11/24	17/4		0.14(0.05)		1.40(0.18)	
T1 (41)	16/31	25/10		0.15(0.04)		1.27(0.17)	
T2 (45)	16/38	29/7	0.6‡	0.25(0.06)	0.7	1.32(0.17)	0.04
T3 (25)	12/18	13/7	0.1§	0.20(0.08)		1.01(0.23)	
T4 (8)	5/4	3/4		0.19(0.18)		0.37(0.22)	
N0 (129)	50/103	79/22	0.6‡	0.18(0.03)		1.29(0.09)	
N1–2 (18)	10/12	8/4	0.4§	0.22(0.12)	0.7	0.72(0.21)	0.04
M0 (141)	56/113	85/28	0.2‡	0.20(0.03)		1.25(0.09)	
M1 (6)	4/5	2/1	0.9§	0.01(0.01)	0.2	0.54(0.25)	0.03
Papillary (131)	53/107	78/24	0.8‡	0.24(0.12)		1.30(0.09)	
Nodular (16)	7/11	9/5	0.1§	0.18(0.03)	0.4	0.63(0.26)	0.02
Grade 1 (57)	24/50	33/7		0.21(0.05)		1.40(0.13)	
Grade 2 (68)	26/49	42/19	0.8‡	0.14(0.03)	0.3	1.20(0.14)	0.04
Grade 3 (22)	10/17	12/5	0.05§	0.27(0.10)		0.84(0.22)	

*χ^2 test; † analysis of variance (over two groups), t-test (two groups); ‡ CA-242; § CA-50.

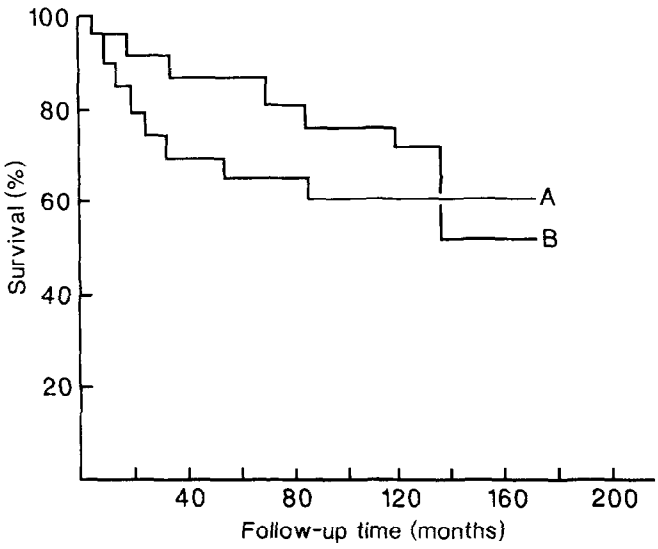

Fig. 2. The survival of patients categorised according to expression of CA-242. The difference in survival between the groups is significant ($\chi^2 = 4.2$, $P = 0.0398$). Curve A: CA-242 (−), $n = 87$; Curve B; CA-242 (+), $n = 60$.

significant differences were observed in staining between the different malignancy grades or other features of malignancy in TCC. The extent of the primary tumour and its ability to invade were not significantly related to expression of CA-242. In grade 2 or grade 3 carcinomas positive cells appeared as single positive cells or in groups and the positivity found in pancreatic tumours exhibited similar features [16]. In contrast, expression of the CA-50 antigen in the same bladder tumour biopsy specimens was significantly related to malignant histological features, clinical classification and invasive potential [6]. In addition, in survival analysis deletion of CA-50 antigen had higher prognostic potential than the expression of CA-242 and, particularly in superficial tumours, the expression of CA-50 seems to have independent prognostic value [6].

Overall, the expression of CA-242 was clearly weaker and only a low number of cells were positive for this antigen in comparison to CA-50 [6]. CA-242 antigen exhibited heterogeneous tissue expression and positive cells appeared as individual positive cells or in groups which supports the heterogeneity of bladder neoplasms as also observed by other methods [12]. Furthermore, the intense expression of CA-242 in cancer cells as in normal umbrella cells suggests a disturbance in coordinated cell maturation and development. The positivity for CA-242 was mainly located on cell membranes in the same way as the expression of CA-50 [6] or other blood group-related antigens in bladder tumours [2–6].

Normal bladder mucosa expressed CA-242 which suggests that the expression of CA-242 is a normal phenomenon like the expression of CA-50, Lewis^a, and other blood group-related antigens in bladder cancer [1–6]. In prostatic ducts lined by transitional epithelium, all cells were positive while normal prostatic epithelium did not show any positivity. This indicates differences in tissue expression of CA-242 between different organ systems. Moreover, colonic adenocarcinoma showed positivity like one adenocarcinoma which was derived from the bladder wall, which suggests disturbance in cell polarity and a relation to secretory function since in normal secretory epithelium CA-242 was expressed only in the apical cell border. Thus, the expression of CA-242 is not only related to cell differentiation. The function of cells and their location also seem to affect the expression of this antigen. The deletion of CA-242 was associated with unfavorable prognosis as occurs with the deletion of other blood group-related antigens [2, 6]. However, the expression of CA-242 may be influenced by genetic factors like the expression of blood group-related carbohydrate antigens in general [4, 5, 17]. This characteristic may act as a confounding factor in survival analysis and a general disadvantage of blood group antigens is their dependence on genetic factors [2, 5]. Consequently, the use of Lewis^a or related antigens has been recommended in place of A,B,H antigens since the prevalence of Lewis antigens in the general population is high [5].

