Expression of Cytokeratins in Early Neoplastic Epithelial Lesions of the Uterine Cervix


Department of Pathology and *Institute of Obstetrics and Gynecology,
St. Radboud Hospital, University of Nijmegen, The Netherlands

Summary: Polyclonal and monoclonal antibodies to cytokeratin polypeptides were used to study the expression of these intermediate filament proteins in normal, squamous metaplastic, and neoplastic epithelium of the uterine cervix, in order to investigate the morphogenesis of early epithelial changes preceding cervical squamous cell carcinoma. A polyclonal keratin antiserum showed a positive reaction in all different epithelial cell types of the uterine cervix. A positive reaction was also found in subcolumnar reserve cell hyperplasia, in squamous metaplastic and dysplastic cells, and in (squamous) carcinoma in situ. A monoclonal antibody specific for columnar epithelium (RGE 53) gave a positive reaction in endocervical columnar cells and in some immature metaplastic cells but was negative in subcolumnar reserve cells, squamous (metaplastic) cells, dysplastic cells, and most cases of carcinoma in situ. Another monoclonal cytokeratin antibody (RKSE 60) pointed to early keratinization in light microscopically nonkeratinizing squamous (metaplastic) and dysplastic epithelium. A possible overlap in staining patterns of RGE 53 and RKSE 60 was seen in some cases of immature metaplasia. Morphologic changes occurring in the transformation zone upon dedifferentiation are accompanied by alterations in cytokeratin expression. Similarities in cytokeratin expression were found between dysplasia and carcinoma in situ on one hand and subcolumnar reserve cell hyperplasia and squamous metaplasia on the other. This study favors an epithelial origin and a squamoid nature of subcolumnar reserve cells. Key Words: Uterine cervix—Dysplasia—Carcinoma in situ—Cervical intraepithelial neoplasia—Cytokeratins—Immunohistochemistry.

To place the subject of our study in its proper perspective, a detailed description of the morphology of the human uterine cervix and the pathways involved in squamous metaplasia, dysplasia, and carcinoma in situ (CIS) is necessary.

In the normal human uterine cervix two morphologically distinct types of epithelium, i.e., the ectocervical stratified squamous and the endocervical columnar epithelium, meet at the squamocolumnar junction (SCJ). The anatomic position of the SCJ is influenced by hormonal stimuli (especially estrogens) and is related...
to age (1–3). During the reproductive years the SCJ is located most frequently on the cervical portio, the clinically visible part of the uterine cervix, resulting in an eversion of the endocervix. Probably due to local environmental and hormonal factors, the original endocervical columnar epithelium will be replaced by squamous epithelium (4,5), thus outlining the transformation zone (TZ; 3,4,6–9; also designated transitional zone; 1–3,9,10–12).

The histogenesis of the TZ has been described as involving two possible pathways. The first pathway consists of the migration of squamous epithelium from the native portio epithelium towards the endocervical mucosa. In the second pathway reserve cells, which appear under the columnar epithelial cells of the everted endocervical mucosa, are transformed into squamous metaplastic cells (1–3,13). The native and metaplastic squamous epithelial tissues will fuse and then form the lining of the TZ providing a new SCJ at the proximal border. The distal extension of the TZ is marked by the so-called last cervical gland (14). Thus, the TZ formed in this way reaches from the original to the new SCJ. According to many authors, the TZ represents the place of origin of potentially premalignant epithelial lesions in the uterine cervix and defines the area of their topographic distribution (1–7,9,11,12,14,15).

Since squamous transformation of everted endocervical columnar epithelium is found in a very large number of women, it is regarded as a normal physiological process (3), and according to Christopherson (4) and Christopherson and Gray (5), the usual stimulus for its development is evidently not a carcinogen. Proliferation and transformation of subcolumnar reserve cells (SRC) is regarded as the most important mechanism leading to squamous transformation in the TZ (3–5,9,11,13,14,16–21) and normally results in a mature squamous (metaplastic) epithelium (3,14). On the other hand, the process of squamous metaplasia may be atypical or proceed in a disorderly fashion leading to dysplasia and (squamous) CIS (4–6,14,19), more recently designated cervical intraepithelial neoplasia (CIN; 3,7,8,14,22–26).

