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T-Cell Receptor ζ-Chain Expression on Tumor-infiltrating Lymphocytes from Renal Cell Carcinoma[1]

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ABSTRACT

Loss of the T-cell receptor-associated ζ chain in tumor-infiltrating lymphocytes (TILs) has been proposed as one mechanism of acquired immunosuppression in cancer patients. Recent reports suggest that ζ-chain loss may be related to contaminating monocyte/macrophage protease activity. Using flow cytometry and Western blot analysis, we have confirmed the expression of ζ chain in matched peripheral blood mononuclear cells and TILs from eight patients with primary renal cell carcinoma, when the cells were exposed to sufficient quantity of protease inhibitors. A small decrease in ζ-chain expression was found in three TIL samples. The loss of ζ-chain expression that was noted by others may be related to differences in laboratory method, and the small changes we have noted are unlikely to be sufficient in explaining the immunosuppression of TILs.

INTRODUCTION

The observation that human cancers have selective lymphocytic infiltration has intrigued immunologists and oncologists over the years and forms one foundation for postulating that the immune system may be involved with tumor regulation (1). This has led to numerous clinical attempts to use the immune system as a therapeutic approach. Although many cancers have TILs[3], these tumors grow progressively, suggesting that TILs are incapable of *de novo* cytolysis and are anergic. *In vitro* expansion of TIL populations recovers tumor-specific CTL activity, demonstrating the existence of potentially functional immune effector cells in human tumors. A search for the possible causes of immunosuppression of TILs has led to evaluation of TCR signal transduction pathways (2–6).

Finke *et al.* (2) first reported the loss of ζ-chain expression in TILs from RCCs, and they were followed by other investigators reproducing the same data in other tumor types (6). These data suggested that ζ-chain loss was a common phenomenon and an explanation for the lack of cytolytic activity of TILs (7, 8). However, in the last year, first Noda *et al.* (9) then Franco *et al.* (10) reported that the losses of ζ chain in TILs and PBLs from tumor-bearing mice, as measured by Western blot analysis, were mainly due to partial degradation of the signal transduction molecules by contaminating granulocytes and monocytes during protein extraction and did not necessarily occur *in vivo*.

The controversy over the expression of ζ-chain in TILs is critical to the further development of immunotherapeutic T cell-mediated approaches. Thus, we chose to evaluate the expression of ζ chain in TILs from RCC samples using two parallel systems: dual-channel immunofluorescence fluorescence-activated cell sorting analysis and Western blot analysis.

MATERIALS AND METHODS

Lymphocytes. TILs and PBMs were obtained from eight patients with RCC and from five healthy volunteers. Excised primary RCCs were sampled and processed under sterile conditions at the time of surgery. Samples were transported in HBSS and were trimmed of skin, fat, and necrotic tissue. Tumors were minced in cold HBSS and strained though a mesh. Residual solid pieces were digested in an enzyme solution of collagenase and DNase. The two different tumor preparations were stored and processed separately. The mixture of TILs and renal cells was separated by centrifugation over a discontinuous Ficoll-Hypaque gradient and cryopreserved by freezing in human AB serum with 10% DMSO using a Nalgene alcohol freezer at −70°C over 24 h and stored at −140°C. Blood samples were obtained within 24–72 h of tumor surgery. PBMs were separated by centrifugation over a single-density gradient cushion of Ficoll-Hypaque and cryopreserved in a manner identical to that described for tumor tissue. In preparation for testing, cell suspensions were thawed rapidly and washed twice with HBSS (11).

mAbs. mAbs were purchased as FITC (green fluorescence) or PE (red fluorescence) conjugates. Leu-4-FITC (anti-CD3; pan-T cell) was obtained from Becton Dickinson (Mountain View, CA); PE-conjugated mAbs to CD4 and CD8, as well as isotype controls mIgG1-PE and IgG2a-FITC, were purchased from DAKO (Carpinteria, CA); and anti-ζ-chain (TIA-2 IgG1) was purchased from Coulter Immunology (Hialeah, FL) and conjugated with FITC.

