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Papillomavirus Infection of the Cervix. III: Relationship of the Presence of Viral Structural Proteins to the Expression of Involucrin

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Summary: Forty-two cervical biopsies with cervical intraepithelial neoplasia were compared with respect to the expression of human papillomavirus (HPV) structural proteins and the expression of the cellular structural protein involucrin, a marker of suprabasal squamous differentiation. HPV structural protein and involucrin expression displayed an inverse correlation with the severity of dysplasia. Both of these proteins were detected in 11 of 28 cases (39%) of mild and moderate dysplasia, but in only two of 14 (14%) cases of severe dysplasia. This difference was statistically significant ($p < 0.001$). The presence of HPV was also associated with expression of involucrin in the full thickness of the epithelium, including the basal layer, and an altered staining pattern in the more superficial cells, particularly the koilocytotic cells.

These findings support the hypothesis that squamous differentiation is required for the expression of viral structural proteins and that HPV infection begins in the basal epithelium. The study also demonstrates the utility of involucrin staining in differentiating virus-induced cytologic atypia from true neoplasia. **Key Words:** Papillomavirus—Involucrin—Dysplasia—Carcinoma *in situ*.

At the First International Congress on Exfoliative Cytology in 1961, carcinoma *in situ* (CIS) of the cervix was defined as a lesion in which undifferentiated cells occupied the full thickness of the epithelium (1). Dysplasia was defined as "all other disturbances of differentiation." Cervical dysplasia has long been regarded as a precursor of cervical cancer, although it has been recognized that the majority of dysplasias regress and that only a small proportion progress to invasive car-

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cinoma. Recent immunocytochemical studies of cervical lesions traditionally classified as dysplasia have demonstrated human papillomavirus (HPV) structural antigens in these lesions (2,3). Furthermore, the presence of HPV is associated with a proliferation of "undifferentiated" basal and parabasal cells and differentiated cells that show characteristic cytologic atypia. HPV structural proteins, identified by immunocytochemistry, and viral particles, identified by electron microscopy, are detected only in the differentiated squamous cells. These studies therefore suggest that lesions classified as dysplasia may represent a spectrum of morphologic changes that include both virus-induced and neoplastic lesions (3,4). At the extremes of the spectrum, viral atypia and CIS are readily distinguished, but an intermediate group exists in which it is impossible to determine where virus-associated atypia ends and neoplasia supervenes.

Previous observations have indicated that the expression of the structural protein involucrin is a marker of normal suprabasal differentiation in non-neoplastic squamous epithelium (5-8). Moreover, the synthesis of involucrin is not directly related to keratinization.

Immunocytochemical detection of involucrin therefore offers a method of assessing squamous differentiation that is not apparent by conventional light microscopy. Since involucrin is abnormal in neoplastic squamous epithelial cells (9), involucrin immunoreactivity is useful in distinguishing potentially neoplastic lesions from immature squamous metaplasia and other non-neoplastic atypias (10,11). Because squamous differentiation appears to be a requisite for the synthesis of HPV structural proteins and virion assembly, an immunocytochemical analysis was undertaken in order to correlate the presence of HPV structural proteins and involucrin in all grades of cervical dysplasia and CIS.

MATERIALS AND METHODS

Cervical biopsies, conizations, and hysterectomies showing cervical dysplasia and CIS were obtained from the surgical pathology files of the Georgetown University Hospital. All tissue was formalin-fixed and paraffin-embedded. Forty-two cases were selected on the basis of showing both positive and negative results for HPV more or less distributed in all grades of dysplasia. None of the cases had significant inflammation, a factor known to affect involucrin expression (10).

Cervical dysplasia was evaluated on hematoxylin and eosin sections according to the criteria and terminology proposed by the World Health Organization (12). Each case was classified according to the most severe area in each specimen, but the presence of lesser degrees of dysplasia was also recorded.

