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Expression of $CD4^+CD25^{high}CD127^{low/-}$ Regulatory T Cells in Transitional Cell Carcinoma Patients and its Significance

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To evaluate the expressions of $CD4^+CD25^{high}CD127^{low/-}$ regulatory T cells (Tregs) in peripheral blood from patients with transitional cell carcinoma (TCC) in urinary system, we investigated the proportion of Treg population in $CD4^+$ T from 93 patients with TCC, 38 with benign urinary diseases, and 37 healthy subjects by using flow cytometric analysis and analyzing different clinicopathologic characteristics and the changes before and after operation. We found that the proportion of Treg in peripheral blood from patients with TCC was significantly increased as compared with the other two groups. There was a strong correlation between the proportion of Treg and tumor recurrence, quantity, lymph node metastasis ($P<0.01$), as well as pathological stage; no correlation was found between the proportion of Treg and clinical TNM stage ($P>0.05$). The proportion of Treg was also different before and after operation ($P<0.05$). This suggest that $CD4^+CD25^+CD127^{low/-}$ regulatory T cells may be responsible for immune suppression in TCC patients. The resection of tumor can decrease the proportion of Treg in peripheral blood. J. Clin. Lab. Anal. 23:197–201, 2009.

Key words: transitional cell carcinoma; regulatory T cells; urologic diseases; cellular immunity

INTRODUCTION

$CD4^+CD25^{high}CD127^{low/-}$ regulatory T cells (Tregs) are indispensable in human immunoregulation and immunosuppression. According to recent reports, Treg also plays a significant role in suppression of specific and nonspecific anti-tumor immune response by affecting the function of $CD8^+$CTL and NK cell (1,2). Tregs are subdivided into natural and induced Tregs, and both natural and induced Tregs contribute to tumor tolerance though contributions of each to tumor-related immune dysfunction remain little understood. Recent studies have revealed that a subset of $CD4^+$ T cells, referred to as $CD4^+CD25^{high}Foxp3^+$ naturally occurring regulatory T cells, may accumulate in the tumor environment and inhibit activation and differentiation of $CD4^+CD25^-$ or $CD8^+$ T cells and the function of natural killer cells, thereby hindering tumor rejection. Thus, $CD4^+CD25^{high}Foxp3^+$ is becoming a new research topic. However Foxp3 is a nuclear protein, it is of limited value in the isolation of Tregs because breaking the membrane is a necessary procedure before marking the protein, and it is a complex operation. Conversely, recent reports have shown that low levels of the IL-7 receptor-chain (CD127) are expressed on Treg cell surfaces. Banham et al. (3) have proven that the expression of CD127 is inversely correlated with Foxp3 expression and with the suppressive function of $CD25^{high}$ Tregs. There were also other results that showed the low expression of CD127 being correlated with the immunoregulation function of $CD4^+CD25^{high}$Treg (4), so CD127 can be used as one of the important markers of regulatory T cells.

Tumors employ myriad mechanisms to defeat host immunity. Many such mechanisms ultimately induce functional tumor $CD4^+CD25^{high}CD127^{low/-}$Treg accumulation. $CD4^+CD25^{high}CD127^{low/-}$Tregs are able to suppress effector T cell proliferation, which contributes to many kind of tumor progression including transitional cell carcinoma (TCC). Therefore, $CD4^+CD25^{high}CD127^{low/-}$ can be a useful selective biomarker for the enrichment of human Treg cells and also for in vitro assays in TCC.

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This study analyzed the frequency of $CD4^+$ $CD25^{high}CD127^{low/-}$ regulatory T cells in peripheral blood of pre- and postsurgical operation of 93 patients with TCC in urinary system and discussed the relationship between the level of Treg and different clinicopathologic characteristics.

MATERIALS AND METHODS

Clinical Materials

Ninety-three patients with TCC in urinary system, who received operations in Friendship Hospital from September 2008 to February 2009, were enrolled into this study. None of them received radiotherapy, chemotherapy, or immunotherapy before their blood samples were collected. Also, 38 patients with benign urogenital diseases and 37 healthy donors were included in this study. Prior to sample collection, appropriate permission was given from the research ethical committee. Peripheral blood was collected from each patient and healthy donors. Patient data is summarized in Table 1.

