EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Apoptosis in Woman Uterine Cervix in Pathologies Associated with Human Papillomavirus

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The level of apoptosis in uterine cervical tissue was evaluated in healthy women and in patients with various pathologies. No signs of apoptosis were found in unchanged stratified epithelium, condyloma latum, and condyloma acuminatum. The level of apoptosis decreased with progression of neoplastic epithelial transformations, usually no apoptosis was observed in samples of stage III cervical intraepithelial neoplasms. The development of preinvasive carcinoma was accompanied by activation of apoptotic processes most pronounced in the upper third of the epithelium. In some stage I and stage I-II cervical intraepithelial neoplasms, apoptosis and elimination of the basal layer cells caused rejection of the epithelium which can explain regression of this pathology at the initial stages. The prevalence of human papillomavirus infection directly correlated with neoplastic changes in the cervical epithelium.

Key Words: *uterine cervix; apoptosis; condyloma; cervical intraepithelial neoplasm; carcinoma; TUNEL*

Diagnostics and treatment of diseases caused by and associated with human papillomavirus (HPV) attract considerable attention because of high oncogenic potential of this pathogen. Virus invasion and persistence in basal cells of the cervical squamous stratified epithelium (SSE) and realization of genetic program of virus reproduction lead to intensification of proliferative processes in the epithelium resulting in the formation of condyloma acuminatum, condyloma latum, and cervical intraepithelial neoplasia (CIN) of various severity.

CIN and cervical carcinoma are considered to be the stages of the same pathological process strictly associated with HPV infection. CIN can completely regress, persist for a long time without progress, or transform into invasive carcinoma [1]. Studies including from 894 to 17946 patients showed that CIN I regressed in 55-57%, persisted in 27-32%, and progressed in 11% cases, respectively, while for CIN II the corresponding values are 43-54%, 16-35%, and 16-30%. Transformation into cervical carcinoma was observed in 1% CIN I, 2.5% CIN II, and 4.2% CIN III [2,4,10,17]. Infection with HPV of high-oncogenic types increases the probability of neoplastic transformation to 12.3%, 20%, and 55.2% for CIN I, II, and III, respectively [9].

Some authorities believe that apoptosis prevents precancer processes and cervical carcinoma by eliminating cells with signs of neoplastic transformations or genetic and other defects stimulating cancer development. Therefore, inhibition or blockade of apoptosis under certain pathological conditions causes rapid and uncontrolled proliferation of anaplastic cells [8,15].

DNA fragmentation to oligonucleosomes is the most typical feature of apoptosis [18,19]. A TUNEL

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technique is one of the most informative methods for the assessment of apoptosis intensity [5].

The present study was aimed at investigation of the relationships between pathological changes in the uterine cervix, HPV infection, and the level of apoptosis.

MATERIALS AND METHODS

Thirty-four tissue samples obtained by biopsy of pathological cervical regions from women of reproductive age were examined.

Human papilloma virus was revealed and typed by PCR (non-selective test for the presence of HPV followed by the identification of 6/11 and 16/18 HPV types) and DNA hybridization using a HPV Hybrid Capture test-system (DIGENE) identifying HPV DNA with a moderate (6, 11, 42, 43, and 44 types) and high (16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68) oncogenic potential [16,20].

For evaluation of the intensity of apoptosis tissue specimens were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 24 h and embedded in paraffin. The sections (5 μ) were fixed on 0.01% polylysine-coated glasses, deparaffinized with toluene (2×5 min), absolute ethanol (3×5 min), 70% ethanol, and PBS.

To reveal the apoptotic cells in the cervical tissue the DNA fragments were detected *in situ* with a TUNEL technique based on binding of biotin-labeled deoxynucleotide triphosphates to free 3'terminals of DNA fragments formed after DNA cleavage by cell nucleases [5]. After reaction with streptavidin-peroxidase conjugate using diaminobenzidine as a substrate, specific biotin labels were visualized microscopically. The nuclei containing fragmented DNA looked brown, the intensity of staining was proportional to the intensity of apoptotic DNA degradation. The data obtained with the TUNEL technique were used as an index of the intensity and localization of apoptosis in the epithelial and subepithelial connective tissues. Staining intensity was classified to weak, moderate, and high.

