Normalization of regulatory T cells, serum TGF-β1, and LTN after 5-aminolevulinic acid-photodynamic therapy in patients with condyloma acuminate

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Abstract. The purpose of this study was to investigate the changes in peripheral blood regulatory T (Treg) cells, serum transforming growth factor-β1 (TGF-β1), and lymphotactin (LTN) following treatment of patients with condyloma acuminata (CA) with 5-aminolevulinic acid-photodynamic. A total of 46 patients with CA were selected as the experimental group and 43 healthy individuals were included in the control group. Before the treatment, the CA patients had a higher number of $CD4^+CD25^+Foxp3^+$ Treg cells and $CD4^+CD25^+$ Treg cells than the healthy group. CA patients also had lower levels of serum TGF-β1 and LTN than the healthy controls. After the treatment, the number of $CD4^+CD25^+Foxp3^+$ Treg cells and $CD4^+CD25^+$ Treg cells decreased significantly in the CA patients and normalized to the levels in the control group after 3 weeks. The treatment also elevated the levels of serum TGF-β1 and LTN in the CA patients, which were close to the values in the control group after 3 weeks. The results showed that low levels of serum TGF-β1 and LTN played important roles in the occurrence and development of CA and cellular immune functions were closely related to the occurrence and development of CA.

Introduction

Condyloma acuminata (CA) is a disease caused by the human papillomavirus (HPV). Clinical manifestations in patients include genital, anal, and perineal papillary, cauliflower-like, or cristate growths (1). WHO statistics show that the global annual incidence of CA is ~30 million cases. CA accounted for 13% of the visits to sexually transmitted disease (STD) clinics in Europe and North America. The high-risk population of CA consists of sexually active 20-40-year old individuals and CA is mainly transmitted by sexual contact (2,3). The incubation period of CA is 1-8 months and the disease is highly contagious. CA is a commonly seen, highly recurrent, and difficult to cure STD, imposing serious physical and psychological impacts on patients (4).

Currently, common CA treatments include microwave, laser, freezing, and electrocautery, which are used to remove as much of the visible warts as possible. However, CA can easily reappear after these treatments (5). 5-Aminolevulinic acid photodynamic therapy can selectively kill tumor cells and fast proliferating cells. In recent years, 5-aminolevulinic acid photodynamic therapy has been applied to treat CA with good therapeutic efficacy, minimal invasion, low recurrence rate, and few side effects (6). A large number of studies suggested that HPV infection was related to immune deficiency or absence in the host cells. Transforming growth factor-β1 (TGF-β1) can inhibit the activity of immune cells, and is involved in cell growth and metabolism (7). Lymphocyte chemokine [lymphotactin (LTN)] is an immune regulatory factor involved in inflammatory responses, playing an important role in immune regulation and immune injury (8).

The aim of the present study was to examine peripheral regulatory T (Treg) cells, serum TGF-β1, and LTN in patients with CA before and after 5-aminolevulinic acid photodynamic treatment to provide a mechanistic understanding for the treatment of CA.

Materials and methods

Subject information. A random sampling method was used to select 46 CA patients treated in Hangzhou First People's Hospital (Hangzhou, China) from November 2014 to October 2015 as the experimental group. A total of 43 healthy individuals were recruited in the control group. Inclusion criteria for the study were: Patients diagnosed with CA based on their clinical and pathological symptoms and positive HPV-DNA tests, patients had no history of recurrent CA and patients provided signed informed consent. Exclusion criteria for the study included, patients that had photosensitive

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Table I. Basic clinical information for the two groups.

Characteristics	Experimental group (n=46)	Control group (n=43)	t/χ^2	P-value
Gender, male/female	22/24	20/23	0.015	0.901
Age, year	20-45	22-45		
Average age, year	31.74±3.43	31.13±3.56	0.837	0.404
Course of disease, months	9.59±2.17	-		
Number of warts	5.56±1.43	-		
BMI index	20.36±2.73	20.74±2.58	0.686	0.494
Education, no. (%)				
Middle school and below	6 (13.04)	5 (11.62)	0.001	0.998
High school or technical secondary school	24 (52.17)	27 (62.79)	0.167	0.675
College and above	16 (34.78)	11 (25.58)	0.049	0.823

disorders or drug allergies, patients with severe immune dysfunctions, and patients who were pregnant or lactating. The basic clinical parameters for the two groups were comparable with no significant differences (Table I).