Analysis of Surface Antigens by Flow Cytometry. Cell suspensions were stained with antibody to external epitopes of CD3, CD4, and CD8 molecules at 4°C for 30 min and washed twice in cold PBS. Cells were fixed with 0.1% paraformaldehyde and allowed to rest overnight at 4°C to permit the cell membrane to stabilize. Cells were permeabilized with 1 ml of 100% ethanol at 4°C for 20 min and then washed twice with cold PBS. Anti-ζ-chain antibody and relative FITC control antibody were allowed to react with cell preparations for 30 min, and cells were washed and analyzed the same day on a Becton Dickinson FACScan using 488-nm argon laser. Lymphocytes were examined by setting bitmap gates on a plot of forward *versus* 90-degree light scatter, which excluded tumor cells (1). Additional gates were set using CD3 fluorescence to identify T-cell populations. By using the appropriate optical filters, green fluorescence was measured at 530 nm and red fluorescence was measured at 585 nm using logarithmic amplifiers spanning four logarithmic decades for the full (1024-channel) scale. Cross-over of green fluorescence into the red detection window was compensated for by analogue subtraction at the preamplifier stage.

Analysis of two-color staining was performed using the Lysis II program (Becton Dickinson) on Hewlett-Packard 340 software.

Immunoblotting. The ζ-chain of the CD3-TCR complex was detected by Western blot analysis using 0.5×10^6 to 1×10^6 cell equivalents/lane. PBMs and TILs (1×10^8/ml) were lysed for 5 min at 4°C. Samples were lysed with the standard lysis buffer which contained 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 2 mM Tris (pH 7.4; Sigma), 300 mM NaCl, 1 mM sodium orthovanadate (Sigma), 5 mM EDTA (Sigma), 10 mg/ml leupeptin (Sigma), 10 mg/ml aprotinin (Sigma), and 10 mM *p*-nitrophenylguanidinobenzoate (Sigma). Samples were also lysed with an alternate buffer which contained added protease inhibitors. The alternate buffer contained 0.5% Triton X-100, 50 mM HEPES (pH 7.2; Sigma), 150 mM NaCl, 1 mM sodium orthovanadate, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (Sigma) in ethanol, 100 mg/ml chymostatin in DMSO (Sigma), and 100 mg/ml trypsin/chymotrypsin inhibitor (Sigma) in PBS. The lysates were electrophoresed under nonreducing conditions using 15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), trans-

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[3] The abbreviations used are: TIL, tumor-infiltrating lymphocyte; TCR, T-cell receptor; RCC, renal cell carcinoma; PBL, peripheral blood lymphocyte; PBM, peripheral blood mononuclear cell; mAb, monoclonal antibody; PE, phycoerythrin; TIM, tumor-infiltrating mononuclear cell.

ferred to nitrocellulose (0.2 mM; Schleicher and Schuell, Keene, NH) in transfer buffer (0.025 M Tris base, 0.192 M glycine, 0.1% SDS, and 20% methanol) at 60 V (constant voltage) for 2 h at 4°C. The nitrocellulose was blocked in PBS-3% BSA overnight at 4°C. Immunoblots were developed by incubating with TIA-2 (Coulter Immunology) at 0.5 mg/ml (in PBS-1% BSA) for 2 h at 4°C, whereas the control blots were developed with CD3ε (DAKO) at 0.5 mg/ml (in PBS-1% BSA) for 2 h at 4°C. All blots were washed with PBS-0.05% Tween (Bio-Rad) for four washes of 15 min each and then incubated with secondary antibodies. The TIA-2 blots were incubated with goat antimouse horseradish peroxidase (Bio-Rad) at 0.5 mg/ml (in PBS-1% BSA), and the anti-CD3 blots were incubated with goat antirabbit horseradish peroxidase (DAKO) at 0.5 mg/ml (in PBS-1% BSA) for 1 h at room temperature; then they were washed with PBS-Tween for two washes of 5 min each and three washes of 10 min each. The immobilized proteins were detected by chemiluminescence using enhanced chemiluminescence (Amersham, Arlington Heights, IL), exposed to X-ray film (Fuji), and developed in a Kodak developer.