Deparaffinized sections were incubated with proteinase K (20 μg/ml, Sigma) for 30 min at room temperature and washed with PBS. Endogenous peroxidase activity was inhibited by 5-min incubation with 2% H_2O_2 in PBS. Endogenous biotin activity was blocked with a Biotin blocking system (DAKO). DNA was labeled by incubation with 0.2 nmol biotin-16-d-uridine triphosphate and 10 units of terminal deoxynucleotidyl transferase (both from Sigma) in 100 μl buffer containing 0.2 M sodium cacodylate (Serva), 25 mM Tris-HCl (Sigma), pH 6.6, 0.25 mg/ml bovine serum albumin (BSA), 2 mM $CoCl_2$ (both Sigma), and 0.1 mM DTT (ICN) for 1 h at 37°C. Nonspecific avidin binding was blocked by 40-min incubation with 2% BSA in PBS at 37°C. Then the sections were incubated for 40 min at 37°C with avidin-peroxidase conjugate (0.6 μg/ml DAKO) prepared in PBS with 0.05% Twin-20 (PSBT) and 1% BSA. After 30-min washout with PSBT and 20-min washout with PBS on a shaker, the sections were washed with distilled water and stained with diaminobenzidine (DAKO) dissolved in PBS. Deparaffinized sections incubated with DNase I (10 U/100 μl per section, Promega) in a buffer containing 25 mM Tris-HCl, pH 8, 0.5 mM $MgCl_2$, and 5 mM $CaCl_2$ (both from Sigma) for 30 min at room temperature served as a positive control. The sections incubated without 16-d-uridine triphosphate served as a negative control.

Correlation analysis was performed [16]. Correlation was significant ($p<0.01$), if the absolute value of the correlation coefficient r was equal or above 0.7.

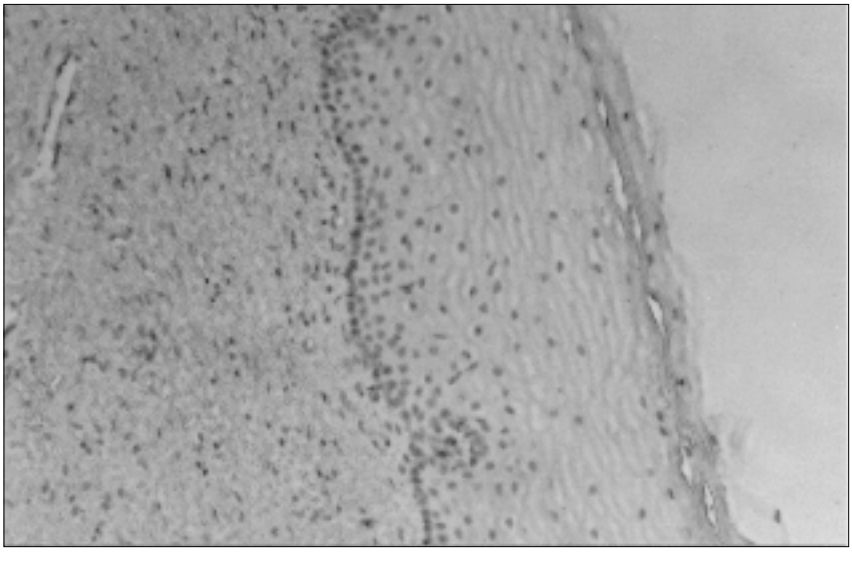

Fig. 1. Fragment of uterine cervix with unchanged stratified squamous epithelium. Immunohisochemical staining with 3,3'-diaminobenzidine and hematoxylin. TUNEL staining for apoptosis. Human papilloma virus (HPV) and apoptosis are absent.

