ORIGINAL ARTICLE

Changes in distribution and ultrastructure of Langerhans cells in condyloma acuminatum tissues, and analysis of the underlying mechanism

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ABSTRACT

Objective: To determine the changes in distribution and function of Langerhans cells (LCs) in condyloma acuminatum (CA) tissues, as well as the possible underlying mechanism.

Methods: The expression of CD1a and E-cadherin in 26 CA lesions and 10 normal prepuce tissues was determined using the streptavidin peroxidase method. The mRNA expression of CD1a and E-cadherin was determined using reverse transcriptase polymerase chain reaction. The ultrastructure of LCs was observed under a transmission electron microscope (TEM).

Results: In the CA tissues, the number of CD1a-positive LCs was significantly decreased (10.66 ± 11.71) compared with the number in normal prepuce tissues (p < 0.05). The LCs in the CA tissues had smaller cell bodies with fewer and shortened cytodendrites. The CD1a mRNA expression in the CA tissues significantly decreased compared with that in the normal tissues (0.4066 ± 0.2671 vs. 0.7444 ± 0.3667; p < 0.01). The average E-cadherin staining score in the CA tissues was significantly lower than that in the normal tissues (2.36 ± 1.41 vs. 7.67 ± 1.64; p < 0.01). The E-cadherin mRNA expression in the CA tissues was also significantly lower than that in the normal tissues (0.1737 ± 0.1083 vs. 0.3786 ± 0.1460; p < 0.01). A positive correlation was observed between the LC number and E-cadherin expression density (r = 0.8381, p < 0.05), as well as between the mRNA expression of CD1a and E-cadherin (r = 0.8381, p < 0.05). TEM observation revealed changes in the morphology and quantity of subcellular organelles (Birbeck granules) in the CA tissues.

Conclusion: CA lesions are characterized by a decreased number of LCs and antigen presentation dysfunction. The decrease in the number of LCs may be correlated with reduced E-cadherin expression, which hinders intraepidermal LC retention.

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Introduction

Condyloma acuminatum (CA), a common sexually transmitted disease, is characterized by mucocutaneous benign hyperplasia attributed to human papilloma viral (HPV) infection.1 In the United States, the number of clinically diagnosed CA cases reaches approximately 500,000 to 1,000,000 annually, accounting for 1% of the sexually active population.2 Current therapies for CA, such as freezing, lasers, fulgerizing, and chemical corrosion, fail to achieve satisfactory results.3 Therefore, radical treatments for CA and methods to prevent or reduce its recurrence remain a clinical puzzle.

To date, more than 130 HPV subtypes have been discovered, more than 40 of which have been found to induce perianal protinal CA.4 HPV is usually classified into low-risk and high-risk types. The low-risk types (HPV 6, 11, 40, 42, 43, and so on) are correlated with benign diseases such as genital or skin warts, whereas the high-risk types (HPV 16, 18, 31, 33, 35, and so on) are mainly responsible for lesions such as cervical intraepithelial neoplasia (CIN), squamous cell carcinoma (SCC), and so on.5

The immune system plays a critical role in determining the natural progression of HPV infections and the consequences of HPV-associated diseases, with the failure to establish an effective
immune response resulting in the long-term presence and recurrence of CA. Effective cell-mediated immune responses to HPV infection involve viral antigen presentation, immunoreactive activation, and virus-infected target cell removal by effector cells. Patients with persistent HPV infection and those with HPV-induced cervical cancer present with immune evasion or deficiency. This is because changes in the number and functions of infected local dendritic cells (DCs) result in immune supervising system damage; thus, immune responses cannot be effectively activated and virus-infected cells are difficult to remove. Therefore, these changes play an important role in the long-term persistence of viral infection and tumorogenesis.

