Can Keratin 8 and 17 Immunohistochemistry Be of Diagnostic Value in Cervical Cytology?

A Feasibility Study

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BACKGROUND. Based on results from evaluation of tissue sections from premalignant lesions of the uterine cervix, the authors examined the hypothesis that immunostaining of Papanicolaou-stained cytologic smears with monoclonal antibodies to keratins 8 and 17 allows detection of cervical intraepithelial neoplasia (CIN) with progressive potential. They also investigated whether detection of these two keratin subtypes could be of help in the analysis of normal and/or poor quality cytology smears.

METHODS. Sixty-one Papanicolaou-stained smears, representing 25 normal smears, 8 CIN 1, 7 CIN 2, 18 CIN 3, and 3 cervical carcinomas, were stained with CAM 5.2 and E3, which are capable of detecting keratin 8 and 17, respectively. The percentages of immunoreactive normal, metaplastic, dysplastic, and malignant epithelial cells were determined.

RESULTS. In normal cervical smears, keratin 8 was detected in endocervical columnar cells and sporadically in immature squamous metaplastic cells. Keratin 17 was identified in reserve cells and frequently in immature squamous metaplastic cells. In CIN, the number of cases in which keratin 8 was present increased with the severity of the lesion. Keratin 17 was found in the majority of CIN lesions, irrespective of grade. Intensity of immunostaining and number of cells stained per lesion varied and were also not related to the severity of CIN.

CONCLUSIONS. The use of the keratin 8 antibody in normal cervical smears enabled the detection of endocervical cells in cases where they were thought to be absent, particularly in cases with severe inflammation. Staining with keratin 17 enabled the identification of reserve cells or immature metaplastic cells, which were often misinterpreted as parabasal cells. The application of antibodies to these subtypes of keratins in cervical cytology can to a certain extent help in the identification of CIN and may in future be tested in automated screening. Cancer (Cancer Cytopathol) 1999;87:87–92. © 1999 American Cancer Society.

KEYWORDS: monoclonal antibodies, keratin 8, keratin 17, cervical intraepithelial neoplasia, cervical carcinoma.

Keratin phenotyping of normal, premalignant, and malignant epithelium of the uterine cervix has revealed interesting changes in the expression patterns of keratins 8 and 17 on progression of cervical intraepithelial neoplasia (CIN). In tissue studies, keratin 8 was found to occur in normal endocervical columnar cells but not in ectocervical squamous epithelium. It was found to display a maturation-related expression pattern in immature squamous metaplastic epithelium, being absent in more mature or fully matured squamous metaplastic epithelium. In formalin fixed, paraffin embedded tissues, the number of lesions expressing keratin 8 was less than...
observed in fresh frozen tissue specimens. Based on observations in fresh frozen tissue, keratin 8 was detected in a minority of CIN 1 and CIN 2 lesions and in approximately 80% of CIN 3 lesions. This observation prompted the hypothesis that the persistent presence of keratin 8 in a CIN lesion indicated that this lesion was progressive in nature. This hypothesis was supported by the observation that keratin 8 was invariably present in cervical carcinomas.

Keratin 17 was found in endocervical reserve cells and in immature squamous metaplastic epithelium, but it was absent in endocervical columnar cells, ectocervical squamous epithelium, and mature squamous metaplastic epithelium. It is noteworthy that with increasing severity of CIN, keratin 17 was more frequently found and the intensity of immunostaining was also increased. In cervical carcinoma, keratin 17 was always detected. These observations prompted us to propose the theory that the combined presence of keratins 8 and 17 in CIN was a reflection of its progressive potential. Based on these findings, we investigated whether immunostaining of Papanicolaou-stained cytology smears with monoclonal antibodies to keratins 8 and 17 could be used to detect CIN in these specimens. Furthermore, we examined whether or not keratin phenotyping of cervical smears could contribute to the accuracy of cytodiagnosis and to our understanding of the pathogenesis of CIN and cervical carcinoma.

