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EXPRESSION OF THE INITIATOR CASPASE-8 IN THE EPITHELIUM OF PHYSIOLOGICAL ECTOPY IN YOUNG WOMEN, INFECTED WITH HUMAN PAPILLOMAVIRUSES

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Abstract

More and more often clinical cases of severe cervical lesions and cervical cancer are observed against the background of physiological ectopy at a young age. In most of them, Human papillomaviruses (HPVs) are detected at the examination, with severe intraepithelial lesions and initial forms of cancer identifying highly oncogenic strains of HPVs. **The purpose** of the study was to study the expression of the initiator caspase-8 in the epithelium of physiological ectopy in young women infected with HPVs. **Material and methods.** A randomized prospective cohort study was conducted on 180 patients aged 18-25 years, of which 150 were with physiological cervical ectopy and 30 without pathological changes in the cervix. The survey included extended colposcopy, HPV-screening, Pap-test, the quantitative determination of the level of human caspase-8 in lysates of cervical epithelial cells. **Results.** In patients with physiological ectopy of the cervix and the detection of high-oncogenic HPVs there was a low expression of caspase-8 (0.18 ± 0.01 ng/ml), which was less in comparison with the same in patients with physiological ectopy of the cervix and with low-oncogenic HPVs (1.72 ± 0.02 ng / ml) in 9.55 times, in women with physiological ectopy of the cervix and without infection of the HPVs (1.76±0.03 ng/ml) – in 9.78 times, in control group without physiological ectopy of cervical epithelium and without HPVs (1,78±0,04

ng/ml) – in 9,89 times. **Conclusions.** High-oncogenic HPV is actively involved in blocking apoptosis and reducing the activity of the enzyme initiating caspase-8, which leads to the disregulation of the cellular differentiation cycle, exfoliation, and disturbance of physiological metaplasia in the transformation zone.

Key words: physiological cervical ectopy, Human papillomaviruses, extended colposcopy, HPV-screening, Pap-test, caspase-8, physiological metaplasia, apoptosis.

The problem of early diagnosis of precancerous changes and cervical cancer has been relevant for many years [12]. This is due to the high incidence and mortality from cervical cancer, including the fact that in recent decades there has been an increase in the incidence of cervical cancer in young women under the age of 30 years. Scientists around the world are trying to find answers to questions about trigger factors in triggering mechanisms of cervical cancer: why do some women with Human papillomaviruses (HPVs)-associated cervical pathology get malignancy within a few months, and others for several years, and for some are not observed.

Of particular interest today is the tactics of conducting young patients with physiological (congenital) cervical ectopy, complicated by HPVs-infection. Traditionally, the opinion was expressed that the congenital cervical ectopy in women at a young age should be considered a dishormonal condition. Therefore, either an observation or a hormonal correction with estrogen-progestogens is proposed [12, 13]. However, according to many authors, more and more often clinical cases of severe cervical lesions and cervical cancer are observed against the background of physiological ectopy at a young age [13]. In most of them, HPVs are detected at the examination, with severe intraepithelial lesions and initial forms of cancer identifying highly concomitant strains of HPVs [1, 2].

HPVs are small, double-stranded DNA viruses. HPV infection occurs on the surface of the epithelium in the basal layer, and the life cycle of the virus is distinguished according to the differentiation program of the infected cells. The HPV family encompasses ~200 categories [11], of which 40 categories have been isolated from the genital tract [16].

Epidemiological and virological studies confirm that at least 95% of all squamous cell carcinomas of the cervix contain HPV DNA. The frequency of detection of HPV DNA increases with the aggravation of the degree of dysplasia and the transition of dysplastic changes in the epithelium into a tumor [9].

On the basis of HPV clinical associations, HPVs may be divided into two types: high- or low- oncogenic risk. High-risk HPVs participate in the development of cervical neoplasia in persistently infected females [8].

