Title

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Authors

Warhol, Michael J
Antonioli, Donald A
Pinkus, Geraldine S
et al.

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IMMUNOPEROXIDASE STAINING FOR INVOLUCRIN:

A Potential Diagnostic Aid in Cervicovaginal Pathology

Michael J. Warhol, MD Donald A. Antonioli, MD,†*
Geraldine S. Pinkus, MD, Louis Burke, MD,‡ and Robert H. Rice, PhD§*

Involucrin, a protein subunit of keratinocyte cross-linked envelopes, is a distinctive marker for suprabasal differentiation in stratified squamous epithelium. Immunoperoxidase staining for involucrin was used to evaluate paraffin sections of tissue obtained by colposcopically directed biopsies of infectious, metaplastic, and dysplastic lesions of the cervix and vagina. Areas of normal squamous epithelium, papillary and flat condyloma acuminatum, and mature and immature squamous metaplasia showed positive staining in 99 per cent of samples lacking significant inflammation and in 60 per cent of those with moderate or severe inflammation. In contrast, only 19 per cent of the squamous cell dysplasias, even those without much inflammation, showed positive staining, and no area with moderate or severe inflammation showed positive staining. These findings indicate that expression of involucrin is modulated by cellular pathologic features and microenvironment. We suggest that immunoperoxidase staining for involucrin may be useful in distinguishing mild dysplasia from immature metaplasia and flat condyloma in some biopsy specimens in which routine histologic examination yields an indeterminate diagnosis. Hum Pathol 13:1095–1099, 1982.

The histologic evaluation of colposcopically directed biopsy specimens is the most practical and effective technique currently available for detecting squamous intraepithelial neoplasia of the uterine cervix. However, the usefulness of the technique is limited by certain inherent difficulties. The interpretation of cervical dysplasia remains partially subjective. Immature squamous metaplasia may be difficult to distinguish from dysplasia, and viral infections may induce cytologic changes that mimic dysplasia.[1–4]

Distinctive protein markers of differentiation in squamous epithelium are potentially useful in resolving uncertainties in interpretation of cervical biopsy specimens. Maturing cells of human stratified squamous epithelia (epidermis, vagina, esophagus, conjunctiva) synthesize immediately beneath the plasma membrane a protein envelope stabilized by ε-(γ-glutamyl)-lysine isopeptide cross linking.[13,14] This envelope, unrelated to keratin, is the 10-nm-thick marginal band visible in electron micrographs.[15] Involucrin is a major structural subunit of this envelope.[9] Immunochemical and biochemical studies in both tissue section and cultures of human squamous epithelium show that involucrin is absent from basal cells but appears in considerable amounts as the cells mature.[7–9] Therefore, involucrin is a marker for squamous epithelium and reflects suprabasal differentiation.[8] Cultured cells from human squamous carcinomas of epidermis and oral cavity are defective in envelope synthesis.[16]

The purpose of this study was to determine the presence and distribution of involucrin in a variety of benign and malignant lesions of the cervix and vagina. The observations noted previously suggested that immunochemical staining for involucrin might be helpful in evaluating the changes in squamous epithelium in surgical specimens. We hypothesized that involucrin would be detected in normally developing squamous metaplastic cells within the cervical transformation zone but that this marker would be absent from improperly differentiating dysplastic epithelial cells.

MATERIALS AND METHODS

Biopsy specimens were obtained from patients referred to the Gynecologic-Oncology Clinic at the Beth Israel Hospital. A complete history was obtained from each patient, with particular reference to contraceptive use, pregnancy, and prior cervicovaginal surgery or cauterization. A complete gynecologic examination, including colposcopy and colpophotography, was performed on all patients, and smears were taken from the endocervical canal and cervical portio of each.

Areas from the cervix and vagina were selected for biopsy on the basis of the colposcopic findings. The colposcopic appearances were classified as normal, leukoplakia, aceto-white epithelium, punctation, mosaic, and atypical blood vessels, based on standard criteria.[10] Abnormal areas were graded I, II, or III on the basis of increasing severity of change in color, surface contour, and vascular pattern. Biopsy specimens from all types and grades of lesions were evaluated.