Treatment. The treatment sites were disinfected before the treatments. 5-Aminolevulinic acid drops were placed on membranes or cotton balls to cover the warts and kept there for 3 h. The covers were removed and the affected areas were washed. An XD-635AB photodynamic laser treatment machine (Guilin Xingda photoelectric medical equipment Co., Ltd., Guilin, China) was used to irradiate the warts. The output wavelength was set at 635±3 nm and the output power was 100 m. The total energy density was ~120 J/cm^2. The areas treated with 5-aminolevulinic acid were fully illuminated. Each spot was exposed for 25 min. When the warts were in the rectum or urethra, laser irradiation required the use of a disposable sleeve to wrap the columnar optical fiber. Paraffin oil was placed on the sleeve and the columnar optical fiber was inserted through the anus or urethra to reach the wart sites. The optical fiber was fixed with tape before the irradiation treatment began. The treatment was carried out once a week, and 3 times for a complete course. Two courses were conducted. The peripheral blood Treg cells and serum TNF-β1 and LTN were monitored during the course of the treatment.

Instruments. Microplate reader (Thermo Fisher Scientific, Inc., Shanghai, China); autoclave (Heto Co., San Diego, CA, USA); low temperature refrigerator (Sanyo, Osaka, Japan); desktop centrifugal machine (Anhui USTC ZonKia Scientific Instruments Co., Ltd., Anhui, China); and flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) were used in the present study.

Reagents. The reagents used were: Mouse monoclonal CD4-FITC anti-human antibody and isotypes (dilution, 1:100; cat. no. CD4001); mouse monoclonal CD25-PE anti-human antibody and isotypes (dilution, 1:100; cat. no. 12-0257-42) (both from eBioscience, Inc., San Diego, CA, USA); MagCellect human CD4$^+$CD25$^+$ Treg cell isolation kit (BD Biosciences); human serum TGF-β1 kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China); human serum lymphocyte chemokine kit (Shanghai XiTang Biological Technology Co., Ltd., Shanghai, China); and PBS buffer (Wuhan Boster Biological Technology, Ltd.).

Cell isolation. Blood (3-5 ml) was collected from both groups early in the morning after 8 h fasting. CD4-FITC monoclonal mouse anti-human and CD25-PE monoclonal mouse anti-human antibodies were added to the test tubes. Peripheral blood mononuclear cells (PBMC) were adjusted to a concentration of 1-5x10^6/ml with PBS buffer, and 10 µl magnetic-labeled anti-human CD25 monoclonal antibody was added. After 15 min incubation at 4˚C, the cell suspension was collected after the blood went through the MS column. CD4$^+$CD25$^+$ Treg cells were obtained by positive selection.

Detection of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells by flow cytometry. PBMC was centrifuged by density gradient centrifugation, and adjusted to 1x10^6/ml. CD4-FITC monoclonal mouse anti-human (20 µl) and 20 µl CD25-PE monoclonal mouse anti-human antibodies were added to 200 µl PBMC. After 30 min incubation at 20˚C in the dark, 2 ml hemolysin was added and the reaction was incubated for 5 min. The supernatant was discarded after centrifugation at 1,200 x g for 5 min. The pellets were washed twice with PBS buffer, centrifuged at 1,200 x g for 5 min, and the supernatant was discarded. Subsequently, 2% normal rat serum of (100 µl) was added and the reaction was kept in the dark for 20 min. PE-labeled anti-human Foxp3 antibody (10 µl) was added followed by incubation in the dark for 30 min. After two washes with PBS, the samples were loaded on the flow cytometry for testing.

