Combined influence of adjuvant therapy and interval after surgery on peripheral CD4+ T lymphocytes in patients with esophageal squamous cell carcinoma

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Abstract. The aim of this study was to investigate possible differences in cellular immunity between chemo- and/or radiotherapy groups during a long interval after surgery in esophageal squamous cell carcinoma (ESCC) patients. Cellular immunity was assessed as peripheral lymphocyte subsets in response to chemotherapy (CT), radiotherapy (RT) and CT+RT by flow cytometric analysis. There were 139 blood samples obtained at different time points relative to surgery from 73 patients with ESCC. The changes in the absolute and relative proportions of lymphocyte phenotypes were significant among the adjuvant therapy groups. There were significant differences in the absolute counts of CD4+ and CD8+ T cells among the interval groups, and a lower CD4/CD8 ratio was found in patients following a prolonged interval. RT alone had a profound effect on the absolute counts of CD3+, CD4+ and CD8+ T cells compared with the other groups. CD4+ T cells exhibited a decreasing trend during a long interval, leading to a prolonged T-cell imbalance after surgery. Univariate analysis revealed that the interaction of the type of adjuvant therapy and the interval after surgery was correlated only with the percentage of CD4+ T cells. The percentage of CD4+ T cells can be used as an indicator of the cellular immunity after surgery in ESCC patients. However, natural killer cells consistently remained suppressed in ESCC patients following adjuvant therapy after surgery. These findings confirm an interaction between adjuvant therapy and the interval after surgery on peripheral CD4+ T cells, and implies that adjuvant therapy may have selective influence on the cellular immunity of ESCC patients after surgery.

Introduction

By discriminating between self and non-self, our immune system protects us from invading microbes, including bacteria, viruses and parasites. The immune system has several cellular components that can theoretically be recruited for protection from tumors. It can detect early lesions through immunosurveillance and eliminate them, and is thought to maintain tumors in a state of equilibrium. Yet, tumor cells with either reduced immunogenicity or with increased capacity to attenuate the immune response can escape equilibrium and progress (1,2).

Functional antitumor immunity requires a response initiated by potent antigen-presenting cells, such as dendritic cells, and the engagement of a variety of effector cells, not only cytotoxic T cells, but also T-helper cells and B cells, which initiate antibody immunity (3). CD4+ and CD8+ T cells are the adaptive components of cell-mediated immunity. These cells express a comprehensive repertoire of antigen-specific receptors (cell surface immunoglobulin receptors for B cells and cell surface T cell receptors for T cells) that can recognize over one million distinct antigens (4). To treat patients with advanced disease and larger tumor burdens, it might, therefore, be necessary to achieve and maintain T cell levels that are greater than the 1-2% that is currently attainable (5). Therefore, T cell number is a crucial parameter in experimental and clinical models of adoptive cell therapy.

Esophageal cancer is a highly aggressive malignancy with a very poor prognosis. Surgery, radiation and chemotherapy are the mainstay of esophageal squamous cell carcinoma (ESCC) management (6). Different cancer adjuvant therapies confer selective influence on immune recovery (7). The combination of chemotherapy, fluorouracil and cisplatin, and radiation has improved the outcome for patients with esophageal cancer (8). Postoperative alterations in host immune functions after major

