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Human Papillomavirus (HPV) and Epstein-Barr Virus (EBV) Cervical Infections in Women with Normal and Abnormal Cytology

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A b s t r a c t

In 48 adult women, subdivided into group 1 with no cervical intraepithelial neoplasia (CIN-negative) and group 2 (CIN-positive), endocervical scrapes were tested for the presence EBV DNA and HPV DNA using PCR-ELISA. In addition, attempts were made to detect HPV 16 and HPV 18 using other PCR amplification techniques. In parallel, in biopsies of uterine cervix obtained from group 2 patients, presence of EBER was documented by RNA *in situ* hybridization (ISH). Sera of all patients were tested for anti-EBV antibodies. In group 1, presence of EBV DNA was noted in the material obtained from 8 women (30.8%), while HPV DNA was detected in 2 women (7.7%). In group 2, EBV DNA was present in the material obtained from 11 patients (50%), including 7 (31.8%) with HPV DNA also identified. In 5 women (22.7%) of group 2 only HPV DNA was detected. The identifical HPV DNA in all cases belonged to HPV 16 type. Both in group 1 and in group 2, all patients were found to carry serum IgG-anti-VCA and IgG-anti-EBNA antibodies. The results allow to conclude that, co-infection with EBV and HPV 16 may be of cervical significance in etiopathogenesis of uterine cervical cancer.

K e y w o r d s: human papillomavirus, Epstein-Barr virus, cervical neoplasia, carcinogenesis

Introduction

At present, both human papillomavirus (HPV) and Epstein-Barr virus (EBV) infections are known to be associated with potential development of epithelioid malignancies (zur Hausen, 1991; Anagnostopoulos *et al*., 1996; Szkaradkiewicz, 2003). Currently, more than 100 types of HPV have been recognised, among which HPV 16 and HPV 18 represent the major causative agent of uterine cervical cancer (Bosch *et al*., 1995; Bosch *et al*., 2002). In the process of carcinogenesis, two oncoproteins, representing products of E6 and E7 HPV genes seem to play a significant role. E6 is a protein capable of inactivating p53 gene while activity of E7 protein leads to inactivation of pRb gene, the key controller of cell cycle at the G1 phase (Munger *et al*., 1992). As demonstrated by *in vivo* experiments, E6 and E7 may act synergistically, inducing tumour development (Munger *et al*., 1992; Furumoto *et al.*, 2002). Another virus, the infectious mononucleosis-inducing EBV, may also play a significant role in carcinogenesis. Such a role for EBV has been documented in the etiopathogenesis of endemically manifested nasopharyngeal carcinoma (NPC) (Le Roux *et al*., 1998), and recent studies point to its association with some cases of oropharyngeal cancers (Szkaradkiewicz *et al*., 2002), and with pathogenesis of cervical tumours (Landers *et al.*, 1993). In the process of neoplastic transformation of epithelial cells, an important part seems to be fulfilled by the latent membrane protein-1 (LMP-1), produced by EBV both in the course of a productive and a latent infection. A direct effect of the interaction involves activation of cellular gene transcription, mediated by NFκB, and expression of the anti-apoptotic bcl-2 gene (Wensing *et al.*, 2000).

Considering the above data, in the present study we investigated development of uterine cervix infections with HPV and/or EBV in women with normal and abnormal cytology.

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Experimental

Materials and Methods

Patients. Results of gynaecological, cytological and histological studies permitted to distinguish 2 groups of women: group 1 including 26 women at the age of 21–43 years (mean 29,9 ± 5,9), which were free of cervical intraepithelial neoplasia (CIN-negative, Papanicolau II°), and group 2 including 22 patients at the age of 24–44 years (mean 35 ± 6,3) with histologically demonstrated cervical intraepithelial neoplasia (CIN-positive). Considering the histology, in the group 2 the subgroup 2a of 12 patients with mild dysplasia (CIN I) or a moderate dysplasia (CIN II) and the subgroup 2b of 10 patients with severe dysplasia or with carcinoma *in situ* (CIN III) were distinguished.