Intraepidermal Langerhans cells (LCs) are immature skin DCs. They process, handle, and present glycolipid and lipid antigens to provide the necessary first and second signals for the activation of antigen-specific T cells, and therefore function as initiators of local specific cell-mediated immune responses and as effective T cell activators. In HPV 16/18–induced CIN and SCC tissues, the antigen presentation function of LC decreases, manifested by decreases in the number and density of LCs and failure in the upregulation of LC surface activity markers and the secreted cytokines, which are responsible for inducing Th1-type cell-mediated immune responses. Epithelial cadherin (E-cadherin) mediates the adherence between epidermal keratinocytes (KCs) and LCs, and it performs a vital role in deciding the localization and retention of LC in the epidermis. Changes in LCs are correlated with changes in the E-cadherin synthesized by HPV 16/18–infected epidermal KC. The changes in E-cadherin play a significant role in the metastasis, intraepidermal retention, activation, and differentiation of LCs. In addition, they greatly influence the KC atypical hyperplasia and malignant transformation induced by high-risk HPV. HPV immune evasion also occurs in progenital local CA skin lesions caused by low-risk HPV 6/11. However, whether similar changes in the number and function of LCs in CIN and SCC also occur under such conditions, and if so, what the detailed mechanism is remain unreported.

To explore the changes in local LCs in CA tissues, as well as the underlying molecule-associated mechanism, we first determined the molecular and mRNA expression of CD1a and E-cadherin in progenital HPV 6/11–induced CA tissues and then observed the LC ultrastructure. This study aims to provide a possible basis for immunotherapy for CA.

Patients and methods

Patients

Twenty-six tissues were collected from CA patients who received treatment at the First Affiliated Hospital of Nanjing Medical University between February 2010 and June 2011. All patients met the CA diagnostic criteria and were pathologically confirmed. Of the 26 patients, 16 were male and 10 were female, with an average age of 30 years (ranging from 18 years to 42 years). Their disease courses ranged from 3 weeks to 9 months. The tissues were sampled from the prepuce and coronary sulcus in males and the vulva in females. Ten normal tissue samples after circumcision comprised the controls. The patients involved had an average age of 26 years. Participants with immunity-influencing diseases such as autoimmune diseases, viral hepatitis, tuberculosis, and diabetes were excluded. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from all participants.

Part of the specimens were immobilized in 10% formaldehyde solution for 24 hours, dehydrated, and then embedded with paraffin. Immunohistochemistry and hematoxylin-and-eosin staining were performed for final diagnosis. The remainder of the specimens were instantly placed in RNA later liquid and then stored at –70°C for total RNA extraction. Five specimens from the experimental group and two from the control group were placed in 2.5% glutaraldehyde solution prepared using 0.1 mol/L phosphate buffered saline (PBS, pH = 7.3) and then stored at 4°C for transmission electron microscopy (TEM).

Primer design

The sequences of CD1a (NM001763) and E-cadherin (NM004360) were obtained from GenBank (National Center for Biotechnology Information, NCBI, Bethesda, MD, USA). The corresponding primers were designed using Premier Primer 5 software (PREMIER Biosoft, Palo Alto, CA, USA). The CD1a primers were 5′-GACCTGCACCTGGAAAGGTC-3′ (upstream) and 5′-CCGTATCTTGAGCAGACTTG-3′ (downstream), with an amplification product length (APL) of 151 bp. The E-cadherin primers were 5′-CCCACGCTACAAGGGTCT-3′ (upstream) and 5′-CTGGGAATGGGGGACAT-3′ (downstream), with an APL of 94 bp. The primers for the β-globin internal reference were 5′-CACTTCTACCACTGTCCTCACC-3′ (upstream) and 5′-GAAGACCAAGGACACGTAC-3′ (downstream), with an APL of 268 bp. The primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China).

Immunohistochemistry

The specimens were placed in 10% paraformaldehyde, routinely embedded in paraffin, and then serially sectioned (5 μm). The streptavidin peroxidase method was adopted. The primary antibodies were used at a 1:100 working concentration, and the primary antibodies were replaced with PBS in the negative control. The sections were stained with diaminobenzidine solution, counterstained with hematoxylin, and then mounted for microscopic observation and outcome determination.