RESULTS

The expression of ζ-chain in $CD3^+$ PBLs and TILs from RCCs, as well as in PBLs from normal healthy donors, were investigated by flow cytometric and Western blot analysis. With both techniques, we were able to detect similar high-level expression of ζ-chain in $CD3^+$ lymphocytes from all three groups (Table 1 and Figs. 1 and 2).

Table 1 depicts ζ-chain expression in $CD3^+$ cells analyzed by flow cytometry and Western blot. Patients' TILs are matched with PBLs.

Table 1 *ζ-chain expression in $CD3^+$ lymphocytes from patients with RCC and normal individuals*

Subject	ζ-chain expression in TILs (%)*	ζ-chain expression in PBIs (%)*
Patient		
1	85.1	96.7
2	98.4	94.5
3	75.6	94.8
4	97.7	100.0
5	65.6	88.5
6	99.0	99.3
7	93.7	100.0
8	93.9	94.4
Mean	88.6	96.0
Median	93.8	95.9
Normal individual		
1		98.8
2		96.9
3		61.2
4		97.2
Mean		88.5
Median		97.1

* Percent positive cells.

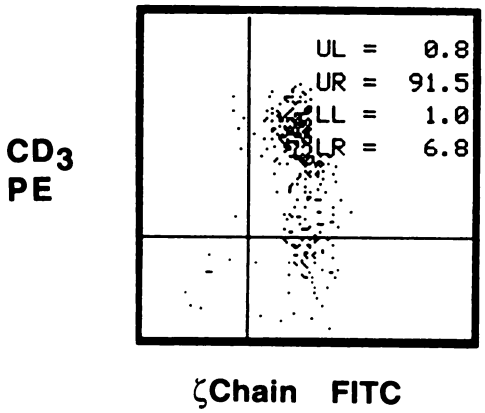

Fig. 1. Example of flow cytometry analysis of CD3 lymphocytes positive for ζ-chain expression in TILs.

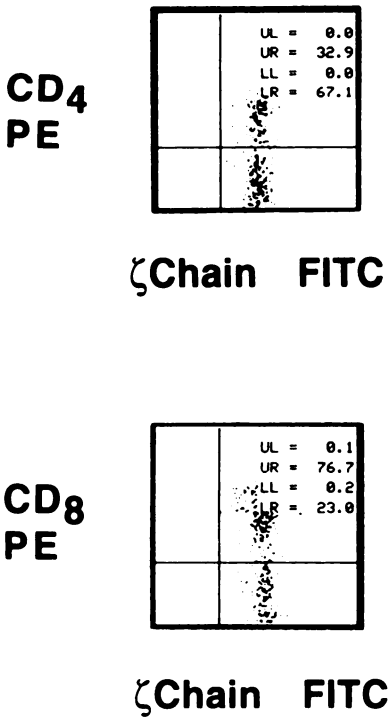

Fig. 2. Examples of ζ-chain expression in CD4 and CD8 populations, as determined by flow cytometry.

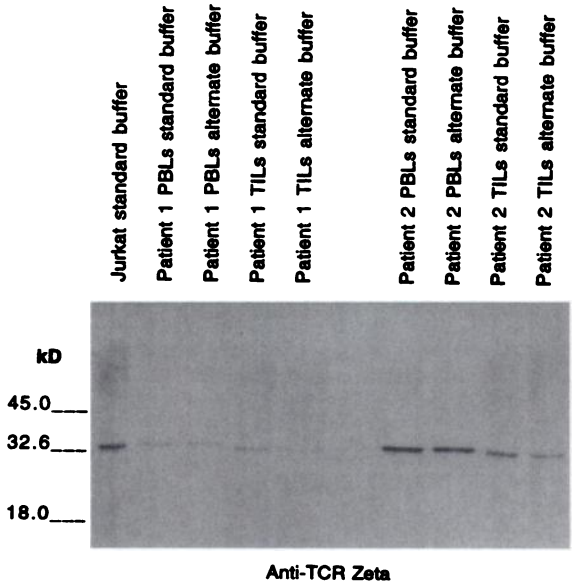

Fig. 3. Western blot analysis for CD3 and ζ-chain expression in TIMs and matched PBMs. Jurkat cell expression for ζ-chain was used as positive control.

