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BASIC RESEARCH

Reparative Spheroids in HPV-Associated Chronic Cervicitis

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Abstract

Background: Spheroid cell structures (SCS) described in cell culture are used to study cell-cell and cell-matrix interactions. However, the role of the SCS in the repair process *in vivo* remains unexplored. ***The aim*** of the study was to examine the cellular composition of the spherical structures and their functional significance in the repair of the squamous epithelium in human papilloma virus-associated chronic cervicitis (HPV-CC).

Methods and Results: The cytology and biopsy materials from 223 patients with HPV-CC were subjected to molecular testing for HPV DNA by Real-Time Polymerase Chain Reaction (Real-Time PCR) with genotyping and chromogenic *in situ* hybridization (CISH), as well as immunocytological and immunohistochemical analyses of p16INK4A, Ki67, SMA, Vimentin, CD34, E-cadherin, Oct4, CD44, CKW markers. In the stem cell niche zone, these spheroid structures were discovered having proliferative activity and showing signs of producing stem cells involved in the repair of the cervical mucosa in HPV-CC.

Conclusion: The persistence of the HPV in the stem cell niche zone cells in the cervix determines the chronization of inflammation in this area, with the ability to perform pathological repair. The immunophenotype of the spheroid cell structures in the HPV-CC includes cells with signs of stem cells ('stemness') and the mesenchymal-epithelial transition.

Keywords: *stem cell niche lesion; mesenchymal-epithelial transition; chronization of inflammation.*

Introduction

The SCS over the recent years have captured the attention of many researchers. More than 2000 works according to PubMed 01.07.2013 have been focused on this problem. Vast majorities of studies have been conducted on the cultures of non-transformed and cancer cells (3D culture models) and fetal tissues; a few studies were performed with animal experiments with the transfer of the SCS in the peritoneal cavity. However, the first mention of these multicellular spheroid structures was in the '80s of the previous century [1-3]. These cellular structures were described under various names, such as, reparative modules and epithelial-mesenchymal spheroids (EMSs) [4], micro-mass [5], mammosphere [6], and micro-tissue structures [7]. The spheroids were obtained by culturing the chondrocytes [8], hepatocytes [9], and tumor cells of various tissues [10-12].

According to E. Fennema et al. [13], the spheroid structures could be used to study the cell-cell and cell-matrix interactions, the properties of the progenitor and stem cells, as well as to create transplants. In addition, according to I. Saburina [4], the plasticity of the EMSs may serve as the donor of the epithelium for the stroma in the damaged areas.

"Granulation tissue" described by R. Virchow consists of loops of the newly produced capillary-type blood vessels and mesenchymal elements forming macroscopically visible "granules", possibly an analog of the cellular spheroids in the tissue culture. However, "the spheroid structures" in the granulation tissue are composed solely of mesenchymal derivatives. We observed EMSs formation in the squamous epithelium, in the diseases of the cervix associated with HPV [14]. However, the question regarding their cellular composition and functional significance still remains open.

Material and Methods

A clinical-morphological and molecular-biological investigation of the materials obtained from 223 women of reproductive age with chronic cervicitis associated with high-risk types of human papilloma virus (HR-HPV) was performed. Group 1 consisted of 48 patients with HR-HPV and no other pathology of the cervix. Group 2 comprised 60 patients with HR-HPV-CC in combination with cervical intraepithelial neoplasia (CIN 1). Group 3 included 115 patients with HR-HPV-CC in combination

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with CIN 2-3. The women ranged in age from 28 to 43 years. All the women were examined and treated at the Center in 2012.

All the patients underwent molecular HPV DNA testing with genotyping of the virus, cytology testing (using the liquid-based cytology method) and cervical biopsy with immunocytological and immunohistochemical analyses of p16INK4A, Ki67.

