Application of Antibodies to Intermediate Filament Proteins as Tissue-Specific Probes in the Flow Cytometric Analysis of Complex Tumors

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The flow cytometric (FCM) analysis of carcinomas is often hampered by the presence of stromal and inflammatory cells in the cell suspensions obtained from such neoplasms. Therefore, an FCM method was developed to distinguish epithelial from nonepithelial cells by using polyclonal and monoclonal antibodies to (cyto)keratins, the epithelial type of intermediate filament proteins. Using a model system of cultured bladder carcinoma (T24) and leukemia (MOLT-4) cells, we tested our hypothesis and procedures by labeling cell mixtures with these antibodies. After incubation with an appropriate intermediate filament antibody and propidium iodide staining, the DNA content and distribution of T24 cells could be analyzed separately from MOLT-4 cells. When applied to cell suspensions of endometrial carcinomas, bladder carcinomas and Gravitz tumors, only the epithelial (primarily carcinoma) cells were stained for cytokeratin; these cells could thus be analyzed separately from stromal, inflammatory and other nonepithelial cells. In this way, a more accurate FCM analysis of the malignant fraction within a tumor can be achieved.
All eukaryotic cells contain an intracellular network of protein filaments with varying diameters; these filaments constitute the so-called cytoskeleton. In addition to microfilaments and microtubules, a class of 10-nm filaments can be recognized. These intermediate-sized filaments, which comprise a considerable part of the water-insoluble cytoskeleton, have been elaborately described and are known to be tissue specific.

Recent investigations have shown that all types of epithelial cells contain cytokeratins while mesenchymal cells contain vimentin as their intermediate filament protein. Muscle cells contain desmin intermediate filaments while neural tissues contain glial fibrillary acidic protein (GFAP) or neurofilaments.

Antibodies to these different types of intermediate filament proteins can be used in the immunohistochemical characterization of normal tissues, cell cultures and benign and malignant tumors. For example, carcinomas normally contain only cytokeratins while malignant lymphomas contain only vimentin intermediate filaments.

When frozen sections of normal human tissues and tumors are incubated with antibodies to cytokeratins, only epithelial cells, benign and malignant, are stained positive in the indirect immunofluorescence technique. For example, transitional-cell carcinomas of the bladder are positive for cytokeratin

Figure 1
Immunofluorescence micrographs of (A) bladder carcinoma stained for cytokeratin with a rabbit antiserum to skin keratin (×100), (B) a well-differentiated endometrial carcinoma stained for cytokeratin (×250) and (C) bladder carcinoma stained for vimentin with a rabbit antiserum to call lens vimentin (×100). Note that epithelial tumor cells are only stained for cytokeratin while stromal components are only stained for vimentin.

Figure 2
Immunocytochemical demonstration of cytokeratin intermediate filaments in cultured T24 bladder carcinoma cells using a monoclonal antibody to cytokeratin 18 (RGE53).
(Figure 1A); the same is true for endometrial carcinoma (Figure 1B). Furthermore, when applied to cells in tissue culture, again only cells of epithelial origin seem to contain cytokeratins.1-12 For example, when applied to T24 bladder carcinoma cells grown on a coverslip, cytokeratin antibodies detect an intracellular filamentous network, in cells fixed in methanol and acetone (Figure 2). MOLT-4 leukemia cells do not stain with the cytokeratin antibodies. An antibody to vimentin, the mesenchymal type of intermediate filament protein, does not normally stain epithelial cells in situ. In general, fibroblasts, blood cells and inflammatory cells, blood vessel endothelium and some vascular smooth muscle cells are positive for vimentin (Figure 1C). Solid carcinomas do not normally stain for vimentin (Figure 1C). One exception to this rule, however, is represented by renal cell adenocarcinoma. The malignant cells of this type of neoplasm can coexpress cytokeratins and vimentin (Figure 3).9

Moreover, since cytokeratins consist of a number of biochemically and immunologically different proteins, which occur in tissue-specific combinations,13 monoclonal antibodies to these different cytokeratin types allow the distinction between different kinds of epithelial cells.3,10,13 For example, monoclonal antibodies to cytokeratin 18 have been prepared that are able to distinguish squamous epithelium (cytokeratin 18-negative) from glandular and columnar epithelium, which contains cytokeratin 18.8-19 It is obvious that such tissue markers are valuable tools, not only in the immunohistologic classification of normal and malignant tissues,13-21 but also in cytology17 and cytometry.

Flow cytometric (FCM) analysis of cell suspensions has proven to be useful as a technique for the estimation and quantitation of several cellular components. Special attention has been focused on the measurement of the DNA content in cells, which may be used to estimate the ploidy and proliferative capacity of tumors.8,9 However, analysis of mixed cell populations, which are normally obtained from tumors, gives rise to data that are sometimes difficult to interpret. The tumor cell suspensions usually contain variable amounts of other cell types, such as inflammatory and stromal cells.