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surgical interventions have been extensively described and investigated for carcinoma of the esophagus (9). Either radio-therapy- or chemotherapy-induced apoptosis of tumor cells has been shown to carry concurrent effects on the immune microenvironment of the tumor (10). Cytotoxic chemotherapy exerts a systemic effect and is used to cytoreduce established tumors and eradicate micrometastatic disease. Chemotherapy can be used to groom the local tumor microenvironment to optimally support a productive immune response. Cytotoxic chemotherapeutic agents might, in some cases, directly modulate the immune response. Chemotherapy has been shown to potentially reduce the number of T-regulatory cells in the environment, promote the expansion of tumor-specific memory T cells and enhance the expression of immune-recognition molecules on the surface of the tumor cell (11). Antimetabolite drugs such as 5-fluorouracil (5-FU), which is a fluorinated derivative of pyrimidines, and cisplatin, a platinum-based DNA damaging anticancer drug, are widely used in the treatment of ESCC patients. Platinum compounds have immune-suppressive effects when tested at a low dose in preclinical models, impairing T cell function, whereas other chemotherapy agents might be devoid of such effects and can even be harnessed to enhance an immune response (12). Preoperative combination radiochemotherapy of patients with ESCC results in the suppression of T lymphocyte functions. The proliferative defects of T cells after radiochemotherapy may be linked to an impaired immune surveillance of cancer and to a higher risk of surgical complications associated with esophagectomy (13). Research found no significant changes in the absolute numbers of T cells and monocytes or in their phenotyping, but a decreasing ratio of CD4+ to CD8+ T cells with advancement of tumor stage in adenocarcinoma of the esophagus was observed (14). A previous study suggested that cellular immunity, especially cytotoxicity, shortly after esophagectomy may be greatly impaired by the surgical stress of esophagectomy and the added effect of chemotherapy (15), and cooperation of CD4+ and CD8+ T cells appears to drastically improve the prognosis of patients with ESCC (16).

Much has been learned about the potential of the immune system to control cancer, and adjuvant therapy is known to alter immune responses significantly (17). Yet, little has been investigated about how the type of cancer therapy and interval after sugery interact with altered immune responses in ESCC patients after surgery. The interaction among the tumor, the stroma and the immune system may play a critical role in patient outcome. Flow cytometric evaluation of the proportions and absolute numbers of lymphocytes and their subsets in the peripheral circulation of cancer patients is important and might provide insights into the redistribution of those lymphocyte subsets that may mediate the antitumor defense (18). Nevertheless, different treatments might have diverse impacts on the immune system, and improving the understanding of circulating lymphocytes could benefit the choice of intervention (19). This study was conducted to clarify the cellular immune response to postoperative adjuvant therapy and interval in patients with ESCC. Cellular immunity was assessed as the counts of leukocytes, lymphocytes and subsets of circulating lymphocytes immediately in response to adjuvant therapy by flow cytometry before adjuvant treatment at 1-3 days of the next cycle for ESCC patient.

Materials and methods

Patients. Between January 2000 and January 2009, 73 consecutive patients with histologically proven ESCC who were scheduled for surgical resection with curative intent consented to participate in the present study. The patients were all treated in the same surgery and oncology unit of ChangZhou Tumor Hospital, according to the National Treatment Guidelines which was used to categorize the patients in the different treatment groups in relation to tumor size, lymph node involvement and relevant clinical data. All patients had locally resectable disease without distant metastases on preoperative investigations, and none had received neoadjuvant chemotherapy or irradiation. Routine preoperative evaluation included upper gastrointestinal endoscopy with biopsy, endoscopic ultrasonography and external ultrasonography of the neck and upper abdomen, indirect laryngoscopy and chest radiography. All specimens of surgical resection were evaluated by an experienced gastrointestinal pathologist, in accordance with the criteria of the International Union Against Cancer, including stage, classification and grade (20). After discharge, patients were seen regularly in the outpatient clinic (every 3 months for 2 years and every 6 months thereafter). Additional diagnostic procedures were performed only when indicated. Approximately 60-70 Gy total radiation, fractioned in 1.8-2.0 Gy doses (6 MeV photons) was delivered to the ESCC patients after surgery. Patients with lymph node involvement had additional radiation delivered to the adjacent lymph nodes. Thirteen patients received chemotherapy (CT) alone (CT group), 43 patients received radiotherapy (RT) alone (RT group) and 17 received a combination of radiation and chemotherapy (RT+CT group) with 5-fluorouracil and cisplain. None of the patients received other adjuvant immunotherapy.

Patients were treated in accordance with the Helsinki Declaration on the participation of human subjects in medical research. The study was approved by the institutional medical ethics committee, and all study participants gave written informed consent.

Blood sampling. On admission to the hospital (1-3 days prior to adjuvant therapy), peripheral venous blood samples were obtained in ethylenediamine tetra-acetic acid (EDTA) tubes for white blood cells (WBCs), differential counts and flow cytometric analysis. Adjuvant therapy was carried out until haematological recovery, defined as total WBCs $>500/\mu l$ and platelets $>20,000/\mu l$. To minimize diurnal influences, all blood samples were drawn between 8 and 9 a.m. There were 139 blood samples in total obtained at different time points relative to surgery from 73 patients with ESCC.

