Prognostic Significance of T Helper 1 and 2 and T Cytotoxic 1 and 2 Cells in Patients with Non-small Cell Lung Cancer

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Abstract. *Background: We report the prognostic significance of peripheral and tumor-infiltrating Th1, Th2, Tc1 and Tc2 cells in lung cancer patients. Materials and Methods: We evaluated the rates of interferon (IFN)-γ+/CD4+ cells (Th1), interleukin (IL)-4+ /CD4+ cells (Th2), IFN-γ+/CD8+ cells (Tc1), IL-4+ /CD8+ cells (Tc2), and the ratio of Th1 to Th2 and that of Tc1 to Tc2 among peripheral blood lymphocytes (PBL) and tumor-infiltrating lymphocytes (TIL), in 51 consecutive patients with non-small cell lung cancer, by detecting the intracellular cytokine production using three-color flow cytometry. Results: Patients with a low Th1/Th2 ratio in peripheral blood lymphocytes had a significantly better prognosis than those with a high Th1/Th2 ratio (5-year survival rate: low: 74.7% vs. high: 50.3%; p=0.038). Patients with a low Th1/Th2 ratio in peripheral blood had a significantly better prognosis than those with a high Th1/Th2 ratio in pathological Stage II or III (5-year survival rate: low: 66.6% vs. high: 18.2%; p=0.018). Conclusion: A high Th1/Th2 ratio in peripheral blood is a negative prognostic factor, especially in pathological Stage II or III non-small cell lung cancer patients.*

T helper type 1 (Th1) cells produce interferon (IFN)-γ to activate cell-mediated immunity (1-3). T helper type 2 (Th2) cells produce interleukin (IL)-4 to activate humoral immunity. Cytotoxic T cells are also divided into subtypes, T cytotoxic 1 (Tc1) and 2 (Tc2) cells, according to cytokines released from these cells (4). We previously reported that the ratio of Th1 to Th2 cells increased in tumor-infiltrating lymphocytes, compared with peripheral blood (5, 6).

There are several reports on the relationship between patient prognosis and serum type 1 / type 2 cytokine levels using enzyme-linked immunosorbent assay or cytokine gene expression (7-12). However, serum cytokine levels are influenced by many kinds of host or cancer cells and they do not directly reflect the balance of Th1/ Th2 or Tc1/Tc2 cells. There have been no reports on the relationship between the state of T cell differentiation into Th1, Th2, Tc1 and Tc2 cells in peripheral blood or within tumor and prognoses of lung cancer patients.

In the present study, we evaluated the significance of the balance between Th1, Th2, Tc1 and Tc2 cells in peripheral blood and tumor on prognoses of patients undergoing surgery for non-small cell lung cancer. Based on the results of this study, the possibility of new therapeutic strategies, are discussed.

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Materials and Methods

Materials. Fifty-one consecutive patients with non-small cell lung cancer resected surgically in the Organ Regeneration Surgery, Faculty of Medicine, Tottori University, Japan and other affiliated hospitals, between April 1997 and March 1998, were studied. The patients ranged in age from 47 to 78 years (mean, 67.2 years) and comprised 37 males and 14 females. No patients had any neoadjuvant therapy before the surgery.

Histologically, 24 tumors were adenocarcinomas, 20 were squamous cell carcinomas and 7 were other types. Thirty-two cases were found to be at pathologically (p) Stage I, 5 at Stage II and 14 at Stage III. Complete resection with dissection of hilar and mediastinal lymph nodes was undergone by 49 patients. Incomplete resection was undergone by 2 patients. Postoperative chemotherapy was done for 13 of Stage II or III patients who are equal to or younger than 75 years old. Adjuvant chemotherapy did not affect patient survival. The mean follow-up period was 42.5 months, ranging from 5 to 70 months. The peripheral blood of 15 normal subjects was analyzed as controls. The control subjects ranged in age from 47 to 77 years (mean 66.9 years) and consisted of 11 males and 4 females. All patients and healthy controls provided informed written consent to participate in the study.

Cell separation and culture. Heparinized blood, 10 ml, obtained from each patient and control subject, was collected before surgery. Peripheral blood mononuclear cells (PBMC) were collected.