The biopsy specimens were fixed in 10 per cent neutral buffered formalin and processed routinely

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* Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts.
† Department of Pathology, Beth Israel Hospital, and Harvard Medical School, Boston.
‡ Department of Obstetrics and Gynecology, Beth Israel Hospital, and Harvard Medical School, Boston.
§ Laboratory of Toxicology, Harvard School of Public Health, Boston.
Address correspondence and reprint requests to Dr. Warhol, Department of Pathology, Brigham and Women's Hospital, 75 Francis St, Boston, MA 02115.

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TABLE 1. CLINICAL PROFILE OF FIFTY WOMEN FROM WHOM BIOPSY MATERIAL WAS OBTAINED FOR STUDY OF INVOLUCRIN

Age:		
Range, 18–61 yr		
Mean, 28 yr		
Contraceptive use:	40	(80%)
Oral	34*	
Diaphragm	12*	
History of Pregnancy:	35/48	(73%)
Prior Therapy to Cervix:	8	(16%)
Laser	5	
Cryoprobe	2	
Laser and Cryoprobe	1	
Reason for Referral to Clinic:		
Abnormal Pap smear or biopsy	45	(90%)
Abnormal colposcopy	3	
History of in utero diethylstilbestrol exposure	2	

* Use of both oral contraceptives and diaphragm at different times in six patients.

with the surgical pathology material. Each study specimen was step-sectioned and sampled at six levels: levels 1, 3, and 5 were stained with hematoxylin-eosin, and levels 2, 4, and 6 were stained with immunoperoxidase for involucrin.

The rabbit antihuman involucrin used was from the same preparation described and employed previously.[9] It was raised against involucrin chromatographically purified from cultured human epidermal cells and shown to be homogeneous in molecular weight and isoelectric point by gel electrophoresis. In Ouchterlony immunodiffusion the antiserum formed a precipitin band with as little as 0.2 μm of purified involucrin, whereas specific binding to other proteins of keratinocytes or dermal fibroblasts was not detectable. No binding to involucrin was detected in preimmune serum or in antiserum absorbed with purified envelopes.

The immunoperoxidase reaction was performed as described previously.[11] Paraffin sections were incubated with rabbit antihuman involucrin (dilution 1:1000) for 30 min, followed by sequential 30-min incubations with swine anti-rabbit immunoglobulin antiserum (Dakopatts, Copenhagen, Denmark; US distributor: Accurate Chemical and Scientific Corp, Westbury, New York) and peroxidase rabbit anti-peroxidase (PAP) immune complexes (Cappel Laboratories, Cochranville, Pennsylvania). Antibody localization was determined on the basis of peroxidase activity, effected by incubation of the slides with a solution containing 3,3′-diaminobenzidine tetrahydrochloride (6 mg/10 ml of 0.1-molar tromethamine buffer at pH 7.4), and hydrogen peroxide (0.1 ml/10 ml tromethamine buffer). Sections were counterstained with hematoxylin and dehydrated and mounted with Permount. Control sections substituting preimmune rabbit serum or antihuman involucrin serum that had been adsorbed with purified envelopes were processed in all cases and were consistently negative. Sections of normal skin were employed as a positive control and revealed only suprabasal immunoreactivity with no background staining.

The hematoxylin-eosin and immunoperoxidase sections were examined by two different observers. Using standard criteria,[1,4] the histologic findings were classified as normal squamous epithelium; squamous metaplasia of glandular tissue (in endocervix and in vaginal adenosis); nonglycogenated squamous mucosa (representing advanced squamous metaplasia)[12]; condyloma acuminatum; or cervical intraepithelial neoplasia (CIN), subdivided as grade I (mild dysplasia), grade II (moderate dysplasia), or grade III (severe dysplasia: carcinoma in situ). Specimens were classified as indeterminate if the preliminary histologic findings were equivocal in separating a CIN lesion from changes caused by inflammation, condyloma, or immature metaplasia. The inflammation in each biopsy specimen was quantified as 0 to 3+, and its location (epithelium, stroma; focal versus diffuse) and type (polymorphonuclear or mononuclear) were noted.