Haematological analysis. The Beckman Coulter ACT DiV Analyser (Global Medical Instrumentation, Inc., Ramsey, MN, USA) was used for determining the absolute number of WBCs and lymphocytes in peripheral venous blood samples, collected in EDTA tubes. 4C Plus Cell Control (Beckman Coulter, Miami, FL, USA) was used to monitor the performance of the Coulter machine. To determine the absolute number for each cell subset reported here as specific cells/ml of blood, the percentage fraction among lymphocytes was multiplied by the number of lymphocytes/l as determined by the lymphocyte count on the same sample.

Flow cytometric analysis. Analysis of the peripheral blood lymphocyte phenotypes was performed on an FACS Calibur flow cytometer (FACScan; Becton Dickinson, San Jose, CA, USA) using the Multiset software package according to the instructions of the manufacturer. A two-color direct immunofluorescence flow cytometric assay was carried out using a Simultest IMK-Lymphocyte kit (Becton-Dickinson) for enumeration of mature human T (CD3$^+$), helper/inducer T (CD3$^+$CD4$^+$), suppressor/cytotoxic T (CD3$^+$CD8$^+$) and natural killer (NK) cells and (CD3$^-$CD16$^+$ and/or CD56$^+$) lymphocytes. Samples were stained and analyzed within 6 h of collection following the manufacturer's instructions. Fresh cells were used whenever possible, failing which cryopreserved cells, ficolled to remove dead cells and debris, were used. For each antibody panel, 5 μl was added to the blood cell samples. The samples were incubated in ice for 30 min in the dark. The red blood cells were lysed with ammonium chloride (10%). After two washing steps, the cell pellets were resuspended in 130 μl PBS with 10% fetal calf serum. A minimum of 2,000 and 5,000 lymphocytes were measured for T cell and for NK, respectively. An isotype-negative control optimized the settings of the fluorescence detectors for each subject. For analyses, a gate was set for lymphocytes using forward vs. the side light scattering property of the cells. CD4$^+$ and CD8$^+$ cells were gated on side scatter and PerCP fluorescence in order to obtain a minimum of 1,500 CD4$^+$ or CD8$^+$ T lymphocytes from the lymphocyte gate. The analysis gate for CD4 or CD8 subsets was set on CD4$^+$ or CD8$^+$ bright events to avoid contaminating CD3$^-$CD4$^+$ monocytes and CD3$^-$CD8$^+$ NK cells. The FACScan was calibrated with CaliBrite fluorescent beads and FACScomp software weekly.

Statistical analysis. Data were entered and analyzed by Excel database and SPSS statistical software (ver. 17.0). Median and reference ranges (percentile 25.0-75.0) were calculated for each marker. Comparisons between groups were made using the Mann-Whitney U test (two group differences) or the Kruskal-Wallis test (three or more group differences) for continuous variables. The cell subset percentage or counts between the types of adjuvant therapy and intervals after surgery was compared using univariate analysis. Differences were considered significant when the P-value was <0.05.

Results

Patient characteristics. The characteristics of all the patients included in this study are shown in Table I. The cohort of 73 patients included 51 men and 22 women with a median age of 61 years (range, 38-86 years) at the time of diagnosis. Height ranged from 145 to 178 cm, with a median height of 165 cm. Weight ranged from 35 to 85 kg, with a median weight of 55 kg. Histologically, 15 (20.6%) tumors were well-differentiated, 45 (61.6%) were moderately differentiated and 13 (17.8%) were poorly differentiated. Thirty-eight cases were in stage II, 20 were in stage III and 15 were in stage IV.

In 14 patients (19.2%), cancer was localized within the lower third of the esophagus; 49 cases (67.1%) were localized within the middle of the esophagus; 10 (13.7%) cases were localized within the upper esophagus. Lymph node metastasis, as determined by histological examination, was noted in

38 (52.1%) cases. All patients received continuous postoperative adjuvant therapy (CT alone, RT alone, both CT+RT) in accordance with our management protocol. In the CT group (n=13, 17.8%), the median patient age was 56 years (range, 44-62 years) and 10 patients were male. In the RT group (n=43, 58.9%), the median patient age was 67 years (range, 46-86 years) and 28 patients were male. In the CT+RT group (n=17, 23.3%), the median patient age was 59 years (range, 38-79 years) and 13 patients were male. The median interval between the last dose of adjuvant therapy and surgical resection was 6 months (range, 0-53 months).

