TISSUE-SPECIFIC MARKERS IN FLOW CYTOMETRY OF UROLOGICAL CANCERS: CYTOKERATINS IN BLADDER CARCINOMA


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Thirty-eight transitional-cell carcinomas (TCC) were analyzed by flow cytometry (FCM) using propidium iodide for DNA analysis and antibodies to cytokeratin by indirect immunofluorescence. By means of two-dimensional FCM analysis, cytokeratin-positive tumor cells could be analysed separately from cytokeratin-negative stromal and inflammatory cells. This resulted in an 18% increase in sensitivity of FCM detection of aneuploidy (10/38 samples with one-parameter DNA analysis versus 15/38 samples with two-parameter DNA and cytokeratin analysis). In addition, S-phase could be determined in the 15 aneuploid samples by means of two-parameter analysis where this was not possible using only DNA content because of the overlap of diploid and aneuploid populations. FCM analysis allowed quantification of the percentage of tumor cells expressing cytokeratin 18 which has previously been shown to correlate quantitatively with higher grade, higher stage TCC. The quantitative measurement of tumor-cell expression of cytokeratin 18 by FCM analysis appears to provide additional information of potential prognostic value, independent of tumor-cell ploidy and proliferative fractions.

Flow cytometry (FCM) has been used extensively as a means of characterizing the DNA content of tumors and assessing their proliferative fraction (Brylany et al., 1983; Laerum and Farstrand, 1981). The routine clinical use of FCM analysis of tumor-cell DNA content and distribution has provided an important new parameter for assessing patient prognosis and response to therapy (Herman and Vooijs, 1982; Herman et al., 1984). These data have also proved of value in urological tumors (Baich et al., 1982; Devine et al., 1982; Gustafson et al., 1982; Klein et al., 1982a,b; Tribukait et al., 1982; Wijkstrom et al., 1984). A persistent problem in the interpretation of DNA data derived from FCM analysis of tumor specimens has been the variable admixture of non-tumor cells, primarily of stromal and inflammatory origin, with the tumor cells. The presence of these non-tumor cells has caused variable results in the interpretation of aneuploidy and proliferative fraction of solid tumor specimens (Herman and Vooijs, 1982).

We have recently described a technique for analysing the DNA content of pure tumor-cell populations in spite of the presence of stromal and inflammatory cells in single-cell suspensions from tumors (Oud et al., 1985; Ramaekers et al., 1984a,b, 1985a). This technique exploits the fact that intermediate filament proteins are highly tissue-specific. Thus, carcinoma cells can be distinguished by their expression of cytokeratin proteins from stromal and inflammatory cells which do not contain cytokeratin proteins.

Further application of immunopathologic techniques to transitional-cell carcinoma has demonstrated that expression of cytokeratin is altered by neoplastic changes in transitional epithelium (Ramaekers et al., 1985b). In particular, the 45 kd cytokeratin 18, generally found in glandular and other "simple" epithelia, is found in the superficial "umbrella" cells of normal transitional epithelium but not in the deeper cell layers. However, development of urothelial carcinomas is associated with an increased number of urothelial cells expressing this cytokeratin protein, as demonstrated by a highly specific monoclonal antibody (RGE53) (Ramaekers et al., 1985b).

In the present study, we report the use of FCM to analyse the DNA content and distribution of transitional-cell carcinomas of the bladder, excluding analysis of admixed stromal and inflammatory cells by immunofluorescent labelling of carcinoma cells with antibodies to cytokeratin. In addition, further FCM analysis made possible the quantitative measurement of the 45 kd cytokeratin 18 expression by bladder carcinoma cells. These two measurements considerably extend the reliability and information content of FCM analysis of bladder cancer.

MATERIAL AND METHODS

Thirty-eight transurethral resection specimens of bladder carcinomas were used as well as a lymph-node metastasis. The material was divided into 3 parts: one part was fixed in formalin and embedded in paraffin for routine histology, one was snap-frozen in liquid nitrogen for immunohistochemical staining, and the third was used for preparation of a single-cell suspension for FCM.

Routine histological classification was done from H. and E.-stained paraffin sections according to the WHO grading system (WHO, 1973). Clinical staging was performed in accordance with the rules of the International Union Against Cancer (Harner, 1982).

A single-cell suspension of the fresh tissue was prepared as described before (Herman et al., 1983). In brief, the tissue was minced with scissors in a sterile centrifuge tube and incubated with a mixture of collagenase Type II (1176 U/ml, Worthington, Freehold, NJ) and DNase I (200 U/ml, Sigma, St. Louis, MO) at 37°C for 2 hr. After enzymatic incubation, McCoy's wash (Gibco, Paisley, U.K.) was added and the cell suspension was passed through a 300-μm metal sieve, a 25-gauge needle and a gas-sterilized 100-μm pore size nylon filter (Ortho Diagnostic Systems, Becton, Belgium). The cells were centrifuged at room temperature at 400g for 5 min, after which the supernatant was discarded but fluid sticking to the wall of the tubes was discarded.