Labeling of CD4$^+$CD25$^+$ Treg cells by flow cytometry. Whole blood direct fluorescent antibody staining was employed. Specific fluorescent monoclonal antibody (20 µl) was added to 100 µl of EDTA anticoagulated whole blood. A fluorescent-labeled irrelevant monoclonal antibody was added to the control tube. Hemolysin (2 ml) was added after 15 min staining in the dark at 20˚C. The reaction was incubated for 5 min and centrifuged for 5 min at 12,000 x g. The supernatant was discarded, and the pellet was washed twice with PBS. Then, 300 µl of 1% paraformaldehyde was added and the samples were ready for flow cytometry.

Table II. Peripheral blood Treg cells between the experimental and control groups.

Group	No.	$CD4^+CD25^+$ Treg cells, %	$CD4^+CD25^+Foxp3^+$ Treg cells, %
Control	43	10.61±1.53	5.83±1.34
Experimental	46	15.34±2.67	13.79±2.45
T		10.159	18.831
P-value		<0.0001	<0.0001

Treg, regulatory T.

Table III. Peripheral blood Treg cells in patients at different time points.

Index	Before treatment	1 week after treatment	3 weeks after treatment	7 weeks after treatment	F	P-value
$CD4^+CD25^+$ Treg	15.34±2.67	13.79±2.12	12.84±1.74	10.79±1.64	38.429	<0.001
$CD4^+CD25^+Foxp3^+$ Treg	13.79±2.45	12.34±2.17	10.69±1.85	6.72±1.43	35.62	<0.001

Treg, regulatory T.

Detection of TGF-β1. Levels of serum TGF-β1 and LTN were tested by enzyme-linked immunosorbent assay (ELISA). Diluted standards and samples were added to the wells. The plate was covered and incubated at 37˚C for 90 min. Biotin-labeled anti-human TGF-β1 antibody was then added followed by another incubation at 37˚C for 60 min. After 3 washes with PBS, the ABC working solution was added and the reactions were incubated for 30 min at 37˚C. The wells were washed 5 times with PBS, then the TMB chromogen solution was added and the plate was incubated for 30 min in the dark at 37˚C. The TMB stop solution was added, and the OD values were read at 450 nm within 15 min in a microplate reader.

Detection of LTN. The standards and the test samples were added to the wells. The plate was covered and incubated at 37˚C for 120 min. After six washes with PBS buffer, the primary antibody from the ELISA kit (Biosharp, Hefei, China) was added and the incubation lasted for 60 min at 37˚C. The enzyme-conjugated secondary antibody was added after six washes with PBS buffer, and the incubation was carried out at 37˚C for 30 min. The substrate solution was added after another six washes with PBS buffer and the plate was incubated at 37˚C for 15 min. After the stop solution was added, the OD value was measured within 15 min at 450 nm with a microplate reader.

Detection of peripheral Treg cells. Blood (2-5 ml) was collected from the two groups before the treatment and 1, 3 and 7 weeks after the first treatment. The peripheral blood $CD4^+CD25^+$ Treg cells and $CD4^+CD25^+Foxp3^+$ Treg levels of cells were counted by flow cytometry.

Detection of serum TGF-β1 and LTN. ELISA was applied to monitor the levels of serum TGF-β1 and LTN before the treatment, and 1, 3 and 7 weeks after the first treatment. Procedures strictly followed the manufacturer's instructions.

Statistical analysis. SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) was used to process the data. Variable data are expressed as mean ± standard deviation and compared using the t-test. Attributes data were analyzed using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of the peripheral blood Treg cells between groups. We collected blood from the experimental and control groups and detected the Treg cells with the desired markers (Table II). The number of $CD4^+CD25^+$ Treg cells and $CD4^+CD25^+Foxp3^+$ Treg cells in peripheral blood were significantly higher in the experimental group compared with the control group (Table II).

Peripheral blood Treg cells at different time points. Following the baseline study, we then examined Treg cells after treating patients in the experimental group. The results showed that the content of $CD4^+CD25^+$ Treg cells and $CD4^+CD25^+Foxp3^+$ Treg cells was significantly lower after the treatment compared with that before the treatment (Table III). The levels of $CD4^+CD25^+Foxp3^+$ Treg cells and $CD4^+CD25^+$ Treg cells progressively decreased at 1, 3 and 7 weeks after the first treatment (Table III). The analysis after three weeks showed values for $CD4^+CD25^+$ Treg cells similar to the control group (Tables II and III). The values for $CD4^+CD25^+Foxp3^+$ Treg cells after treatment also normalized after 3 weeks, but they remained slightly higher than those for the control group. A downward trend appeared over time following the first treatment (Fig. 1).