Analysis of the percentages of each lymphocyte subset in ESCC patients with postoperative adjuvant therapy. Using the single-platform method, we initially compared the percentages of each lymphocyte subset in ESCC patients with postoperative adjuvant therapy. Table II shows the distribution of lymphocyte subsets in the peripheral blood of ESCC patients with postoperative chemotherapy and/or radiotherapy. We found that the percentages of T lymphocytes were associated with the types of postoperative adjuvant therapy. There were significant differences between the percentages of each lymphocyte subset (CD3$^+$, CD4$^+$, CD8$^+$ and NK) and the types of adjuvant therapy (P=0.001, P=0.01, P=0.006 and P=0.037, respectively). The percentages of CD3$^+$ and CD8$^+$ were relatively lower in the RT group than those in the CT and CT+RT groups. The percentage of NK cells and the CD4/CD8 ratio were relatively higher in the RT group than the percentage of NK cells and the CD4/CD8 ratio in the CT and CT+RT groups. But the percentage of CD4$^+$ was relatively higher in the CT group than the percentage in the RT and CT+RT groups. Comparing the CT and RT groups, differences in the percentages of CD3$^+$, CD4$^+$, CD8$^+$ and NK cells were observed. Comparing the CT and CT+RT groups, differences in the percentage of CD4$^+$ and the CD4/CD8 ratio were also observed. Meanwhile, there were significant differences in the percentages of CD3$^+$, CD8$^+$ and the CD4/CD8 ratio between the RT and CT+RT groups.

Analysis of the absolute counts of each lymphocyte subset and WBCs in the ESCC patients with postoperative adjuvant therapy. To examine the correlation between cell immune reactions and postoperative adjuvant therapy, we compared the absolute counts of each lymphocyte subset in different adjuvant therapy groups (Table III). We found no significant differences in the absolute counts of NK and lymphocyte blood cells (LBCs) among the adjuvant therapy groups (P=0.291 and P=0.130, respectively). Significant differences were noted in the absolute counts of CD3$^+$, CD4$^+$, CD8$^+$ and WBCs among the adjuvant therapy groups (P=0.001, P=0.012, P=0.003 and P=0.006, respectively). Surprisingly, we found that the absolute count of WBCs was increased in the RT group as compared to that in the CT and CT+RT groups, while the absolute counts of CD3$^+$, CD4$^+$ and CD8$^+$ were relatively lower. This implies that radiotherapy has an important impaired response on the absolute counts of the lymphocyte subsets.

Analysis of the lymphocyte subsets and WBCs in ESCC patients during the interval after surgery. To examine the correlation between cell immune reactions during the interval

Table I. Clinicopathological parameters in patients with esophageal squamous cell carcinoma.

Variable[a]	Total	CT (n=13)	RT (n=43)	CT+RT (n=17)
Gender				
Male	51	10	28	13
Female	22	3	15	4
Location				
Upper esophagus	10	0	6	4
Middle esophagus	49	9	31	9
Lower esophagus	14	4	6	4
Differentiation[b]				
Poorly	13	3	6	4
Moderately	45	6	30	9
Well	15	4	7	4
Nodal metastasis				
Absent	38	5	25	8
Present	35	8	18	9
Smoking				
Yes	37	6	23	8
No	36	7	20	9
Alcohol				
Yes	51	8	31	12
No	22	5	12	5
T stage				
II	38	3	28	7
III	20	3	10	7
IV	15	7	5	3
Median height (range), in cm		165 (158-174)	165 (145-175)	168 (154-178)
Median weight (range), in kg		54 (44-62)	56 (35-85)	56 (47-73)
Median age (range), in years		56 (47-63)	67 (46-86)	59 (38-79)

[a]At the time of initial diagnosis. [b]Determined at the time of surgery. CT, chemotherapy; RT, radiotherapy.