RESULTS

No signs of DNA degradation and inflammatory infiltrates were found in the control group comprising the samples of unchanged SME and stroma (Fig. 1).

Numerous koilocytes, but not apoptotic cells with their specific nuclear staining were observed in the epithelial tissue from condyloma acuminatum samples.

Irrespective of the presence of HPV or its type, the samples of condyloma contained a considerable number of koilocytes (with two or more nuclei) and dyskeratocytes in the superficial layer. In some cases, acanthosis with hyperplasia of both the basal and parabasal layers was observed. No signs of apoptotic DNA degradation were found in cells of the striated epithelium and subepithelial connective tissue.

The character of apoptosis in CIN I and I-II samples could be different (Fig. 2). In some cases (6 samples, among which 3 HPV positive, 1 with HPV of moderate oncogenicity), the count of the basal layer cells with heavy nuclear staining increased from single cells to 100% while moving from the normal to neoplastic SME. In I CIN sample (Fig. 2, *a*) apoptotic cells with typical loss of polarity were found in the border area between the normal and transformed epithelium. Rejection of the entire epithelial layer was observed starting from the area with single apoptotic cells and finishing in the area with 100% apoptosis, where all the neighboring cells of the basal epithelial layer were apoptotic (Fig. 2, *b*). It should be noted that along this zone, neither stroma, nor other SME layers, except basal, contained cells with specific staining, whereas clusters of intensely stained lymphocytes and plasmacytes were scattered in deep stromal layers (Fig. 2, *c*). The increase in the number of apoptotic cells in the basal SME layer correlated with the intensity of focal inflammatory infiltrion. Infiltrates were located near the basal membrane and contained mononuclear cells, primarily plasmacytes, small lymphocytes, and granulocytes. Lymphoid infiltration was accompanied by fibroblast proliferation. Cell composition of the infiltrate was typical of chronic inflammation. Examination of other sections of the same sample revealed focal lymphoid infiltration not only in the stroma, but also in SME with morphological signs of CIN I.

Development of CIN I and I-II could be accompanied by different apoptotic manifestations (Fig. 2, *c*). Single cells with weakly stained nuclei were found only in the parabasal layer of the epithelium with morphological signs of CIN I and I-II, whereas no signs of apoptosis or rejection of pathologically transformed SME were observed in other parts of the section. As a rule, in the presence of HPV with moderate oncogenicity, CIN I and I-II areas with the lowest level of apoptosis bordered with more pronounced changes in epithelial tissue, typical of CIN III.

Finally, CIN I-II with pronounced epithelial dystrophy in some regions (1 HPV positive and 1 HPV-negative samples) could be accompanied by inflammatory infiltration of the stroma without activation of programmed cell death as evidenced by the presence of only single apoptotic cells with moderate staining in the stroma.

In samples of CIN II and in one sample of CIN III (6 samples; 4 HPV-positive cases, 2 with moderate and 2 with high oncogenicity), we found single apoptotic cells (Fig. 3, *a*) located in the superficial epithelial layers. The intensity of staining of their nuclei varied from weak to moderate, no apoptosis was observed in the stroma.

Apoptotic cells were absent in the majority of CIN III samples (5 samples; HPV of moderate and high oncogenicity, 3 cases each) (Fig. 3, *b*).

Preinvasive carcinoma combined with severe displasia (CIN III) was characterized by different features of the apoptotic process (2 samples: 1 HPV-negative, 1 HPV-positive with HPV of high oncogenicity). In the examined sections areas without apoptosis (data not shown), with few moderately stained apoptotic cells chaotically scattered in the anaplastic epithelium (Fig. 4, *a*), and with numerous heavily stained cells located preferentially in the upper third of the epithelial plate (Fig. 4, *b*) could be distinguished. Superficial localization and chaotic distribution of the cells with fragmented DNA differ this type of apoptosis from regular death of basal epithelial cells in CIN I samples. A high level of apoptosis in cells of invasive carcinoma was previously described by K. Kokawa *et al.* [9].