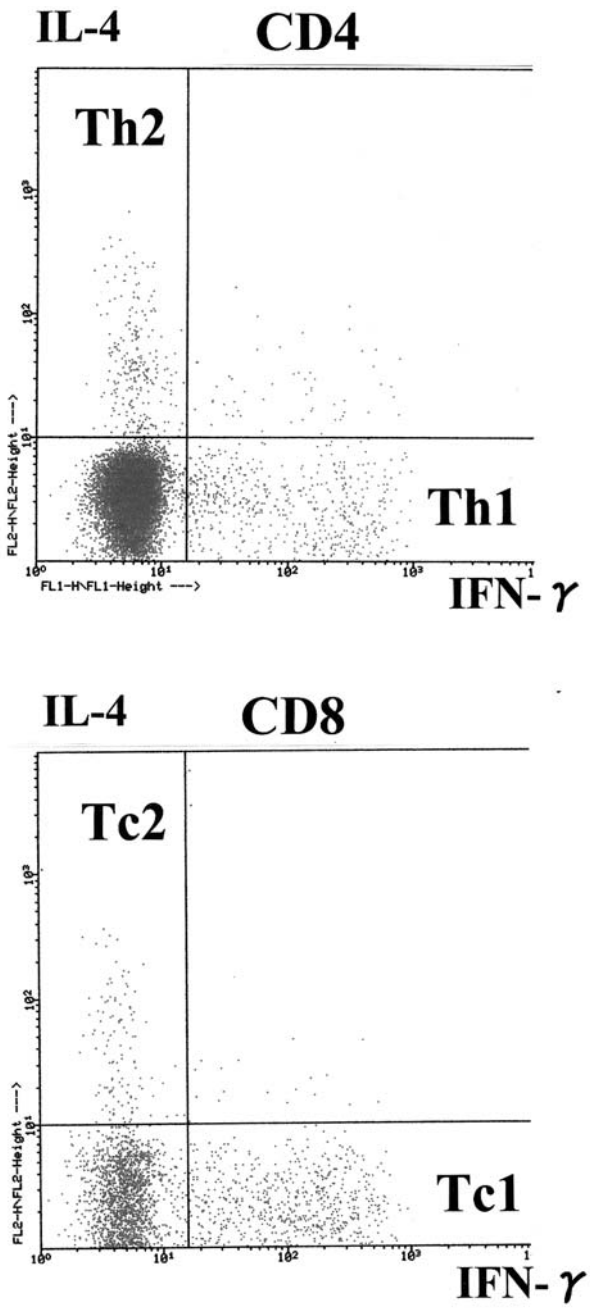

Figure 1. *Dot plot analysis of intracellular cytokine detection with flow cytometry. We defined the cell populations as follows. Populations on CD4+ cells: Th1; IFN-γ-positive and IL-4-negative, Th2; IFN-γ-negative and IL-4-positive. Populations on CD8+ cells: Tc1; IFN-γ-positive and IL-4-negative, Tc2; IFN-γ-negative and IL-4-positive.*

As was reported previously, tumors were obtained aseptically during the resection of the primary lung cancers. We separated the mononuclear cell population from the tumors using the Ficoll gradient, as reported previously (5).

Mononuclear cells were stimulated with phorbol 12- myristate 13- acetate (PMA) 25 ng/ml + ionomycin 1 μmol/l in the presence

Table I. *Five-year survival rate according to clinicopathological factors (Univariate-log rank test).*

	N	5-year survival rate (%)	*P*
Total	51	63.0	
Age			0.857
≤70	33	63.1	
>70	18	62.7	
Gender			0.246
Male	37	58.1	
Female	14	76.9	
Smoking history			0.120
Positive	31	50.0	
Negative	20	72.4	
Histology			0.266
Adenocarcimoma	24	67.9	
Squamous cell carcinoma	20	65.0	
Other types	7	42.9	
pT			0.289
1	25	70.6	
2	18	60.6	
3, 4	8	42.9	
pN			<0.0001*
0	38	77.7	
1, 2	13	20.5	
Stage			0.006*
I	33	78.6	
II, III	18	31.2	
Tumor size			0.075
≤30mm	28	70	
>30mm	23	46.8	