Equipments and Agents

Peripheral blood leukocytes (PBL) were isolated by centrifugation through a standard density gradient. Anticoagulate peripheral blood were resuspended in PBS supplemented with 2% bovine serum albumin at a leukocyte concentration of $5–6 \times 10^5$ cells/ml. Cell surface marker analysis was performed by using flow cytometry. Fluorochrome-labeled mouse anti-human monoclonal antibodies targeted against CD4-FITC, CD25-PC5, CD127-PE (Beckman Coulter, Miami, FL); together with appropriate isotype controls IgG1-FITC, IgG1-PE, IgG1-PC5 (Beckman Coulter) were used. Erythrocyte splitting liquor Optilyse C and EPICS XL4 flow cytometry machine are also bought from Beckman Coulter Company.

$CD4^+CD25^{high}CD127^{low/-}$Treg Assay

A total of 2 ml peripheral total blood was collected from healthy donors, TCC patients, and patients with benign urogenital diseases. For TCC patients, another 2 ml peripheral total blood was collected within 24–48 hr after operation and 2–4 weeks after operation for the second time. All blood was kept in EDTA-K_3 anticoagulation vacuum tube at room temperature and tested by flow cytometry within 24 hr.

First, we prepared anticoagulate peripheral total blood 100 μl, supplemented with fluorochrome-labeled mouse anti-human monoclonal antibodies targeted against CD4-FITC, CD25-PC5, CD127-PE, together with appropriate isotype controls, supplemented with IgG1-FITC, IgG1-PE, and IgG1PC5. Second, all the six units were incubated for 15–20 min at room temperature, with 2 ml of PBS added, they were centrifuged for 5 min at the speed of 2,000 r/min. Finally, they were washed twice with PBS before tested by flow cytometry.

Lymphoid cells were gated according to their forward and side light scatter properties. Lymphoid cells were gated for analysis of CD4, CD25, and CD127. The mean percentage of expression of a particular marker on PBL was compared between the subjects in each category (healthy controls, TCC patients, and benign urinary diseases patients).

Statistical Analysis

We used SPSS software package 10.0 (SPSS, Chicago, IL) to investigate the data. The data of normal distribution were expressed as the mean ± SD while the data of partial distribution were expressed as the median (25–75%). Statistical significance was determined using Student's *t*-test and analysis of variance and a *P*-value of 0.05 or less was considered significant.

RESULTS

The proportion of $CD4^+CD25^{high}CD127^{low/-}$Treg is shown in Table 2. The proportion of Treg in patients with TCC group (Fig. 1) was significantly higher than group of patients with benign urogenital disease ($P<0.01$) and healthy donor group ($P<0.01$) (Fig. 2). However, the proportion of $CD4^+CD25^{high}CD127^{low/-}$ Treg showed no difference between the two groups of benign urogenital diseases and healthy donors.

For TCC patients, the proportion of Treg population in peripheral blood after operation is fewer than before

TABLE 1. Patient Characteristics

Item	TCC ($n=93$)	Benign ($n=38$)	Health ($n=37$)
Gender			
Male	60	30	25
Female	33	8	12
Age (yr)	64.9 ± 10.5	56.7 ± 15.4	57.4 ± 12.2

TABLE 2. Treg Population in Peripheral Blood of the Three Groups

Groups	Cases (*n*)	Treg (%)
Transitional cell carcinoma patients	93	1.660 (1.125–2.275)**
Benign urogenital diseases patients	38	0.353 ± 0.272
Healthy donors	37	0.310 ± 0.173

**$P<0.01$. The proportion of Treg was significantly higher among patients with TCC group than the other two groups.

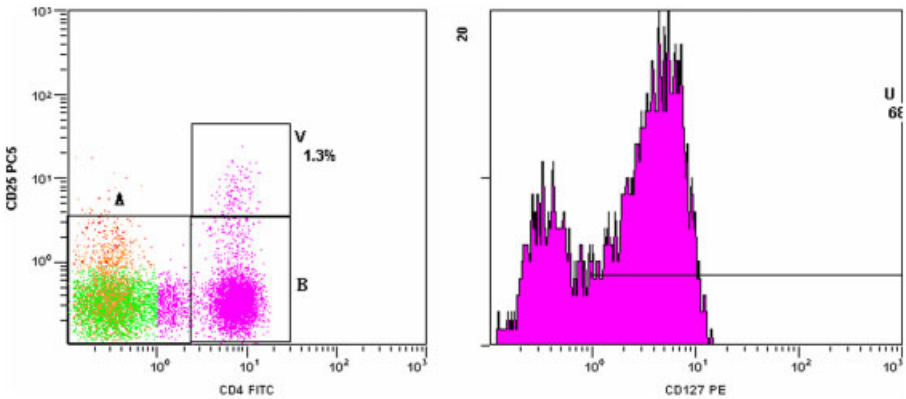

Fig. 1. Three-color flow cytometric analysis of PBLs from a TCC patient. PBLs were stained with CD4, CD25 and CD127. A group cells are gated from CD4$^-$ cells. CD25 expression in CD4$^+$CD25$^{-/low}$ cells. V group cells are gated from CD4$^+$CD25^{high} cells, and the histograms show plot are gated from CD127 positive cells from CD4$^+$CD125^{high} cells. This figure appears in color online.