**MATERIALS AND METHODS**

**Cytologic Specimens**

The cytologic material used in this study had been stored for 11–13 years and was retrieved from the archives of the Department of Pathology at the SSDZ/Reinier De Graaf Hospital, Delft, The Netherlands. It comprised 25 Papanicolaou-stained cervical smears with no abnormalities, 8 smears with cytologic findings consistent with CIN 1, 7 smears with CIN 2, 18 with CIN 3, and 3 with cervical squamous cell carcinoma. Cases with reasonable numbers of diagnostic cells were selected.

**Antibodies**

1. CAM 5.2 (IgG2a) was supplied by Becton Dickinson (San Jose, CA). In immunoblotting studies, CAM 5.2 was shown to be reactive with keratin 8 and to a minor degree with keratin 7, but not with keratins 18 and 19, as suggested previously. In tissue studies it reacts with columnar cells of the endocervix. CAM 5.2 does not stain keratinizing or nonkeratinizing squamous epithelium.

2. E3 (IgG 2b) was supplied by DAKO A/S (Glostrup, Denmark). The E3 keratin antibody is specific for keratin 17. It reacts with endocervical reserve cells and also with immature squamous metaplastic epithelium.

The primary monoclonal antibodies were tested on separate “normal” cervical smears to determine the optimal antibody dilutions, which were 1:400 for CAM 5.2 and 1:4 for E3.

**Staining Procedure**

All smears used in this study were Papanicolaou-stained (hematoxylin, Orange G, E50, Merck, Germany) for routine cervical screening at least 10 years prior to retrieval. In the current study the smears were all rescreened. Areas of the smear containing reserve cells, squamous metaplastic cells, and columnar cells were marked on the coverslip, as were dysplastic cell groups in smears consistent with CIN. Photocopies were taken of the slide, allowing relocation of the marked groups after immunohistochemical staining procedures had been performed. The coverslips were removed by immersing the slides in xylol for at least 24 hours. In some cases this process was accelerated by a microwave step for 2 minutes at 700 W. Each slide was then divided in half by a paraffin bar across the middle of the slide perpendicular to the long axis. In this way two wells were formed.

Cells were rehydrated in a descending alcohol series (95%, 70%, and 50%), after which they were rinsed in distilled water for 10 minutes and phosphate-buffered saline (PBS, pH 7.4) at room temperature. Each slide was then incubated for 10 minutes in 5% bovine serum albumin (Sigma, St. Louis, MO) in PBS. One-half of the slide was incubated with CAM 5.2 and the other half with E3.

**TABLE 1**

<table>
<thead>
<tr>
<th>Keratin 8</th>
<th>Keratin 17</th>
<th>K 8 and 17</th>
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</thead>
<tbody>
<tr>
<td>Ectocervical squamous epithelial cells</td>
<td>0/25*</td>
<td>2/25*</td>
</tr>
<tr>
<td>Endocervical columnar cells</td>
<td>25/25</td>
<td>0/25</td>
</tr>
<tr>
<td>Reserve cells</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Squamous metaplastic cells</td>
<td>3/21</td>
<td>10/21</td>
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</tbody>
</table>

* No. of positive cases over the total number of cases tested.
with 3-amino-9-ethylcarbozol AEC (0.5 mg/mL Sigma); Step 7, rinsing in tap water for 30 minutes; Step 8, counterstaining with haematoxylin, a repeat rinsing, and coverslip with glycerin/gelatine. Marks were then replaced on the slides with use of the photocopies. The level of immunostaining was evaluated by counting the number of immunostained cells and the total number of cells per epithelial cell type. In this way percentages of metaplastic, dysplastic, and neoplastic cells immunoreactive for the two antibodies were determined.

RESULTS
Normal Cervical Epithelial Cells in Cytology Smears
Results for normal cervical epithelial cells are given in Table 1. Keratin 8 was not detected in ectocervical squamous epithelial cells (Fig. 1a). In 2 cases a few superficial ectocervical cells stained weakly with the keratin 17 antibody. Strong immunoreactivity was observed with CAM 5.2 in both cell sheets and in dispersed endocervical columnar cells (Fig. 1b). Groups of endocervical columnar cells present in inflammatory infiltrates, which could not be classified previously, showed intense keratin 8 staining that highlighted their external contours and enabled identification (Fig. 1b). Without exception, these cells were negative for the keratin 17 antibody (Fig. 2a).