The SRC cells, which apparently have the potential to differentiate into a squamous or columnar type of cell (3–5,9,13,16,18–21,27–29) are thought to play a central role in the development of the spectrum of epithelial lesions which may progress to cervical cancer (1,2,4,5,16,18,20,28–30). Basal cells of the ectocervical stratified squamous epithelium are probably involved in the development of ectocervical dysplasia and CIS (14,15,30).

The modern concept of the morphogenesis of cervical squamous cell carcinoma essentially comprises three possible pathways. Besides the pathway that primarily involves the ectocervical mucosa and leads to keratinizing squamous cell carcinoma, two other pathways are considered. These are related to the original endocervical mucosa. For both pathways the occurrence of SRC hyperplasia has been suggested to be the first step in the formation of either nonkeratinizing squamous cell carcinoma (through squamous metaplasia, dysplasia, and CIS) or anaplastic carcinoma (through atypical SRC hyperplasia and anaplastic CIS) (9,31). Squamous metaplasia, dysplasia, and CIS, occurring in the TZ, constitute morphologic appearances different from that of the originally columnar epithelium. Generally these alterations have a squamoid appearance. In the past two decades, to gain a better understanding of the development of cervical cancer, cell biological and biochemical investigations have been performed in addition to epidemiological, clinical, and histological studies (7,19,20,32,33).
Recently, antibodies to cytokeratins have been applied in the study of the cervical mucosa and premalignant and malignant epithelial lesions of the uterine cervix (21,34–39), but the examination of the pattern of cytokeratin expression in the spectrum of epithelial lesions that may progress to cervical cancer has not been the main issue of studies published so far. Cytokeratins constitute the intermediate filament type specific for epithelial cells. Nineteen distinct cytokeratin polypeptides have been recognized in human epithelial tissues. These cytokeratin polypeptides are not distributed randomly throughout different epithelia but occur in tissue-specific combinations (35). As a result, several of these cytokeratins may be considered as markers for different directions of epithelial differentiation (40), and therefore monoclonal antibodies directed against distinct cytokeratin components allow the immunohistochemical identification of several types of epithelial tissues. Recently, we have described a monoclonal cytokeratin antibody (RGE 53) that specifically recognizes columnar epithelial cells and tumors derived therefrom (41) and a monoclonal cytokeratin antibody (RKSE 60) which specifically stains keratinizing squamous epithelial cells (42). The aim of this study was to examine a possible relationship between the expression of different types of cytokeratin polypeptides and early epithelial changes preceding cervical squamous cell carcinoma.

MATERIALS AND METHODS

Patterns of cytokeratin expression in normal ectocervical and endocervical epithelial tissues, in metaplastic epithelium, and in SRC and SRC hyperplasia were examined using fresh tissue from hysterectomy specimens in which no dysplastic epithelial lesions or CIS were present. A total of 24 uteri was found to be suitable for this purpose, and one tissue specimen from each uterus was used. The youngest patient was 28 years old and the oldest patient was 64 years old.

Cytokeratin expression in dysplastic lesions and CIS was studied in a total of 82 fresh cervical biopsies, which were obtained from a group of 32 patients who had shown cytologic features consistent with dysplasia and/or CIS in previous cytologic examinations. Biopsy specimens were taken from several patients in the past, but these specimens are not included in this study. The age of these patients ranged from 23 to 63 years. The biopsies were taken under colposcopic control.

Pieces of tissue from both the hysterectomy specimens and the cervical biopsies were cut on a cryostat, and the 4–7 μm–thick frozen sections obtained in this way were stained with hematoxylin and eosin and screened for suitable areas for immunohistochemical studies. Appropriate parallel sections were fixed in methanol (−20°C, 5 to 10 min) and acetone (room temperature, 10 to 30 s).