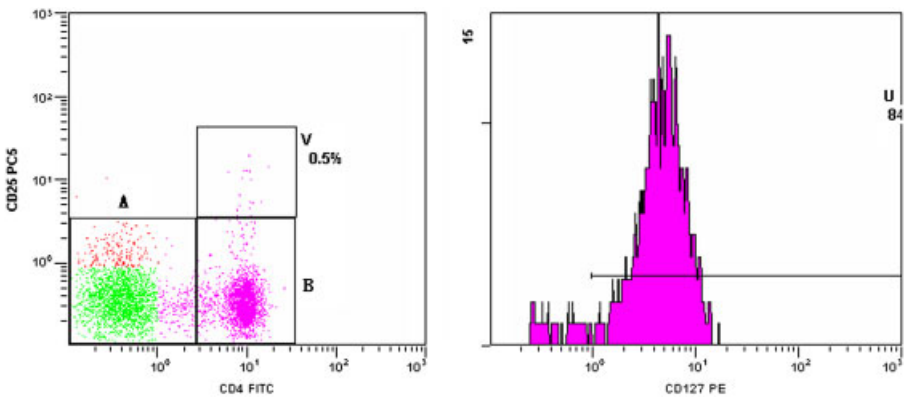

Fig. 2. Three-color flow cytometric analysis of PBLs from a healthy donor. PBLs were stained with CD4, CD25 and CD127. A group of cells are gated from CD^{4-} cells. CD25 expression in CD4$^+$ T cells is divided into high, low, and negative categories. B group cells are gated from CD4$^-$CD25$^{-/low}$ cells. V group cells are gated from CD4$^+$CD25^{high} cells, and the histograms show plot are gated from CD127 positive cells from CD4$^+$CD125^{high} cells. This figure appears in color online.

TABLE 3. Treg Population in Peripheral Blood of Pre- and Postoperative Patients for Transitional Cell Carcinoma

Groups	Cases (n)	Treg (%)
Preoperation	93	1.660 (1.125–2.275)
Postoperation (24–48 hr)	93	1.395 (0.985–2.228)*
Postoperation (after 2–4 weeks)	77	1.420 (0.934–2.256)*

*$P<0.05$. Compared with postoperation group, the proportion of Treg for preoperation group was higher.

($P<0.05$); however, there was no difference between 24–48 hr and 2–4 weeks after operation for Treg population ($P>0.05$) (Table 3).

We next addressed whether the proportion of CD4$^+$ CD25^{high}CD127$^{low/-}$Treg in TCC patients had any difference compared with patients with benign urogenital diseases and healthy controls in different clinicopathologic characteristics. As listed in Table 4, we could find out there was no difference between Superficial TCC (Tis Ta T1) and TCC infiltrate muscle or serosa membrane (T2, T3) ($P>0.05$), and no difference was found between TCC infiltrate muscle or serosa membrane and TCC with adjacent tissue invasion ($P>0.05$) either. However, the distribution of Treg in advanced disease (G3) significantly increased compared with early tumor stage (G1 and G2) in isolated PBMCs ($P<0.01$). Furthermore, there was also significant correlations of Treg expressions with lymph node metastasis, recurrence, and number of tumor ($P<0.01$). However, the proportion of Treg did not differ with different tumor locations.

DISCUSSION

In recent years, several investigators have reported that the number of Treg in peripheral blood and/or tumors increases in patients with malignant tumor such as gastric cancer (5), hepatocellular carcinoma (6), lymphoma (7), renal cell carcinoma (8), prostate cancer (9), etc. However, little is known about the

TABLE 4. Relationship Among the Level of Treg in Different Clinicopathologic Characteristics

