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Dendritic cell subsets and immunological milieu in inflammatory human papilloma virus-related skin lesions

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ABSTRACT

Background: Human papilloma virus (HPV)-related warts persist, evading host immune surveillance, but sometimes disappear with inflammation.
Objectives: To elucidate the immune evasion mechanisms of HPV, we have examined the density, dynamics, and subsets of dendritic cell (DC) types in non-inflammatory or inflammatory HPV-related skin lesions such as warts and Bowen's disease (HPV-Bowen), and compared the epidermal expression levels of macrophage inflammatory protein (MIP)-3α and E-cadherin.
Methods: The expression of various DC markers, MIP-3α, and E-cadherin in the tissue samples obtained from patients with warts, HPV-Bowen and HPV-unrelated skin diseases was evaluated by immunohistochemistry. MIP-3α gene expression levels were examined in warts and HPV-Bowen by *in situ* hybridization (ISH) and real-time quantitative polymerase chain reaction (RT-qPCR).
Results: The numbers of Langerhans cells (LCs) and the expression levels of MIP-3α and E-cadherin were decreased in non-inflammatory warts and HPV-Bowen, as compared with normal skin. Both epidermal LCs and MIP-3α expression reappeared in inflammatory warts, associated with dermal infiltrates composed of many cytotoxic T cells and various subsets of DCs, while cellular infiltrates in HPV-Bowen contained many B cells and plasma cells with sparse infiltration of DCs. The upregulation of MIP-3α gene expression was confirmed in the inflammatory warts and HPV-Bowen by ISH and RT-qPCR.
Conclusions: The depletion of LCs in the non-inflammatory warts and HPV-Bowen is associated with a down-regulation of expression levels of MIP-3α and E-cadherin in the lesional keratinocytes. MIP-3α expression is upregulated in lesional keratinocytes of inflammatory warts, with the subsequent recruitment of various DC subsets and cytotoxic T cells, whereas plasma cell-rich infiltration was induced in HPV-Bowen.

1. Introduction

Human papilloma virus (HPV) induces benign and malignant mucocutaneous diseases, including common warts, myrmecia, genital warts, epidermodysplasia verruciformis, and Bowen's disease [1,2]. Although HPV-encoded antigens can be detected in the living layers of the epidermis, the HPV-infected cells evade the host immune surveillance. It has been unclear why immune cells are unresponsive against HPV. Previous studies have reported the depletion of Langerhans cells (LCs) in common warts and plantar warts, despite the presence of considerable numbers of LCs in the perilesional skin [3]. Besides HPV-induced lesions, LC numbers were decreased in viral infections caused by molluscum contagiosum virus [3]. The mechanism of the LC depletion remains to be answered.

Since LCs are professional antigen-presenting cells in the epidermis, the depletion of LCs may inhibit the induction of immune responses against HPV-infected cells and allow them to persist in the living layers of skin, even though the cells express HPV-specific antigens. In addition, it has been previously reported that HPV-related warts disappear simultaneously, associated with a sudden onset of inflammatory changes mainly mediated by CD8+ T cells, together with the migration of LCs [4,5]. These observations suggest that the recruitment of LCs might be an essential role in the induction of antigen-specific T cells directed against HPV-related antigens. Recent investigations have clarified the presence of

Abbreviations: HPV, human papilloma virus; DC, dendritic cell; MIP-3α, macrophage inflammatory protein-3α; HPV-Bowen, human papilloma virus-related Bowen's disease; LC, Langerhans cell; pDC, plasmacytoid dendritic cell; DC-LAMP, dendritic cell-lysosomal-associated membrane protein; iNOS, inducible nitric oxide synthase; Tip-DCs, tumor necrosis factor and inducible nitric oxide synthase-producing dendritic cell.

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various subsets of dendritic cells (DCs) with different biological functions and chemotactic ligands [6].

In order to clarify the dynamic alterations of DCs in HPV-related skin lesions, we first examined the density of epidermal LCs in non-inflammatory or inflammatory warts and HPV-related Bowen's disease (HPV-Bowen), and further studied DC subtypes migrating to the inflammatory warts and HPV-Bowen. We then examined the expression levels of macrophage inflammatory protein (MIP)-3α, a chemokine for epidermal LCs, and E-cadherin, an adhesion molecule expressed on the surfaces of keratinocytes and LCs that retains LCs within the epidermis.

2. Materials and methods

2.1. Materials

All tissue materials were obtained for diagnostic or therapeutic purposes, and utilized for the present study with an approval of ethical committee in Okayama University Hospital (No. 1034). Tissue specimens were obtained from HPV-associated non-inflammatory ($n = 26$) and inflammatory ($n = 24$) warts. Of 50 samples, the association of HPV was proven by polymerase chain reaction (PCR) amplification in 25 samples, and by immunostain in 25 samples. A total of 12 surgically removed samples of HPV-Bowen, including non-inflammatory ($n = 3$) and inflammatory ($n = 9$) samples, were used for the present study. HPV 16 and 33 were amplified in 10 and 2 samples, respectively.

Control samples include 27 samples of non-inflammatory ($n = 10$) and inflammatory ($n = 17$) seborrheic keratosis, and 12 normal skin samples obtained from the periphery of surgically resected skin tumors.