Paraffin sections were utilized for all immunocytochemical techniques. The detailed description of the staining reaction for papillomavirus antigens has been previously described (3). The primary antiserum, reactive with papillomavirus genus-specific structural antigens, was obtained from Dako Corp., Santa Barbara, California. Sections (4 μ m) were deparaffinized and reacted with the anti-HPV-1 serum at a dilution of 1:500 overnight at 4°C. This was followed by an incubation with swine antirabbit immunoglobulin (Dakopatts, Accurate Chemical and Scientific Corp., Westbury, NY) (dilution 1:40) for 30 min, and a 30-min incubation with rabbit PAP complex (dilution 1:100). The reaction was developed by the addition of 0.05% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in Tris

TABLE 1. *Correlation of koilocytosis with involucrin and HPV expression*

	Total no. of areas	No. of areas (+) for HPV
Koilocytosis only	42	15 (36%)
Koilocytosis and involucrin ^a	16	13 (81%)

HPV, human papillomavirus.

^a Only cases containing full-thickness involucrin staining are included.

HCl (pH 7.6) for 5–8 min. Sections were examined without a hematoxylin counterstain.

Immunohistochemical localization of involucrin was performed as previously described (10). Briefly, deparaffinized sections were incubated for 30 min at room temperature with rabbit antihuman involucrin (dilution 1:500 and 1:1,000). This was followed by swine antirabbit immunoglobulin (dilution 1:30) for 30 min (Dakopatts, Copenhagen, Denmark; U.S. distributor—Accurate Chemical and Scientific Corp., Westbury, NY), and horseradish peroxidase rabbit antiperoxidase immune complexes (dilution 1:100). Peroxidase activity was detected by incubation of the slides with a solution of 3,3'-diaminobenzidine tetrahydrochloride (6 mg/10 ml of 0.1 M Tris buffer at pH 7.4) and hydrogen peroxide. Sections were counterstained by methyl green and mounted with permount. Anti-involucrin antiserum adsorbed with purified envelopes and rabbit preimmune serum were used as negative controls. A section of normal human skin was employed as a positive control.

The staining pattern for involucrin was evaluated by an observer (MJW) without previous knowledge of the histologic grade or results of HPV staining. Involucrin staining in squamous areas was recorded as to the presence and distribution of immunoreactive areas. The extent of staining (suprabasal or full thickness) and the patterns of staining (diffuse cytoplasmic, membranous, or granular) were noted. These staining patterns were then correlated with conventional light microscopy and HPV staining. Direct comparisons were made between areas of positive HPV staining and corresponding areas stained with anti-involucrin.

RESULTS

The results of this study are summarized in Tables 1 and 2. All 42 cases had areas of dysplasia by routine H&E examination.

Mild dysplasia (15 cases)

The mild dysplasias histologically exhibited a thickened epithelium due to pronounced proliferation of basal and parabasal cells. Cytologic abnormalities included koilocytotic atypia in cells of the intermediate zone and cells with flattened pyknotic nuclei and scanty cytoplasm in the most superficial layers.

There was localization of HPV in eight (53%) cases and involucrin in 10 (67%) cases. Both HPV and involucrin were present in the same area of the same specimen in eight of these cases. Koilocytosis was present in 12 cases (80%). It

TABLE 2. Correlation of morphologic features of PVA with the presence of PV structural antigens and involucrin proteins in all grades of cervical dysplasias and CIS cases ($n = 42$)

	No. of cases with PVA	No. of cases with PV antigen	No. of cases with involucrin	No. of cases with PV antigen and involucrin
Mild dysplasia (15 cases)	12 (80%)	8 (53%)	10 (66%)	8 (53%)
Moderate dysplasia (13 cases)	9 (69%)	3 (23%)	4 (30%)	3 (23%)
Severe dysplasia-CIS (14 cases)	8 (57%)	4 (29%) ^a	2 (14%) ^a	2 (14%)
Total	29	15	16	13

CIS, carcinoma *in situ*; PV, papillomavirus; PVA, papillomavirus-induced atypia.

^a PV antigens and involucrin were confined to areas of mild to moderate dysplasia adjacent to the high grade lesions.

was found concomitantly with HPV in eight cases, with involucrin in 10 cases, and in all cases in which both HPV and involucrin were present.