The levels of ζ-chain expression in normal healthy donors' PBLs were used as controls. Representative flow analysis of TILs is depicted in Fig. 1, which shows $CD3^+$ cells along the PE (*Y*) axis and ζ-chain-positive cells along the FITC (*X*) axis. Once the gates were set, additional analysis was performed on subsets of $CD3^+$ cells, as seen in Fig. 2. $CD4^+$ and $CD8^+$ cells were analyzed in this fashion in four samples.

Although the mean number of $CD3^+$ ζ-chain-positive cells was lower in TILs compared to that in PBLs, this difference was not statistically significant ($P = 0.07$, paired *t* test). The lower TIL ζ-chain expression was seen in three of the eight patients and was

Table 2 *ζ-chain expression in CD4 and CD8 subsets in TILs and PBLs from patients with RCC and normal individuals*

	Expression in TILs (%)*		Expression in PBLs (%)*	
Patient no.	CD4	CD8	CD4	CD8
4	97.9	96.0	NO	NO
5	100.0	64.6	79.9	55.3
6	100.0	99.8	99.1	98.7
8	85.7	85.5	93.6	97.3
Normal individuals			100.0	90.5

* Percent positive cells.

statistically significant ($P = 0.03$, paired t test; Table 1). One implication of this observation is that there may be a selective decrease in ζ-chain expression in a small subset of TILs. Analysis by Western blot with protease inhibitors (Fig. 3) confirms the presence of ζ-chain in both TIMs and PBMs, in accordance with flow cytometric analysis. Expression of ζ-chain in patients' PBMs was similar to that seen in normal subjects' PBMs ($P = 0.86$, Wilcoxon test). This was true for $CD4^+$ and $CD8^+$ subsets ($P = 0.65$ and $P = 0.95$, respectively).

Analysis of ζ-chain expression in $CD4^+$ and $CD8^+$ subsets in four patients reveals discordance in only one patient (patient 5) with a decrease in ζ-chain in $CD8^+$ subsets. Overall, there did not appear to be a difference in CD8 and CD4 expression of ζ-chain (Table 2).

DISCUSSION

We have shown that dual-fluorescence analysis of membrane-bound molecules (CD3, CD4, and CD8) and cytoplasmic molecules (ζ-chain) can be performed with regularity by flow cytometric techniques and is a useful method for identifying subsets of TIMs. The uniform reduced CD3 ζ-chain expression reported by several authors in mononuclear cells infiltrating human cancers may represent an overreporting of this finding, possibly due to a procedural artifact (6, 7, 9). This loss of ζ-chain may arise from the release of proteases from granulocytes and monocytes/macrophages during protein extraction for Western blot analysis or during cell permeabilization for flow cytometry of nonselect populations.

More recently, a greater variability of ζ-chain expression in TILs from RCC has been reported. Tartour *et al.* (12) found decreases of ζ-chain expression in four of 13 TIL samples. The same group found that ζ-chain expression, determined by Western blot, increased to normal levels as TILs were expanded *in vitro* with IL-2, in contrast with TILs obtained from patients treated with high-dose IL-2, which had persistent low ζ-chain expression (3, 12). These data could suggest that the reexpression of the ζ-chain in populations of T cells expanded in IL-2, compared with the same specimens before culture (day 0), could be due to a population of T cells depleted of monocytes that contain proteases (13). Furthermore, Wang *et al.* (14) found normal levels of CD3ε chain, TCR ζ-chain, and the three tyrosine kinases, p56 (Lck), p59 (Fyn), and ZAP-70, in TILs from B-cell non-Hodgkin's lymphoma. In addition, an animal model also demonstrates normal expression of p56 (Lck), p59 (Fyn), ZAP-70, and CD3-ζ chain in T cells from tumor-bearing mice (15).

We did not quantify the amount of ζ-chain in our study, although mean fluorescent intensity was similar for PBMs and TIMs, suggesting similar levels of expression. The majority of TIMs expressed ζ-chain, and the levels of decrease in expression that were seen in three patients, although significantly different from the levels in PBMs, are small and do not easily explain the overall anergic state of TILs *in situ*.

This work demonstrates that local tumor anergy is a complex process that may involve different pathways. Understanding this process is critical to furthering T cell-mediated antitumor therapies in the future.

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