The analysis showed that at least CA-242 offers no advantages in terms of survival over CA-50 or A,B,O blood group antigens [3, 4]. The same cohort has been analysed for other prognostic factors (stage, grade, DNA ploidy, S-phase fraction, mitotic frequency) and they clearly show that clinical classification, conventional histology and modern quantitative techniques are more accurate and simple predictors [12, 13]. Consequently, we conclude that the analysis of CA-242 expression in transitional cell bladder tumours has hardly any significance in a clinical context.

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Cytotoxic Effect of Interferon on Primary Malignant Tumour Cells. Studies in Various Malignancies

Dan Grandér, Bo Xu and Stefan Einhorn

It is a well established fact that interferon (IFN) can inhibit cell growth, but only recently has it been found that IFN can exert a direct cytotoxic effect on primary tumour cells. This was shown in malignant cells from patients with multiple myeloma. In this study the influence of IFN on the viability of primary malignant cells from patients with different malignancies was studied. As previously described a direct cytotoxic effect of IFN on multiple myeloma cells was observed. No major effects on cell viability could be found in malignant cells from patients with lymphoma, chronic lymphocytic leukaemia, hairy cell leukaemia, chronic myelogenous leukaemia and carcinoma. This indicates that the direct cytotoxic effect of IFN in multiple myeloma may be relatively specific for this malignancy. It could be due to a specific differentiation stage in the myeloma cells, specific genetic alterations and/or abrogation of an autocrine/paracrine loop.
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INTRODUCTION

INTERFERONS (IFN) have been shown to induce remissions in a variety of malignancies [1]. The mechanisms behind IFN antitumour action are still unclear and may vary in different malignancies. Theoretically, IFN could act by an indirect host mediated mechanism or by a direct effect on the tumour cell, for instance by cell growth inhibition or by induction of differentiation (reviewed in [2]).

By the use of a dye exclusion assay we have previously shown that IFN can exert a direct cytotoxic effect, unrelated to cell growth inhibition, on primary malignant cells from patients with multiple myeloma [3, 4]. This may be one major reason why some patients with myeloma respond to IFN therapy. The exact mechanism behind the cytotoxic effect is still unknown.

In this study we have examined whether a similar effect could be obtained with IFN in other malignancies, and we have mainly focused our interest on other B cell malignancies. In most of the diseases tested, clinical studies have shown that IFN can induce remissions [1].

MATERIALS AND METHODS

Malignant cells

Bone marrow samples were obtained from 44 patients with multiple myeloma. 4 of the myeloma patients were on therapy with natural IFN-α (7×10^6 U/m^2/day for 5 consecutive days repeated every third week), but had not received IFN injections within 7 days prior to sampling. Peripheral blood samples were obtained from 6 patients with chronic lymphocytic leukaemia (CLL) and 1 patient with hairy cell leukaemia. Lymph nodes were obtained from 7 patients with B cell lymphomas (1 centroblastic, 1 centrocytic and 5 centroblastic/centrocytic lymphomas). Blood and bone marrow were obtained from 11 patients with chronic myelogenous leukaemia (CML). Ascites fluid was obtained from 3 patients with ovarian carcinoma. A surgical specimen from a patient with gastric cancer was also studied. None of the patients with malignancies other than myeloma were on IFN therapy at the time of sampling.

Preparation and culture of cells

Preparation of tumour cells from ascites, surgical specimens and lymph nodes has been described elsewhere [5]. Briefly, a cell suspension was prepared by treatment of finely minced tumour tissue with collagenase (3 mg/ml) and DNAse (0.2 mg/ml) for 30 min. Heparinised peripheral blood and bone marrow samples were centrifuged on a layer of Lymphoprep. All cells were subsequently collected and washed twice by centrifugation in medium (RPMI 1640 with 1% L-glutamine and 10% fetal calf serum for bone marrow cells from multiple myeloma patients and minimum essential medium, Eagle modified with 1% L-glutamine, 10% human AB-rhesus positive serum for all other cells). Subsequently, 1×10^6 cells were incubated for 4 days in round-bottomed 5-ml plastic tubes (Falcon 2058, Lincoln Park, New Jersey) with 1 ml of medium, at 37°C in the absence or presence of IFN (5000 U/ml), if not otherwise stated. For malignancies other than myeloma, the results obtained with

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