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allowed to cover the pellet. The pellet was vortexed vigorously and the cells were resuspended in the small volume of McCoy’s wash. Then 70% ethanol (–20°C) was added rapidly under constant vortexing or shaking. The final cell concentration was about 3 × 10^6 cells/mL ethanol. At this stage the cells could be stored at –20°C for several days.

**Immunohistochemical staining procedures and antibodies**

Frozen sections of normal human bladder tissues, primary TCC’s and tumors, as well as a TCC lymph-node metastasis, were incubated with antibodies to cytokeratin by the indirect immunofluorescence technique (Ramaekers et al., 1983b). Antibodies used in this study were: (1) An affinity-purified polyclonal rabbit antisemur to human skin keratins (pKer) which reacts with virtually all epithelial tissues, but not with non-epithelial tissues. The antisemur was diluted 1:5 in PBS for staining of cells in suspension and 1:25 for staining of frozen sections. (2) RGE53, a monoclonal antibody to cytokeratin 18, one of the 19 cytokeratins present in human epithelial tissues (Debus et al., 1982; Moll et al., 1982). This antibody is specific for glandular epithelia and does not react with squamous epithelia (Ramaekers et al., 1983b). Undiluted culturing supernatant was used for labelling of cell suspensions and frozen sections. Ethanol-fixed cell suspensions from the bladder carcinomas were split into 4 fractions. One sample was stained with propidium iodide (PI) as described below and used for DNA measurements. A second sample was used for the calculation of the DNA-index, using chicken red blood cells (CRBC) as internal standard (Hamilton et al., 1980; Tannenbaum et al., 1978).

The third and fourth samples were stained separately with the antibodies pKer and RGE53 as follows. About 1 × 10^6 cells in ethanol (70%, –20°C) were centrifuged at room temperature (1.5 min, 400 g) and the pellet was resuspended with 1 ml of 5% fetal calf serum (FCS; Gibco) in buffer A (0.01 g NaCl, 0.2 g KCl, 1.44 g NaHPO4, 0.2 g KH2PO4 per 1,000 ml water, pH = 7.4) and pelleted again. The pellet was resuspended in 0.2 ml of PBS containing 10% FCS in Buffer A (pKer), and incubated for 30 min at room temperature with regular shaking. Then the cells were washed 3 times in 1.0 ml Buffer A containing 5% FCS. After the last washing step the cell pellet was resuspended in 0.5 ml of FITC-conjugated secondary antibody, diluted 1:25 in Buffer A containing 5% FCS. As second antibody, either FITC-conjugated goat-anti-rabbit IgG or FITC-conjugated rabbit-anti-mouse IgG (Nordic, Tilburg, The Netherlands) were used. After incubation for 30 min at room temperature with regular shaking, the cells were washed as described above. The cell pellet was then resuspended in 1 ml of a PI solution (20 mg/ml A-grade in 150 mM sodium phosphate buffer, pH 7.4; Calbiochem—Boehringer, La Jolla, CA). To 1 ml of this cell suspension, 0.1 ml of a stock solution of RNase (1% type A in sodium phosphate buffer; Sigma) was added and the suspension was incubated for exactly 10 min at 37°C. Then the cell suspension was filtered through a 100-μ nylon filter (Ortho). Cells were kept in the dark at room temperature prior to FCM analysis.

**FCM analysis**

Cell analysis and sorting were performed using a Cytofluorograph 50H (Ortho Instruments, Westwood, MA). Fluorochromes PI and FITC were excited at 488 nm with an argon ion laser (Spectra Physics, Mountain View, CA). Fluorescence was measured simultaneously using a 515–530-nm bandpass filter and a 635-nm longpass filter for FITC and PI, respectively. A correction was made for leakage of FITC fluorescence into the PI channel. All data were stored in correlated (list) mode on a PDP 11/34 computer (Digital Equipment Corporation, Marlboro, MA) for subsequent data analysis. The normal human G1 DNA-index in our laboratory is 2.65 to 2.75 times the fluorescence intensity of CRBC (Hiddeman et al., 1984). Samples with a DNA index of the second peak of more than 1.110 (mean normal G1 = 0.990 with a s.d. = 0.040) were classified as aneuploid. Cell cycle analysis was done for characterization of the G1, S and G2/M phases (Baisch et al., 1975).