following postoperative adjuvant therapy, we compared the percentages and absolute counts of each lymphocyte subset and WBCs in the different interval groups (Tables IV and V). The time duration between surgery and blood collection for this study was variable, ranging from 0 to 53 months, classified by five groups (I, ≤3 months; II, 4-6 months; III, 7-12 months; IV, 13-24 months; V, >24 months). We found that the percentages of CD3⁺, CD4⁺ and CD8⁺ were significant different among the interval groups (P=0.013, P=0.042 and P=0.040, respectively). However, there were no significant differences in the percentages and absolute counts of NK cells among the interval groups (P=0.492 and P=0.360, respectively). Notably, that the CD4/CD8 ratio exhibited a decreasing trend with prolonged interval (P=0.010). We found significant differences in the absolute counts of CD4⁺, CD8⁺ and WBCs among the interval groups (P=0.015, P=0.030 and P=0.005 respectively). The absolute counts of all the lymphocyte subsets (CD3⁺, CD4⁺, CD8⁺ and NK) and LBCs, except for WBCs, were relatively lower in the >24 month group than the counts in the other groups. This implies that

the absolute counts of the lymphocyte subsets decreased with interval during postoperative adjuvant therapy.

Combined influence of adjuvant therapy and interval after surgery on lymphocyte subsets and WBCs in ESCC patients. We examined whether the percentages and counts of T lymphocyte subsets changed during the postoperative interval when following adjuvant chemotherapy and/or radiotherapy. For this reason, a more detailed analysis of the relationship between cell subsets and interval after surgery in different adjuvant therapy groups was undertaken (Figs. 1 and 2). Univariate analysis revealed that the interaction between the type of adjuvant therapy and interval correlated only with the percentage of CD4⁺ (Fig. 1B, P<0.001). Meanwhile, we found that interval was one of the factors contributing to the percentages of CD3⁺, CD8⁺ and the CD4/CD8 ratio (P=0.018, P=0.017 and P=0.011, respectively), while the type of adjuvant therapy was one of the factors contributing to the percentages of CD3⁺ and NK cells (P=0.042 and P=0.027). The absolute counts of NK cells and LBCs were unrelated to interval in

Table II. The percentage of lymphocyte subsets in different treatment groups after surgery in ESCC.

	Patients (n=139)	CT (n=41)	RT (n=66)	CT+RT (n=32)	P-value[a]
CD3^{+} (%)	57.8 (46.9-69.6)	66.3[b] (53.4-82.0)	51.7[c] (44.6-62.2)	62.1 (50.1-69.5)	0.001
CD4^{+} (%)	27.7 (19.8-37.2)	32.0[b,d] (23.8-44.4)	27.7 (19.2-33.9)	23.9 (17.6-32.7)	0.010
CD8^{+} (%)	24.7 (18.6-33.4)	27.3[b] (21.5-32.5)	21.5[c] (16.0-30.9)	30.2 (22.3-40.7)	0.006
CD4/CD8 (ratio)	1.1 (0.7-1.7)	1.1[d] (0.8-2.0)	1.3[c] (0.8-1.9)	0.8 (0.5-1.3)	0.022
NK (%)	18.5 (11.1-27.4)	16.4[b] (8.0-20.8)	20.2 (12.4-32.3)	19.9 (10.3-30.1)	0.037

Median values are shown with the 25th and 75th percentiles in parentheses. [a]Kruskall-Wallis test (three or more group differences). [b]CT vs. RT; [c]RT vs. CT+RT; [d]CT vs. CT+RT; p<0.05, by using the Mann-Whitney U test (two group differences). CT, chemotherapy; RT, radiotherapy; NK, natural killer cells.

