IL-10 Expression is Associated with Lack of HPV-16 E7 Specific Th1 Response in Cervical Cancer Patients

Asociación de la expresión de IL-10 con la pérdida de repuesta específica Th1 a VPH-16 E7 en pacientes con cáncer de cuello uterino

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Abstract

Objective: To examine the association between the presence of HPV specific T cell response in relation to HLA class I, HLA-G and IL-10 expression in the tumor cells of cervical cancer patients. Materials and methods: Lymphocytes were isolated from 18 cervical cancer patients and stimulated with autologous HPV16 E7-pulsed monocyte-derived dendritic cells or directly with synthetic peptides: E7_{51-70}, E7_{65-84}, and E7_{79-98}. The cells were stained for CD4, CD69, intracellular IFN-γ and IL-4 cytokines and analyzed by flow cytometry. HLA-I, HLA-G and IL-10 expression on tumor cells was analyzed by immunohistochemistry. Results: HLA class I expression was completely lost in 11/18 and downregulated in 4/18 of patients. HLA G expression was observed in 4/18 of patients and IL-10 expression was observed in 6/17 of patients. An inverse association between IL-10 expression and a lack of HPV-specific immune response was observed (P = 0.041). No clear associations between HLA class I or HLA-G expression and specific immune response were observed. Conclusions: Our results underline the importance of other immune factors, like IL-10 expression, that could influence the antitumoral response. These results could have important implications during development of new therapeutic strategies like immunotherapy.

Key words: T-lymphocytes, helper-inducer; HLA antigens; tumor escape; uterine cervical neoplasms, papilloma

Resumen

Objetivo: Examinar la asociación entre la respuesta celular anti-VPH y la expresión de HLA clase I, HLA-G e IL-10 en células tumorales. Materiales y métodos: Linfocitos fueron aislados de 18 pacientes con CCU y estimulados con células dendríticas pulsadas con la proteína E7VPH-16 o directamente con péptidos sintéticos: E7_{51-70}, E7_{65-84} y E7_{79-98}. Las células fueron marcadas para CD4, CD69, citoquinas intracelulares IFN-γ y IL-4 y analizadas por citometría. La expresión de HLA clase I, HLA-G e IL-10 fue analizada por inmunohistoquímica. Resultados: Una pérdida total de la expresión de HLA clase I se observó en 11/18 y regulación negativamente en 4/18 pacientes. La expresión HLA-G se observó en 4/18 pacientes mientras que la expresión IL-10 en 6/17 pacientes. Se observó una asociación inversa entre la expresión de...
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Introduction

Cervical cancer is the second most common cause of death by cancer in women worldwide. Human Papillomavirus (HPV) infection is considered a major cause of cervical cancer and is detected in 99.7% of cases (1,2). Among high-risk HPV types, HPV 16 is the most prevalent accounting for approximately 50% of all cervical cancers (2,3). HPV-16 E6 and E7 genes are frequently coexpressed in tumor cells and their oncogenic effect is mediated by the binding of these viral oncoproteins to the products of tumor suppressor genes p53 and pRb respectively causing deregulation of the cell cycle (4,5). The viral transcripts of these proteins have been the most abundant found in HPV-16 positive cervical cancer biopsies to date; therefore, these oncoproteins are attractive targets for T cell-mediated immunotherapy (4,6).

Although the infection with high risk HPV (HR-HPV) is an important risk factor in cervical cancer etiopathogenesis, there is increasing evidence that the immune system plays a pivotal role in determining the outcome of HPV infections (7). It has been shown that immunosuppressed individuals are more likely to have persistent HPV infections and to develop cervical intraepithelial HPV-associated neoplasia lesions (8-10). Thus, the ability to avoid immune attack is also linked to the transforming potential of HPV and the rapid progression of human cancer (7).

Lymphoproliferative responses to specific HPV16 E6 and E7 peptides appear to be associated with the clearance of HPV infection and with the regression of cervical lesions (11-13). However, the infection of cervical epithelia by HPV is not always associated with a strong local inflammatory reaction (14) and cervical cancer may be the final stage of a persistent oncogenic HPV infection during which the host’s immune system fails to eliminate the virus (15,16).