**RESULTS**

**Immunohistochemistry**

Frozen sections of normal bladder, primary TCC and a TCC lymph-node metastasis were incubated with polyclonal and monoclonal antibodies pKer and RGE53. In normal bladder tissue, all epithelial cells are stained with the polyclonal cytokeratin antibody pKer, while only the superficial layer of transitional epithelium is stained with the antibody to cytokeratin 18 (RGE53) (Fig. 1a,b). In low-grade, low-stage tumors a comparable staining pattern is seen (Fig. 1c,d), while in the high-grade, high-stage tumors most epithelial tumor cells express cytokeratin 18 (Fig. 1e,f). In a lymph-node metastasis all epithelial cells expressed cytokeratin 18 (Fig. 1g,h).

**Labelling of tumor cells in single-cell suspensions and FCM analysis**

When ethanol-fixed cell suspensions from 38 bladder TCC were incubated with the polyclonal cytokeratin antibody (pKer) and thereafter with an appropriate FITC-conjugated second antibody and PI, the epithelial tumor cells could be distinguished from stromal and inflammatory cells. Analysis of the cytokeratin and PI-positive bladder cells revealed tumor cells ranging in DNA-index from 0.94 to 3.58 (normal human diploid equals 1.0). An example of such a two-parameter FCM analysis is shown in Figure 2. In the one-parameter analysis an abnormal DNA stemline is shown in this case of TCC (Fig. 2a). Two-parameter analysis for PI and pKer in a two-dimensional projection (Fig. 2b) allows the stromal and inflammatory cells (Fig. 2c) to be clearly distinguished from the cytokeratin-positive aneuploid tumor cells (Fig. 2d) and also from cytokeratin-positive but diploid normal bladder epithelial cells, although a percentage of the aneuploid cell fraction is negative for pKer. This aneuploid fraction negative for cytokeratin is represented by naked nuclei from TCC tumor tissue, as judged from microscopic inspection of sorted fractions from the cell suspension. The proliferative fraction (S- and G2/M phase) of the tumor can now be estimated after labelling with pKer.
**Figure 1** - Immunofluorescence micrographs of normal human bladder epithelium (a,b), a low-grade, low-stage TCC (c,d), a high-grade, high-stage TCC (e,f) and a lymph-node metastasis of a TCC (g,h) stained with the polyclonal anticytokeratin antiserum pKer (a,c,e,g) or with the monoclonal anticytokeratin 18 antibody RGE53 (b,d,f,h). Note the increase in the relative number of RGE53-positive cells as a function of neoplastic progression. Bar indicates 50 μm.

**Detection of small numbers of tumor cells and estimation of their ploidy**

In the case of some tumor-cell suspensions, containing only small numbers of aneuploid cells, it was not possible to detect the tumor cells using the one-parameter DNA analysis of the sample stained only with PI (Fig. 3a). However, after application of the two-parameter technique, we could detect aneuploidy in 15/38 samples instead of the 10/38 samples detected by
using one-parameter DNA analysis (Fig. 3b). This represents an increase of 17.9% in the detection of aneuploidy (5/28 samples considered diploid by single parameter analysis) with a confidence value of 6.06-36.89% at 95% and 4.07-42.8% at 99%.

The two-parameter labelling technique also increased the evaluable of samples containing large amounts of debris, usually a result of surgical and/or preparative procedures. Again epithelial cells can be detected and analysed separately. These cells can be sorted and identified as tumor cells in the microscope.

Furthermore cytokeratin antibodies in FCM were used for the recognition of slightly aneuploid cells in the cell population. Such cell fractions were recognized as a shoulder in the first G1-peak in some specimens (Fig. 3c). By means of the two-parameter technique and a two-dimensional projection of the DNA content (PI) and the cytokeratin content (pKer), it was possible to select the epithelial cells (Fig. 3d) from the non-epithelial cells and analyse these fractions separately for their DNA content and proliferative fraction.

Cell-cycle analysis

In those samples (20/38) which did not contain excessive debris or cell clumps, we could estimate the
number of cells in S-phase. The percentage of cells in S-phase could be correlated with the histological grade of the tumors (Fig. 4a). We found a significant increase in the percentage of cells in S-phase with increasing histologic grade of the tumor. It should also be noted that almost all of the grade-III tumors are aneuploid, while the 2 aneuploid grade-II tumors show an S-phase fraction higher than the mean value estimated for all grade-II TCC's.

