Immunochemical Demonstration of Keratin and Vimentin in Cytologic Aspirates

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Antibodies to intermediate filament proteins were used to characterize tumor cells present in peritoneal and pleural effusions and in thin needle aspirates from palpable lymph nodes. Metastatic adenocarcinoma cells (breast, ovary, endometrium, cervix, colon and stomach) as well as squamous-cell carcinomas and mesotheliomas stained specifically with antibodies to keratin while mesenchymally derived tumor cells (lymphomas, melanoma, fibrosarcoma and neurofibrosarcoma) were positive only for vimentin. Especially in cases of lymph node aspirates, keratin staining in cells was a direct indication of metastatic carcinoma.

Antibodies to these different components of the cytoskeleton can thus be used in cytopathologic diagnosis when a definitive diagnosis cannot be made on the basis of conventional cytologic features.

Materials and Methods

Cell Preparations

The cytologic samples used in this study consisted of material from effusions and fine needle aspirates of lymph nodes. Samples of human malignant peritoneal and pleural effusions were examined using

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This study was supported by the Netherlands Cancer Foundation (Queen Wilhelmina Fund) grant NUKC 1981-12.

Received for publication August 19, 1983.
Accepted for publication December 13, 1983.
Papanicolaou-stained conventional smears and were shown to contain either epithelial tumor cells (metastatic carcinomas of the breast, ovary, endometrium, cervix, large intestine and stomach as well as squamous-cell carcinoma) or nonepithelial tumor cells (lymphoma, melanoma, fibrosarcoma and neurofibrosarcoma) in addition to mesothelial cells and blood cells. Some of the preparations used in these experiments had been stored at −40°C for up to four years.

Thin needle aspirates were obtained from palpable lymph nodes. The cells present in these aspirates were smeared on glass slides using conventional techniques or brought onto glass slides using a pressure-fixation technique ("Cytopress") as described by Oud et al.11 These preparations were shown to contain cells from squamous-cell carcinomas, adenocarcinoma or lymphoma in addition to normal lymphocytes and other blood cells.

**Antisera and Indirect Immunofluorescence Technique**

Three antibody preparations were used in this study: (1) An antiserum directed against human foot callus keratin was raised in rabbits, as described earlier.12,13 (2) A rabbit antiserum directed against calf lens vimentin was prepared and tested as described by Ramaekers et al.,12,13 who have also described the specificity of its reactions.16,17 (3) A mouse monoclonal antibody (RGE 53), directed against keratin isolated from HeLa cells and shown to react specifically with glandular epithelial cells from various organs but not with stratified epithelia or nonepithelial tissues,18 was also used. This antibody reacts with adenocarcinomas but not with squamous-cell carcinomas, sarcomas or lymphomas.

In addition to the sera described above, preimmune sera, preabsorbed sera and an antiserum directed against desmin were tested in parallel control experiments on preparations from which sufficient material was available. The desmin antibodies were raised in rabbits and directed against chicken gizzard muscle desmin prepared using a modification of the method described by Geisler and Weber.7

The indirect immunofluorescence technique with these antisera was performed as follows: Cell smears were fixed in cold methanol and acetone and incubated with the first antibody. After incubation for 30 minutes in a humidified box at room temperature, the slides were washed with phosphate-buffered saline (PBS) containing 0.25% Triton X-100 in two subsequent washing steps of 10 minutes each and thereafter washed for 30 minutes with PBS alone. The cell smears were then incubated for another 30 minutes with the second antibody (fluorescein-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG, diluted 1:20, obtained from Nordic Immunology, Tilburg, the Netherlands). After a second series of washes, the slides were stained with hematoxylin for 60 seconds and mounted with 50% glycerol in PBS (pH 7.4). In some cases, washing steps with PBS buffers containing either 100 mM MgCl₂ or 100 mM KCl were applied in order to minimize nonspecific binding. No significant decrease in keratin or vimentin staining was observed in these control experiments. When the vimentin antiserum was absorbed with a crude vimentin preparation from bovine lens, fibrillar staining was diminished. Absorption of the vimentin antiserum with a keratin preparation from human skin had no such effect.

Cells were viewed with a Leitz Dialux EB 20 microscope (E. Leitz, Wetzlar, West Germany) equipped with epifluorescent illumination (XBO 100 bulb), using the appropriate filter systems for fluorescein fluorescence. Pictures were taken with a Leitz Fluotar 40× objective using an automated Leitz camera (ASA setting of 400 or 800) and Kodak Tri-X film (Eastman Kodak, Rochester, New York).