In areas of preinvasive carcinoma of cervical glands (Fig. 4, *c*) we found a few cells with moderate apoptosis and chaotic localization. No signs of apoptosis was revealed in the stroma.

A sample (HPV of moderate and high oncogenicity) containing areas of CIN III and cervical carcinoma with minimal invasion showed no apoptosis.

Progression of CIN from stage I to stage III directly correlated with the presence of HPV and its oncogenic potential (Table 1). A decrease in apoptotic manifestations with CIN development and high positive correlation between the stage of neoplasia and the presence of oncogenic HPV types are in line with published data on negative viral regulation of apoptosis in cervical epithelial cells [13] and on the increased probability of unfavorable CIN development under conditions of HPV infection [17]. At the same time, the absence of HPV in one of the two examined samples of preinvasive carcinoma confirms the data of J. T. Cox [3] on the absence of HPV in 15% cervical carcinoma cases.

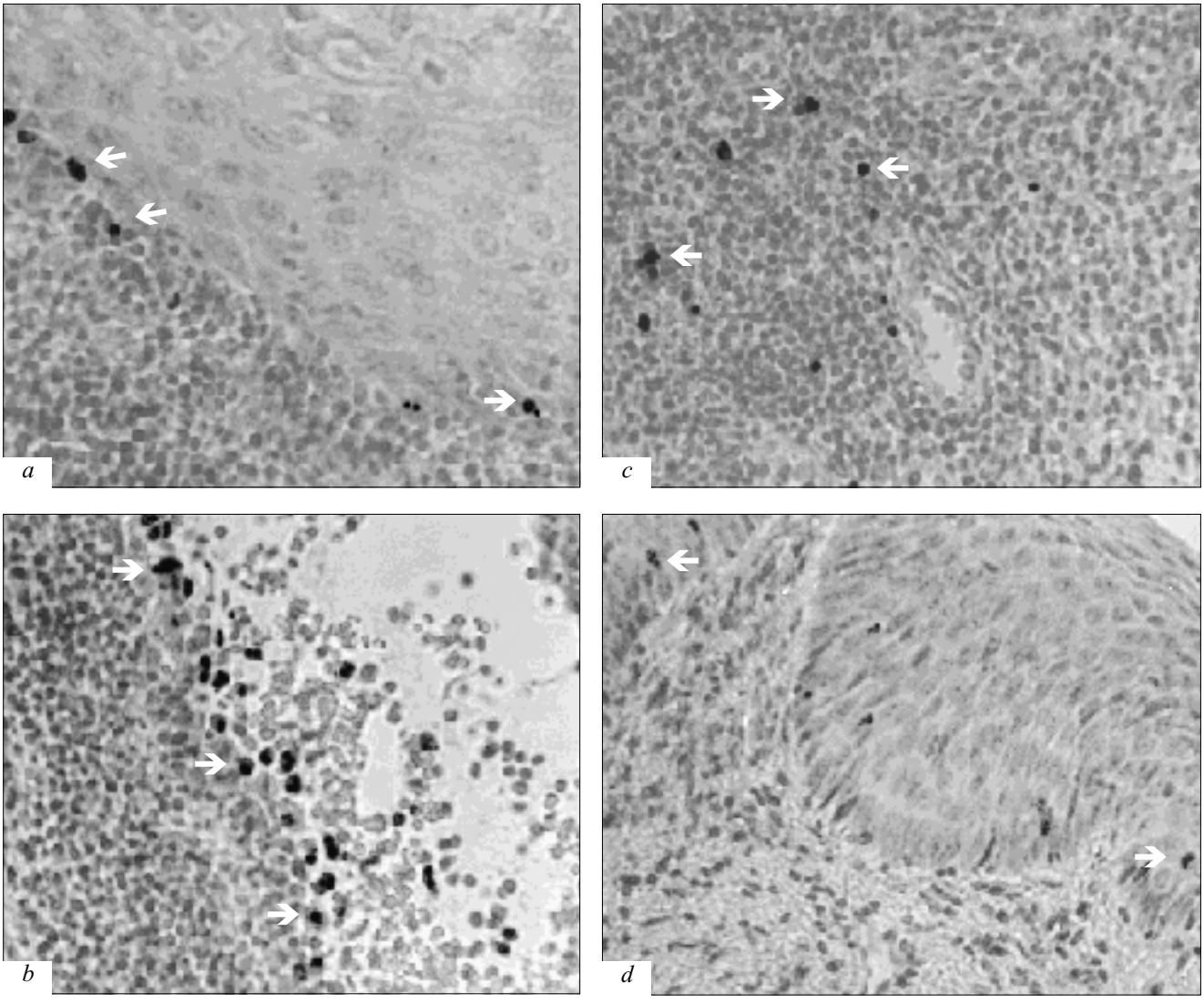

Fig. 2. Apoptosis in the uterine cervix with the morphological signs of CIN I and I-II. *a*) single cells with intense staining in the basal epithelial layer and inflammatory lymphoid infiltration in the stroma, no HPV; *b*) rejection of the epithelial layer along the border of the intensely stained apoptotic cells of the basal layer. On the left — pronounced lymphoid infiltration of the subepithelial connective tissue, no HPV; *c*) inflammatory lymphoid infiltration and clusters of intensely stained lymphocytes and plasmacytes in deep stromal layers, no HPV; *d*) single cells with weak staining in the