Immunoperoxidase staining was interpreted as present or absent, and the distribution of suprabasal staining was noted with respect to the presence of inflammation and intraepithelial neoplasia. The results of the histologic evaluation were then compared with those of the immunoperoxidase studies to detect correlations.

TABLE 2. HISTOLOGIC FEATURES OF 100 BIOPSY SPECIMENS

	Major or Sole Feature (No. of Specimens)	Noted with Other Pathologic Features (No. of Specimens)	Total No. of Specimens
Normal squamous epithelium	4	46	50
Immature squamous metaplasia	26	2	28
Nonglycogenated mucosa (mature metaplasia)	16	3	19
Condyloma accuminatum	11	0	11
Cervical intraepithelial dysplasia:			
Grade I	6	1	7
Grade II	16	0	16
Grade III	11	0	11
Indeterminate	10	0	10
TOTAL	100	52	152

TABLE 3. COMPARISON OF HISTOLOGIC FEATURES AND IMMUNOPEROXIDASE RESULTS (152 AREAS IN 100 SPECIMENS)*

	No. of Lesions Positive for Involucrin		No. of Lesions in Which Staining for Involucrin was Related to Amount of Inflammation			
			0 and 1+ Inflammation		*2+ and 3+ Inflammation*	
Normal squamous epithelium (50 lesions)	46	(92)	40/40	(100)	6/10	(60)
Condyloma accuminatum (11 lesions)	11	(100)	9/9	(100)	2/2‡	(100)
Squamous metaplasia† (47 lesions)	40	(85)	33/34	(97)	7/13	(54)
Indeterminate (10 lesions)	4	(40)	4/8	(50)	0/2	(0)
Cervical intraepithelial neoplasia, all grades (34 lesions)	4	(12)	4/21	(19)	0/13	(0)
TOTAL (152 lesions)	105	(69)	90/112	(80)	15/40	(38)

* Percentages are given in parentheses.
† Includes immature squamous metaplasia and nonglycogenated mucosa.
‡ Inflammation was 2+ in both cases.

RESULTS

The clinical profile of the 50 patients is summarized in table 1. Of the 100 biopsy specimens, 90 were obtained from the cervix and 10 from the upper third of the vagina. Table 2 summarizes the predominant and incidental histologic lesions in each of the specimens. There were 152 separate areas for which histologic findings were to be compared with immunoperoxidase results. One third of these areas were normal squamous epithelium and served as controls. Dysplastic lesions accounted for 22.4 per cent of the areas, 38.1 per cent represented metaplasias and condylomas, and the remaining 6.6 per cent were indeterminate on initial evaluation.

Comparison of the histologic diagnoses with findings on involucrin staining is shown in table 3. Normal squamous epithelium (fig. 1), non-neoplastic lesions such as flat and papillary condylomas (figs. 2 and 3), and the various degrees of squamous metaplasia (fig. 4) showed staining for involucrin with suprabasal distribution, except areas accompanied by prominent inflammation, which characteristically was moderate (2+) or severe (3+), was diffuse, contained neutrophils, and involved the epithelium. Cervical intraepithelial neoplasia was associated with negative staining in 81 per cent of cases (fig. 5). There was no significant difference in the percentage of biopsy specimens with negative staining among the different grades of CIN lesions (grade I, 86 per cent; grade II, 88 per cent; grade III, 91 per cent). Staining was absent from all CIN lesions accompanied by moderate or severe inflammation. On chi-square analysis, the difference in positive involucrin staining between the infectious or metaplastic lesions and the CIN lesions was highly significant ($P < 0.001$).