Endocervical scrapes for cytological examination were collected from a disc of the vaginal portion and from the external orifice of the canal using Cytobrush Cell Collector (Medscand Medical). The material was applied to the microscope slide and immediately fixed with the Cytofix preparation (Biochetest). The preparations were classified as described by Papanicolau (Papanicolau, 1954). In all the patients in whom cytological III° result was obtained, samples of uterine cervix disc were also obtained and histological examination was performed.

Material for virological studies was sampled from a disc of uterine portion and from external orifice of cervical canal using brush collectors with a transportation medium (Cervical Sampler, Digene).

In all studied women of groups 1 and 2 samples of 2 ml peripheral blood were obtained to perform serological tests for presence of anti-EBV antibodies.

Extraction of DNA. DNA was isolated from the uterine cervix material using the Perfect gDNA Blood Mini Isolation Kit (Eppendorf). Presence of genomic DNA was verified employing a primer pair for β-globin, coding a region of 326 bp (TIB MolBiol, Poznań).

Determination of EBV DNA. EBV-DNA was detected using PCR-ELISA technique (Sharp Signal System; Digene). PCR reaction was conducted according to Digene procedure, using EBV1 primer and biotinylated EBV2 primer. The obtained biotinylated PCR product was estimated using a microplate colorimetric technique with single-stranded RNA probe. The immobilised hybrids were detected using anti-RNA-DNA antibodies, conjugated with alkaline phosphatase. The absorbance was recorded using Behring Microstrip Reader, at $\lambda = 405$ nm. The cut-off value was calculated as recommended in the manufacturer's instruction: $2 \times \overline{X}NPCR + 0.100$, where $\overline{X}NPCR$ represented mean absorbance for PCR product in the negative control. In our studies, $\overline{X}NPCR = 0.098$, which corresponded to the cut-off value of 0.296.

Determination of HPV DNA. HPV-DNA was detected using PCR-ELISA technique (Sharp Signal System; Digene). PCR reaction was conducted according to Digene instruction with the defined primers (MY09 and the biotinylated primer MY11) for HPV types: 6/11/42/43/44 and 16/18/31/33/35/39/45/51/52/56/58. The obtained biotinylated PCR product was analysed by a colorimetric microplate technique using a complementary single-stranded probe. The immobilised hybrids were detected using alkaline phosphatase-conjugated anti-RNA-DNA antibodies. The absorbance was quantitated using the Behring Microstrip Reader, at $\lambda = 405$ nm. The cut-off value was calculated as recommended by the instruction: $2 \times \overline{X}NPCR + 0.100$, where $\overline{X}NPCR$ represents mean absorbance for PCR product in the negative control. In our studies, $\overline{X}NPCR = 0.086$, which corresponded to positive cut-off value of 0.272.

Determination of HPV 16 and HPV 18. DNA sequences specific for HPV 16 (160 bp) and 18 (240 bp) genomes were detected using PCR amplification employing primers homologous to E6 region (TIB Molbiol). The detection was performed in 2% agarose gel, using ethidium bromide (Sigma).

Detection of EBER (EBER 1 and EBER 2) in tissue material. EBV DNA product in the form of untranslated RNA (EBER 1 and EBER 2) particles was detected in tissue material using *in situ* hybridization (ISH) (Howe *et al*., 1986). The tissue material was fixed in formalin and embedded in paraffin. It originated from uterine cervix samples obtained from women with Papanicolau-stained cytological smears graded III°. Five μm thick sections were deparaffinised and digested with proteinase K for 30 min at 37°C, washed in DEPC. This was followed by inactivation of proteinase K in 0.4% PFD solution for 20 min at 4°C. The hybridisation was performed using fluorescein-labelled RNA probe of 15 nucleotides in length (PNA Probe/FITC; DakoCytomation) for 15 h at 37°C. After a thorough washing in SWS solution (DakoCytomation) the product was detected using anti-FITC/AP antibodies. BCIP/NBT (PNA ISH Detection Kit; DakoCytomation) was used as a substrate.