HPV structural antigens were localized in cells exhibiting koilocytosis in the intermediate layers and in cells with flattened pyknotic nuclei in the superficial layers (Fig. 1). In these cases involucrin was detected in all layers of epithelium including the basal epithelium (Fig. 2). In contrast, in normal squamous epithe-

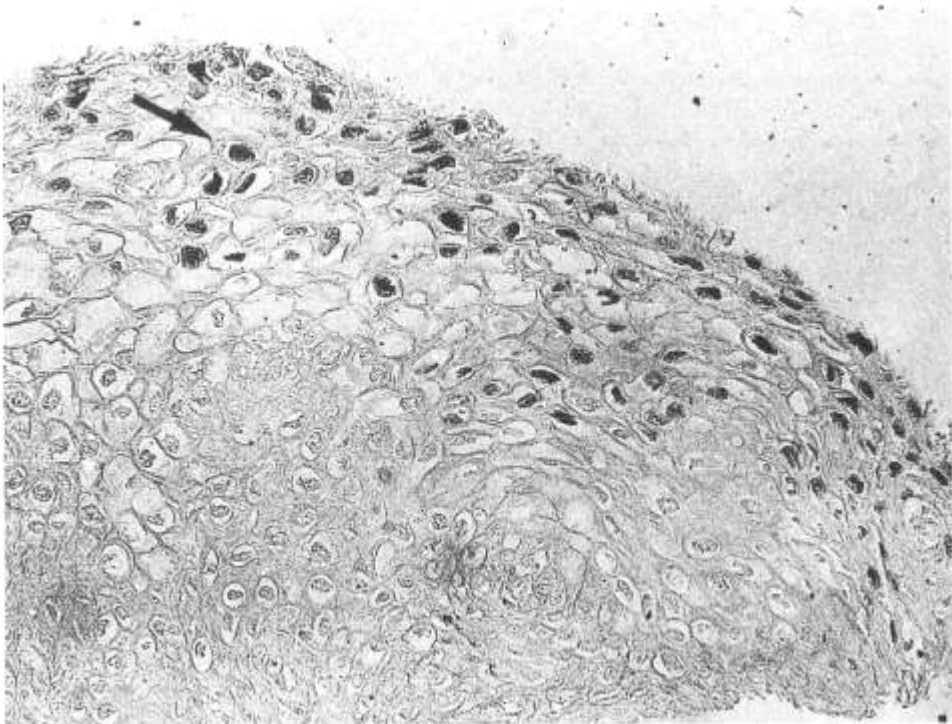


FIG. 1. Mild dysplasia stained with anti-papillomavirus antisera, no counterstain. Human papillomavirus (HPV) structural proteins (black deposit) are localized to nuclei of superficial epithelial cells. An arrow points to one of the positive cells (anti-HPV immunoperoxidase, no counterstain, $\times 125$).