The ability to persist for a long time is possible in connection with the presence of HPV numerous mechanisms that affect the formation of antiviral immune defense and contribute to the deviation of the virus from immunological surveillance and apoptosis of infected cells [14].

The extrinsic apoptotic signaling pathway may be activated, as a part of the host response, and 'death receptors' on the cell surface may be induced by extracellular signals during HPV infection. The death receptors include tumor necrosis factor (TNF) receptor-1 (TNFR-1), Fas/CD95 and TNF-related apoptosis-inducing ligand (TRAIL) receptor (DR4 and DR5), and belong to the TNFR family. The receptors stimulate caspases-8 and -10, leading to the formation of the death-inducing signaling complex. The death-inducing signaling complex also activates the downstream executioner caspases, such as caspase-3 and -7, and triggers apoptosis. In addition to the TNF pathway, it has also been shown that HPV-16 E6 is capable of inhibiting apoptosis stimulated by Fas and TRAIL pathways [7].

All types of HPV share a common genomic structure and encode eight proteins, including six early proteins: E1, E2, E4, E5, E6 and E7, and two late proteins. In particular, E5, E6 and E7 oncoproteins of the high-risk strains are considered to be antiapoptotic oncoproteins, and the main contributors to malignant transformation [4].

HPV E5 inhibits death receptor-mediated apoptosis in human keratinocytes. HPV E5 is capable of downregulating the total amount of Fas receptor and decreasing Fas location, as well as altering the formation of DISC induced by TRAIL; thus, E5 is able to impair Fas ligand (FasL) - and TRAIL-mediated apoptosis [7].

The E6 oncoprotein is involved in two pathways associated with apoptosis, including p53 inactivation and blocking apoptosis [6]. The E6 protein targets intrinsic and extrinsic signaling, protecting the infected cells from multiple apoptotic stimuli and cross-activation between the two pathways.

HPV-16 E7 protein inhibits TNF-α-mediated apoptosis in normal human fibroblasts by upregulating the expression of the inhibitor of apoptosis (IAP) protein, c-IAP2, and by a mechanism involving the suppression of caspase 8 activation [15]. The HPV-16 E7 oncoprotein induces p53-dependent and independent apoptosis. E7 leads to antiapoptotic pRb degradation via a mechanism that involves association with and reprogramming of the cullin 2 ubiquitin ligase complex, indicating that E7 may trigger apoptosis [7].

Caspases is a family of cysteine proteases, which consists of more than 10 related proteins. Caspases are synthesized in the form of inactive proenzymes. Activation of caspases in the process of apoptosis leads to the cleavage of the most important cellular substrates, accelerating the sharp morphological changes in apoptosis. Apoptosis induced by CD95 (Fas / APO-1) and TNF activates caspase-8 (MACH / FLICE / Mch5), thus providing a direct link between the cell death receptors and caspases, i.e. caspase-8 is at the apex of the apoptotic cascade. The CASP8 gene contains at least 11 exons overlapping approximately 30 kb of the 2q33-34 region of the human chromosome. Caspase-8 is critical for generating death cell signals to eliminate potentially malignant cells. The genetic variation of CASP8 can affect the susceptibility to cancer. The absence of caspase-8 may predispose the cells to further oncogenic mutations or allow spontaneous oncogenic changes to accumulate more easily. The exact genetic mechanism (mechanisms) by which the deficit of caspase-8 promotes transformation remains little investigated [3, 10].

The purpose of the study was to study the expression of the initiator caspase-8 in the epithelium of physiological ectopy in young women infected with HPVs.