*Significant value

Table II. *Five-year survival rate according to Th1, Th2, Tc1, and Tc2 levels in peripheral blood lymphocytes (Univariate-log rank test).*

	N	5-year survival rate(%)	*P*
Th1 (%)			0.427
<12.8	31	68.4	
≥12.8	20	57.9	
Th2 (%)			0.604
<3.7	30	69.1	
≥3.7	21	57.4	
Th1/Th2 ratio			0.038*
<4.0	29	74.7	
≥4.0	22	50.3	
Tc1 (%)			0.155
<38.2	28	77.3	
≥38.2	23	54.2	
Tc2 (%)			0.689
<4.7	33	64.4	
≥4.7	18	69.2	
Tc1/Tc2 ratio			0.054
<19.0	30	75.5	
≥19.0	21	46.9	

*Significant value

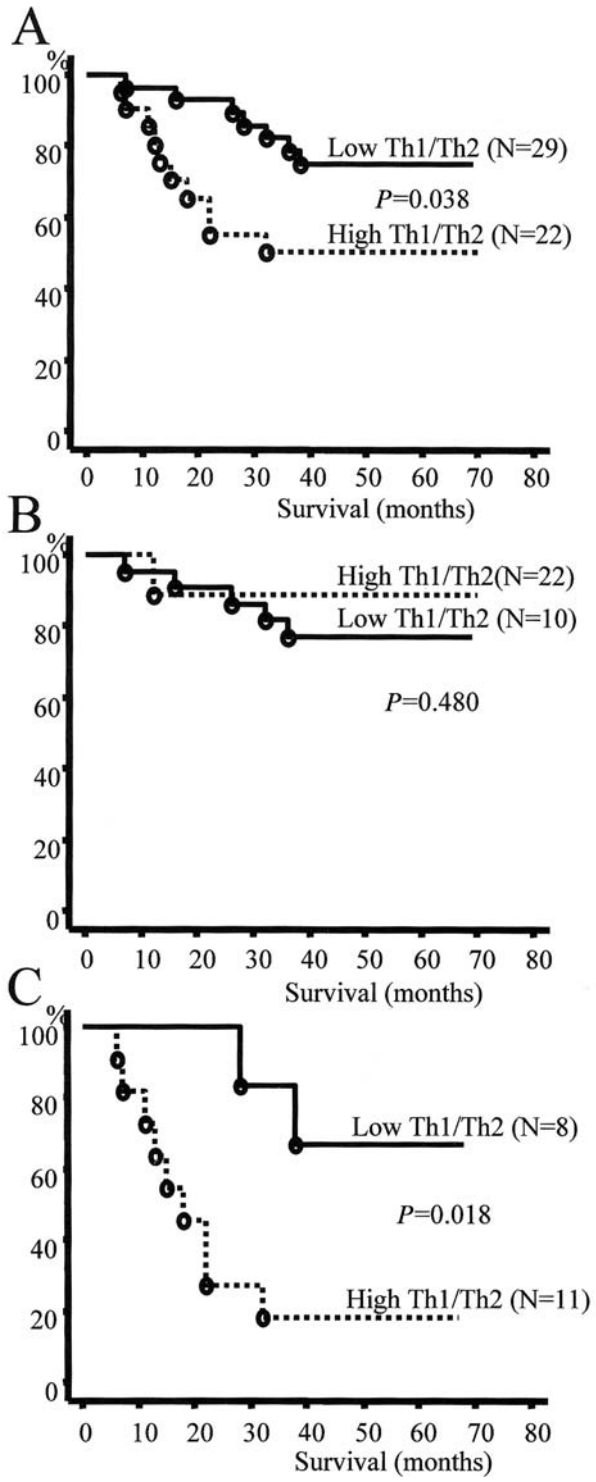

Figure 2. *Survival curves according to the Th1/Th2 ratios in patients with A: All, B: pathologically (p-) Stage I, C; p-Stage II or III non-small cell lung cancer. A: Low Th1/Th2 ratio was significantly associated with a worse prognosis (5-year survival rate: low: 74.7%, high: 50.4%, p=0.038). B: The Th1/Th2 ratio was not significantly associated with any prognosis (5-year survival rate: low: 77.0%, high: 88.9%, p=0.480). C: Low Th1/Th2 ratio was significantly associated with a worse prognosis (5-year survival rate: low: 66.7%, high: 18.2%, p=0.018).*