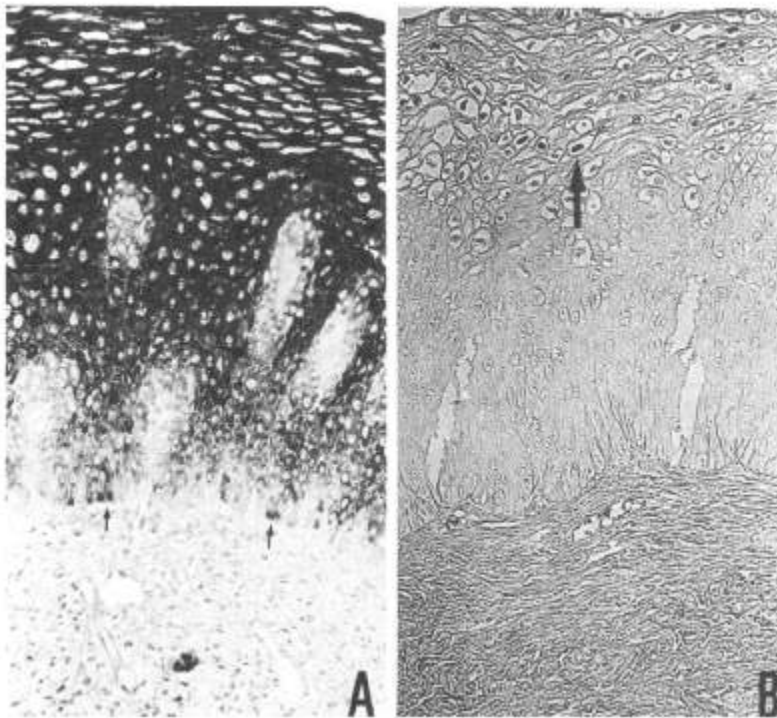


FIG. 2. A: Mild dysplasia showing intense full-thickness staining for involucrin, including staining of basal epithelium (small arrows) (anti-involucrin, 1:1,000, immunoperoxidase, methyl green counterstain, $\times 50$). B: A section of the same area stained with anti-HPV (human papillomavirus). HPV proteins are localized to the nuclei of the superficial epithelial cells (large arrow) (anti-HPV, immunoperoxidase, no counterstain, $\times 50$).

lium involucrin is confined to the intermediate and superficial layers (Fig. 3). In the koilocytotic cells, involucrin was distributed along the periphery of the cells producing a "membranous" staining pattern. This peripheral staining pattern suggested profound alterations in the cell cytoplasm. In other cells the staining was more granular and diffusely distributed in the cytoplasm (Fig. 4).

Other histologic findings suggestive of HPV infection, specifically epithelial "spikes" and individual cell keratinization, were noted in four and six cases, respectively. These cases all exhibited staining for HPV antigens. Involucrin staining patterns in these cases were normal in areas without cytologic atypia.

Moderate dysplasia (13 cases)

The moderate dysplasias differed from the mild dysplasias by exhibiting both a greater degree of cellular proliferation and more pronounced atypia. Mitotic figures, including atypical mitoses, were more frequent. These changes occupied one-half to two-thirds the thickness of the epithelium. There was localization of HPV in three (23%) cases and involucrin in four (30%). Both HPV and involucrin were localized in the same area of the specimen in three (23%) of these cases. One case that exhibited a full-thickness pattern of involucrin staining was negative for HPV. Koilocytosis was present in nine cases (69%). In all the cases exhibiting both HPV and involucrin staining, localization of these antigens occurred in the areas of koilocytotic atypia. The pattern of involucrin staining in cases containing HPV antigens was similar to that described for the mild dysplasias. There was