Material and methods

A randomized prospective cohort study was conducted on 180 patients aged 18-25 years, of which 150 were with physiological cervical ectopy and 30 without pathological changes in the cervix. Diagnosis was established using extended colposcopy. All patients were screened for the presence of HPVs. Depending on the results, 3 groups of patients were formed: group I (n = 50) – patients with cervical ectopy and high-oncogenic risk HPVs infection, group II (n = 50) – patients with cervical ectopy and low-oncogenic risk HPVs infection, group III (n = 50) – patients with cervical ectopy and without HPVs infection. A control group of women K (n = 30) was also recruited, in which no pathological changes in the cervix were detected according to the extended colposcopy, and at the time of the survey there were negative indicators for the HPVs. In patients of all groups in anamnesis there were no births, abortions, conservative, ablative and surgical treatment of the cervix.

Extended colposcopy was performed using the colposcope "MK 200" (Ukraine, Cherkassy). The evaluation of the obtained data was carried out according to the International Classification of Colposcopic Terms, IFCPC, Rio de Janeiro (2011) [11].

The cytomorphological study was carried out according to Papanicolaou's method and was evaluated according to the Terminology System Bethesda (2001) [11]. In evaluating cytological preparations, attention was drawn to the presence of cytological signs of HPVs infection.

The proteolytic activity of caspase-8 was determined in the lysates of epithelial cells from the scrap of the cervical epithelium. In women with cervical ectopy, it was a cut-off of cells from the zone of transformation, in patients of the control group – scrapes of cells of exocervix.

The following extraction protocol was used to prepare lysate cells. The cell suspension was precipitated by centrifugation. Cells were washed once with PBS. The precipitate was resuspended in lysis buffer, incubated for 60 minutes at room temperature under neat shaking. The extracts were transferred to microcentrifuge tubes and centrifuged at 1000 turnovers per minute for 15 min. The quantitative determination of the level of human caspase-8 resulted in the use of enzyme-linked enzymes (Bender MedSystems, ELISA).

Antibodies to caspase-8 were sorbed in microplate wells. Samples of the test material and standards containing caspase-8 interact with captivating antibodies that were sorbed in the microplate wells. As the primary exciting antibodies, detective rabbit polyclonal antibodies to caspase-8 were used. The detectable antibodies that remained unbound were removed by washing. Then, rabbit IgG antibodies that were conjugated to horseradish peroxidase (anti-IgG-HRP) were introduced into the wells. After incubation, the microplate wells were washed to remove anti-IgG-HRP and subjected to a solution of the substrate that interacts with HRP. The reaction was stopped by the addition of a stop solution, and then the absorbance of the wells was calculated at a wavelength of 450 nm. The amount of converted substrate, which was determined colorimetrically, by measuring absorption, was proportional to the caspase -8 concentration. Based on the values obtained for the 7 standards, a calibration curve was constructed, and the caspase-8 concentrations in the samples under study were determined by interpolation from the calibration curve.

ll participants were informed about participation in the study and gave voluntary consent.

Statistical processing of the results was carried out using Statistics 13.0 (Dell StatSoft, USA). The average (M), the standard deviation error (SE) was determined. The parametric and non-parametric criteria (t-criterion of the Student, the U-criterion of Wilcoxon-Mann-Whitney, χ^2-criterion, odds ratio) were used for comparison of the data.

Results and discussion

The age distribution in the groups was homogeneous: group I – 21,27 ± 0,23 years, group II – 21,14 ± 0,19, group III – 21,02 0,32, group K – 21,33 ± 0, 41. According to the social status, gynecological, reproductive and infectious anamnesis, the study groups did not have statistically significant differences.

According to the Pap-test, it was found that in patients of group I in the majority of cases, in comparison with group II, changes were observed for HPVs (koylocytosis, dyskeratosis, dual core cells), 68.0% and 48.0% of women respectively (OR 2.32; 95% CI 1.02-5.19) (Table 1). Patients of groups III and K had no cytological characteristics of HPV, according to the Pap-test. Hyperkeratosis and parakeratosis were found in cytograms of the same frequency in all three groups: I – 44.0% of patients, II – 38.0%, III – 38.0%.