**Electron Microscopy**

For electron microscopy, cells from ascites or pleural fluid were centrifuged and the pelleted cells fixed for 1 to 1.5 hours in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2; 415 mOsm) at 4°C, rinsed in this buffer and postfixed for 1 hour in 1% osmic acid in the same phosphate buffer. Following dehydration in an ascending series of aqueous ethanol, the specimens were transferred via a mixture of propylene oxide and epoxy resin (1:1) into pure Epon 812 as an embedding medium. After polymerization, appropriate areas were selected under a phase contrast microscope and sectioned with glass knives on a Reichert OM U3 (C. Reichert, Vienna, Austria). The sections were picked up on copper grids, double contrasted with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope (Philips, Eindhoven, the Netherlands).

**Results**

**Body Cavity Effusions**

Table 1 summarizes the pertinent pathologic and immunohistochemical data from 48 cases of malignant ascites and pleural fluids. The metastatic tumor cells of epithelial and nonepithelial origins present in these
Table 1  Patterns of Keratin and Vimentin Filament Expression in Benign and Malignant Cells in Effusions (Ascites and Pleural Fluid)*

<table>
<thead>
<tr>
<th>Cytologic diagnosis</th>
<th>No. of cases</th>
<th>Keratin</th>
<th>Vimentin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous-cell carcinoma</td>
<td>2</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Adenosquamous carcinoma of the cervix</td>
<td>1</td>
<td>++</td>
<td>–/+</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>11</td>
<td>+/+</td>
<td>–/+</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>11</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>5</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>2</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Stomach carcinoma</td>
<td>2</td>
<td>+/++</td>
<td>+/++</td>
</tr>
<tr>
<td>Thyroid carcinoma</td>
<td>1</td>
<td>+/++</td>
<td>+/++</td>
</tr>
<tr>
<td>Parotid carcinoma</td>
<td>1</td>
<td>–/+</td>
<td>+/++</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>3</td>
<td>+/++</td>
<td>+/++</td>
</tr>
<tr>
<td>Benign mesothelial cells</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>4</td>
<td>–</td>
<td>+/+</td>
</tr>
<tr>
<td>Leukemia</td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Fibrosarcoma of the larynx</td>
<td>1</td>
<td>–</td>
<td>+/++</td>
</tr>
<tr>
<td>Neurofibrosarcoma</td>
<td>2</td>
<td>–</td>
<td>+/++</td>
</tr>
<tr>
<td>Macrophages and lymphocytes</td>
<td></td>
<td>–</td>
<td>+/++</td>
</tr>
</tbody>
</table>

*The degree of reactivity to the antisera is shown ranging from negative (–) to strongly positive (++).† Various numbers of epithelial tumor cells were positive, ranging from 1% to 50%.

preparations, varying in the numbers of cells thus affected; this phenomenon has been described in more detail in an earlier report.††

For illustrative purposes, Figure 4 shows electron micrographs of ovarian carcinoma cells present in ascites. These cells were treated with Triton X-100 before fixation in order to remove most of the soluble effusions were examined for their intermediate filament proteins.

Figures 1 to 3, 5 and 6 depict some typical examples of both epithelial and nonepithelial tumor cells present in cytologic preparations and incubated with the diverse antisera. It can be seen in Figures 1A to 1C that metastatic tumor cells from a squamous-cell carcinoma present in a peritoneal effusion were strongly positive for keratin but negative for vimentin. Macrophages adhering to the epithelial cell cluster stained strongly positive for vimentin but were negative for keratin. In the interpretation of these preparations, it was of great help that cell nuclei could be counterstained with hematoxylin. This staining procedure did not seem to interact with or influence the fluorescence pattern.