The outcomes were determined based on the criteria as follows:

1. CD1a expression was observed under light microscopy. The cells with buffy-stained membranous or cytoplasmic dendritic processes were CD1a positive LCs. Five high-power fields with the most densely distributed LCs were randomly selected, and their LCs were counted. The mean of the CD1a-positive cell counts was obtained.

2. E-cadherin expression was observed under the light microscope. The cells with continuous linear or mottling yellow-to-buffy deposited particles in the membrane or plasma were positively stained cells. The intensity of E-cadherin expression was calculated using semiquantitative scoring based on staining intensity and distribution range: (1) Staining intensity (A value). A score was given according to staining intensity (3 for brown, 2 for buffy, and 1 for yellowish staining). (2) Distribution range (B value): According to the proportion of stained epidermal cells, a score was given (3 for >66%, 2 for 33–66%, and 1 for <33%).

Reverse transcriptase polymerase chain reaction

Total RNA was extracted using TRI reagent (Molecular Research Center Company, Cincinnati, OH, USA). The A260/A280 ratios of all the extracted RNA fell between 1.8 and 2.0 by an ultraviolet
spectrophotometer, which suggests that the obtained total RNA was not degraded or contaminated. cDNA was synthesized after reverse transcription according to the manufacturer’s instructions. PCR was performed and the total reaction system of PCR was 50 μL. The amplification conditions consisted of 35 cycles of 94°C for 3 minutes, 59°C for 45 seconds, and 72°C for 1 minute, followed by a final extension step at 72°C for 7 minutes. The amplification products were subjected to agarose gel electrophoresis, and the gel images were analyzed using an Image Master VDC analyzer (Amersham Biosciences, Piscataway Township, NJ, USA). Densitometric analysis was performed using Total Lab software (TotalLab Limited, Newcastle upon Tyne, Tyne and Wear NE1 2JE, UK). The integrated optical density (IOD) value of the electrophoresis band was used to represent the quantity of the PCR products. The relative mRNA level of the target gene was calculated based on the ratio between its IOD value and that of the internal reference.

**Preparation and observation of LC electron microscopic specimens**

Freshly excised normal prepuces and CA tissues were cut into 3 mm × 1 mm × 1 mm pieces. They were fixed with 2.5% glutaraldehyde and 1% osmic acid, gradiently dehydrated with acetone, and embedded and polymerized in Epon812 resin. Semi-thin sections were localized at the skin epidermis, and ultra-thin sections at 70 nm were made. The sections were subjected to double electron staining with uranyl acetate and lead citrate, and then observed under a JEM-1200 transmission electron microscope (JEOL, Akishima, Tokyo, JP).

**Statistical analysis**

All data were processed and analyzed using Microsoft Excel 2003 and SPSS 10.0 (SPSS, Dawson Creek, BC V1G 3Z3, CA). T-tests and linear correlation analysis were performed for independent sample. The value p < 0.05 was considered statistically significant.

**Results**

**CD1a expression**

In the normal prepuce tissues, numerous intraepidermal CD1a-positive LCs were embedded among the cells in the basal and prickle layers, with most cells located in the middle and inferior parts of the epidermis (Figure 1A). The cells were irregularly shaped with some round or oval cells. Each cell had three to four long and thin processes; some cells even had secondary processes (Figure 1B). The CD1a-positive LC count was 21.59 ± 10.48 per high-power field. By contrast, the intraepidermal CD1a-positive LCs were relatively rare in the CA tissues, with most cells distributed in the middle part of the epidermis (Figure 1C). Cell bodies were small, and processes noticeably were reduced, shortened, or not observed at all. Second-order processes were seldom observed. Typical DCs were rare, which were even manifested by spotted or strip-shaped cellular process structures (Figure 1D). Local CD1a-positive LCs were absent in some CA specimens. The CD1a-positive LC count was 10.66 ± 11.71 per high-power field, which is significantly less than that in the control group (p < 0.05). The results are summarized in Table 1.