Negative for intraepithelial lesion or malignancy (NILM) had 24.0 % of patients in group I (OR_{I-III} 0.05, 95% CI 0.02-0.14), 44.0 % of group II (OR_{II-III} 0.13; 95% CI 0.05-0.34) and 86.0 % of group III. Atypical squamous cells undetermined significance (ASCUS) were found in 50.0 %, 38.0 %, 14.0 % of patients, respectively. Inflammatory type of smear (reactive and reparative changes) of the epithelial layer of the cervix was observed in 20.0 % of patients in group I, 18.0 % and 12.0 %) of groups II and III, respectively. Low-grade squamous intraepithelial lesions (LSIL) were found in 18.0 % and 14.0 % of patients in groups I and II; high grade squamous intraepithelial lesion (HSIL) were reported in 8.0 % and 4.0 % of women in groups I and II. In patients of the control group, according to the cytological data, squamous intraepithelial lesions were not present.

Table 1

Cytological characteristics of scrapings from the area of cervical transformation in patients with physiological ectopy of the cervix

Cytological data	Group I, n=50		Group II, n=50		Group III, n=50	
	n	%	n	%	n	%
Coilocytosis	34	68,0 ^{II,III}	24	48,0 ^{I,III}	0	0,0 ^{I,II}
Hyperkeratosis and dyskeratosis	26	52,0	24	48,0	18	36,0
NILM	12	24,0 ^{II,III}	22	44,0 ^{I,III}	43	86,0 ^{I,II}
ASCUS	25	50,0 ^{III}	19	38,0 ^{III}	7	14,0 ^{I,II}
Inflammatory changes	10	20,0	9	18,0	6	12,0
LSIL	9	18,0	7	14,0	0	0,0
HSIL	4	8,0	2	4,0	0	0,0

Note: $^{I, II, III}$ – a statistically significant difference with group I, II, III ($p<0,05$).

According to the data of extended colposcopy in group I with HPV infection, in 78.0% cases, there were severe lesions (grade II of abnormal colposcopic picture) such as sharp

border; inner border sign; ridge sign; dense acetowhite epithelium; coarse mosaic; coarse punctuation; rapid appearance of acetowhitening; cuffed crypt (gland) openings; loop-shaped vessels (Table 2).

Table 2

Colposcopic findings in patients of the studied groups

Colposcopic findings	Група I, n=50		Група II, n=50		Група III, n=50	
	Абс.	%	Абс.	%	Абс.	%
Fine mosaic	34	68,0 ^{II,III}	18	36,0^{I,III}	2	4,0 ^{I,II}
Fine punctation	11	22,0^{II}	26	52,0^{I,III}	5	10,0
Coarse mosaic	21	42,0 ^{II,III}	6	12,0 ^{I,III}	0	0,0 ^{I,II}
Coarse punctuation	11	22,0 ^{II,III}	3	6,0 ^{I}	0	0,0 ^{I}
Leukoplakia	17	34,0 ^{II,III}	5	10,0 ^{I,III}	0	0,0 ^{I,II}
Loop-shaped vessels	22	44,0 ^{II,III}	12	24,0 ^{I,III}	0	0,0 ^{I,II}
Intraepithelial condiloma	13	26,0 ^{II,III}	2	4,0 ^{I}	0	0,0 ^{I}
Crests	9	18,0 ^{II,III}	2	4,0 ^{I}	0	0,0 ^{I}

Note: $^{I, II, III}$ – a statistically significant difference with group I, II, III ($p<0,05$).

As can be seen from table 2, women with physiological cervical ectopy infected with high-oncogenic risk HPV compared to patients with physiological cervical ectopy infected with low-oncogenic HPV, revealed a greater 1.89 times the frequency of fine mosaics (OR 3.78, 95% CI 1.65-8, 65); in 3.50 – coarse mosaics (OR 5.31; 95% CI 1.91-14.75); in 3.67 – coarse punctuation (OR 4.42; 95% CI 1.15-16.97); in 3.40 – leukoplakia (OR 4.64; 95% CI 1.55-13.84); in 1,83 – loop-shaped vessels using a green filter (OR 3.73; 95% CI 1.45-9.60); in 6.50 – intraepithelial warts (OR 8.43; 95% CI 1.79-36.70); in 4.50 – crests (OR 5.27; 95% CI 1.08-25.79).