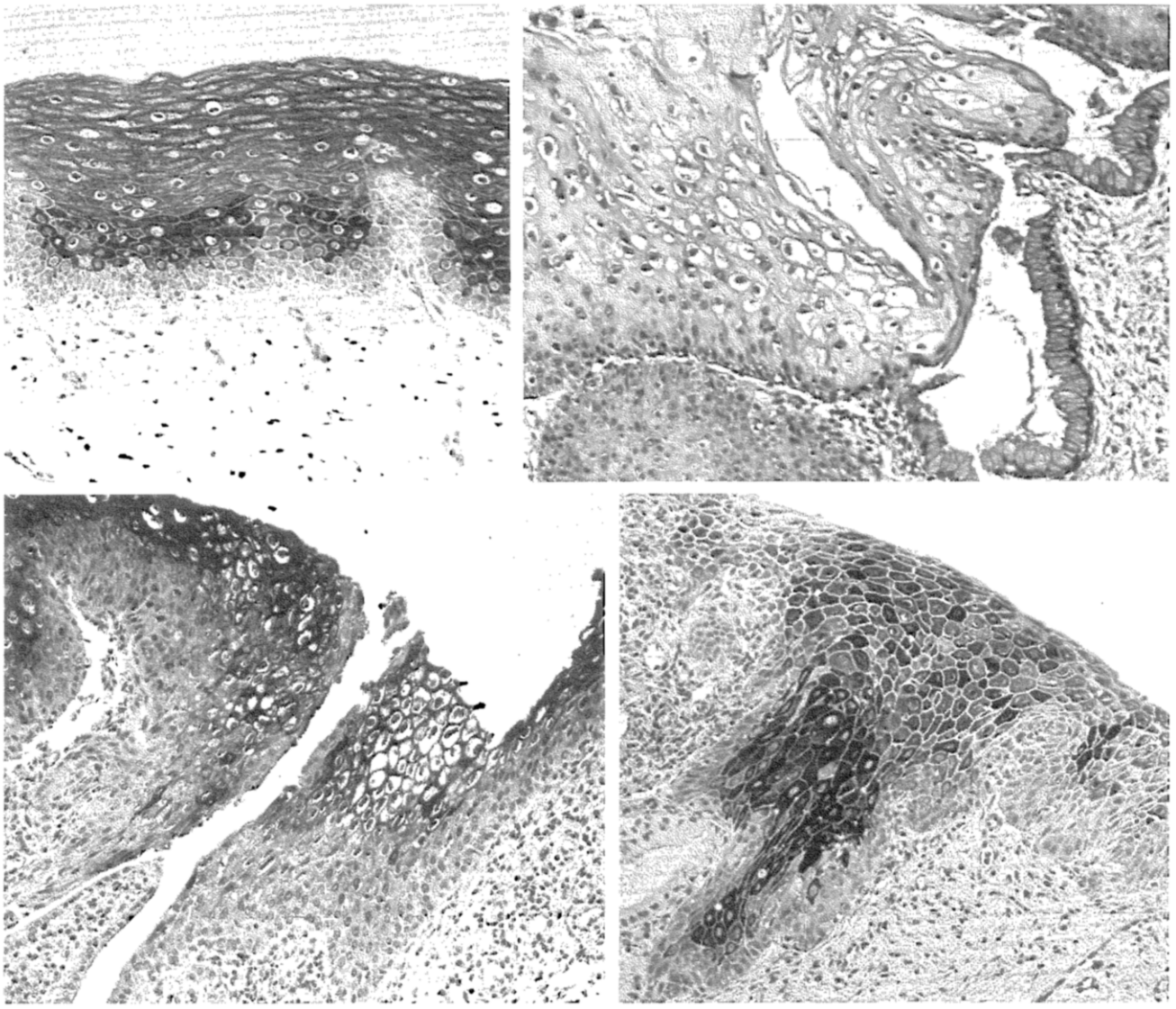
The features of the ten indeterminate lesions, all from the cervix, are summarized in table 4. In two cases, specimens 60 and 72, there was difficulty in distinguishing CIN from epithelial atypia associated with inflammation. Both cases in which staining was scored negative were classified CIN. In the other eight cases, which had slight or no inflammation, there was difficulty in the differential diagnosis of flat condyloma or immature squamous metaplasia versus CIN. The immunoperoxidase results appeared to be at variance with the histologic interpretation in four instances (specimens 85, 88, 90, and 93), in three of which (specimens 85, 90, and 93) the discrepancy involved the interpretation of condyloma-like changes.