There are several mechanisms that could explain the complex cancer–host immune interactions and the immune escape of the cancer cells in the tumor milieu. One of the major mechanisms used by tumor to escape immunosurveillance is the MHC class I molecules downregulation. In cervical cancer, a minimum of 73% loss HLA class I is estimated (17-20). It has also been reported that certain tumor cells may secrete immunosuppressive factors like IL-10 and TGF-B, which might help to downregulate tumor-specific immune responses in the microenvironment of the tumor (21). Moreover, it has been also hypothesized that HLA-G expression in cancer cells plays a role in the evasion of immunosurveillance mediated by host’s T-lymphocytes and NK cells (22,23).

To comprehend the contribution of these mechanisms to the escape of tumor cells from the actions of HPV-specific T cells, we examined the association between the presence of HPV specific T cell response in relation to HLA class I, HLA G and IL-10 expression at the tumor cell surface.

Materials and methods

Patients

Eighteen HPV 16 positive patients with invasive cervical cancer staged II A to IIIB according to FIGO (International Federation of Gynecologists and Obstetricians), being treated as outpatients at the gynecological clinic of the National Cancer Institute, in Bogotá (Colombia), were enrolled in the study. Patients were not included if they had undergone any treatment before radiotherapy (RT)
or if they had prior or concurrent second malignancies. During the first gynecological examination, cervical biopsies and scrapes were collected from each patient, processed and frozen -70°C until use. On a second visit, 60 ml of heparinized venous blood were obtained from HPV-16 positive patients before treatment. All patients underwent primary RT, with or without concurrent cisplatin, according to the institutional radiotherapy protocols (24). This study was approved by the Institutional Medical Ethics Committee, and informed consent was obtained from patients.

**HPV testing**

Cells from cervical scrapes were detached from the spatula and brush by vortex and subsequently centrifuged at 3,000 g for 10 min. The cell pellet was suspended in 1 ml of buffer Tris-HCl 10mM pH 8.3 and stored at −70°C until use. To assess the quality of the target DNA all pellets were prescreened by using a 209 base pair amplifying ß–globin PCR using PCO3 and PCO5 primers as described by Molano et al. 2002. HPV typing was performed by PCR by using generic primers GP5+/GP6+ (25). The specific detection of HPV-16 was carried out by PCR amplifying a 561pb segment of the E6 gene. The sequences of primers used were 5´AAACTAAGGGCGTAACCG 3´ and 5´TGTAGGTGTATCTCCATGC 3´. As a positive control, SiHa DNA cells were used.

**Detection of HPV-16 E7 specific T helper immune responses**

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and used either for DCs generation or peptide stimulation (26). The monocytes were separated from PBMCs by negative selection by using magnetic-activated cells sorting (MACS) beads and MACS® separation columns (MACS®, Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Monocyte purity was determined by flow cytometry analysis of CD14 expression, which must be between 80% and 90% of the total number of cells. The monocyte-depleted PBMCs were cryopreserved in fetal bovine serum (FBS, GIBCO, Grand Island, NY) containing 10% dimethyl sulfoxide until use. Dendritic cells (DCs) were obtained by in vitro differentiation of monocytes as was previously described (26, 27). Briefly, Monocytes were cultured in RPMI-1640 supplemented with 1000 U/ml rhGM-CSF (BD Biosciences), 200 U/ml rhIL-4 (SIGMA) and 10% of FBS (GIBCO) at 37°C in 5% CO2 and 95% humidity for 5 days. Fresh medium supplemented with cytokines was added to the cultures on day 3. To determinate the immature DCs (iDCs), the level of expression of CD1a, CD86, HLA-DR and CD83 markers was measured at 5 days. The non-adherent cells corresponding to immature DCs were harvested and used for antigen loading. The endotoxin levels from media and all reagents used to obtain DCs were below 0.03 EU/ml.