Serum TGF-β1 and LTN levels between the groups. Compared with the control group, the experimental group had significantly lower levels of TGF-β1 and LTN (Table IV).

Table IV. Serum transforming growth factor-β1 and lymphotactin between the experimental and control groups.

Group	No.	Transforming growth factor-β1, ng/ml	Lymphotactin, pg/ml
Experimental	46	9.19±3.63	267.83±79.34
Control	43	12.34±4.67	435.79±128.45
t-test		9.593	38.841
P-value		<0.0001	<0.0001

Table V. Serum TGF-β1 and LTN levels in patients at different time points.

Index	Before treatment	1 week after treatment	3 weeks after treatment	7 weeks after treatment	F	P-value
TGF-β1, ng /ml	9.19±3.63	9.89±3.65	10.83±3.34	11.93±3.24	29.94	<0.001
LTN, pg/ml	267.83±79.34	312.34±82.67	375.79±98.45	415.72±115.23	19.62	<0.001

TGF-β1, transforming growth factor-β1; LTN, lymphotactin.

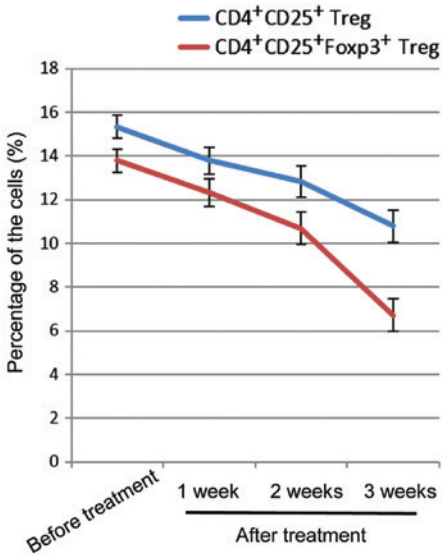

Figure 1. The trend of $CD4^+CD25^+Foxp3^+$ Treg cells and $CD4^+CD25^+$ Treg cells in study subjects. Treg, regulatory T.

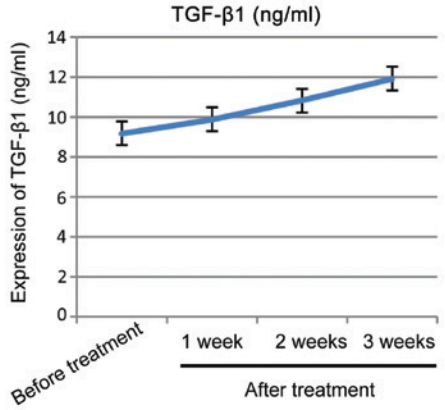

Figure 2. Trend of serum transforming growth factor-β1 (TGF-β1) level in study subjects.

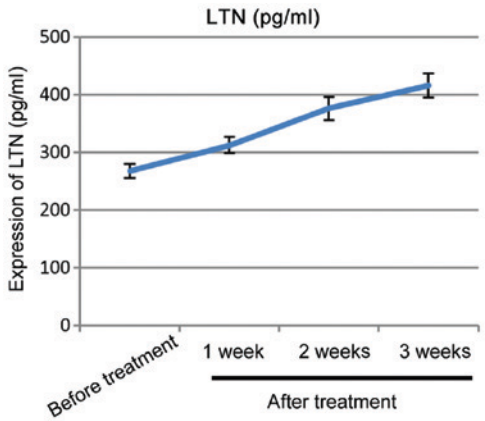

Figure 3. Trend of serum lymphotactin (LTN) level in study subjects.

Serum TGF-β1 and LTN levels in patients at different time points. The levels of serum TGF-β1 and LTN increased after the treatment compared with before the treatment (Table V). Serum TGF-β1 and LTN levels increased progressively at 1, 3 and 7 weeks after the first treatment. Both indexes showed a rising trend along with the treatments (Figs. 2 and 3).