Expression of cytokeratin 18 by tumor cells

We have reported above (see also Ramakers et al., 1985b) that when frozen sections of TCC are stained with RGE53, tumors of higher grade and higher stage contained increased numbers of cells expressing cytokeratin 18, as shown by an RGE53-positive staining pattern. In the analysis of low-grade, low-stage TCC with both polyclonal (pKer) and monoclonal (RGE53) antibodies in combination with PI, variable numbers of cells showed a positive staining reaction with the polyclonal cytokeratin antibody. Only a low percentage of these tumor cells expressed cytokeratin 18 (as detected by RGE53) in these low-grade tumors. In contrast, high-grade, high-stage tumors showed quite a different staining pattern. In all samples, again variable numbers of cells were positive for the polyclonal
cytokeratin antibody. Within these high-grade tumors a higher percentage of the tumor cells also expressed cytokeratin 18. In a lymph-node metastasis of a TCC all tumor cells were aneuploid while there was a complete overlap in the fractions staining with the polyclonal and monoclonal antibodies to cytokeratin. As a control, a sample with RAM-FITC was analysed and all cells were negative with this control staining. A correlation between the percentage of cells expressing cytokeratin 18 (as related to the total of epithelial pKer-positive cells) with the histological tumor grade is shown in Figure 4b. An increase in cells expressing cytokeratin 18 is seen with increasing tumor grade, independent of S-phase or ploidy of the tumor cells.

**DISCUSSION**

Flow cytometric analysis of TCC offers new diagnostic tools for the clinician and provides new prognostic values (Lovett et al., 1984; Herman et al., 1984). The use of tissue-specific markers in combination with DNA analysis in flow cytometry may give more reliable analysis of epithelial tumors. When polyclonal and monoclonal antibodies to cytokeratins are used, normal epithelial cells and carcinoma cells can be labelled specifically by the indirect immunofluorescence technique. Non-epithelial cells, stromal cells, blood cells and inflammatory cells do not stain with cytokeratin antibodies. Therefore, these antibodies are already widely used as tissue markers for histopathology and cytopathology (Ramaekers et al., 1983b). Flow cytometric analysis of mixed cell populations, such as are obtained from tumors, produces results which are often difficult to interpret because of the admixture of cells of interest, usually carcinoma cells, with other cells such as inflammatory and stromal cells. In some cases these cell populations can be easily distinguished, but most tumor material is still difficult to analyse and to interpret by FCM (Herman et al.,
1984). Because of the ubiquitous presence of cell-type-specific intermediate filament proteins, immunofluorescence of these structures using specific antibodies can be used for FCM analysis of many solid tumors (Ramaekers et al., 1984a,b, 1985b). All carcinomas can be distinguished from non-epithelial cells by broad-specificity cytokeratin antibodies and specific epithelial cell types can be further recognized by means of monoclonal antibodies against particular subclasses of cytokeratin.

Three advantages of the application of cytokeratin antibodies in FCM analysis have become obvious from the present experiments. First, in those cases of carcinoma having only a very small fraction of cells with an abnormal stemline, these cells can be detected by the two-parameter analysis. Even in samples containing a large amount of debris, which are not evaluable by one-parameter FCM analysis, cells with an abnormal DNA content are detectable by two-parameter FCM analysis. When the epithelial fraction is not clearly visible as a peak in the one-parameter DNA histogram, its exact position can still be determined by recognition of the epithelial cells through the epithelium-specific cytokeratin markers. This offers the opportunity to detect abnormal, aneuploid stemline cells in more tumors than is possible with one-parameter FCM analysis. Second, the two-parameter analysis offers the possibility to study the cell fraction of interest, in our case the carcinoma cells, and to analyse the cell cycle of the tumor. In the analysis of TCC's we have found a significant increase of cells in S-phase in correlation with increased grade of tumor cells. These values of the S-phase fractions of TCC's are theoretically more reliable than those published by Tribukait et al. (1982), since we have only used S-phase values of the epithelial pKer-positive cell population and not the S+ G0/M fraction of the total cell population. It remains to be evaluated whether or not this refinement in FCM methods gives a better correlation with prognosis than the methods used so far. In all estimations one has to keep in mind that a certain percentage of the tumor cells lose their cytoplasm during preparation of the cell suspension and thus are cytokeratin-negative. This phenomenon, however, does not interfere with the calculation of the percentage of cytokeratin-positive tumor cells. Its influence on the estimation of the proliferative fraction of TCC's is under study. A third advantage of potential clinical value of the two-parameter analysis of tumors lies in the differential staining patterns of the tumor cells with different types of cytokeratin antibodies. An increase in cells expressing cytokeratin 18 was seen with an increase in malignancy in frozen sections of TCC's (Ramaekers et al., 1985b). This phenomenon has now been quantified following FCM analysis of these tumors. In low-grade, low-stage TCC only a small percentage of the tumor cells express cytokeratin 18 (as detected by RGE53), while in a lymph-node metastasis all tumor cells express cytokeratin 18. In fact, this latter result further extends and confirms our earlier observations in primary TCC's (Ramaekers et al., 1985b). The correlation between the percentage of tumor cells expressing cytokeratin 18 and an increase in malignancy, expressed as grade of the tumor, although not yet seen for each individual tumor, may offer the clinician a new prognostic criterion in the evaluation of TCC.

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