Immature DCs (iDCs) were harvested and pulsed with 10 µg/ml HPV-16 E7 protein or 10 µg/ml of MBP protein or incubated with 100 ng/ml of LPS (Escherichia coli 026:B6, Sigma) in RPMI 1640 containing 10% of FBS, and incubated for 48 hours. In order to obtain optimal DC maturation, iDCs were incubated with 100 ng/ml LPS for 24 hr or 48 hr, two hours after HPV-16 E7 protein and MBP addition. Mature DCs (mDCs) were analysed by flow cytometry for the expression of CD1a, CD86, HLA-DR and CD83. Cryopreserved monocyte-depleted PBMCs were thawed and cocultivated with E7-pulsed or MBP-pulsed autologous DCs for 12 hours (ratios from 5:1 to 10:1). As a positive control, 1.25 µg/ml SEB (staphylococcal enterotoxin B, Sigma) were added to LPS-stimulated autologous DC cocultures, and LPS-stimulated autologous DC cocultures without antigen were used as a negative control. Between five and eight hours before harvesting cells for analysis, 10 µg/ml of Brefeldin A (Sigma) were added. For peptide stimulation, PBMCs were suspended in an AIM-V medium supplemented with 10% FBS and stimulated separately with 10µg/ml of each HPV-16 E7 peptide (Invitrogen Life Technologies). The sequences of the peptides were: E751-70: HYNIVTFCCKCDSTLRLCVQ; E765-84: LRLCVQSTHVDIRTLEDLLM and E779-98: LEDLLMGTLGIVCPICSQKP. As a negative control, a peptide (20-residue long) from MSA-2 (merozoite surface antigen 2 of *P. falciparum*) protein was used (KNESKNTTFINNAYNMSIR). As a positive control, the PBMCs were stimulated
with SEB (1.25µg/ml), and as a negative control the PBMCs were cultured in medium alone. From five to eight hours before harvesting the cells for analysis, 10 µg/ml of Brefeldin A were added.

**Antibodies and reagents**

For the cytometry analysis we used the following monoclonal antibodies (mAbs): anti-CD14 conjugated with fluorescein isothiocyanate (FITC; M5E2), anti-CD1a conjugated with phycoerythrin (PE; HI149), anti-CD83 FITC (HB15e), anti-HLA-DR PE (G46-6), anti-CD86 FITC (2331), anti-CD69 conjugated with allophycocyanin (L78), anti-CD4 conjugated with peridinin chlorophyll protein (SK3), anti-IFN-γ FITC (4S.B3), anti-IL-4 PE (8D4-8) and isotype controls FITC and PE (MOPC-21 and UPC-10) (all purchased from BD Biosciences, San Diego, CA and San Jose, CA). The recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) was purchased from BD. Recombinant human IL-4 and lipopolysaccharide (LPS; Escherichia coli 026:B6) were purchased from SIGMA (St Louis, MO).

**Flow cytometry analysis**

After stimulation with each antigen, lymphocytes were harvested and stained for superficial antigen expression by using directly conjugated MAbs anti-CD4, and anti-CD69 for 30 min. at 4°C. For analysis of intracellular cytokines expression, the cells were washed with PBS and fixed and treated with Cytofix/Cytoperm (Pharmeringen) for 20 min. at 4°C. They were then washed once with Perm/Wash 1X (Pharmeringen) and incubated with anti-INFγ and anti-IL-4 for 30 min at 4°C. In the final step they were washed twice with Perm/Wash 1X and suspended in 200µl of PBS-Paraformaldehyde 0.05% solution. Control cells were stained with the respective isotype controls. All monoclonal antibodies (purchased from BD Biosciences) were previously titrated, and optimal concentrations were used. The analysis of stained cells was performed using a four-color FACSCalibur™ flow cytometer (BD Immunocytometry Systems). Between 30,000 and 50,000 gated CD4+ events were acquired and analyzed by using Cell Quest Pro™ software (26).