Discussion

CA is a viral STD caused by HPV infection. Epithelial tissues can easily be infected, so CA warts centralize on the outer genital and anal areas. Previous findings have shown that CA occurrence, development, and prognosis were closely related to the immune responses (9). Currently, the clinical treatment of CA includes drug therapy (foot grass leaf esters and trichloroacetic acid), physical therapy (microwave, freezing, electrocautery and laser), and surgical resection (10). Physical and surgical treatments are somewhat invasive and the pain complicates the collaboration from patients, resulting in

incomplete debridement and longer wound healing after treatment. The corrosive substances used in the drug therapy increase the damage to the normal skin around the warts, making it more difficult to treat. In addition, the limited penetration of drugs complicates the treatment of larger lesions and CA could easily reoccur (11,12). The 5-aminolevulinic acid photodynamic therapy was first developed by Canadian scholars in the 1990s. The idea was to use a specific wavelength of light to irradiate the topical photosensitizer previously applied to the warts. The reactive oxygen species generated from the photochemistry and photobiology reactions would kill the pathologic tissues while the adjacent normal tissue would not be affected (13). 5-Aminolevulinic acid photodynamic therapy was especially useful for warts located at mucosal sites (glans, urethra, vagina and anus), which showed good therapeutic effectiveness, high clearance rate, and quick healing of local lesions, and had no effect on patient daily life (14).

In the mid-1990s, Japanese scholars first proposed the concept of Treg subset cells. Previous findings have identified the existence of abnormal Treg cells in HPV infection. Treg cells are critical for the pathogenesis of autoimmune, cancer, and immune tolerance diseases (15). Treg cells not only suppress the immune responses of the host cell but also induce immune tolerance cells. $CD4^+CD25^+$ Treg cells are immune-modulatory cells that regulate the immune system and maintain immunologic homeostasis. $CD4^+CD25^+$ Treg cells modulate the immune response in patients with CA by inhibiting cytokine secretion and the proliferation of effector cells (16). The possible mechanism for the CA lesions to achieve immune escape was that $CD4^+CD25^+Foxp3^+$ Treg cells had localized aggregation and, together with related factors, contributed to the development of CA. The increased level of $CD4^+CD25^+Foxp3^+$ Treg cells in CA patients have been widely reported (17). This study showed that CA patients had significantly more $CD4^+CD25^+Foxp3^+$ Treg cells and $CD4^+CD25^+$ Treg cells than the healthy control group, which was consistent with previous studies. Both indexes decreased after the treatment. The treatment worked by inhibiting or removing Treg cells to induce the specific immune responses to HPV and inhibit HPV infection.

TGF-β1 is a growth factor with critical biological activities. Among these, TGF-β1 plays a major role in the cellular immune system. It had been shown that TGF-β1 expression level correlated to the degree of CA wart proliferation. TGF-β1 inhibits epithelial cell proliferation and regulates growth, differentiation, apoptosis, and immune responses. Reduced level of TGF-β1 in CA patients leads to the excessive proliferation of skin mucous membranes in the presence of HPV infection, resulting in the formation of warts (18). LTN is produced by $CD8^+$ T cells, NK cells, and other immune cells. LTN attract inflammatory cells to move to the site of HPV infection, and induce the activation of leukocytes (19). LTN can regulate the balance of the immune system and also plays an adjuvant role in the immune system by participating in the mediation of Th1 and Th2 cells. HPV infection in CA patients induced changes in the expression of LTN, the resulting cytokines chemotactic effect was involved in the pathogenesis of HPV (20). The results of our study showed that the experimental group had significantly lower TGF-β1 and LTN levels, but the levels rose after the treatment, indicating that both played key roles in the immune clearance of the virus in CA patients.

In summary, low level of serum TGF-β1 and LTN contributed to the occurrence, development, and prognosis of CA. During the virus infection, the number of Treg cells increased in peripheral blood. These indexes were corrected by the treatments, supporting the role of these markers in the occurrence and treatment of CA.

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