According to the results of the analysis of the studies of caspase-8 expression in the cervical epithelium, it was found that in the group I in patients with physiological ectopy of the cervix and the detection of high-oncogenic HPV there was a low expression of caspase-8 (0.18 ± 0.01 ng/ml), which was less in comparison with the same in group II in patients with physiological ectopy of the cervix and with low-oncogenic HPV (1.72 ± 0.02 ng / ml) in 9.55 times, in group III with physiological ectopy of the cervix and without infection of the HPV

(1.76±0.03 ng/ml) – in 9.78 times, in group K without physiological ectopy of cervical epithelium and without HPV (1,78±0,04 ng/ml) – in 9,89 times.

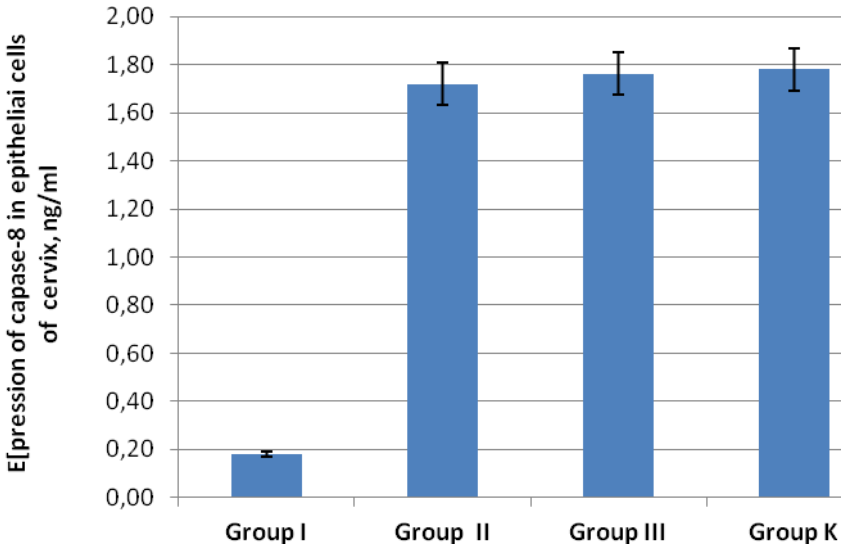

Fig. Expression of caspases-8 in the epithelial cells of the cervix

This once again confirms the role of high-oncogenic HPV in blocking apoptosis and reducing the activity of the enzyme initiating caspase-8, which leads to the disregulation of the cycle of cell differentiation and harmonization of maturation and exfoliation of the epithelium. The same events explain the integration of the virus into the host cell genome and create ideal conditions for the persistence of the viral infection, which in turn can lead to the development of neoplastic transformation of the cervical epithelium, and also indicates the formation of its irreversible changes that occur during the extended colposcopy. Normally, the physiological ectopy of the cervical epithelium is accompanied by processes of benign metaplasia, which should be completed by the age of 23-25, but in the presence of any endogenous or exogenous initiating factors, epithelization processes are slowed down or unhealthy epithelization is observed, especially when it is observed when infected with high-oncogenic HPV, the role which has already been proven in the development of precancerous changes in the cervical epithelium.

Conclusions

High virus contagiousness, histopathological features of the cervix in the presence of congenital ectopy, hormonal and immunological shifts, harmful habits, as well as high sexual activity make young women very vulnerable to HPV. High-oncogenic HPV is actively involved in blocking apoptosis and reducing the activity of the enzyme initiating caspase-8,

which leads to the disregulation of the cellular differentiation cycle, exfoliation, and disturbance of physiological metaplasia in the transformation zone.

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