A New Indicator of Favorable Prognosis in Locally Advanced Renal Cell Carcinomas: γδ T-Cells in Peripheral Blood

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Abstract. Background: Although human γδ T-cells that express Vγ2Vδ2-bearing T-cell receptor (Vγ2Vδ2 T-cells) have recently received considerable attention in the development of novel cancer immunotherapies, consensus has not yet been reached regarding the physiological relevance of this T-cell subset in the context of cancer immunosurveillance. Clinical trials of adoptive immunotherapy using autologous Vγ2Vδ2 T-cells have been applied to patients with advanced renal cell carcinoma (RCC) and some clinical benefits have been reported. In the present study, we investigated the correlation between the proportion of γδ T-cells in peripheral blood before surgery in patients with locally advanced RCC and those clinical outcomes. Patients and Methods: Of 41 patients who underwent surgery for RCC, 13 patients had stage III disease without metastasis. These stage III patients were stratified into two groups based on the peripheral γδ T-cell proportion being greater or less than 8.7% before surgery and were followed up for up to 137 months (median 129 months). Results: Remarkably, an obvious difference was found in the overall survival and cause-specific survival rate between the two groups. In 6 patients with a higher proportion of γδ T-cells, one patient had lung metastasis, but there were no cancer-related deaths. In contrast, 5 out of 7 patients with a lower proportion of γδ T-cells died during the study and 4 out of 7 patients died due to RCC. Conclusion: An increase in the proportion of peripheral γδ T-cells is a favorable prognostic factor for patients with locally advanced RCC.

Human Vγ2Vδ2 T-cells recognize nonpeptide antigens and exhibit cytotoxicity against various tumor cell lines and may play an important role in future immunosurveillance systems. Vγ2Vδ2 T-Cells are major subsets of peripheral blood γδ T-cells. Vγ2Vδ2 T-cells are also activated by synthesized pyrophosphomonoester derivatives and nitrogen-containing bisphosphonates, such as zoledronic acid, with interleukin-2 (IL-2) in vitro. Based on these findings, Vγ2Vδ2 T-cells have recently attracted considerable attention in the development of novel cancer immunotherapies, and several different approaches have been designed and employed in clinical trials (1-3).

We previously reported that a patient with lung metastasis after radical nephrectomy for renal cell carcinoma (RCC) had six cycles of adoptive immunotherapy using autologous in vitro-activated Vγ2Vδ2 T-cells followed by low-dose IL-2 and zoledronic acid intravenous infusion. Complete response was achieved, which has been maintained for 3 years without any additional treatment (4). We also reported that Vγ2Vδ2 T-cells exert effects on a variety of tumor cells (5) and there is an increase in the number of Vγ2Vδ2 T-cells in some patients with RCC (6). However, consensus has not been reached regarding the physiological relevance of Vγ2Vδ2 T-cells in the context of immunosurveillance for cancer. We therefore investigated the prognostic significance of the proportion of γδ T-cells in peripheral blood in patients with locally advanced RCC.
Patients and Methods

Patients. From December 1997 to August 1998, 41 patients underwent surgery for RCC at the Department of Urology, Tokyo Women’s Medical University Hospital. We have already reported that 10 out of the 41 RCC patients showed increased proportions of peripheral blood γδ T-cells in T-cells (6). These patients were followed up at the Tokyo Women’s Medical University Hospital and our associated hospitals for up to 137 months after the surgery. Of 41 patients, 29 were men and 12 were women, who ranged in age from 33 to 78 (median age, 59) years at the time of surgery. Also included were 32 healthy individuals, ranging in age from 19 to 73 (median age, 40) years, who had never suffered from malignancies or bacterial infections, such as tuberculosis, typhoid, and tularemia, as we have already reported (6). Computed tomography (CT) was conducted twice yearly after the surgery for up to 5 years and then once a year for at least 10 years.

Isolation of peripheral blood mononuclear cells (PBMC-) and flow cytometric analysis. PBMCs were isolated from the heparinized peripheral blood of both RCC patients and healthy subjects by Ficoll-Conray density-gradient centrifugation performed at 1500 rpm for 30 minutes, as described previously (6). The following monoclonal antibodies (mAbs) were used to identify fresh PBMCs during immunofluorescence analysis: Fluorescein isothiocyanate (FITC)-conjugated-anti-Vδ2 chain mAb (Immu389; Beckman Coulter, CA, USA; or 15D, Serotech Ltd, Kidlington, Oxford, UK), FITC-conjugated-anti-Vγ2 chain mAb (Immu360; Beckman Coulter), FITC-conjugated-anti-pan-γ/δT-cell receptor mAb (Immu515; Beckman Coulter), Phycoerythrin (PE)-conjugated-anti-CD3 mAb (SK7; Becton Dickinson Immunocytemetry Systems, San Jose, CA, USA). The stained T-cells were examined by two-color flow cytometric analysis using an EPICS CS or XL flow cytometer (Beckman Coulter), as described previously (6). During all staining procedures, the cells were kept on ice.