Figures 2 and 3 show the staining patterns of cells from a breast carcinoma present in pleural fluid and from an ovarian carcinoma present in ascitic fluid, respectively. Again, only the epithelial cells present in these preparations (both carcinoma cells and mesothelial cells) were positive for keratin. Macrophages, lymphocytes and other blood cells were negative for the keratin antiserum but reacted strongly with the antivimentin antiserum. Erythrocytes were, however, negative with the antivimentin antibody. Strikingly, both tumor cells and mesothelial cells were partly positive for vimentin. Coexpression of keratin and vimentin has been observed in several

Figure 1
(A) Metastatic tumor cells from a squamous-cell carcinoma present in a pleural effusion (hematoxylin, × 400). (B) Same tumor cells as in Figure 1A, visualized in the immunofluorescence microscope after incubation with the keratin antiserum, show a positive reaction for keratin (× 400). (C) Tumor cells from the same effusion, visualized in the immunofluorescence microscope after incubation with the vimentin antiserum, are negative for vimentin; note the presence of vimentin-positive macrophages (× 400).
cytoplasmic constituents. With this procedure, intracytoplasmic filaments become readily visible. Figure 4 shows that filaments with a diameter of about 10 nm (intermediate-sized filaments) are localized in a relatively high concentration around the nucleus. Furthermore, some of the 10 nm intermediate filaments interact with the desmosomal plaques. These filaments are known to be keratin-containing tonofilaments.

The results summarized in Table I show that metastatic carcinoma cells originating from endometrium, cervix, colon, stomach, thyroid, mesothelium and, to a lesser extent, from the parotid gland contain keratin as a marker of their epithelial nature.

All nonepithelial tumor cells present in effusions were negative for the keratin antiserum but positive for the antiserum directed against vimentin. Macrophages and lymphocytes, as well as tumor cells of mesenchymal origin, could be shown to exclusively express vimentin-type intermediate-sized filaments. These neoplastic cells included those from lymphomas, a leukemia, a fibrosarcoma of the larynx (Figure

Figure 2
(A) Metastatic breast carcinoma cells present in a pleural fluid are (B) positive for keratin and (C) positive for vimentin (× 350). Details are as in Figure 1.

Figure 3
(A) Metastatic carcinoma cells from an ovarian tumor present in ascites are (B) positive for keratin and (C) positive for vimentin (× 350). Details are as in Figure 1.
Figure 4
Electron-microscopic demonstration of intermediate filaments and desmosomes in (A) nontreated ovarian carcinoma cells (× 27,500) and (B) a cytoskeleton preparation of Triton-X-100-extracted ovarian carcinoma cells obtained from ascites (× 33,500).

5), a melanoma and two neurofibrosarcomas (Figure 6); all were positive for vimentin but negative for keratin. This finding is in complete agreement with data obtained for solid melanomas and neurofibrosarcomas, which also contain only the vimentin-type of intermediate filaments.16–18

The monoclonal antibody to keratin from glandular epithelium (RGE 53) was tested on some preparations of body fluids. Only cells from adenocarcinomas and benign mesothelial cells gave a positive reaction with this antibody. From these experiments it was concluded that RGE 53 reacted similarly on cells present in effusions as it does on cells in solid tumors.18

Lymph Node Aspirates
Cells present in thin needle aspirates from palpable lymph nodes were incubated with the polyclonal and monoclonal antibodies to keratin, as well as with the antibody to vimentin, using cell smears or preparations made by the pressure-fixation technique. Table II summarizes some significant pathologic and immunocytochemical data obtained with seven cases of lymph node aspirates. In all cases, lymphocytes were negative for keratin but (weakly) positive for vimentin. When examining frozen sections of total lymph nodes, we have found that no keratin-positive cells occur in normal lymph follicles.16–18 As a result, the detection of keratin-positive cells in aspirates from a lymph node is a very strong indication of the presence of metastatic carcinoma cells. Table II and Figures 7 and 8 show some typical examples of staining patterns of cell preparations from lymph nodes containing malignant cells. It is obvious that epithelial cells present in these preparations all showed strong reactions with the polyclonal antikeratin antiserum but were negative for vimentin. No keratin-positive cells were observed in the case of a chronic lymphadenitis; instead, all cells were vimentin positive. The monoclonal keratin antibody RGE 53 was also used in these cases. As expected from our results on frozen sections of several types of epithelial tumors,19 the antibody did not react with cells from metastatic squamous-cell carcinomas or with lymphocytes. A rather strong reaction, however, was seen in cells from two cases of adenocarcinoma. The case of a carcinoma from the stomach present also in the esophagus (Figure 8) illustrates dramatically how these antibodies to intermediate filament proteins can be strong tools for use in cytology. Using conven-
tional staining procedures, no tumor cells could be recognized in smears from lymph node aspirates of this patient with a previously diagnosed adenocarcinoma of the esophagus. Using both the polyclonal and monoclonal antikeratin antibodies, tumor cells were readily seen as strongly fluorescent groups in an almost negative background.