DISCUSSION

In this study we investigated the potential usefulness in cervicovaginal pathology of immunochemical staining for the structural protein involucrin, particularly in differentiating benign non-neoplastic lesions from those with dysplastic features. The known chemical and biologic features of involucrin suggested that it might be especially useful for this purpose.

In lesions clearly identifiable histologically as condyloma acuminatum, staining was strongly positive above the basal layer, with distribution and intensity mimicking that in normal squamous epithelium (fig. 3). By this criterion the viral lesions have normal cytoplasmic differentiation. Therefore, involucrin staining is potentially useful in separating condylomas from true mild dysplasias (CIN grade I). Recent reports have suggested that the majority of lesions diagnosed as CIN grade I are, in fact, flat condylomas.[2,3] The histologic distinction between the two is often difficult, particularly if the basal layer is not completely normal and contains enlarged cells or cells not in a totally parallel array. Indeed, three of our four indeterminate cases involving condylomatous change had positive staining, suggesting that the histologic diagnosis of CIN grade I was inappropriate.

Our data show an 81 per cent correlation between the histologic diagnosis of CIN and lack of staining, indicating that immunohistochemical staining for involucrin can be of assistance in distinguishing neoplastic from non-neoplastic lesions of the cervix. The correlation was equally good for all grades of CIN, including carcinoma-in-situ. A small proportion of CIN lesions were scored positive for involucrin,



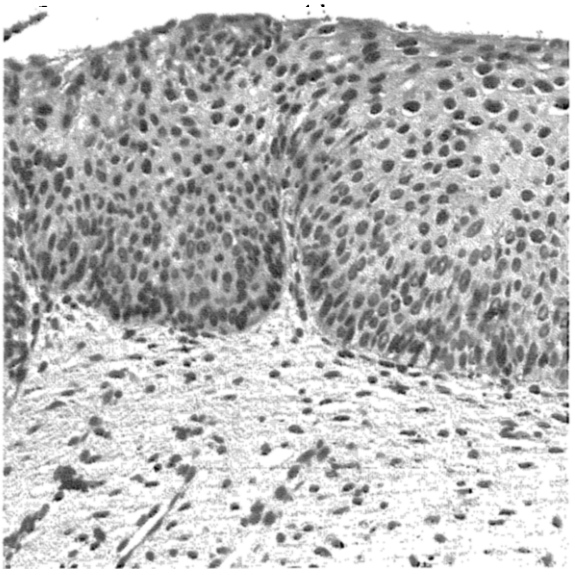


Figure 1. (*top left*). Vaginal mucosa showing normal squamous epithelium with suprabasal staining for involucrin. (Immunoperoxidase technique; hematoxylin counterstain. ×40.)

Figure 2. (*top right*). Cervical biopsy specimen with flat condyloma acuminatum with marked koilocytosis. (Hematoxylin-eosin stain. ×250.)

Figure 3 (*middle left*). Cervical biopsy specimen with flat condyloma showing a normal pattern of suprabasal staining for involucrin. (Immunoperoxidase technique; hematoxylin counterstain. ×100.)

Figure 4 (*Middle right*). Squamous metaplasia with a normal pattern of suprabasal staining for involucrin. (Anti-involucrin immunoperoxidase technique; hematoxylin counterstain. ×100.)