Table III. *Five-year survival rate according to Th1, Th2, Tc1 and Tc2 levels in tumor infiltrating lymphocytes (Univariate-log rank test).*

	N	5-year survival rate(%)	*P*
Th1(%)			0.307
<29.5	24	56.9	
≥29.5	31	71.8	
Th2(%)			0.694
<2.7	25	68.2	
≥2.7	26	62.2	
Th1/Th2 ratio			0.699
<14.9	31	63.3	
≥14.9	20	68.2	
Tc1(%)			0.486
<61.3	18	71.4	
≥61.3	33	61.7	
Tc2(%)			0.884
<2.3	29	66.8	
≥2.3	22	61.9	
Tc1/Tc2 ratio			0.653
<45.4	30	61.4	
≥45.4	21	71.4	

Table IV. *Multivariate analysis of prognostic factors.*

Prognostic Factor	Coefficient	Standard error	Hazard rate	*P* (score test)
Tumor size	-0.10	0.72	-0.13	0.91
pN	-1.96	0.57	-3.475	0.0005*
Th1/Th2 in PBL	-0.50	0.56	-0.89	0.52
Tc1/Tc2 in PBL	-0.38	0.60	-0.64	0.62

*Significant value

of 10 μg/ml brefeldin A (Sigma) in 24-well plates at a density of $2 \sim 10^6$ cells/ml for 5 hours, at 37˚C with 5% CO_2 in RPMI1640 supplemented with FCS.

Antibodies. Phycoerythrin cyanine 5 (PE-Cy5)-conjugated anti-CD4, PE-Cy5-conjugated anti-CD8, PE-Cy5-conjugated anti-mouse IgG1 (Coulter, Hialeah, FL, USA), fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ, phycoerythrin (PE)-conjugated anti-IL-4 (Pharmingen, San Diego, CA, USA), unlabelled anti-IFN-γ (Pharmingen) and unlabelled anti-IL-4 (Pharmingen) were all used for analyzing cell surface antigens and intracellular cytokines.

Intracellular cytokine staining. Intracellular cytokine staining was performed according to the method of Jung *et al.* and Picker *et al.* (13, 14). Anti-CD4-PECy5 or anti-CD8-PECy5 antibodies were added to the lymphocytes and incubated for 20 minutes, with the cells then being washed. After the cell pellet had been fixed and permeabilized using Fix and Perm (Caltag, San Francisco, CA, USA), anti-IFN-γFITC and anti-IL-4 PE were added. As controls for the intracellular cytokine detection, mouse IgG1 FITC was added to a number of cell pellets instead of the

anti-CD4-PECy5 or anti-CD8-PECy5 antibodies. A molar excess of unlabelled antibodies (anti-IFN-γ and anti-IL-4 antibody, Pharmingen) was used for blocking special binding after solution B had been applied. After staining, all samples were washed with PBS/BSA once, and the pellets were resuspended with 250 μl of 0.5% paraformaldehyde.

Flow cytometry. The samples were analyzed on a FACSort flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA) using LYSIS II software. Twenty thousand cells were analyzed for the cell surface markers and intracellular cytokine analysis. We defined the cell populations as follows: Populations on CD4+ cells: Th1; IFN-γ-positive and IL-4-negative, Th2; IFN-γ-negative and IL-4-positive. Populations on CD8+ cells: Tc1; IFN-γ-positive and IL-4-negative, Tc2; IFN-γ-negative and IL-4-positive (Figure 1).

We compared the patient prognoses between the two groups divided by the mean values of Th1, Th2, Tc1 and Tc2, and the Th1/Th2 and Tc1/Tc2 ratios.

Statistical analysis. Patient survival was analyzed using Kaplan-Meier's method. Differences were analyzed using the log-rank test. A multivariate analysis was made using Cox proportional hazard model. Statistical significance was defined as $p<0.05$.