The cervical samples were collected using an endocervical brush and placed in SurePath medium. The cervical samples were used for liquid-based cytology and DNA testing in the Cobas system (Roche Molecular Systems, USA). The Cobas system is a fully automated system that detects DNA on three separate channels: for HPV-16, HPV-18 and the pooled HPV of 12 genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).

Liquid-based cytology was performed in the B&D TriPath system. The specimens were subjected to Papanicolaou staining. The results were evaluated based on the Bethesda classification (Wright TCJr, et al., 2006). In parallel, the same material was studied using immunocytochemistry CINtec PLUS cytology kit (Roche, Germany) to detect the expression of p16INK4A and Ki67. Furthermore, we were trying to detecting the Oct4 expression (MRQ-10, 1:100, Cell Marque, USA).

The biopsy material was presented as cervical scrapings in the cases of CC in combination with CIN1, and with the operating pictures (the remote cervix cones) in the cases of CC in combination with CIN 2-3 to study HPV-CC in the surrounding intact cervical mucosa. Biopsies were fixed in 10% neutral formalin and embedded in paraffin. The serial paraffin sections were stained with hematoxylin and eosin staining; the immunohistochemical detection of the p16INK4A, Ki67 (Roche, Germany), SMA (SP171, 1:50, Springbioscience, USA), Vimentin (SP20, 1:200, Springbioscience, USA), CD34 (QBEnd/10, 1:100, Cell Marque, USA), E-cadherin (EP700Y, RTU, Cell Marque, USA), Oct4 (MRQ-10, 1:100, Cell Marque, USA), CD44 (DF1485, 1:50, Dako, Denmark), CKWSS (1:500, Dako, Denmark), and p16INK4a (E6H4, CINtec histology kit, Roche, Germany) expression was performed using conventional methods. We used the Dako REAL EnVision Detection System (Dako, Denmark) kit for secondary antibodies. Positive and negative control reactions were performed.

In addition, in the serial paraffin sections (n=27), we determined the localization of the HR-HPV DNA (16, 18, 31, 33, and 35) by CISH using ZytoFast PLUS (Germany) kit. Hybridization was performed with digoxigenin-labeled DNA probes. The hybridization results were visualized by the primary anti-digoxigenin antibodies, which were detected by the secondary antibodies. The reaction with diaminobenzidine led to the formation of the resistant brown-colored stain that was seen under the light microscope. Positive and negative controls were performed, which were represented by the histological slices of the cervix tested for the presence of HPV using Real-Time PCR.

Results

In the first phase, we evaluated the distribution of HR-HPV among those patients with HPV-CC from groups 1-3. Evidence of the HPV genotypes was found in these groups. For example, HPV-16 and HPV-18 were not found in the Group 1 patients. All the 48(100%) patients were infected with the pooled HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). In Group 2, the Cobas test revealed HPV-16 DNA in 12 (20%) women, whereas the combination of HPV-16 DNA with the other 12 types was determined in 48 (80%) women. In Group 3, the individual HPV-16 DNA was identified in 100 (87%) women, whereas the combination of the HPV-16 DNA with the other 12 types was identified in 15 (13%) women.

In the second phase, the cytological diagnosis of CC was performed. In the cytology samples of Group 1 patients, we found koilocytes and signs of chronic inflammation as evidence of the reactive epithelial changes in combination with the symptoms of leukopedesis and the presence of inflammatory cells in the cytology samples (lymphocytes, macrophages, eosinophils, fibroblasts). In the groups with the HR-HPV-CC with CIN combination, a combination of these changes was observed with the presence of atypical squamous cells with dyskaryosis, hyperchromia and polymorphism of the nuclei with the formation of multicellular clusters in the samples. In the control group, only the cytologic signs of CC were recorded. In the immunocytochemical study, solitary proliferating Ki67- positive cells of the squamous and glandular epithelium, as well as solitary senescent cells expressing p16 were found in the HVP-CC patients. In the samples of HR-HPV-CC women with CIN1, on double staining, atypical cells simultaneously expressing p16 and Ki67 markers were identified, which comprised from 15 to 100% of the squamous epithelium. We found multicellular clusters consisting of monomorphic cells with no signs of atypia, which expressed Ki67, Oct4 (Fig. 1a-b), an embryonic stem cell marker [19] and p16INK4A- negative cells in 5 cases of Group 1.