parabasal epithelial layer, HPV of moderate oncogenicity. Here and in Fig. 3 and 4: immunohystochemical staining with 3,3'-diaminobenzidine and hematoxylin, staining for apoptosis with a TUNEL technique. Arrows indicate cells with specific staining.

Our findings suggest rejection of pathologically transformed epithelium via the mechanism of programmed cell death and elimination of basal layer cells is a possible pathway of CIN regression, at least at its initial stages. It cannot be excluded that immunocompetent cells of stromal infiltrate play a role of regulators and/or effectors in this process. Cellular composition of the infiltrate is indicative of the presence of active chronic inflammation accompanied by fibroblast activation and the appearance of granulocytes which agrees with published data [14]. Therefore, our data confirm the presence of inflammatory reaction in the region of active apoptosis proposed by K. Miwa *et al*. [11].

If apoptosis is suppressed and no spontaneous regression of neoplasm occurs, CIN progresses to the next stage. Apoptosis remains at a low level through the entire period of neoplasm development from CIN I to cervical carcinoma. This is illustrated by a decrease in apoptosis intensity in areas of CIN I and I-II bordering with CIN III.

Proceeding from our data we can neither propose the mechanisms of spontaneous regression of CIN II and III, nor claim that the rejection of pathologically transformed epithelium observed in CIN I is sufficient for favorable outcome of the neoplasm. At the same time, our findings provide a basis for additional study of the role of apoptosis in the prevention of cervical cancer.