The epithelial lesions were classified according to the proposals of the World Health Organization (43) into mild, moderate, or severe dysplasia and/or (squamous) CIS. Epithelial lesions of intermediate grade were frequently encountered. Furthermore, different degrees of severity were found next to each other in the same biopsy in 14 cases, accounting for the total of 96 dysplastic lesions and/or CIS studied in 82 biopsies: mild dysplasia (7), mild to moderate dysplasia (15), moderate dysplasia (20), moderate to severe dysplasia (15), severe dysplasia (25), severe dysplasia to CIS and CIS (14). According to the CIN classification, grade

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1 refers to mild dysplasia, grade 2 to moderate dysplasia, and grade 3 to severe
dysplasia and CIS (3,25).

Air-dried tissue sections were rehydrated in phosphate-buffered saline, pH 7.4,
and incubated for the indirect immunofluorescence technique. Incubation pro-
cedures for single and double labeling have been described before (42,44–47).

The following primary antibody preparations were used in this study.
1. An affinity purified polyclonal rabbit antiserum directed against human skin
keratins. For preparation and specificity testing see Ramaekers et al. (42,44–46).
This antiserum reacts with cytokeratins in virtually all epithelial tissues but not
with nonepithelial tissues.

2. The mouse monoclonal antibody RGE 53 (Euro-Diagnostics B.V., Apel-
doorn, The Netherlands) directed against cytokeratin 18. Preparation and speci-
ficity testing of this antibody have been described earlier (41). RGE 53 specifically
recognizes glandular epithelial cells from breast, digestive, respiratory, and uro-
genital tracts, endocrine and exocrine tissues, and mesothelial cells. No reaction
is found in stratified squamous epithelial or nonepithelial tissues. RGE 53 is an
IgG1.

3. The mouse monoclonal antibody RKSE 60 (Euro-Diagnostics B.V.) directed
against human skin keratins. Preparation and specificity testing of this antibody
were described previously (42,48). RKSE 60 recognizes only keratinizing strati-
fied squamous epithelial tissues. No reaction is found in nonkeratinizing stratified
squamous and glandular epithelium. RKSE 60 is an IgG1.

4. In addition to cytokeratin expression, the presence of vimentin, another type
of intermediate filament protein, was examined in the tissue sections. A mono-
clonal antibody to calf lens vimentin was purchased from Euro-Diagnostics B.V.
This antibody recognizes cells of mesenchymal origin, such as fibroblasts, en-
dotheial cells, lymphoid cells, and cells from cartilage and bone. In immuno-
blotting assays this antibody only recognizes vimentin. In cultured cells a fibrillar
staining pattern is observed identical to that seen with a polyclonal antiserum to
calf lens vimentin as described before (44). Generally, no reaction is found in
epithelial cells.

As second antibodies, fluorescein isothiocyanate (FITC) conjugated goat anti-
rabbit, goat anti-mouse, or rabbit anti-mouse IgGs (Nordic Immunology, Tilburg,
The Netherlands) and Texas Red conjugated sheep F(ab')2 anti-mouse Ig (New
England Nuclear, Boston, MA, U.S.A.) were used. Overlap between the FITC
and Texas Red channels was checked by using tissue sections labeled with a single
second antibody. Incubations with the second antibodies alone were used as
negative controls.

RESULTS

The staining patterns observed in cervical tissues with the antibodies described
above are summarized in Table 1. Normal ectocervical squamous cells, endo-
cervical columnar cells, and SRC all showed a positive reaction with the poly-
clonal keratin antiserum (Figs. 1a, c, and e). In many instances SRC seemed to
show a stronger reaction with the polyclonal keratin antiserum than the endo-
cervical columnar cells did. In the ectocervical stratified squamous epithelium,
an increased staining intensity (Fig. 2c) was seen in the superficial layers as
compared to the basal layers.