In four slides reserve cells were found. Both the keratin 8 antibody (Fig. 1c) and the keratin 17 antibody (Fig. 2b) were strongly expressed in these cases.

Naked nuclei, classified by Boon et al.11 as reserve cells, demonstrated no immunoreactivity with either antibody (Fig. 2c).

Squamous metaplastic cells were found in 21 smears. Keratin 8 was detected in some dispersed cells in 3 cases (Fig. 1d), and the keratin 17 antibody stained scattered cells in 10 cases with moderate intensity.
Cervical Intraepithelial Neoplasia

Results for CIN are given in Table 2. Of the 8 cases with CIN 1, 4 displayed keratin 8 positivity in approximately 10% of the cells (Fig. 3a) and 5 showed immunoreactivity for keratin 17 (Fig. 4a), with approximately 60% of the cells positive. Intensity of staining for both antibodies was mild to moderate.

In CIN 2 keratin 8 was expressed in 4 of 7 cases (Fig. 3b), in which an average of 15% of cells were positive with moderate intensity. The keratin 17 antibody stained cells in 6 of 7 cases; 40% of cells stained intensely (Fig. 4b). Twelve of the 18 smears with CIN 3 were immunoreactive for keratin 8 (Fig. 3c); approximately 10% of cells stained with mild-to-moderate intensity. Fourteen of 17 cases were immunoreactive for keratin 17 (Fig. 4c), with an average of 25% of cells intensely immunoreactive.

Squamous Cell Carcinoma

These results are shown in Table 2 and Figure 5. Keratin 8 was detected in 2 of the 3 cases of squamous cell carcinoma. Approximately 25% of the cells stained intensely (Fig. 5b). Keratin 17 stained a similar percentage of cells in the same cases (Fig. 3c).

Combined Results of Keratin 8 and 17 Immunostaining

In approximately 25% of the cases of CIN 1, CIN 2, and CIN 3, both antibodies were expressed.

### TABLE 2

<table>
<thead>
<tr>
<th>Keratin 8</th>
<th>Keratin 17</th>
<th>K 8 + 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN 1</td>
<td>4/8</td>
<td>10%</td>
</tr>
<tr>
<td>CIN 2</td>
<td>4/7</td>
<td>15%</td>
</tr>
<tr>
<td>CIN 3</td>
<td>12/18</td>
<td>10%</td>
</tr>
<tr>
<td>Cervical nonkeratinizing squamous cell carcinoma</td>
<td>2/3</td>
<td>25%</td>
</tr>
</tbody>
</table>

*No. of positive cases over total no. of cases tested.

% of positive cells.

Intensity of immunoreaction: +, weak; ++, moderate; ++++, strong.
DISCUSSION
Comparison of Keratin Expression Patterns in Cervical Cytology Smears and in Cervical Tissue Sections

The monoclonal antibodies to keratin 8 and 17 used in this study gave excellent staining results in routinely Papanicolaou-stained cervical smears that had been stored for longer than 10 years. Antigen retrieval procedures were not necessary, and we did not observe any improvement in staining results when we compared the slides that had been subjected to microirradiation (in order to remove the coverslip) with smears in which this treatment was not necessary. Background staining was completely absent, even in inflammatory exudates.

When we compared the keratin expression patterns in cytologic specimens with those previously observed in tissue slides, a number of differences were observed. Keratin 8 expression in tissue specimens with CIN 1 was observed as very minor staining in about 10% of cases, whereas cytologic smears harboring CIN 1 stained in 50% of cases. In cytologic smears harboring CIN 2, the number of cases that stained for keratin 8 had slightly increased compared with the smears from CIN 1 lesions; and again, compared with histologic sections, the percentage of cases staining was very much higher. In cytologic smears with CIN 3 67% of cases expressed keratin 8, with many cells displaying moderate staining intensity, whereas in histologic sections 80% of cases stained for the keratin 8 antibody.