**LC ultramicrostructure**

In the two normal prepuce specimens, the intraepidermal LCs were sporadically distributed in the basal and prickle layers. The cells were polygonal with numerous microvilli-like cell processes on the surface and abundant cytoplasm. Their electron density was lower than that of the surrounding epidermal cells. The cell processes went among epidermal cells but had no contact with them. Intra-cytoplasmic organelles were rare: lysosomes were commonly observed, whereas mitochondria and endoplasmic reticula were rare and no intermediate filaments were observed. The nuclei were irregular with deep clefts. In some LCs, Birbeck granules, the

![Figure 1](image-url) CD1a expression. (A) CD1a expression in the normal prepuce tissues (SP × 100); (B) CD1a expression in the normal prepuce tissues (SP × 400); (C) CD1a expression in the CA tissues (SP × 100); and (D) CD1a expression in the CA tissues (SP × 400).
specific organelle, could be observed. These organelles had regular morphologies: they appeared rod-like or tennis racket-shaped with a length of 400 nm and a width of 50 nm; noticeable transverse striations and middle compact longitudinal lines were observed (Figure 2A).

Meanwhile, in the CA specimens (n = 5), the number of LCs was less than that in the control group: intraepidermic LCs were not observed in three specimens and only rarely in the other two. The cells had reduced volumes and polygonal shapes. Cell processes protruded among the surrounding epidermic cells, but no cell conjunction with the epidermic cells was observed. Intra-cytoplasmic organelles were rare, and the nuclei were irregular with nuclear grooves. Neither Birbeck granules nor virus particles were observed (Figure 2B).

**E-cadherin expression**

In the normal prepuce epidermis, E-cadherin displayed strongly positive staining in the inferior parts of the basal, spiny, and granular layers. The membrane presented continuously linear buffy staining with buffy-stained granular sedimentations in the cytoplasm (Figure 3A). The E-cadherin expression value was 7.67 ± 1.64. In the CA tissues, the intraepidermic E-cadherin staining was noticeably shallower than that in the normal prepuce tissues. In some tissues, apparent staining was even not observed and buffy-stained granules were rare in the cytoplasm (Figure 3B). The E-cadherin expression value of the CA tissues was 2.36 ± 1.41, which is significantly lower than that of the control group (p < 0.01). The results are summarized in Table 1.

**Correlation analysis**

The number of CD1a-positive cells was small in the CA tissues where E-cadherin was poorly expressed; the number was increased in the normal prepuce tissues where E-cadherin was highly expressed. In both normal and CA tissues, the intraepidermic E-cadherin expression density was correlated with the number of CD1a-positive cells (r = 0.9404, p < 0.05). The results are shown in Figure 4.

**mRNA expression**

After PCR amplification, 2% agarose gel electrophoresis, and ethidium bromide staining, the length of the obtained CD1a mRNA was 151 bp and that of the obtained E-cadherin mRNA was 94 bp, taking the DNA marker as the molecular weight standard. Both the PCR products fit the theoretical design (Figure 5). PCR gel images of both groups were quantified and then compared. The results show that the relative mRNA expression levels of CD1a and E-cadherin in the CA tissues were significantly lower than those in the normal prepuce tissues (p < 0.01; Table 1).

**mRNA expression analysis**

Based on the tendency demonstrated by immunohistochemistry, CD1a mRNA expression decreased as the E-cadherin mRNA expression decreased in both the CA tissues and the normal prepuce tissues, which indicates a positive correlation (r = 0.8381, p < 0.05; Figure 6).

**Discussion**

The immune system plays an important role in determining the natural progression of HPV infections and the consequences of its associated diseases. The long-term existence and recurrence of CA is mainly because the host fails to establish effective immune responses to HPV infection. Previous studies on CA mostly focused on general immune dysfunction; however, more attention recently has been given to local skin and mucosal immunity, particularly specific cellular immunity, which is believed to be a more important factor in the development, evolution, and prognosis of CA. LC acts as the initiator of local specific cellular immune responses to HPV.