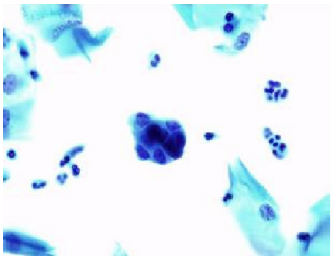

Fig. 1(a).
SCS in liquid-based cytology sample. Stained by Papanicolaou (x 1000)

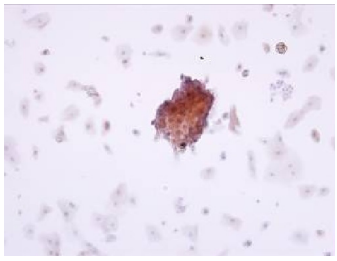

Fig. 1(b).
Oct4+ cells in the liquid-based cytology samples. Immunocytohemical staining (x1000)

In the third phase, we conducted histological and immunohistochemical studies of the biopsy and surgical specimens [20-22]. The histological examination of the cervical mucosa from the HPV-CC women revealed a specific complex of morphological signs, such as lymphohistiocytic infiltration with some plasma cells, as well as the phenomenon of neo-angiogenesis in the lamina propria, leukopedesis of the lymphocytes, acanthosis and koilocytosis in the squamous epithelium.

In 56 cases from among the 223 (25%) HPV-CC patients, the thickness of the squamous epithelium revealed epithelial-mesenchymal SCS composed of the squamous cells of the basal and parabasal types with intact cell-cell contacts in the form of so-called bridges. Squamous cells with the CKWS expression in SCS had the preserved the intercellular contacts called “bridges” (Fig. 1c-d). In the center of several SCS, endothelium was present, located on the basal membrane. In addition, it was possible to see the formation of SCS in the zone of the stem cell niches around the invaginated blood capillaries. On double staining of the p16INK4A and Ki67 in the SCS described, only the expression of Ki67 was detected in some epithelial and endothelial cells. In

the HPV-CC patients, the p16INK4A oncomarker was absent in the SCS cells. Some SCS contained koilocytes and cells with apoptotic changes (Fig. 1e-f). Formations such as SCS were found, although in the CIN area they differed morphologically, and expressed the Ki67 and r16INK4A oncomarkers on double staining.

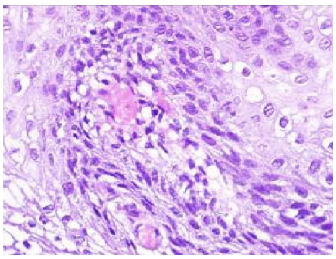

Fig. 1(c).
SCS with koilocytes and the capillary in centre. Stained with H&E (x 600)

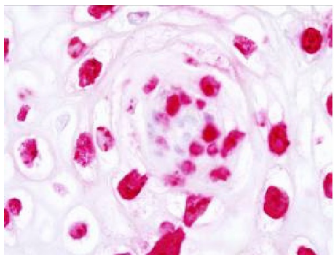

Fig. 1(d).
SCS with proliferation of the Ki67+ cells and the P16INKA4- cells. Immunohistochemical staining (x 600)

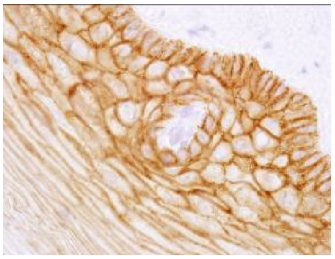

Fig. 1(e).
E-cadherin+ cells in SCS. Immunohistochemical staining (x 600)