Results

There were no significant differences in the proportion of Th1, Th2, Tc1 and Tc2 in PBL between the controls (Th1; 14.9%, Th2; 4.0%, Tc1; 41.8%, Tc2; 4.4%) and lung cancer patients (Th1; 12.8%, Th2; 3.7%, Tc1; 38.2%, Tc2; 4.7%). Univariate analysis of clinical factors and prognoses of lung cancer patients revealed that pN and p-Stage were significant prognostic factors (Table I). No significant differences were observed in prognosis between the presence of a low percentage of Th1 or Th2 cells and a high percentage of those cells in peripheral blood. Patients with a low Th1/Th2 ratio in peripheral blood had a significantly better prognosis, compared with a high ratio (5-year survival rate: low: 74.7% *vs.* high: 50.3%; $p=0.038$) (Table II). No significant differences were observed in prognosis between a low percentage of Tc1 or Tc2 cells and a high percentage of those cells in peripheral blood. A low Tc1/Tc2 ratio tended to have a better prognosis, compared with a high ratio in peripheral blood (5-year survival rate: low: 75.5% *vs.* high: 46.9%; $p=0.054$).

When a comparison of prognosis was made between low and high Th1/Th2 ratios according to pathological stages, no significant difference was found among Stage I patients (5-year survival rate: low: 77.0% *vs.* high: 88.9%; $p=0.480$). However, a low Th1/Th2 ratio was associated with a significantly better prognosis, compared with a high ratio, among Stage II or III patients (5-year survival rate: low: 66.6% *vs.* high: 18.2%; $p=0.018$) (Figure 2).

There were no significant differences in prognosis between low and high levels of the percentage of Th1, Th2, Tc1, or Tc2 cells, the Th1/Th2 ratio, or the Tc1/Tc2 ratio in tumor-infiltrating lymphocytes (Table III).

When pN, Th1/Th2 and Tc1/Tc2 ratios in peripheral blood were examined through multivariate analysis, only pN was found to be associated with significant differences in prognosis. However, neither the Th1/Th2 ratio nor Tc1/Tc2 ratio was an independent prognostic factor (data not shown).

Discussion

Tumor cells specifically produce Th2 cytokines, but not Th1 cytokines (15). It has been reported that Th2 cytokines produced by tumor cells make the cancer patients Th2-dominant (12). We previously reported the state of cell differentiation to Th1, Th2, Tc1 and Tc2 cells in tumors of lung cancer patients using three-color flow cytometry. The results revealed that the percentage of Th1 and Tc1 cells was higher than in peripheral blood (5). The percentage of Th2 and Tc2 cells was significantly lower in tumor than in peripheral blood. These results indicate that Th1 or Tc1 dominant cell differentiation occurred as an innate immunity against tumors.

In the present study, lung cancer patients with a high Th1/Th2 ratio in peripheral blood after surgery had a significantly worse prognosis than those with a low Th1/Th2 ratio. This tendency was more remarkable in p-Stage II or III patients. We suggest that host naïve T lymohocytes differentiate Th1 or Tc1 when they recognize a tumor cell or tumor-derived substances. Th1 and Tc1 cells increase in the tumor at the early stage of lung cancer. However, they increase in the peripheral blood when the tumor or tumor-derived substances appear in the peripheral blood. A high Th1/Th2 ratio in peripheral blood might be the result of the occurrence of tumor-mediated Th1-dominant differentiation in association with tumor-derived substances in peripheral blood.

Multivariate analysis showed that neither the Th1/Th2 ratio nor Tc1/Tc2 ratio was an independent prognostic factor. One of the reasons might be that the Th1/Th2 ratio or Tc1/Tc2 ratio has correlation with pN or p-Stage. Further investigation might be important.

From these results, tumor-specific Th1 might appear in peripheral blood in patients with lung cancer who have poor prognosis, because the Th1 level is high in peripheral blood. If such tumor-specific Th1 can be identified from the peripheral blood of each patient, a protocol of new immunotherapy could possibly be developed. It should be possible to proliferate or activate tumor-specific Th1 selectively and possibly identify tumor antigens from each patient.

Conclusion

Patients with a high Th1/Th2 ratio in peripheral blood had a significantly worse prognosis, compared with those with a low ratio, and this tendency was particularly remarkable in Stage II or III patients.

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