In tissue specimens with CIN 1 expression of keratin 17 was infrequent, whereas in cervical smears two-thirds of CIN 1 cases displayed immunoreactivity. In smears harboring CIN 2 and CIN 3 lesions, the percentage of cases immunoreactive for keratin 17 was comparable to that of tissue specimens, with approximately 80% of cases staining. Again, keratin 17 expression did not significantly increase with the severity of CIN in the smears.

In contrast to previous observations regarding histologic specimens, in this study the number of CIN lesions showing simultaneous expression of keratins 8 and 17 in smears did not show the same dramatic increase with severity of CIN, as approximately 50% of the positive cases were positive for both antibodies irrespective of CIN grade.

On the basis of histologic studies, we and other authors have suggested that expression of keratin 8 alone or in combination with keratin 17 in CIN may signify that a group of lesions could be more aggressive and develop into cervical carcinoma. This was supported by the observation that low grade CIN expressed these keratins in small numbers of cases, whereas in high grade CIN large numbers of cases expressed these two keratins. Additional proof was based on the fact that keratin expression is generally conserved during neoplastic development. This also seems to be the case for cervical carcinoma, which expresses keratins 8 and 17 irrespective of type and grade.

The fact that the percentage of cytologic specimens combining keratins 8 and 17 is higher than the positive fraction estimated from tissue section studies could indicate a relatively high percentage of progression of low grade CIN lesions, as has been suggested by Whittaker et al. However, it is generally thought that only a low percentage of low grade CIN lesions are progressive in nature. The immunohistochemistry protocol we used may have influenced our results. As we did not use an antigen retrieval technique for the cytologic specimens, one could expect immunoreactivity to be even more intense if heat-induced epitope retrieval were used; however, immunoreactivity in cytology specimens is often excellent without a retrieval step.

We observed that keratin immunostaining allowed better insight into the cellular components of a cervical smear. For example, parts of the smear that showed numerous inflammatory cells exhibited groups of cells that could not be categorized in Papanicolaou-stained specimens. These cells showed an intense reactivity with keratin 8 close to their mem-
branes. This made identifying endocervical cells easy. Small cells with relatively large nuclei and a thin cytoplasmic rim that displayed beginning squamous characteristics could be identified as reserve cells or very immature squamous cells on the basis of their keratin 17 expression. Naked nuclei, identified by Boon as reserve cells, displayed no immunoreactivity at all. The cytoplasm of these cells was undoubtedly stripped, and this could of course explain their lack of immunoreactivity. On the other hand, it could also be expected that at least some of these cells had some stainable cytoplasm. We consider these nonimmunoreactive “cells” to be naked nuclei of different cell types from the cervix that may have lost their cytoplasm during smear-taking or processing.

**Future Prospects**

Cervical cytodiagnostics urgently need specific markers capable of distinguishing dysplastic and normal epithelial cells, as well as markers capable of distinguishing progressive CIN lesions from those with more indolent behavior. Studies of other types of (pre)malignancies provide indications that antibodies to the so-called “simple keratins,” such as keratin 8, could become such markers. Lane et al. reported that when keratin 8 positive cells were detected in a smear from the oral cavity, this invariably indicated the presence of a carcinoma of the oral cavity. To study the prognostic value of keratins 8 and 17 in the uterine cervix, cytology expression will have to be examined in successive cervical smears from women with progressive CIN lesions. Only this approach will relate the expression of keratins 8 and 17 to the prognoses of patients with abnormalities of the cervix.

Keratin 8 is found in a large number of dysplastic lesions, but it is also detected in some reserve cells, some cases of immature squamous metaplastic epithelium, and all endocervical cells. Usually these normal cells are easily identified, thus allowing slides with “no abnormalities” that express keratin 8 to be separated from slides showing CIN. Even then, however, a large number of cases of CIN (approximately 40%) are not immunoreactive, limiting the applicability of this method. Automated screening may benefit from case selection on the basis of the method described above. The keratin 17 antibody recognizes in a high number of cases with CIN lesions, but approximately 20–30% of normal cells will be recognized as false-positive and a number of CIN lesions will not stain. Obviously this percentage is far too high, but when keratin 17 positivity is used as an indicator for manual rescreening of the specimens it may be acceptable, and it would therefore be interesting to investigate the applicability of this method.

**REFERENCES**