Statistical analyses. Statistical analyses were conducted to test the differences between two items using the log-rank test and Kaplan-Meier estimator. We used the Stat View 5.0J software package (Abacus Concepts, Inc, CA, USA).

Results

The proportion of γδ T-cells among CD3+ cells derived from healthy individuals was 4.3±2.2% (data not shown). Forty-one RCC patients were divided into groups of RCC development stage according to the UICC 2002 classification (Table I). The flow cytometric results of PBMC from patient no. 6 are shown in Figure 1 as representative data. Predominant expansion of Vγ2Vδ2 T-cells in γδ T-cells was also seen in those from the other patients with expansion of γδ T-cells in PBMCs. Four patients (18.2%) in stage I, 1 patient (50.0%) in stage II and 5 patients (38.5%) in stage III showed an increased proportion of peripheral blood γδ T-cells in their CD3+ cells. The numbers of patients in each stage were different from those numbers that we previously reported because of the different UICC classification (6). We focused on the patients with stage III disease because of their high risk of RCC recurrence (7). Thirteen patients with stage III clear cell RCC were classified into two groups, patients 1-6 and 7-13, according to their having a γδ T-cell proportion
higher or lower than 8.7% (the mean±2×standard deviation (SD) for healthy individuals is 4.3±4.7%, average of healthy controls±2SD) of PBMC. There was no inherent dichotomy in the clinical and pathological characteristics between these two groups (Table II). Surprisingly, obvious differences were observed in the overall and the cause-specific survival rates (Figure 2a and b), but no significant difference was found in the disease-free survival rate by log-rank tests (Figure 3). In patients 1-6, there were no deaths due to cancer during the study and only one patient experienced lung metastasis, 45 months after the surgery. In contrast, among patients 7-13, four patients died due to RCC at 10, 52, 67 and 135 months after the surgery and another due to acute myocardial infarction 105 months after the surgery. In addition, four patients from this group developed metastasis (Table II). Interestingly, the proportion of peripheral blood γδ T-cells was only 0.5% at 18 months after lung metastasis developed in patient no. 7, compared with 2.9% at 6 years after lung metastasis developed in patient no. 1, who is still alive (data not shown). In the patients with stage I and II RCC, whose proportions of peripheral blood γδ T-cells were elevated to more than 8.7% of CD3+ cells, there have been no deaths due to RCC (data not shown).

**Discussion**

While evidence has been accumulating that Vγ2Vδ2-bearing γδ T-cells exert cytotoxic activity against a wide spectrum of tumor cells in vitro as well as in vivo, the physiological role of this T-cell subset remains enigmatic (8). We previously suggested that γδ T-cells may provide innate immunity against RCC, based on the findings that the proportion of γδ T-cells increased with an increase in the disease stage and that the percentage of these cells returned to normal levels after nephrectomy (6). In this study, we clearly demonstrated that increased levels of γδ T-cells were indeed correlated with the overall survival rate in patients with locally advanced RCC. Table II shows there was no significant difference in the actual numbers of lymphocytes between these two groups. The increased proportion of γδ T-cells was due to an increase...
in the number of γδ T-cells. This result strongly supports the hypothesis that γδ T-cells should play a cardinal role in cancer immunosurveillance and indicates that an increase in the proportion of peripheral blood γδ T-cells is a favorable prognostic factor for patients with locally advanced RCC. There was no significant difference in the disease-free survival rate between these two groups. One explanation might be that only a small number of patients were followed up and we should draw up a project to obtain proof of the concept that an increased proportion of γδ T-cells in peripheral blood before radical nephrectomy may prevent recurrence of RCC in a large number of patients. Five out of 41 RCC patients with stage I or II localized RCC showed increased levels of γδ T-cells and it is difficult to explain the roles of γδ T-cells in these cases because none of these patients developed recurrence of RCC. In patient 6, the proportion of γδ T-cells decreased from 19.8% to 4.4% at four months after the surgery and to 2.9% at the time of RCC recurrence. Peripheral blood γδ T-cells did not increase at the time of recurrence. This indicates that recurrent RCC may escape from the immunosurveillance system of γδ T-cells or that γδ T-cells might not be able to respond to RCC because of the immunosuppressive mechanisms of the regulatory cells. Finke et al. reported that immunosuppressive myeloid dendritic cells and regulatory cells increased in peripheral blood of patients with advanced RCC (9, 10). In patient 7, the proportion of γδ T-cells decreased from 3.3% to 0.5% at the time of recurrent RCC. It is difficult to explain whether the metastatic RCC developed because of the decreased proportion of γδ T-cells or because metastatic RCC had immunosuppressive mechanisms which reduced the proportion of γδ T-cells. Nevertheless, whatever mechanisms are involved in decreasing the proportion of γδ T-cells after the development of recurrent RCC, the resulting reduction clearly influences the prognosis. This finding provides an impetus to undertake further investigation for cancer immunotherapy using γδ T-cells.

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