We classified all tissue sections into 2 groups: non-inflammatory and inflammatory lesions. Lesions with nonspecific, scant or focal perivascular infiltrates were defined as "non-inflammatory" ones, and lesions associated with massive or diffuse infiltration were defined as "inflammatory" ones.

2.2. Polymerase chain reaction for HPV DNA typing

The DNA was extracted from skin samples using a DNeasy tissue kit (Qiagen, Hiden, Germany) according to the manufacturer's instructions. The following PCR reaction was carried out using specifically designed primer sets for: *HPV*, sense (GP5+) 5′-TTTGTTACTGTGGTAGATACTAC-3′, antisense (GP6+), 5′-GAAAAA-TAAACTGTAAATCATATTC-3′; β-globin, sense 5′-CAACTTCATC-CACGTTCACC-3′, antisense 5′-GAAGAGCCAAGGACAGGTAC-3′.

Primer sequences and PCR conditions were optimized as previously described [7]. Each experiment was performed with water as a negative PCR control. β-Globin gene primers were used to control the quality of the isolated DNA [8,9]. PCR products were analyzed by electrophoresis on 2% agarose gels and visualized with ethidium bromide.

PCR products were purified with a Gel extraction kit (Qiagen). After purification, products were sequenced in a 310 Automated Sequencer (Applied Biosystems, Foster City, CA, US), using the BigDye® terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol in Central Research Laboratory, Okayama University Medical School. A BLAST search was performed to check the specificity of the DNA sequences (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Immunohistochemistry

Before staining, sections were deparaffinized and rehydrated. For immunostaining with murine monoclonal antibodies (Abs) against human papilloma virus (Dako, Glostrup, Denmark), MIP-3α (R&D Systems, Minneapolis, USA), E-cadherin (Lab vision, Fremont, USA), CD4, CD8 and CD20 (Nichirei, Tokyo, Japan), tissue sections were pretreated by microwaving in 0.01 M citrate buffer, and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were incubated at 4 °C overnight with each one of the Abs. The binding of mouse IgG was revealed by rabbit-peroxidase labeled anti-mouse IgG Ab (IgG; Nichirei) at room temperature for 1 h. The peroxidase activities were revealed using 3-amino-9-ethylcarbazole (AEC) substrate.

Antigen retrieval was carried out by boiling the sections at 97 °C for 35 min in antigen retrieval solution (Dako) in a pressure cooker for Abs against human Langerin (Novocastra, Newcastle, UK), CD83 (Immunotech, Marseille, France), CD123 (Bioscience, San Jose, USA), T cell-restricted intracellular antigen (TIA)-1+ (Immunotech), and granzyme B (Nichirei). Antigen retrieval by immersing in trypsin buffer was performed for immunostaining using DC-lysosomal-associated membrane protein (LAMP) monoclonal Ab (Immunotech). The sections were incubated with each Ab at room temperature in a moist chamber for 1 h. The sections were further reacted with alkaliphosphatase-labeled secondary Ab (LSAB/AP, K0678; Dako) at room temperature. Fuchsin substrate-chromogen was applied to the sections and counterstained with hematoxylin.

As controls, the same skin specimens were incubated with an isotype-matched Ab using identical experimental conditions. The numbers of DCs and lymphocytes and positive cells for cytotoxic molecules were determined in three randomly selected fields (original magnification; ×400) per section. Only Langerin+ cells in the epidermis were counted as LCs. Each count was performed on three sections taken from different levels, and the results are expressed as the mean ± SD of cell numbers per three fields.

Expression levels of E-cadherin and MIP-3α were evaluated as compared with normal skin: 3, stronger and diffuse expression; 2, equal expression; 1, weaker and focal expression; and 0, loss of staining. Each expression level was performed on three sections taken from different levels.

2.4. Two-color immunofluorescence of CD11c and iNOS

Before staining, sections were deparaffinized and rehydrated. Antigen retrieval was carried out by boiling the sections at 97 °C for 35 min in antigen retrieval solution (Dako) in a pressure cooker. Nonspecific Ab binding was blocked by incubation of the sections with 10% normal donkey serum. The sections were incubated at 4 °C overnight with mouse anti-human CD11c (Novocastra, Newcastle, UK) and rabbit anti-human inducible nitric oxide synthase (iNOS), followed by incubation with donkey anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) and donkey anti-rabbit IgG conjugated with Cy3.

2.5. Terminal deoxydyl Tranferase-mediated dUTP Nick End Labeling (TUNEL) assay

Samples from patients were assessed for apoptosis on deparaffinized sections using the Terminal deoxydyl Tranferase-mediated dUTP Nick End Labeling (TUNEL) assay according to the manufacturer's protocol (Promega, Madison, USA).

Three fields were randomly selected for each case (original magnification; ×400). The percentage of apoptotic keratinocytes were defined as the number of TUNEL-positive cells per total number of keratinocytes counted and averaged for each lesion. TUNEL-positive cells in the epidermis were counted.

2.6. In situ hybridization for MIP-3α

Coupled primers were used to amplify the majority of the open reading frame of the MIP-3α gene by PCR. *20*/*MIP*-3α/*CCL20* (sense