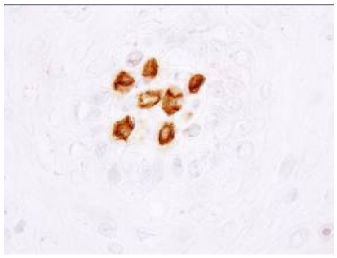

Fig. 1(f).
CD 34+ cells in SCS. Immunohistochemical staining (x 600)

In the fourth phase, we studied the immunophenotype of the SCS in the squamous epithelium of those with HPV-CC. The SCS are clearly visible in the CC areas, due to the greater expression of Oct4, CD34-, CD44-, and E-cadherin compared with the adjacent epithelium (Fig. 1d-e; Fig. 2). Thus, many SCS were localized in the cervical transformation zone, which is the stem cell niche, located directly on the basal membrane. Oct4 was detected as a brown-colored staining of the nuclei and cytoplasm of the cells, located around the perimeter of the SCS or at one of its poles, as well as in certain endothelial cells adjacent to the SCS, and in the connective tissue cells (Fig. 2a-b). The expression of CD34 was observed in the endothelium of the SCS capillaries; the reaction product was localized in the endothelial cell cytoplasm in the center of the SCS or in the invaginated blood capillaries close to the basal membrane.

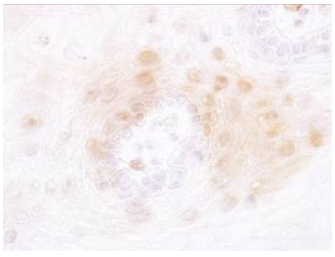

Fig. 2(a).
Oct4+ cells in SCS. Immunohistochemical staining (x 600)

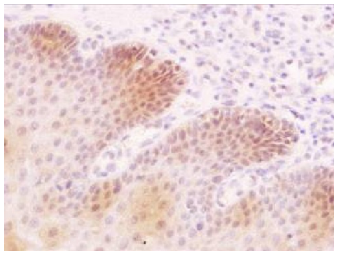

Fig. 2(b).
Oct4+ cells in SCS in the zone of the stem cell niche of the cervix. Immunohistochemical staining (x 600)

Besides, CD44 and E-cadherin were found in the cells of the squamous epithelium of the SCS (Fig. 2c). In this case, the intercellular bridges (desmosomal contacts) were clearly contoured. Mainly, Vimentin was detected in the capillary cells (Fig. 2d); however, Oct4 was found in the cytoplasm and nuclei of some epithelial cells in the SCS as well. The question regarding their origin in the SCS in the squamous epithelium is still not answered, but is probably due to the phenomenon of mesenchymal-epithelial transition (MET) in the SCS of the cervical squamous epithelium, as we discovered. Thus, the SCSs contained the cells with the epithelial immunophenotype (CK WSS+ и E-cadherin+), with the mesenchymal immunophenotype (Vimentin+, SMA+, CK WSS-, and E-cadherin-) and the epithelial-mesenchymal phenotype (Vimentin+, SMA+, CK WSS+, and E-cadherin+).

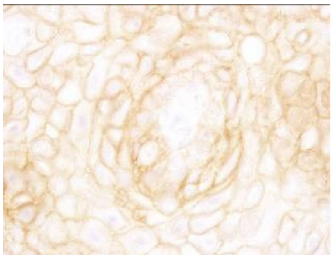

Fig. 2(c).
CD 44+ cells in SCS. Imunohistochemical staining (x 600)

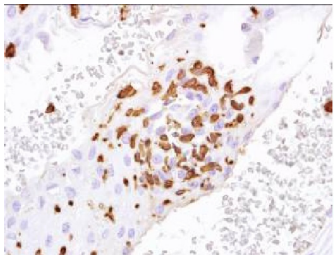

Fig. 2(d).
Vimentin+ cells in SCS. Immunohistochemical staining (x 600)

In the fifth phase, we investigated the localization of HR-HPV DNA (16, 18, 31, 33, and 35) using CISH in the cervical biopsies of patients with CC. Viral DNA was detected in the nuclei of the parabasal and basal epithelial cells, koilocytes, as well as in the capillary endothelium, fibroblastic cells and cells of the inflammatory infiltrate (Fig. 2e-f).