Determination of serum anti-EBV antibodies. Anti-EBV antibodies were quantitated by ELISA. Sera of studied women were tested employing kits for antibodies directed to the early antigen, IgG-anti-EA (ETI-EA-G; DiaSorin), antibodies directed to viral capsid antigen, IgM-anti-VCA (ETI-VCA-M; DiaSorin), IgG-anti-VCA (ETI-VCA-G; DiaSorin) and antibodies reactive with EBV nuclear antigen, IgG-anti-EBNA (ETI-EBNA-G; DiaSorin). Absorbance was read at $\lambda = 450/630$ nm, using the Behring Microstrip Reader. Results were expressed in AU/ml, where AU corresponded to an arbitrary unit established by comparison with antibody standard. Values ≥20 AU/ml in the above described tests were considered positive.

Statistical analysis. Differences in frequencies of positive results were assessed by Fisher's exact test and were considered significant at $P < 0.05$.

Results

Results of viral DNA quantitation by PCR technique in the uterine cervix material in the distinguished groups of women are shown in Table I. In group 1, presence of EBV DNA was disclosed in material obtained from 8 women (30.8%), while HPV DNA was present in 2 women (7.7%). In none of women in the group could presence of both viral genomes, EBV and HPV was disclosed. In group 2, EBV DNA was detected in material obtained from 11 patients (50%), including 7 (31.8%) in whom HPV DNA was also identified (in 3 patients in the subgroup 2a and in 4 patients in the subgroup 2b). In 5 women (22.7%) of group 2 (2 patients of subgroup 2a and 3 patients of subgroup 2b) only HPV DNA was detected. The

Table I
EBV DNA and HPV DNA in group 1 (CIN-negative) women and group 2 (CIN-positive) patients

Studied group/subgroup	Number of cases			
	DNA EBV(+) / HPV(+)	DNA EBV(+) / HPV(–)	DNA EBV(–) / HPV(+)	DNA EBV(–) / HPV(–)
GRUP 1 (n = 26)	0	8	2	16
GRUP 2 (n = 22) subgroup 2a (CIN I or CIN II) (n = 12)	3	1	2	6
subgroup 2b (CIN III) (n = 10)	4	3	3	0

identified HPV DNA in all cases (2 women of group 1 and 12 women of group 2) belonged to HPV 16 type. In 16 women (61.5%) of group 1 and in 6 women (27.3%) of group 2 (subgroup 2a) genomes of EBV and/or HPV could not be detected. HPV DNA or HPV DNA and EBV DNA were significantly more frequent in group 2 ($p<0.05$). On the other hand, no significant differences between the two groups were found in frequencies of EBV DNA ($p>0.05$).

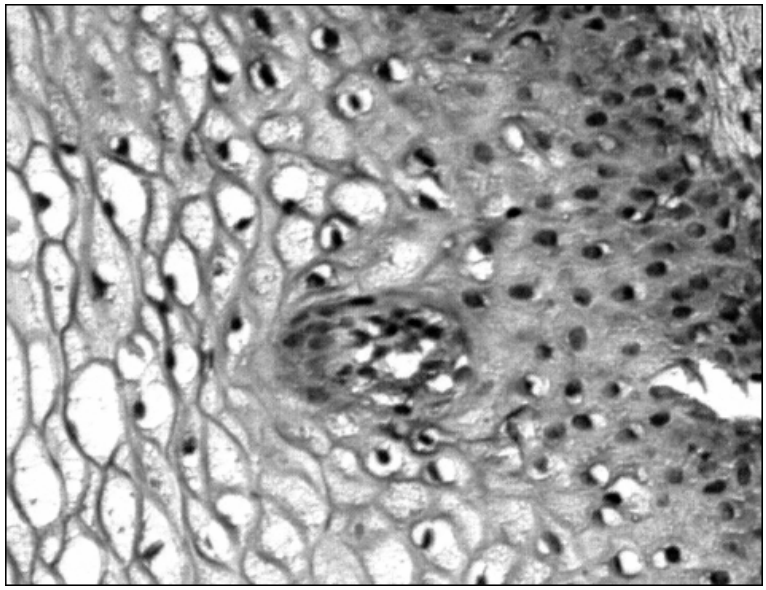

Fig. 1. Mild dysplasia (CIN I) in cervical epithelium.
Note positive reaction for EBER in most of cell nuclei