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**TABLE 1. Patterns of expression of cytokeratins and vimentin in normal tissues and potentially premalignant epithelial lesions of the uterine cervix**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of specimens</th>
<th>Polyclonal cytokeratin</th>
<th>RGE 53</th>
<th>RKSE 60</th>
<th>Monoclonal vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectocervical stratified squamous epithelium</td>
<td>16</td>
<td>+</td>
<td>-</td>
<td>-/+/a</td>
<td>-</td>
</tr>
<tr>
<td>Endocervical columnar epithelium</td>
<td>16</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Subcolumnar reserve cells</td>
<td>12</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectocervical and endocervical stroma</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subcolumnar reserve cell hyperplasia</td>
<td>12</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Squamous metaplastic epithelium</td>
<td>11</td>
<td>+</td>
<td>-</td>
<td>-/+/a</td>
<td>-</td>
</tr>
<tr>
<td>Dysplastic epithelium (mild-moderate-severe dysplasia) and carcinoma in situ</td>
<td>82</td>
<td>+</td>
<td>-/+/a</td>
<td>-/+/a</td>
<td>-</td>
</tr>
</tbody>
</table>

* A variable number of cells was found to be positive.
* A single case of carcinoma in situ was found to be positive.

RGE 53 showed a positive reaction only in the endocervical columnar cells (surface lining and the endocervical clefs), while ectocervical squamous cells and SRC were negative (Figs. 1b and d). From Fig. 1d it can be seen that the cytoplasmic processes of the endocervical columnar cells reach through the SRC layer onto the basal membrane.

In nine cases, solitary cells or clusters of cells in the ectocervical squamous lining were found to be positive for RKSE 60 (Fig. 1f). These cells were distributed randomly throughout the stratified squamous epithelium, however, with a negative reaction in the basal layer in all cases.

In cases of SRC hyperplasia a positive reaction was found with the polyclonal keratin antiserum but not with the monoclonal cytokeratin antibodies RGE 53 and RKSE 60 (Figs. 2a and b).

Frequently, a gradual transition from SRC hyperplasia to immature and mature squamous metaplasia (Fig. 2e) was seen. In both immature and mature squamous metaplastic epithelium, a positive reaction was found with the polyclonal keratin antiserum (Fig. 2d), while RGE 53 was negative (Fig. 2e). In about 75% of the cases of squamous metaplasia, a variable number of cells was found to be positive for RKSE 60 (Fig. 2f). In this respect we have not observed differences between immature and mature squamous metaplasia. In several tissue sections from hysterectomy specimens, a histologically immature metaplasia was seen without a histologically detectable squamoid differentiation. In four of these cases, a small number of immature cells with a positive reaction to both RGE 53 and RKSE 60 was found (Figs. 3a and b). The supposed coexpression of a cytokeratin specific for glandular epithelium and cytokeratins specific for keratinizing squamous epithelium was concluded from observations in parallel sections, since a double label study with RGE 53 and RKSE 60 could not be performed for obvious reasons.

In all patients who showed dysplastic lesions and/or CIS, the lesions were located in and confined to the TZ except in one patient in whom the lesions were found in the (histological) ectocervix as well as in the TZ.

Regardless of the degree of severity, all lesions classified as dysplasia and/or
CIS (Figs. 4a and d; 5a and d), except one, showed similar staining reactions, i.e., a positive reaction with the polyclonal keratin antiserum and a negative reaction with RGE 53, while a variable number of cells was found to be positive for RKSE 60 (Figs. 4b, c, e, and f; 5b, c, e, and f).