We have developed an FCM procedure to distinguish epithelial, primarily tumor cells from nonepithelial cells in such mixed cell populations on the basis of their intermediate filament type. With this method, it is possible to simultaneously analyze the DNA content distributions of the two cell populations. This article reviews our earlier published findings with a model system of mixed cultured cells, in which we tested our hypothesis.15-16 Furthermore, our observations with cell suspensions obtained from gynecologic14 and urologic tumors are briefly summarized.
Materials and Methods

Cells and Tissues

T24 cells are derived from a human transitional-cell carcinoma. These cells grow in tissue culture and are positive for cytokeratin when tested by the indirect immunofluorescence technique. MOLT-4 cells are a culture of T-cells derived from a patient with acute lymphatic leukemia. These cells are negative for cytokeratin.

Cells attached to the bottom of tissue culture flasks were covered with a minimal amount of 0.25% trypsin in phosphate-buffered saline (PBS) or culturing medium without calf serum. After five to ten minutes at 37°C, the cells were suspended in PBS and centrifuged for five minutes at 400 g. The supernatant was discarded, but fluid sticking to the wall of the tubes was allowed to cover the pellet. The pellet was then vortexed vigorously, and the cells were suspended in a small volume of PBS. Then 70% ethanol (−20°C) was added rapidly under constant vortexing or shaking. The final concentration in our experiments was about 3×10⁶ cells/mL of ethanol. At this stage, the cells could be stored at −20°C.

Liquid nitrogen-stored material from normal human endometrium and endometrial carcinomas was homogenized in PBS using a Dounce homogenizer (loose-fitting pestle). The preparation of single-cell suspensions and their fixation was performed as described elsewhere.14 Transitional-cell carcinomas of the bladder and renal-cell tumors were brought into cell suspensions using enzymatic digestion (collagenase plus DNase) as previously described in detail.7 Fixation of these cell suspensions in 70% ethanol was as described above for the cultured cells.

Antibodies

Three antibodies were used in this study. (1) The first antibody was RGE 53, a monoclonal antibody to cytokeratin 18, one of 19 cytokeratins present in human epithelial tissues. This antibody is specific for glandular epithelia and does not react with squamous epithelia.13 Undiluted culturing supernatant was used for the labeling of cell suspensions. (2) An affinity-purified polyclonal rabbit antiserum to human skin keratins that reacts with virtually all epithelial tissues but not with nonepithelial tissues18 was used. This antiserum was diluted 1:5 in PBS. (3) A rabbit antiserum directed against calf lens vimentin, which reacts with cells of mesenchymal origin but normally does not stain epithelial cells,24 was also utilized. This antiserum was diluted 1:20 in PBS.

Labeling of the Cell Suspensions

The immunocytochemical staining procedure was performed at room temperature. The endometrial cell suspensions were labeled as previously described.14

The fixed suspensions from cultured cells, bladder carcinomas, and renal-cell tumors were split into three fractions. One sample was stained with propidium iodide (PI) as described below and used for cell kinetic studies. A second sample was used for estimation of the DNA index, with chicken red blood cells used as an internal standard.5–6 The third sample was labeled as follows.

About 1×10⁶ cells in ethanol (−20°C) were centrifuged at room temperature (15 minutes, 400 g) and the pellet was washed with 1 mL of 5% fetal calf serum (FCS; Gibco Europe B.V., the Netherlands) in buffer A (8.01 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.2 g of KH₂PO₄ in 1,000 mL of distilled water, pH 7.4) and pelleted again. The pellet was resuspended in 0.2 mL of antiserum, either as culturing supernatant or diluted in 5% FCS in buffer A, and incubated for 30 minutes at room temperature with regular shaking. Then the cells were washed three times in 1.0 mL of buffer A containing 5% FCS.

After the last washing step, the cell pellet was resuspended in 0.5 mL of FITC-conjugated second antibody, diluted 1:25 in buffer A containing 5% FCS. As second antibodies, either FITC-goat-anti-rabbit IgG or rabbit-anti-mouse IgG (Nordic, Tilburg, the Netherlands) was used. After incubation for 30 minutes 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 (30 mg/L A-grade in 150 mM sodium phosphate buffer, pH 7.4; Calbiochem, Boehringer Corporation, La Jolla, California, USA).

To 1 mL of this cell suspension, 0.1 mL of a stock solution of RNA-ase (1% type A in sodium phosphate buffer; Sigma, St. Louis, Missouri, USA) was added, and the suspension was incubated for exactly 10 minutes at 37°C. Then the cell suspension was filtered through a 100-μm nylon filter (Ortho Diagnostics Systems, Beersel, Belgium). Cells were kept in the dark at room temperature prior to FCM analysis.