Variables	Cases (*n*)	Treg (%)
TNM stage		
Tis Ta T1	71	1.763 (1.110–2.090)*
T2–T3	15	2.305±1.406
T4	7	2.810±1.150
Risk factor		
Smoke	26	1.650 (1.223–2.295)
Nonsmoke	67	1.660 (1.110–2.280)
Recurrence		
Yes	32	2.418±1.307**
No	61	1.420 (1.080–2.085)
Single/multiple		
Single	51	1.480 (1.050–1.980)
Multiple	42	2.382±1.317**
Pathological stage		
G1	26	1.405 (1.215–2.035)
G2	50	1.525 (1.063–2.068)
G3	17	2.900±1.264**
Location of tumor		
Bladder	72	1.645 (1.143–2.358)
Renal pelvic and ureteral	21	1.980 (0.840–2.270)
Lymph node metastasis		
Yes	10	2.840 (1.358–4.300)**
No	83	1.640 (1.110–2.160)

**$P<0.01$, *$P<0.05$. The proportion of Treg in different clinical pathological charicteristics.

frequency of $CD4^+CD25^{high}CD127^{low/-}$ cells in TCC patients. Immune therapy such as intravesical therapy for superficial bladder cancer on recurrence by Bacillus Calmette-Guerin (BCG) has been introduced into bladder cancer for many years, but a strategy focused on immunoregulation is not popular until recent years. Loskog et al. reported the domination of Tregs in bladder carcinoma tissue (10). To investigate the clinicopathological characteristics in TCC of urinary system comprehensively, we analyzed not only the TNM stage, pathological stage, lymph node metastasis, and quantity of tumor but also the recurrence according to its own feature. Since TCC can be diagnosed easily and quickly compared to cancer from other areas thanks to cystoscopy, most patients visit a doctor the first time there is blood in their urine, and most cases are Ta and T1 stage. Besides, normalized postoperative re-examination prevents recurrent cancer from invading to muscle layer. This leads to a most frequent diagnosis of superficial TCC though some of these patients have been through several times of trans urine resections of bladder tumors and some of them have highly malignant carcinoma in terms of pathological features. In this study we found Treg in peripheral blood had no correlation with clinical TNM stage. However, highly malignant tumors may cause significantly decrease in immune function, which would lead to recurrence and implantation. What was also demonstrated by this study was that pathological stage, recurrence, and quantity of tumor as related to Treg percentage in $CD4^+$T cells. Smoking is one of the potential risk factor for many types of cancer including TCC; however, it does not affect the immune system directly. In addition, the study showed that the proportion of Treg in peripheral blood did not differ between the two groups of smokers and nonsmokers and gave no evidence of any relationship between the location of TCC and the immune system.

Treg can inhibit tumor immunity through disparate pathways and mediate immunologic effects through various means including soluble factors and contact-dependent mechanisms. More Treg were found in peripheral blood and tumors of patients than in healthy ones, and the percentage of Treg in $CD4^+$T cells also increased. In our study the proportion of Treg in postoperation was much lower than in preoperation for the same group ($P<0.05$). Thus, significant recovery of immune system is detected in 24–48 hr. The first time of follow-up was 2–4 weeks after operation and we found there was no difference between 24–48 hr and 2–4 weeks after operation for Treg population ($P>0.05$). However, the recovery of immune function always requires a sufficient time and further follow-up is necessary to determine how the immune function changes.

This study indicated that the proportion of $CD4^+CD25^{high}CD127^{low/-}$Treg in patients with TCC was significantly higher than patients with benign urogenital diseases and healthy donors ($P<0.01$). Regulatory T cells mediate homeostatic peripheral tolerance by suppressing autoreactive T cells. Failure of host antitumor immunity may be caused by exaggerated suppression of tumor-associated antigen-reactive lymphocytes mediated by Treg cells. The increasing proportion of Treg in TCC patients' peripheral blood completely proved this point. And the analysis of $CD4^+CD25^{high}CD127^{low/-}$Treg in TCC patient is contributable to both prognosis and treatment.

Many investigators have studied Treg and reported that the elimination of Treg could inhibit tumor growth in vitro (11,12). Reducing Treg function is also a logical therapeutic strategy because thus doing would allow more effective immune-based therapies, alone or in combination with other therapies. Reports have shown antigen-specific Treg elimination, effector cell threshold raise, restrained Treg trafficking, and effector functions (13). Treg has a strong correlation with TCC recurrence, pathological stage, and lymph node metastasis; therefore, effective Treg cell elimination will be critical for its successful immunotherapy. It is significant to enhance antitumor immunity in order to ensure immunotherapeutic strategies and reduce the recurrence and metastasis. In conclusion, the abovementioned methods may have killing effects on human cancer cells, which

indicates we should remain optimistic that Treg can be helpful in our fight against cancer.

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