**Immunohistochemistry**

Eighteen biopsies from cervical cancer were analyzed for HLA, HLA G and IL-10 expression. Four-µm-thick cryostat sections were mounted on glass slides, air dried overnight, fixed with precooled acetone for 10 min. and stored at −20°C until used. Endogenous peroxidase activity was blocked with 0.3% H2O2 for 15 min. and non specific binding sites were blocked by incubating slides with 20% AB serum/PBS for 20 min. at room temperature. Then the slides were incubated during 30 min. at room temperature with 1:100 mouse anti-HLA I mAb (W6/32, BD Pharmingen), anti-IL-10 mAb (E10, Santa Cruz) or anti-HLA G (4H84, Pharmingen). PBS was used as a negative control. A secondary biotinylated goat antibody to mouse immunoglobulin (DAKO) was applied and incubated at room temperature for 30 min. The slides were then incubated with an avidin-biotin-peroxidase conjugate for 30 min. The reactions were developed with a fresh 3.3’-diaminobenzidine tetrahydrochloride solution. The slides were counterstained with hematoxylin, dehydrated through alcohol, and cleared in xylene before mounting. The slides were reviewed by a pathologist. Three levels of expression were determined: No expression (negative), partial expression (HLA I: low intensity expression, HLA G: focal expression), and strong expression (more than 70% positive).

**Statistical interpretation**

The frequencies of CD4+CD69+INFγ+ or CD4+ CD69+IL-4+ T cells responding to HPV-16 E7 producing INFγ or IL-4 in the responders were obtained by subtracting the percentage of T helper cells responding to the negative control (without antigen) from the percentage of T helper cells responding to the viral antigen. The subjects were considered responders when the differences between the frequencies of T helper cells responding to HPV-16 E7 antigens and to the negative control were statistically significant by the χ2-test. Statistical analysis was performed by using the SPSS 15.0 software. Correlations between HLA class I, HLA G and IL-10 expression and immune response were assessed by using Spearman correlation. For all tests, P-values less than 0.05 were considered statistically significant.
Results

Clinical patient characteristics

A total of 18 HPV-16 positive women with diagnosis of invasive cervical cancer stages II A (n = 1), IIB (n = 4) and IIIB (n = 13) were enrolled in the study. Age range was 28 to 64 years (mean 45, SD± 10.9). Twelve patients (66.5%) received combined chemoradiotherapy (RT-CHT), four patients (33.5%) received only radiotherapy, and one patient did not accept any therapy. Tumor sizes before treatment ranged from 3.5 cm to 7.25 cm. The mean follow-up time was 22.4 months, with a time range from 1.8 to 36.9 months. Disease recurrence was observed in 6/15 of patients (40%) with a mean disease-free time of 15.8 months (range: 0.3 – 34.7 months). Four patients died from the disease in a mean time of 15.6 months.

Specific HPV-16 E7 Th1 response in cervical cancer patients

Specific CD4+ CD69+ INFγ+ immune response against E7 HPV16 protein was observed in half of the patients, while 9 of 17 patients (52.4%) showed a HPV16 E7 79-98 peptide response. Three (17.6%) also showed a specific CD4+ CD69+ IL-4+ response at the same time (Table 1).

HLA class I, HLAG and IL-10 expression in cervical cancer patients

Eleven of eighteen patients (61.1%) displayed a total loss of HLA class I expression, while four (22.2%) displayed a weak expression, thus a downregulation of HLA class I expression was observed in 83.3% of patients (Figure 1). IL-10 expression was observed in 6/17 patients. Five of them (29.4%) showed a strong expression. HLA G expression was observed only in 4/18 of patients, two of which showed a strong expression and were also IL10 positive (11.11%); the other two showed a partial expression (11,11%) (Table 2).

IL-10 expression is associated with the lack of HPV-16 E7 specific Th1 response in cervical cancer patients

When IL-10 expression and specific HPV-16 E7 Th1 response were correlated, a high number of patients (7/11) who did not express IL-10 showed a specific HPV-16 E7 Th1 response, while 5 of 6 who showed IL-10 expression did not show HPV specific T-cells (P=0.041) (Table 2 and figure 2). No association was observed between HLA G expression and the presence of a specific immune response. Although down regulation of HLA class I has been shown to be associated with a diminished immune response, in this study, 5/11 of patients without HLA expression at cervical epithelia showed a specific immune response to E7.