FCM Analysis

Cell analysis and sorting were performed using a Cytofluorograph System 50H (Ortho Instruments, Westwood, Massachusetts, USA). The fluoro-
chromes PI and FITC were excited by a 488-nm wavelength light beam from an argon ion laser (Spectra Physics, Mountain View, California, USA). Fluorescence was measured simultaneously using a 515–530 nm filter and a longpass 630-nm filter for FITC and PI, respectively. A two-color compensation network was used to compensate for color overlap between green and red fluorescence, using a single-stained fraction as reference.

All data were stored in correlated (list) mode on a PDP 11/34 computer (Digital Equipment Corporation, Maynard, Massachusetts, USA) for subsequent data analysis.

The normal human G1 DNA index in our laboratory is 2.65 to 2.75 times the fluorescence intensity of chicken erythrocytes.

In order to show that antibodies to cytokeratins can be used to analyze epithelial fractions of mixed cell populations and to test our hypothesis and methods, we first applied the staining procedure to a model system of mixtures of established tumor cell lines (Figure 4). Mixtures of ethanol-fixed epithelial T24 bladder carcinoma cells and nonepithelial MOLT-4 leukemia cells were labeled with a cytokeratin antibody and thereafter with an appropriate FITC-conjugated second antibody. After PI staining and subsequent FCM analysis for FITC and PI intensity, it was obvious that only the epithelial T24 cells were stained for cytokeratin.

Analysis of pure T24 cells showed a single population of cells with a DNA index of 1.56 and all cells staining for cytokeratins. MOLT-4 cells showed a higher DNA index of 2.03 and no reaction with the antibodies. Mixtures of T24 and MOLT-4 cells could be separated into the individual cell populations on the basis of cytokeratin staining of the T24 cells. DNA histograms of the two different cell types could be obtained by interactively placing a window in the two-dimensional histogram (Figure 4). Comparison of these data with those of the pure cell cultures, analyzed for DNA content and distribution before mixing, revealed identical histograms. The distribution of cells in the different phases of the cell cycle in the two cell populations agreed with the data derived from the pure populations (Table I).

**Table 1** Cell Cycle Analyses of T24 Cells and MOLT-4 Cells in Pure Populations and Mixed Populations

<table>
<thead>
<tr>
<th></th>
<th>Pure population (%)</th>
<th>Mixed population (%)</th>
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<tbody>
<tr>
<td><strong>T24 cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_1$</td>
<td>72.7</td>
<td>73.4</td>
</tr>
<tr>
<td>$S$</td>
<td>19.4</td>
<td>18.6</td>
</tr>
<tr>
<td>$G_0M$</td>
<td>7.8</td>
<td>8.0</td>
</tr>
<tr>
<td><strong>MOLT-4 cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_1$</td>
<td>56.2</td>
<td>56.0</td>
</tr>
<tr>
<td>$S$</td>
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<td>34.6</td>
</tr>
<tr>
<td>$G_0M$</td>
<td>9.8</td>
<td>9.4</td>
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Encouraged by these data we applied the method to cell suspensions from solid normal tissues and tumors, which are always mixtures of different cell types. When cells isolated from the endometrium and stained for cytokeratin and DNA were analyzed by FCM, several cell populations could be distinguished. Cells positive for cytokeratin could be distinguished clearly from negative cells. Furthermore, PI staining distinguished cells containing nuclei from cell debris and loose cytoplasmic fragments. A DNA histogram of the cytokeratin-positive cells could readily be obtained from these data. In this material the position of the endometrial cell G2G1 peak was 2.7 times that of chicken erythrocytes, a normal human diploid ratio.

Similar studies were performed with neoplastic lesions of the endometrium. Figure 5A shows a three-dimensional histogram of cells from a well-differentiated adenocarcinoma of the endometrium, including chicken erythrocytes as an internal standard. Again epithelial cells (RGE53 positive) could be clearly distinguished. In the sample illustrated, the main fraction of the adenocarcinoma cells was found in the diploid region. When a window was placed interactively in this histogram (Figure 5B), RGE53-positive nucleated cells could be separated from RGE53-negative objects and cell debris.

The correctness of the window was checked by sorting samples of cells onto glass slides, and by examination of these preparations in the fluorescence microscope and after routine cytologic staining. In these sorted samples, only cytokeratin-positive and PI-positive cells were found, in contrast to unsorted cell suspensions. Furthermore, cell morphology was well preserved and indicated the presence of mainly (if not only) epithelial tumor cells.14

When ethanol-fixed cell suspensions from bladder transitional-cell carcinomas were incubated with the polyclonal cytokeratin antibody and thereafter with an appropriate FITC-conjugated second antibody and PI, again the carcinoma cells could be separated from stromal and inflammatory cells (Figure 6). However, as can be judged from Figures 6A and 6D, a cytokeratin-negative, aneuploid fraction is often present in these cell suspensions. We sorted this population and, after microscopic inspection, we concluded that it represents naked nuclei, which always occur in enzymatically or mechanically obtained cell suspensions from solid tumors. After comparing several DNA profiles of labeled and unlabeled cells, we further concluded that this phenomenon had no significant impact on the DNA distribution histograms of the tumor.