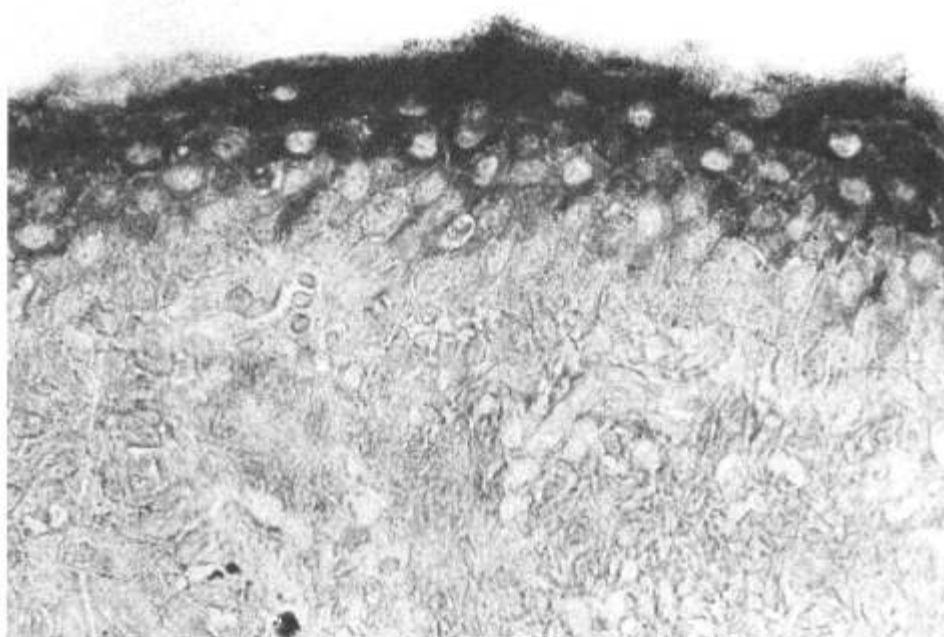


FIG. 3. Squamous epithelium of the cervix exhibiting normal pattern of staining with anti-involucrin. Involucrin is first detected in intermediate layers with increasing intensity in the more superficial layers (anti-involucrin, 1:1,000, immunoperoxidase, methyl green counterstain, $\times 125$).

full-thickness staining including the basal epithelium. In koilocytotic cells the staining was confined to the periphery of the cells.

Severe dysplasia—CIS (14 cases)

In these cases, there was complete or almost complete replacement of the epithelium by proliferating parabasal cells. Numerous mitoses, including atypical mitoses, were frequently observed even in superficial layers. Nuclear irregularity and hyperchromasia were marked. There was localization of HPV in four (29%) cases and involucrin in two (14%) cases. Both HPV and involucrin were localized in two (14%) of these cases. The pattern of HPV and involucrin localization was similar to that seen in the mild and moderate dysplasias. The two cases of HPV antigen localization with areas of absent involucrin staining contained areas of mild and moderate dysplasia in addition to the high grade lesions. The areas of most severe dysplasia lacked HPV and involucrin (Fig. 5). HPV and involucrin were localized to areas of mild to moderate dysplasia only (Fig. 6). The two cases with HPV, but without involucrin, demonstrated the HPV in areas of moderate dysplasia. The areas of severe dysplasia lacked HPV.

Koilocytotic changes were only a slightly less frequent occurrence, being present in eight (57%) cases. These changes, however, were most prominent in areas of mild or moderate dysplasia and absent from areas of severe dysplasia.