Discussion

Genital HPV infection is common among sexually active women. However, only a minor fraction of infected subjects develops progressive cervical epithelial lesions or cancer. Bypassing local immune responses is an important factor for the development of cervical cancer (8,14). In this work we studied

![Figure 1.](image-url)
IL-10 expression is associated with lack of HPV-16 E7 specific Th1 response in cervical cancer patients

As in previous studies, in this work circulating HPV-specific T cells responding to E7 HPV proteins were detected only in half of cervical cancer patients (12,16,26). Potential explanations for this lack of response may include local immune escape mechanisms mediated by downregulation of HLA class I or upregulation of HLA G and IL-10 expression (28).

In a previous study, De Boer et al. showed a strong association between HLA class I expression on tumor cells and the presence of HPV-specific immune response. When HLA class I expression was completely retained, circulating HPV-specific T cells were present, whereas in all cases where HLA class I expression was lost, HPV specific T cells were not detected (28). In this study, HLA class I expression was not correlated with the systemic specific immune response to HPV. Five patients with complete loss of HLA class I showed INFγ specific immune response to HPV. It is possible that this response corresponds to the stimulation of circulating E7 HPV16 precursor T lymphocytes which could have been generated before HLA class I expression.

Table 2. HPV-16 E7 specific Th1 response in relation to HLA class I, HLA G and IL-10 expression.

<table>
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<th>Patients</th>
<th>HPV-16 E779-98 Th1 (INFγ) response</th>
<th>HLA I expression</th>
<th>HLA G expression</th>
<th>IL10 expression</th>
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<td>(-)</td>
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<td>(-)</td>
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<td>17-299</td>
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No expression (-); Partial expression (PE); Strong expression (+); Not determined (ND)

Figure 2. IL-10 expression in relation to HPV-16 E7 specific Th1 response. A lack of HPV-16 E7 specific Th1 response in cervical cancer patients was associated with high IL-10 expression (P=0.041).
I loss in tumor cells in advanced stage (II B-IIIB). On the other hand, the patients that showed HLA class I expression, including those with a partial one, did not show a specific immune response to HPV16-E7 protein. These patients showed IL-10 expression in the tumor cells. These results suggest that the immune response to HPV antigens depends not only on factors like HLA class I expression, but also on other factors in the tumor milieu, like IL-10 expression, that are probably playing a determining role.

It has been shown that the detection of IL-10 and TNF-α in cervical secretions may be an useful indicator of local immune responses and of the stage of the cervical lesions induced by HPV infection (29). In our study, a negative correlation between the IL-10 and a downregulation of HPV 16- E7 specific immune response in cervical cancer patients was observed. 5 of 6 patients who showed an increase in IL-10 expression failed to present HPV-specific T cells responding to E7 HPV protein. These results support the concept that a predominant expression of immunosuppressive cytokines might help to downregulate tumor-specific immune responses in the tumor microenvironment (29).

In cervical cancer, the shift towards immunosuppressive T helper (Th2) cytokine profile and the secretion of IL-10 appears to occur during progression. On the other hand, in addition to down-regulation of HLA class I expression, IL-10 appears to be one of the factors responsible for the up-regulation of HLA-G, another molecule involved in the immunescape. It is possible that the expression of HLA-G account for the induction of Th2-skewing state and the production of IL-10, thus establishing a vicious circle of immune abrogation in cancer. A correlation between the HLA G and interleukin-10 expression in cervical carcinoma has been reported (30). In this study, a low expression of HLG was observed in cervical cancer patients but it was not associated with IL-10 expression. However this study is limited by the small number of patients and clearly other studies will be needed to confirm if IL-10 could be one of the factors responsible for the up-regulation of HLA-G.

The present study shows that IL-10 expression correlated negatively with downregulation of HPV 16 E7 specific T cell response in cervical cancer patients. Our results underline the importance of other immune factors, like IL-10 expression, that could influence the tumoral immune response. These results could have important implications during the development of new therapeutic strategies since immunotherapy directed against HPV might not be effective in patients with upregulation of IL-10 expression.

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Referencias