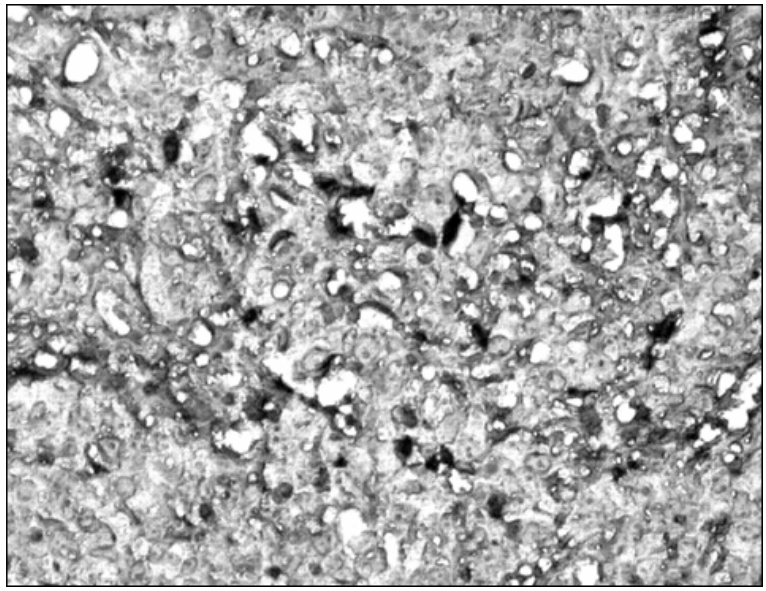

Fig. 2. Cervical carcinoma with individual cell nuclei with EBER

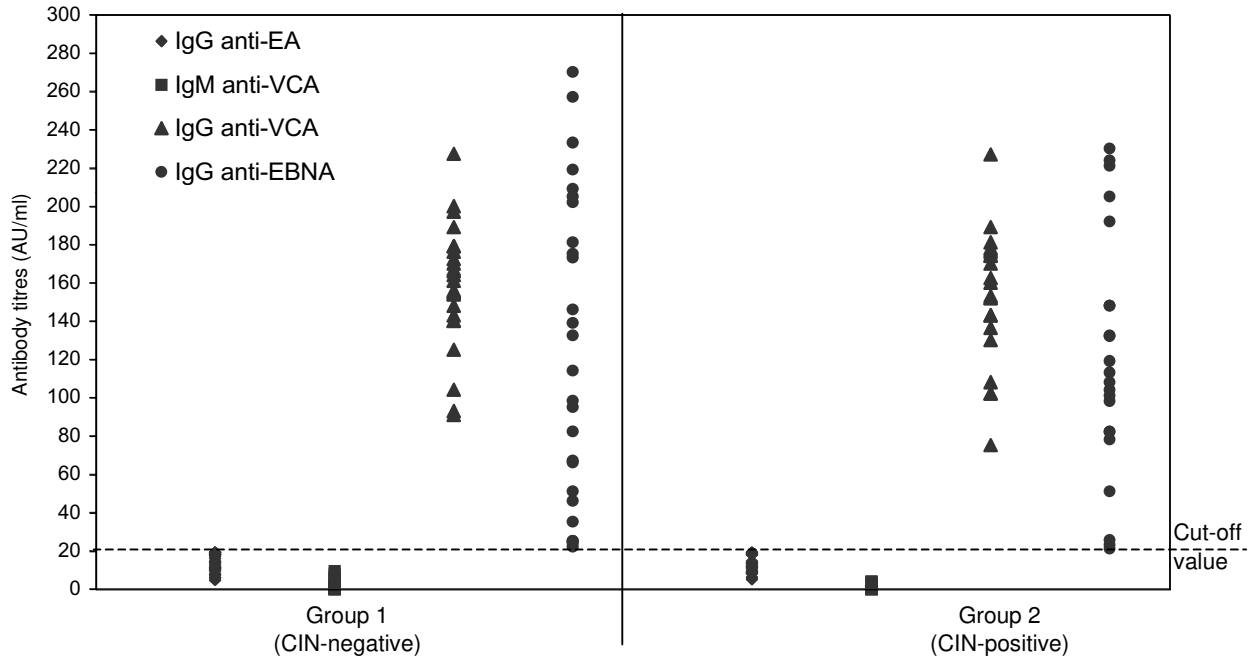

Fig. 3. Anti-EBV serum antibodies (AU/ml) in studied group of women

In turn, in uterine cervix samples obtained from 22 patients of group 2, ISH technique permitted to demonstrate EBER presence in 11 women (4 patients of subgroup 2a and 7 patients of subgroup 2b), in the form of uniform deposits in cell nuclei of the stratified multilayered flat epithelium, manifesting various grades of dysplasia and carcinoma (CIN I, CIN II or CIN III) (Figures 1 and 2). In parallel, EBV DNA was identified by PCR in uterine cervical samples obtained in the women, consistent with identification of the DNA by ISH approach in the patients.