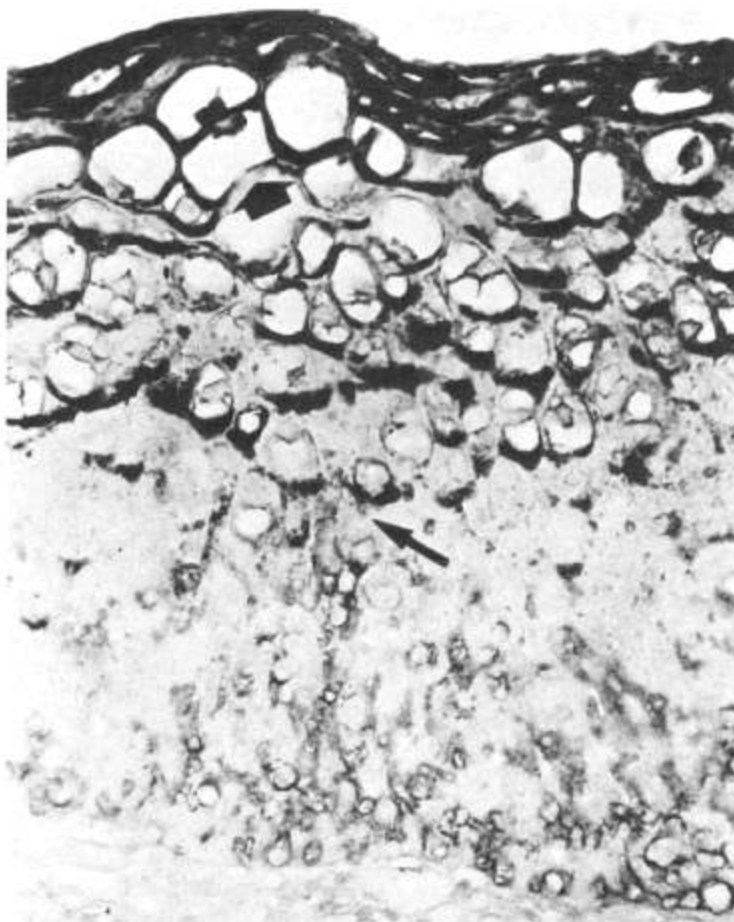


FIG. 4. Mild cervical dysplasia. Anti-involucrin staining pattern in a specimen with positive human papillomavirus staining. Note the localization of involucrin at the periphery of koilocytotic cells in the superficial epithelium (broad arrow) and the granular pattern of cytoplasmic staining in the basal and parabasal cells (narrow arrow) (anti-involucrin, immunoperoxidase study 1:1,000, methyl green counterstain, $\times 125$).

Again there was close correlation between both HPV antigens and koilocytosis, as well as involucrin and koilocytosis (Table 1).

In summary, HPV was detected in 15 of the 42 cases and involucrin immunoreactivity in 16 of 42 cases (Table 2). HPV antigens were localized in nuclei of the superficial cells. The expression of these antigens was focal and clustered. A high correlation existed between the presence of HPV antigen and positive staining for involucrin. None of the cases of mild or moderate dysplasia exhibited staining for HPV without involucrin staining. In the four cases of severe dysplasia-CIS that exhibited HPV antigens, localization of HPV was confined to areas showing mild or moderate dysplasia. In two of these cases involucrin was present in the same areas of mild to moderate dysplasia.

The presence of HPV structural antigens also produced a dramatic qualitative change in the pattern of involucrin staining. In sections of normal cervical tissue,

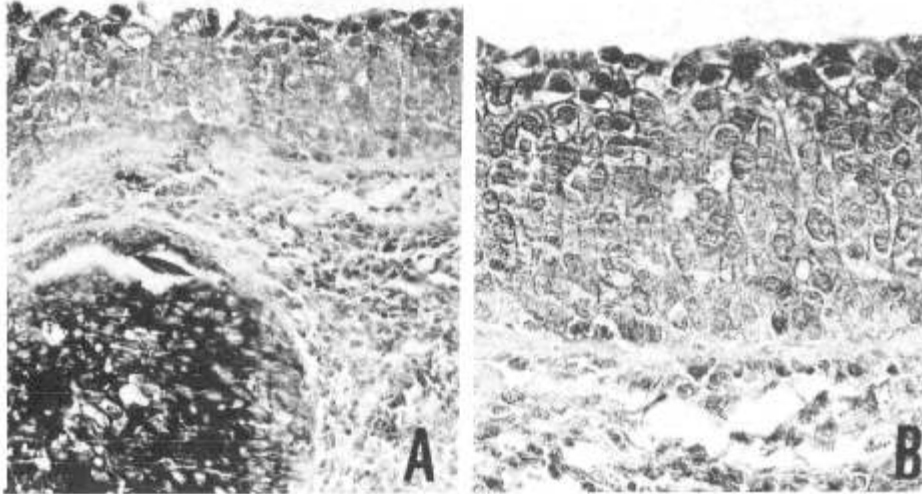


FIG. 5. A: Severe dysplasia-CIS (carcinoma *in situ*) overlying an endocervical gland replaced by metaplastic squamous epithelium (lower left). The metaplastic squamous epithelium is intensely stained with anti-involucrin, but the severe dysplasia-CIS is unstained (anti-involucrin immunoperoxidase 1:1,000, light green counterstain, $\times 50$). B: Higher power of severe dysplasia-CIS showing absence of involucrin (anti-involucrin 1:1,000, immunoperoxidase 1:1,000, methyl green counterstain, $\times 125$).

anti-involucrin produced a diffuse cytoplasmic staining only in superficial squamous epithelium. In contrast, in lesions containing HPV antigens, which invariably were localized in superficial cells, involucrin staining involved the full thickness of the epithelium including the basal layer. The character of the staining pattern was also altered, with localization of involucrin either in a peripheral, membranous pattern or a granular pattern. These changes were consistently observed in areas of coexistent HPV and involucrin expression.