Discussion

Several conclusions can be drawn from the experiments described in this paper.

First, it is obvious from the data in Tables I and II and from Figures 1 to 3 and 5 to 8 that in body cavity effusions and lymph node aspirates only epithelial tumor cells and mesothelial cells react positively with the antikeratin antiserum in the indirect immunofluorescence assay; all nonepithelial cells, both tumor cells and nucleated blood cells, are negative for this antiserum but positive for the vimentin antiserum. This implies that antibodies to keratin can be of help in the cytopathologic diagnosis when a differential diagnosis between epithelial and nonepithelial cells cannot be made on the basis of routine cytologic techniques. We have, however, occasionally observed that clusters of epithelioid-like tumor cells in effusions were negative for keratin while single cells showed a positive reaction for this antibody. This result may be partially explained by the compact nature of these three-dimensional tumor cell groups. However, the cell clusters stain for epithelial membrane antigen (EMA), as described by To et al.26

Second, we have noticed that keratin and vimentin filaments may be expressed simultaneously in metastatic epithelial tumor cells present in body fluids. This is in contrast to carcinoma cells in solid tumors, which have been shown to exclusively contain intermediate filaments of the keratin type.14 Apparently,

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>Keratin</th>
<th>RGE 53</th>
<th>Vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous-cell carcinoma</td>
<td>4</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma of the thyroid</td>
<td>1</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma of cardia</td>
<td>1</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Chronic lymphadenitis</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

*The degree of reactivity to the antisera is shown ranging from negative (-) to strongly positive (++++).
epithelial tumor cells in body fluids adapt to their new growth conditions by the development of a vimentin cytoskeleton. Since the presence or absence of such an additional cytoskeleton may influence the mitotic as well as the motile activity of the cells, it may well have an effect on the growth rate and/or aggressive behavior of a metastatic tumor. The study of this phenomenon may therefore provide valuable information on a possible role of vimentin in tumor metastasis and spread.

Expression of vimentin as an adaptation to growth conditions has also been observed when epithelial cells are brought into tissue culture. It is noteworthy in this respect that the cells from squamous-cell carcinomas and some epithelial cells from an adenosquamous carcinoma did not express vimentin when growing in body fluids. This phenomenon may be explained by the highly differentiated state of these cells, although keratinocytes in culture do coexpress both vimentin and keratin. Also, the onset of vimentin expression may be less rapid in cells from squamous-cell carcinomas than in cells from adeno-

carcinomas. We have already noted that a certain period of time is needed before vimentin coexpression occurs in tumor cells from pleural fluid. In this respect, our finding with cells from an adenosquamous carcinoma of the cervix may support the idea that cells of different morphologic appearance or origin may develop the additional vimentin cytoskeleton more or less rapidly.

Third, the monoclonal antibody directed against keratin from glandular epithelial tissues (RGE 53) can distinguish between cells derived from adenocarcinomas and squamous-cell carcinomas present in aspirates from body cavity fluids and lymph nodes. It is obvious that the application of this antibody in cytopathology can help to make more precise differential diagnosis and can thus give valuable information with respect to the localization of a primary tumor, prognosis and treatment.

Application of the immunohistochemical techniques described above seems most promising in those cases in which lymph node aspirates have to be evaluated. Epithelial tumor cells from these preparations exclusively contain keratin intermediate filaments and do not coexpress vimentin (as is the case in ascites and pleural fluids). Furthermore, the keratin-positive reaction in cells is detected quickly in the immunofluorescence microscope, even in cases in which only a few tumor cells are present in the preparation. This positive reaction then indicates very strongly the presence of metastatic carcinoma cells in the lymph nodes examined.

Acknowledgments
We thank Arie Kant, Anita Huysmans, Tilly Aalders and Olof Moesker for excellent technical assistance.
with the preparation and specificity testing of the antisera described in this study. We thank Yvonne Stammes and Janny van Rennes for typing the manuscript and Dr. Chester Herman for valuable discussions. We are indebted to all collaborators of the Division of Cytopathology for their immeasurable help in routine diagnoses. Theo Hafmans and Ton van Eupen provided help with electron microscopic procedures and photography.

References