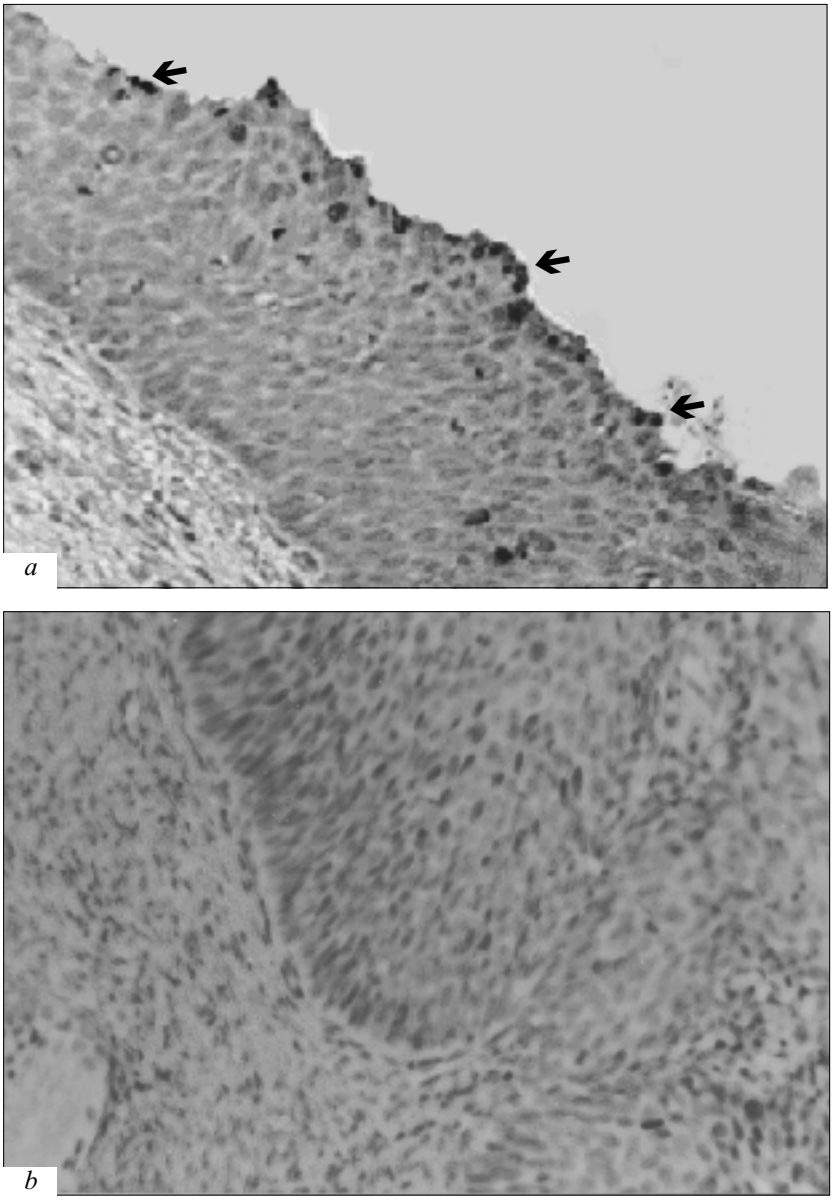

Fig. 3. Apoptosis in the uterine cervix with the morphological symptoms of CIN II (*a*) and III (*b*). *a*) single apoptotic cells predominantly localized in superficial epithelial layers, HPV of moderate oncogenicity; *b*) no cells with specific staining, HPV of high oncogenicity.

TABLE 1. Neoplastic Manifestations and the Presence of HPV in the Cervical Tissue Samples under Study

Index	CIN stage				
	I	I-II	II	III	*r**
The number of samples					
HPV-negative	3	1	2	1	-0.66
HPV-positive	4	3	3	7	0.75
HPV oncogenicity					
moderate	1	2	2	3	0.95
high	0	0	1	5	0.93

Note. *Correlation with the stage of pathology (p<0.01).

In conclusion, the assessment of the intensity, dynamics and localization of apoptosis in the uterine cervix together with the understanding of mechanisms involved in the regulation of apoptosis, among them the oncogenic proteins produced during intracellular development of HPV represent the necessary conditions for future development of therapy based on the induction of apoptosis in pathologically transformed tissues.