Analysis of the cytokeratin-positive and PI-positive bladder cells revealed tumor cells ranging in DNA index from 0.94 to 2.02 (normal human diploid equals 1.0).

In one cell suspension from a bladder carcinoma, very few tumor cells were recognized by one-dimensional FCM DNA analysis (Figure 7A). In con-
Figure 6

FCM analysis of a transitional-cell bladder carcinoma in suspension after labeling for cytokeratin using the polyclonal keratin antiserum (FITC channel) and staining with PI. (A) Two-parameter (FITC for cytokeratin and PI for DNA) analysis showing position of the window containing cytokeratin-positive epithelial cells. The cytokeratin-negative aneuploid fraction was represented by naked nuclei, as judged from microscopic observations. (B) DNA histogram of the total cell suspension. (C) DNA histogram of cytokeratin-positive cells selected by placing a window as illustrated in Figure 6A. (D) DNA histogram of cytokeratin-negative cells.

Contrast, double-labeling (Figure 7B) allowed the cytokeratin-positive aneuploid tumor cells to be clearly distinguished from the stromal cells and also from the cytokeratin-positive but diploid bladder epithelial cells.

When fixed cell suspensions from renal-cell tumors were incubated with the monoclonal or polyclonal antibody to cytokeratin and thereafter with an appropriate FITC-conjugated second antibody and PI, the epithelial component of this tumor could be distinguished from other, nonepithelial, cell types (Figure 8A). Also in cases in which only small numbers of epithelial tumor cells were present in the cell suspensions, aneuploid cells could be detected by the application of the cytokeratin staining procedure. In some cases, labeling of the cells with the antiserum to vimentin revealed an FITC-positive aneuploid subpopulation of cells in addition to diploid renal cells,
stromal cells and inflammatory components (Figure 8B).

**Discussion**

Intermediate filament proteins are intracellular cytoskeletal proteins that are extremely stable and water insoluble. Most important for the understanding of the underlying data presented in the present study is the fact that, in general, cytokeratins occur only in normal and malignant epithelial cells and that vimentin is normally specific for cells of mesenchymal origin (for exceptions see Herman et al9 and Ramaekers et al10). Using, for example, polyclonal and monoclonal antibodies to cytokeratins, normal epithelial cells and carcinoma cells can be labeled specifically using the indirect immunofluorescence technique. Nonepithelial tumor 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.13,17,21

Flow cytometric analysis of mixed cell populations such as are obtained from tumors produces results that are often difficult to interpret because of the variable mixture of cells of interest, usually carcinoma cells, with other cells, such as inflammatory and stromal cells. Although these cell populations can be discriminated in some material, most tumor material continues to be difficult to analyze and interpret.1,2,4,9

Because of the ubiquitous presence of cell type-specific intermediate filaments, immunofluorescence of these structures can be used for FCM analysis of many solid tumors. All carcinoma cells can be distinguished from nonepithelial cells by broad-spectrum cytokeratin antibodies and adenocarcinoma cells from nonglandular epithelial and nonepithelial elements by antibodies specific for glandular cells.19 In addition, antibodies specific for muscle cells (anti-desmin), neural cells (anti-neurofilament) and glial cells (anti-GFAP) are available,21 and the tumors derived from these cell types can thus be specifically distinguished by use of the appropriate antibody. In FCM, these tissue-specific markers allow recognition, analysis and sorting of the specific cell types of interest, excluding stromal and inflammatory elements.

Two advantages of the application of cytokeratin antibodies in such FCM selection procedures have become obvious in the present experiments. First, in those cases of carcinomas in which an aneuploid tumor cell peak could be detected in the one-dimensional DNA profile, labeling of the epithelial
tumor cells with FITC for cytokeratin allowed the analysis of the DNA distribution in the tumor and estimation of its proliferative fraction S-phase separately from the stromal and inflammatory components.

Second, in those tumor fractions in which the malignant cells represent only a small fraction of the cells in suspension, and thus cannot be recognized in the one-parameter analysis, labeling of the cells in suspension clearly displays such minor fractions in a two-parameter analysis, allowing the estimation of their ploidy and proliferative fractions. It should also be stated here that it is essential to have good-quality cell suspensions for successful labeling studies.

In summary, we conclude that antibodies to intraacellular intermediate filament proteins, especially the cytokeratins, can successfully be applied for the flow cytometric analysis of most carcinomas in dealing with the common problem of stromal and inflammatory cell admixture.

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