Figure 5 (*bottom*). Cervical biopsy specimen with cervical intraepithelial neoplasia showing no involucrin staining. (Immunoperoxidase technique; hematoxylin counterstain. ×200.)

however, suggesting some variability in expression of the antigen among the dysplasias. Although staining intensity is difficult to quantify, our impression was that there was less staining in these lesions than in non-neoplastic epithelium, which is consistent with the observation that none of the dysplasias gave positive staining in areas of inflammation although the majority of non-neoplastic areas did (table 3). Further work will be required to determine quantitatively the degree to which involucrin synthesis is defective. The possibility that expression of involucrin may correlate with clinical course merits scrutiny. In the large majority of CIN lesions, the apparent lack of involucrin is indicative of altered differentiation and is consistent with the notion that these cells are neoplastic when the earliest lesions are detected. In contrast, non-neoplastic immature and mature squamous

TABLE 4. SUMMARY OF FEATURES OF INDETERMINATE SPECIMENS

	Problem in Interpretation	Inflammation*	Staining for Involucrin	Final Histologic Diagnosis†
Specimen 34	Immature metaplasia versus CIN II	1+	Negative	CIN II
Specimen 43	Immature metaplasia versus CIN II	None	Positive	Immature metaplasia
Specimen 44	Immature metaplasia versus CIN II	1+	Negative	CIN II
Specimen 60	Inflammatory changes versus CIN I	3+	Negative	CIN II
Specimen 72	Inflammatory changes versus CIN II	3+	Negative	CIN II
Specimen 85	Flat condyloma versus CIN I	1+	Positive	CIN I
Specimen 88	Immature metaplasia versus CIN II	1+	Negative	Immature metaplasia
Specimen 89	Flat condyloma versus CIN I	None	Negative	CIN I
Specimen 90	Flat condyloma versus CIN I	1+	Positive	CIN I
Specimen 93	Flat condyloma versus CIN I	1+	Positive	CIN I

ABBREVIATION: CIN = cervical intraepithelial neoplasia.
* 1+, mild; 2+, moderate; 3+, severe.
† Final histologic diagnosis determined without knowledge of results of staining for involucrin.

metaplasia exhibit a normal content of involucrin as well as normal differentiation.

The absence of staining in regions of severe inflammation (fig. 5), an unexpected observation, may result from local effects of intraepithelial inflammation. Inflammation alters the levels of biologically active molecules (e.g., peptide hormones, prostaglandins) and produces highly reactive chemical species (e.g., superoxide radicals, hydrogen peroxide). We hypothesize that this alteration in the microenvironment reversibly affects expression of proper differentiation. An analogous effect on involucrin content has been observed in cultured keratinocytes as a function of hydrocortisone and vitamin A concentrations in the medium (P. R. Cline, R. H. Rice, unpublished observations, 1981).

In evaluating lesions characterized as indeterminate by routine histologic examination, involucrin staining appeared to be helpful in separating immature metaplasia from dysplasia. Although its usefulness was limited by the presence of inflammation, this difficulty could be surmounted in most cases by antibiotic treatment of the causative microbial infection, with subsequent re-examination, as is now done with Pap smears and colposcopy. Further prospective studies of indeterminate lesions, using spectrophotometric analysis of DNA as the baseline for separating CIN from non-CIN, are needed. The routine histologic findings and immunoperoxidase staining of such lesions can then be more precisely related to the true nature of the lesions and their natural history.

In conclusion, this preliminary study of involucrin was performed on material from patients typical of the referral population of the Gynecologic Oncology Clinic at the Beth Israel Hospital: they were young and sexually active, and there was a high prevalence of contraceptive use among them. The areas chosen for biopsy yielded the spectrum of common metaplastic, infectious, and dysplastic squamous lesions for hematoxylin-eosin and immunoperoxidase evaluation. Our results suggest that 1) in the absence of extensive inflammation, involucrin is present in the cervix and vagina in normal squamous cells, in immature and mature squamous metaplastic cells, and in condyloma acuminatum; 2) involucrin staining is absent in the majority of cases of intraepithelial neoplasia, even when the dysplasia is mild; 3) Involucrin staining is often absent at sites of intense inflammation; and 4) immunoperoxidase staining for involucrin may be useful in resolving the diagnosis of lesions that appear indeterminate on routine hematoxylin-eosin evaluation.

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