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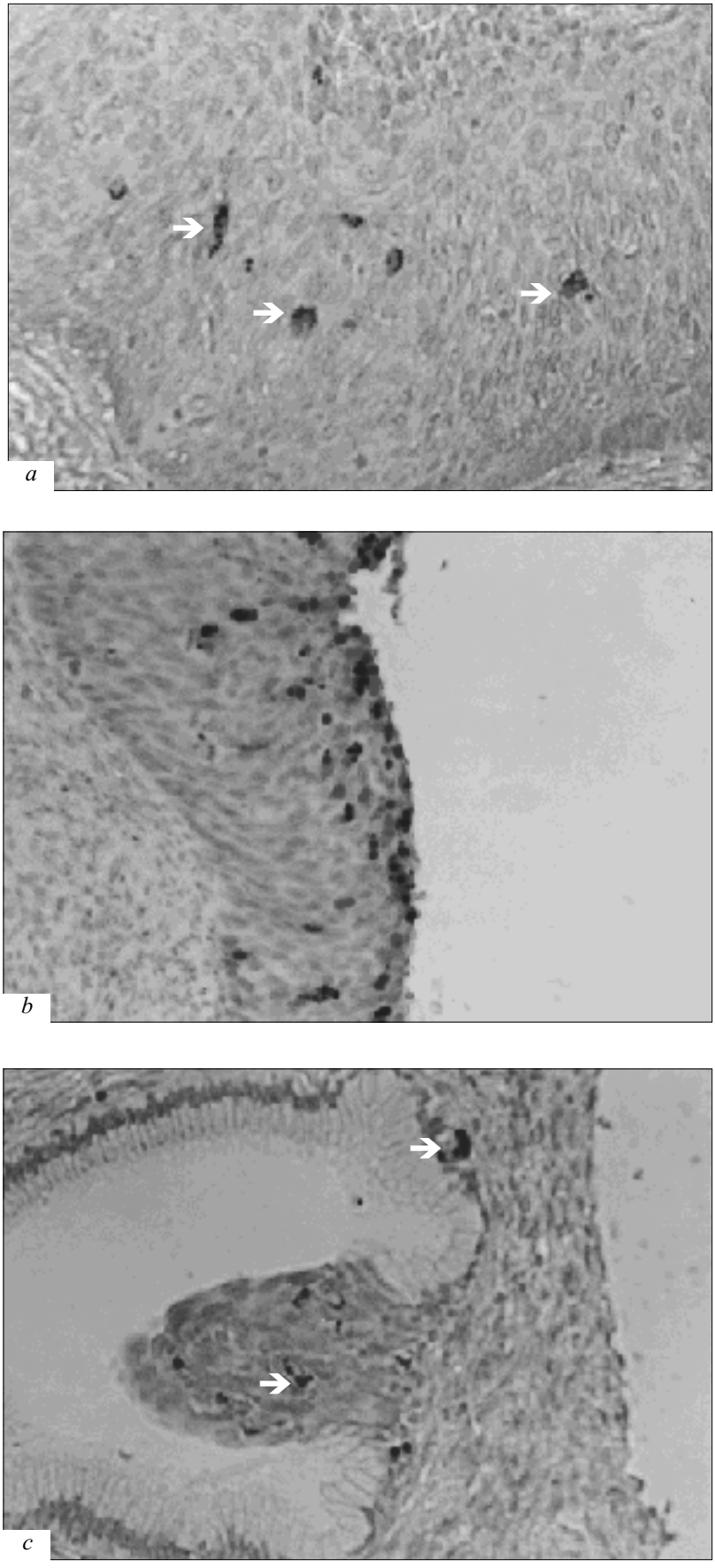

Fig. 4. Apoptosis in the uterine cervix with the morphological signs of CIN III (*a, b*) and preinvasive carcinoma (c). *a*) single cells with moderate staining chaotically scattered in anaplastic epithelium, HPV of high oncogenicity; *b*) numerous cells with intense staining localized in the upper third of epithelium, HPV of high oncogenicity; *c*) cells with moderate staining in preinvasive carcinoma of the cervical gland, no HPV.

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