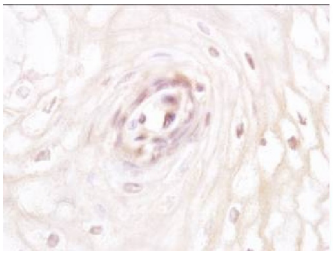

Fig. 2(e).
DNA HPV in the forming SMS. CISH (x 100)

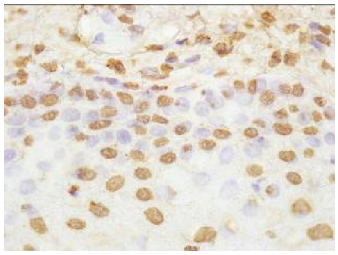

Fig. 2(f).
DNA HPV in the squamous epithelium and the capillary endothelium in the zone of the stem cell niche of the cervix. CISH (x 1000).

Discussion

In the HPV genotyping test we found that the characteristic of the HPV-CC without CIN is the absence of HPV-16. In contrast, the combination of the HPV-CC with CIN 1-3 was characterized by HPV-16 presence, both individually and in combination with the other HR-HPV DNA genotypes. Similar results have been described by several authors [15-17]. The HPV-18 was not detected, which is consistent with the published data on its active participation in the occurrence of hysterocarcinoma [18]. It should be noted that the HPV DNA was detected in the zone of the stem cell niche, where an infection was present not only in the epithelial cells, including SCS, but in the vascular endothelium in the MET areas as well. Thus, it can be assumed that HPV is involved in MET. This assumption is also confirmed by the literature. In the experiments, in the HPV16 E6/E7

infected cell culture, HPV was revealed to induce the phenotypic and molecular programming of the MET in the culture of normal keratinocytes. The fibroblast growth factor was found to inhibit the expression of the epithelial marker E-cadherin and increase the expression of Vimentin, the mesenchymal marker [24-25]. In cervical cancer, Lee et al., also observed MET programming [26].

Therefore, the spheroid structures represented by the cell clones having signs of stem cells and functioning as the reparative formations in the HPV-CC patients, contained HR-HPV types that, probably, can potentiate MET.

The spheroidal structures discovered by us in the HPV-CC were composed of cells with proliferative activity and signs of 'stemness'. Considering the proliferative activity of the SCS, the cellular structure and the lack of p16INK4A expression lead us to suggest that these structures are involved in the repair of the cervical mucosa in HPV-CC. The composition of the reparative SCS in HPV-CC includes those cells with the expression of the stem cells markers, which are involved in squamous epithelium repair.

The phenomenon of epithelial-mesenchymal transition (EMT) has been described in the earlier studies on tissue culture and *in vivo* during the invasion and metastasis of the malignant tumors. MET is the reverse process of EMT, which is discussed in some chronic inflammatory diseases [23]. Our data and literature data confirm the existence of MET in HPV-CC and enables one to assume that the cells with signs of 'stemness' in the SCS may be a result of the MET, possibly, with the participation of the tissue stem cells and, also possibly with bone marrow-originated mesenchymal stem cells.

Findings:

1. The persistence of the HPV in the cells of the stem cell niche zone of the cervix causes the chronization of inflammation here, having the capacity for pathological repair.

2. Regarding the repair of the cervical mucosa in HPV-CC, we found the participation of special epithelial-mesenchymal SCSs, which had been formed in the zone of the stem cell niche and were able to accomplish the proliferative activity.

3. The cells of the spheroid structures in the HPV-CC are of the heterogeneous immunophenotype and include cells with the signs of stem cells and the mesenchymal-epithelial transition which, in turn, suggests the possibility of MET in the repair of epithelium in the HPV-CC.

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