The only exception concerned a case of CIS in which in all tissue blocks obtained from this patient a strong positive reaction with RGE 53 was seen next to
a positive reaction with the polyclonal keratin antiserum and a negative reaction with RKSE 60. Upon review of the hematoxylin and eosin stained sections we found no reason to change our original diagnosis (squamous CIS) into adeno-CIS, although a remarkable columnar-like arrangement of the atypical cells was seen in some areas, especially in the basal layers (data not shown).
The monoclonal antibody to vimentin gave a positive reaction only in mesenchymal cells (e.g., fibroblasts, endothelial cells, inflammatory cells) within the ectocervical and endocervical stroma. All epithelial cells were negative.

In normal cervical stratified squamous, squamous metaplastic, and dysplastic epithelium, a variable number of vimentin-positive dendritic cells was found lying between the epithelial cells (Figs. 6a and b). These cells were negative for cytokeratin and showed a distribution and morphology similar to that observed for vimentin-positive dendritic cells in human epidermis (49), some of which have been identified as Langerhans cells (50).

**DISCUSSION**

The study of the natural history of potentially premalignant epithelial lesions of the uterine cervix has been hampered by a lack of universally accepted definitions, observer disagreements in histopathologic diagnosis, and the unpredictable influence of commonly applied biopsy procedures on the outcome of epithelial lesions (12,13). Studies using noninvasive methods, such as cytology and colposcopy, have been performed in order to avoid the latter obstacles (7,22,23).

Despite the problems mentioned above, many authors hold the view that dysplasia is part of a spectrum of epithelial lesions that may progress to CIS and eventually to invasive cervical cancer (1,2,4,5,9,11-13,28,30,51). A relation among dysplasia, CIS, and invasive cancer has become highly suggestive through epidemiological, clinical, and histological data (2,12,13,52). Although such a course is not obligatory, a concept of progression from mild lesions through lesions with an increasing degree of severity to CIS and invasive cancer is almost unanimously proposed (1-4,6,7,9,11-13,23,24,27,53). However, Burghardt (26) and Burghardt and Ostör (14) claim that dysplasia cannot be the precursor of CIS because of their different topographic distributions. Several authors (2,11,14,15,25,26) have mentioned the possibility of developing invasive cancer directly from lesions that are less severe than CIS.

It has been described extensively that epithelial lesions of varying severity, i.e.,
different degrees of dysplasia and/or CIS, may coexist (1,4,5,9,11,12,30), as was also shown in our material. In these cases the less severe lesions tended to occupy a more distal position in the uterine cervix (see also 3,8,9,11,14,15,26).

Richart (7,8,22,23) and Richart and Barron (24) have introduced the CIN classification for the whole spectrum of these potentially premalignant intraepithelial lesions. In this classification dysplasia and CIS are supposed to form a continuum of epithelial lesions that do not differ in a qualitative but only in a quantitative manner (see also 3,33). Our results are in line with this idea advocated by several authors (3,14,25,26,54), in that we have not found qualitative differences in staining reactions between epithelial lesions of varying degrees of severity.

It is generally accepted that potentially premalignant epithelial lesions of the
uterine cervix are preferably localized in close proximity to the SCJ or, more precisely, in the TZ. In all our patients, except one, the epithelial lesions appeared to be confined to the TZ.

According to the concept of morphogenesis described by Patten (31), the first step in the development of cervical squamous cell carcinoma in the region of the TZ involves a proliferation of SRC. Some authors (18,32) mention a frequent occurrence of SRC in the normal uterine cervix, while others (3–5,14) state that these cells occur only infrequently in the normal uterine cervix.

The nature and origin of SRC have been controversial and actually are still obscure. In the past, several proposals as to the origin of these primitive cells have been made, such as embryonal rests of urogenital origin, basal cells of fetal squamous epithelium, and stromal cells (2,3,14 and references therein). In the
opinion of Stegner (19), these SRC originate from cervical glandular cells (see also 10,18). Our results—i.e., a negative reaction in SRC with the monoclonal antibody RGE 53 (which, however, stained the endocervical columnar cells and their cytoplasmic processes between SRC, Fig. 1d)—seem to preclude this possibility. Similarly, our results rule out a mesenchymal origin of SRC because of the negative reaction for vimentin and a positive reaction for keratin.