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**Table 1** The expression of CD1 and E-cadherin in normal prepuce and CA tissues (x ± s).
infection, as well as the powerful T cell activator. It is closely correlated with the function of the immune surveillance system. In the human epidermis, CD1a is merely expressed in LC where it participates in antigen presentation, similar to MHC-I molecules. CD1a serves as the surface marker for LCs and is commonly used to identify isolated, purified, and in vitro cultured DCs. The ultrastructure of LCs was first described by Birbeck, for which its specific organelle, the Langerhans granule, was also named the Birbeck body (which is tennis racket- or rod-shaped). Cell culture and morphologic observation show that Birbeck bodies may arise from the membrane of LCs and that they may be formed through cell membrane invagination and folding after LC contacts with antigens and then captures them. However, the changes in the LC in the CA tissues remain controversial. Some studies show that the number of intraepidermic LCs in CA tissues is noticeably less than that in normal tissues, whereas another researcher drew a different conclusion. In the present study, CD1a immunohistochemistry showed that the number of LCs decreased with reduced bodies in the CA tissues. The processes were reduced, greatly shortened, or even absent. Typical DCs were rare and their CD1a mRNA expression decreased. In addition, ultrastructural observation obtained consistent results: LCs were rarely observed in the CA tissues; the

**Figure 3** E-cadherin expression. (A) E-cadherin expression in the normal praeputium tissues (SP × 400); (B) E-cadherin expression in the CA tissues (SP × 400); (C) negative control.

**Figure 4** Correlation between CD1 and E-cadherin in the normal praeputium and CA tissues.

**Figure 5** Reverse transcriptase-polymerase chain reaction analysis of CD1a and E-cadherin mRNA products in the normal praeputium and CA tissues (M: 1 kb DNA marker; 1–3: the CA tissues; 4–6: the normal praeputium tissues).
LC bodies reduced without Birbeck bodies observed in the cytoplasm. These findings indicate that changes in LC occur after an HPV infection. The LC changes are manifested in the decreases in cell number and density, as well as in the expression of antigen presentation-associated molecules and the number of antigen presentation-associated organelles. This further suggests that local LC loss and the so-caused decrease in antigen presentation after HPV infection prevents the triggering of specific immune responses, which hinders the immune system from completely removing HPV. This mechanism may also play a certain role in the immune evasion of HPV.

To date, the mechanism underlying local LC loss and dysfunction in CA tissues remains uncertain. Changes in LC in the tissues of high-risk HPV-induced CIN and SCC are, to some extent, correlated with the changes in adhesion molecules and chemical inducers synthesized by infected cervical epithelial cells. Such molecules are significant for the epidermal metastasis, retention, activation, and differentiation of LC. E-cadherin is a calcium ion-dependent intercellular adhesion molecule that functions in cell adhesion and cell morphologic maintenance. It is expressed in the epidermal basal layer as well as on the surfaces of KC and LC on the basal layer to mediate the adhesion between KC and LC. E-cadherin also plays a critical role in LC localization and retention in the epidermal basal layer, which enables LCs to form a grid-like distribution in the middle and inferior parts of the epidermis to perform their immune surveillance function. In the current study, the molecular and mRNA expression of E-cadherin significantly decreased in the KCs in the CA tissues. Furthermore, in the tissues with low E-cadherin expression, the number of LCs also greatly decreased, indicating a positive correlation. These results suggest that a decrease in the number of intraepidermal LCs in CA tissues may be associated with the decrease in E-cadherin expression in the local KCs because the latter causes difficulty in intraepidermal LC retention. The E-cadherin-mediated adhesion between KC and LC is of great significance for the activation and maintenance of specific immune responses to HPV infection. Decreased E-cadherin expression presumably leads to difficulties in the contact and adhesion between LCs and HPV-infected KCs. As a result, LCs develop disorders in viral antigen presentation. This condition further influences the priming of immune responses to HPV and allows HPV to escape immune surveillance by the host. However, the detailed mechanism underlying the changes in the expression and secretion of adhesion molecules by KCs remains to be explored.

References