DISCUSSION

The immunocytochemical localization of HPV structural proteins and involucrin within the same areas of dysplastic epithelium indicates that there is a close correlation between the presence of HPV antigens, involucrin, and the grade of the dysplasia. In mild dysplasia, involucrin was detected in 10 of 15 (66%) cases. Of the 10 cases exhibiting the presence of involucrin, eight contained HPV, suggesting that many mild dysplasias are HPV-induced lesions. The two cases with positive involucrin staining and lack of HPV antigens may represent immature squamous metaplasia. In moderate dysplasia, involucrin was detected in four of 13 (30%) cases and HPV antigens were present in three of these. The close correlation between HPV and involucrin in this group confirms the impression that HPV-induced atypia may account for a significant proportion of lesions classified as moderate dysplasia. In severe dysplasia-CIS, involucrin was localized in two of 14 (14%) cases and HPV in four. In the two cases that contained

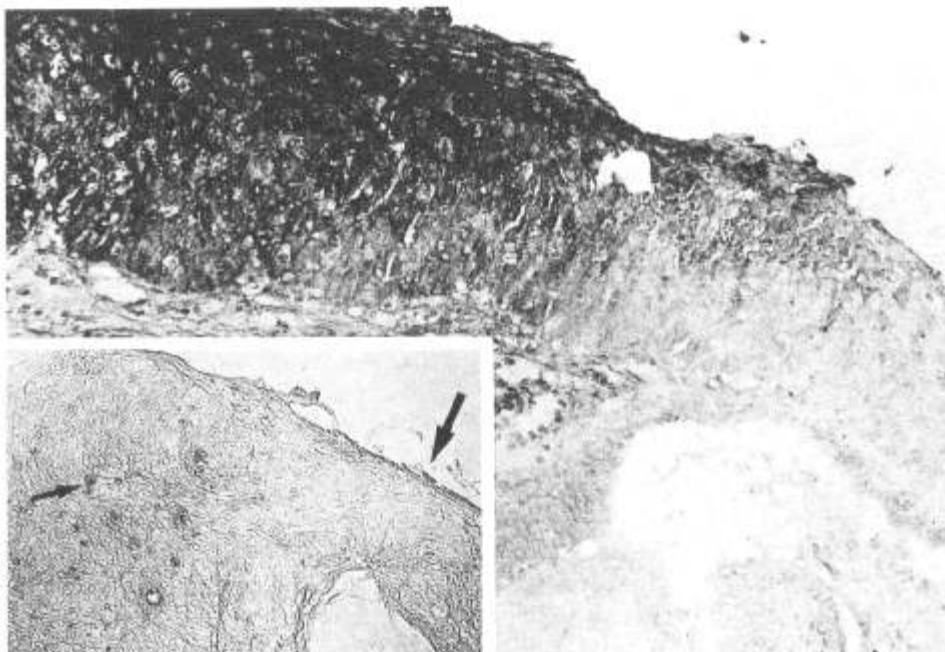


FIG. 6. Mild dysplasia exhibiting intense full-thickness staining for involucrin on the left, adjacent to severe dysplasia showing absence of involucrin on the right (anti-involucrin, 1:1,000, immunoperoxidase technique, methyl green counterstain, $\times 125$). **Inset:** Immunoperoxidase localization of human papillomavirus (HPV) antigens (small arrow) is present in an area of mild dysplasia that exhibits positive involucrin staining adjacent to severe dysplasia in which there is no detectable involucrin (large arrow). This area is also negative for HPV (HPV immunoperoxidase stain, no counterstain, $\times 250$).

involucrin, the localization of both involucrin and HPV was in areas of moderate dysplasia adjacent to the severe dysplasia, not within the area of severe dysplasia. Two cases exhibited HPV in areas of moderate dysplasia. These areas lacked involucrin. Thus, with increasing severity of dysplasia there is a progressive decrease in the localization of involucrin and a loss of expression of HPV antigens.