Results of the search for IgG-anti-EA, IgM-anti-VCA, IgG-anti-VCA and IgG-anti-EBNA antibodies in sera of patients of the two groups are presented in Figure 3. In all studied patients of both group 1 and group 2 presence of serum antibodies of IgG-anti-VCA and IgG-anti-EBNA types was disclosed. On the other hand, in none of the cases could presence of serum IgG-anti-EA or IgM-anti-VCA antibodies be documented.

Discussion

In present study, manifestation of HPV and/or EBV viral infections has been analysed in the two distinguished groups of CIN-negative and CIN-positive women. The studies, performed in all the patients by PCR technique on uterine cervix-sampled material, have demonstrated a significant link between infection with HPV 16 or HPV 16 plus EBV on one hand and cervical intraepithelial neoplasia on the other. On the other hand, incidence of EBV infection has not significantly differed between the two groups of women. Thus, confirming significance of HPV 16 infection in etiopathogenesis of uterine cervix carcinoma, the results have pointed to possible co-operation of EBV in the pathogenesis. The suggestion seem to be confirmed by studies of Sasagawa *et al*. (2000), who demonstrated HPV infection and EBV co-infection in 39% patients with invasive cancer of uterine cervix and documented strong *in situ* expression of latent EBV genes, expressed by production of EBER-1 and of EBNA-2 and LMP-1 proteins. EBV latency has been well documented to involve function of its selected genes (Anagnostopoulos *et al*., 1996). Cells of epithelial tumours linked to EBV infection manifested expression of genes coding for EBER, BART transcripts and EBNA-1 protein only (type I latency), or also of LMP-1 and LMP-2 (type II latency), as well as various variants thereof (I/II type latency). However, expression of all EBV latent genes, including the gene coding for EBNA-2 protein (type III latency) takes place in B cells (Rowe *et al*., 1992; Anagnostopoulos *et al*., 1996). In view of the data it seems probable that the quoted above authors obtained falsely positive result for EBNA-2 protein. On the other hand, mixed infection with HPV could have resulted in activation of various latent EBV genes.

Studies presented in this paper indicate that the infection with EBV alone does not constitute a causal factor for uterine cervix pathology. The conclusion has been corroborated by results of *in situ* hybridisation which documented presence of EBER particles both in cell nuclei of cells manifesting mild dysplasia or moderate dysplasia (CIN I, CIN II), and in cells of severe dysplasia or cancer. The data may confirm in part results of the other authors (Ammatuna *et al*., 2000; de Oliveira *et al*., 1999), who demonstrated no relation between detection of EBV DNA and intraepithelial pathology of uterine cervix.

Analysis of serological tests in the two groups has demonstrated presence of IgG-anti-VCA and IgG-anti-EBNA antibodies. The detected profile of humoral reaction has indicated an experienced in the past EBV infection, and presence of anti-viral antibodies in all the patients is consistent with the ubiquitous presence of EBV in human population (Anagnostopoulos *et al*., 1996; Trzcińska *et al*., 2001). Therefore, it is possible that acute EBV infection is followed by secondary persistent infection in epithelial cells of uterine cervix, which promotes carcinogenesis with additional effects of other factors (co-infection with HPV, genetic factors, environmental effects) (Sixbey *et al*., 1986; Herrmann *et al*., 2003).

All the data obtained allows to suggest that, in most cases, acute EBV infection results in persistent infection of epithelial cells in uterine cervix. While cervical intraepithelial neoplasia is linked mainly to infection with HPV 16, EBV may co-operate with HPV in induction of uterine cervix pathology.

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