With respect to their morphologic appearance, Ferenczy (3) has mentioned an ultrastructural resemblance of SRC to ectocervical basal cells. Interestingly, Löning et al. (21) have noted similarities in antigenic properties between these two types of cells.

Although not extensively discussed in the literature, it seems to be generally accepted that SRC may differentiate into either squamous or columnar cell types. Referring to this bipotential character of SRC, Van Roon et al. (29) described the simultaneous occurrence of both precancerous columnar cell and squamous cell lesions in the uterine cervix. Our immunohistochemical findings are in favor of the view expressed by Boon and Tabbers-Boumeester (9) that SRC possess an innate tendency towards squamous differentiation.

Accordingly, several authors (18,32) have stressed that endocervical columnar cells are capable of mitotic division, which would make the integrity of the endocervical epithelium independent of a continuously proliferating basal cell layer. The proposed bipotential nature of SRC may, however, explain our finding that immature metaplastic cells may coexpress a cytokeratin typical for glandular epithelium next to cytokeratins typical for keratinizing squamous epithelial tissues.

Under nonhyperplastic as well as hyperplastic conditions, SRC show a pattern of cytokeratin expression identical to that found in squamous metaplasia, dysplasia, and CIS, except for the negative reaction with RKSE 60 in SRC in all instances. This pattern differs from that seen in columnar epithelium. Therefore, we conclude that dysplasia and CIS are more likely to be related to SRC hyperplasia and squamous metaplasia than to columnar epithelium.

The histology in the case of the RGE 53—positive CIS was remarkable because of the columnar-like arrangement of the atypical epithelial cells in some areas. We have encountered corresponding morphological findings in an increasing number of cervical biopsies from young women currently under examination. Preliminary follow-up data from these cases point to a special nature of this
particular type of CIS because of its strong tendency to regression. The pattern of cytokeratin expression in this distinctive type of cervical epithelial lesion will be the subject of future studies.

In normal ectocervical stratified squamous epithelium as well as in many instances of squamous metaplasia, dysplasia, and CIS, we have observed a variable number of cells positive for RKSE 60. This antibody has been shown to stain suprabasal cells in human skin and keratinizing cells in squamous cell carcinomas (42). Therefore, the antigens recognized by this antibody may be regarded as markers for “prekeratinization.”

The RKSE 60 positivity in suprabasal cells of human skin is accompanied by the occurrence of a granular cell layer and the formation of a cornified layer at the epidermal surface. Both conditions may occur in the ectocervix, where keratinizing dysplasia is a well-known entity (9,31). It is remarkable, however, that we have never found light microscopic evidence of keratinization at the surface of those epithelial lesions with RKSE 60–positive cells.

In this context it is stressed that terms such as cytokeratin and keratinization should not be confused. Often, pathologists refer to keratin and keratinization as a morphological process in which cells synthesize large amounts of cytoplasmic keratin while the nuclei of cells become pyknotic. However, since nearly all types of epithelial cells have the ability to synthesize cytokeratins, it was recently suggested (9) that keratinizing cells differ from nonkeratinizing cells in the quantity of keratin produced. On the contrary, our observations (42) and those of others (55) show that keratinizing cells produce relatively high amounts of specific cytokeratin polypeptides that are not found in nonkeratinizing cells. Therefore, it is obvious that the occurrence of cytokeratins in any given type of epithelial cell should not be confused with the morphological process of keratinization (see, for example, 34).

The application of monoclonal antibodies specific for different cytokeratin polypeptides in future studies may help to analyze more extensively alterations in cytokeratin expression on changes in cell morphology in the TZ to further elucidate the process of carcinogenesis of the cervical epithelium. In doing so, it is of special interest to clarify the exact nature and origin of SRC because of their apparent role in the development of the spectrum of epithelial lesions that may progress to cervical cancer.

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