Previous studies have shown that as squamous epithelium matures, the plasma membrane becomes more permeable and the cells develop a protein envelope, of which involucrin is one component (5–7). This phenomenon is referred to as “terminal differentiation” and represents the final stage of squamous differentiation (8). Immunocytochemical staining for involucrin has shown that in normal squamous epithelium involucrin is first observed in the intermediate layers with progressively increased intensity and distribution in the superficial layers. It is absent in the basal and parabasal cells. Involucrin expression can, therefore, serve as an index of squamous differentiation. The absence of involucrin in dysplastic squamous epithelial cells indicates a lack of squamous maturation and may represent a disturbance in cellular function associated with early neoplastic transformation since involucrin is not expressed by neoplastic cells (9). In contrast, the expression of keratin is common to all epithelial cells and does not reflect a commitment to squamous differentiation, since the normal basal epithelium of the cervix is composed of cells that are relatively “undifferentiated” but

rich in keratin filaments (13). Moreover, since numerous epithelial neoplasms contain keratin proteins, the expression of keratin has no relationship to neoplastic transformation (14).

It has been well established by both immunocytochemical and electron microscopic studies that the presence of HPV structural proteins and viral particles is confined to the superficial cells in lesions traditionally classified as dysplasia (2,3). This has led some investigators to conclude that HPV infection occurs on the surface of the epithelium and that surface atypia is a manifestation of HPV infection or "condylomatous atypia" in contrast to neoplastic lesions, i.e., "true dysplasia," which arise in the basal layer (4). In a previous study (3) it was shown that in over three-fourths of cervical dysplasias containing HPV antigens there is an underlying proliferation of basal and parabasal cells for which the term "PV associated hyperplasia" was proposed. Based on these findings and molecular hybridization studies (2), it was suggested that HPV infection begins in the basal layer. The finding of involucrin staining involving the full thickness of the epithelium, including the basal layer, strongly supports this hypothesis. In normal maturing squamous epithelium there is a coordination between DNA synthesis and involucrin expression. The basal cells engaged in DNA synthesis do not express involucrin. HPV infection appears to "uncouple" these events and induce a premature expression of cellular structural proteins. Although the expression of cellular structural proteins is correlated with the expression of viral structural proteins, the biochemical relationships between these events remain unclear. The abnormal involucrin staining pattern observed in HPV infection is probably the result of subcellular structural alterations caused by the virus. The granular and peripheral staining patterns observed in cells with koilocytotic atypia probably reflect structural derangements within these cells.

Although koilocytotic atypia is generally regarded as a cytologic marker of PV infection (15-18), a high proportion of lesions that exhibit koilocytosis show no evidence of PV antigens (3). Two possible explanations for this discordance are that koilocytosis is a nonspecific alteration that can be produced by a variety of etiologic agents or that periodic HPV expression may result in a morphologic change that persists after the virus has ceased to synthesize viral structural proteins. In this report it was found that whereas HPV antigens were present in only 36% of lesions showing koilocytosis, HPV was identified in 81% of lesions in which there was full-thickness staining for involucrin in addition to koilocytosis. There was only one case of full-thickness staining for involucrin that lacked koilocytosis. This case also lacked HPV.

This correlative study between involucrin and HPV expression indicates that a requisite for HPV protein synthesis is full-thickness squamous maturation as manifested by the production of involucrin. The cells that express involucrin are not truly neoplastic but are relatively immature squamous cells induced to proliferate by the virus. Despite their immature morphologic appearance, these cells undergo squamous maturation as evidenced by the localization of involucrin. This type of lesion is generally classified as mild or moderate dysplasia, although in some instances lesions can qualify as severe dysplasia according to traditional morphologic criteria. Such lesions may represent viral infections only and may be capable of spontaneous regression. Cells that fail to react for involucrin are not undergoing squamous maturation and are unable to support the synthesis of

viral structural proteins. These cells may be truly neoplastic. Whether the HPV genome itself is capable of inducing true neoplastic transformation, or whether HPV-induced proliferation of immature cells makes them susceptible to other carcinogenic agents, will require further study.

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