Table III. The lymphocyte subsets and WBC counts in different treatment groups after surgery in ESCC

	Total (n=139)	CT (n=41)	RT (n=66)	CT+RT (n=32)	P-value[a]
CD3^{+} (cells/μl)	760.6 (505.2-1063.4)	932.8[b] (699.6-1203.9)	661.0[c] (440.8-900.3)	828.1 (526.7-1304.6)	0.001
CD4^{+} (cells/μl)	355.6 (242.5-537.0)	501.7[b] (287.3-715.4)	328.0 (252.0-426.4)	387.7 (151.0-581.0)	0.012
CD8^{+} (cells/μl)	329.3 (213.9-511.7)	352.0[b] (269.4-504.0)	273.6[c] (165.7-438.4)	377.8 (293.3-684.7)	0.003
NK (cells/μl)	218.9 (116.6-365.6)	184.5 (108.1-304.8)	234.1 (120.3-394.2)	227.6 (120.9-535.8)	0.291
WBCs (x10^9/l)	5.6 (4.2-8.0)	4.3[b,d] (3.7-6.7)	6.1 (4.7-8.0)	5.3 (4.4-8.6)	0.006
LBCs (x10^9/l)	1.4 (0.9-1.7)	1.5[b] (1.2-1.8)	1.2 (0.9-1.6)	1.4 (0.8-2.1)	0.130

Median values are shown with the 25th and 75th percentiles in parentheses. [a]Kruskall-Wallis test (three or more group differences). [b]CT vs. RT; [c]RT vs. CT+RT; [d]CT vs. CT+RT; p<0.05, by using the Mann-Whitney U test (two group differences). CT, chemotherapy; RT, radiotherapy; NK, natural killer cells; WBCs, white blood cells; LBCs, lymphocyte blood cells.

Table IV. The percentage of lymphocyte subsets during various periods after surgery in ESCC patients.

	I (n=52) ≤3 months	II (n=23) 4-6 months	III (n=24) 7-12 months	IV (n=18) 13-24 months	V (n=22) >24 months	P-value[a]
CD3^{+} (%)	56.3[b] (44.0-68.8)	63.5[e] (55.0-73.2)	62.2[f] (52.2-78.9)	60.1[g] (51.5-67.9)	49.7 (38.7-59.9)	0.013
CD4^{+} (%)	29.2[d] (23.6-36.9)	29.5[e] (22.0-42.3)	27.5 (19.3-42.3)	24.0 (20.7-31.0)	19.8 (14.8-37.2)	0.042
CD8^{+} (%)	20.1[b,c] (14.6-30.4)	24.8 (20.0-39.1)	28.2 (23.3-34.7)	31.6 (24.3-38.8)	26.1 (17.8-30.9)	0.040
CD4/CD8 (ratio)	1.4[b,c,d] (0.9-2.1)	1.3 (0.7-1.9)	1.0 (0.6-1.6)	0.9 (0.5-1.0)	0.9 (0.7-1.2)	0.010
NK (%)	18.5 (10.2-26.5)	14.3 (8.8-22.5)	16.9 (8.4-28.4)	21.9 (14.2-30.4)	19.9 (14.0-25.2)	0.492

Median values are shown with 25th and 75th percentiles in parentheses. [a]Kruskall-Wallis test (three or more group differences). [b]I vs. III; [c]I vs. IV; [d]I vs. V; [e]II vs. V; [f]III vs. V; [g]IV vs. V; p<0.05, by using the Mann-Whitney U test (two group differences). NK, natural killer cells.

both adjuvant groups. Nevertheless, the interval was one of the factors contributing to the absolute counts of CD8^{+} and WBCs (P=0.017 and P=0.027); the type of adjuvant therapy was one of the factors contributing to the absolute counts of CD3^{+} and CD4^{+} (P=0.006 and P=0.006). Overall, it should be noted that the combination of the interval and adjuvant therapy was the main factor contributing to the percentage of CD4^{+} in postoperative ESCC patients. Notably, there was a similar trend for the percentage of CD4^{+} during 13-24 months after surgery in ESCC patients. During the postoperative

adjuvant therapy, an interval (month)-dependent decrease in the percentage of CD4^{+} T cells was found in the CT and CT+RT groups throughout the five-year interval after surgery (Fig. 1F, R=-0.525 and R=-0.387), except for the RT group. The percentage of CD4^{+} in the RT group was negatively correlated with the follow-up interval (months) after surgery. This indicates a long-term effect on CD4^{+} T cells during RT after surgery in ESCC patients; a minimum of at least more than two years. The percentage of CD4^{+} T cells appeared to be steadily regulated during this interval in the RT group.
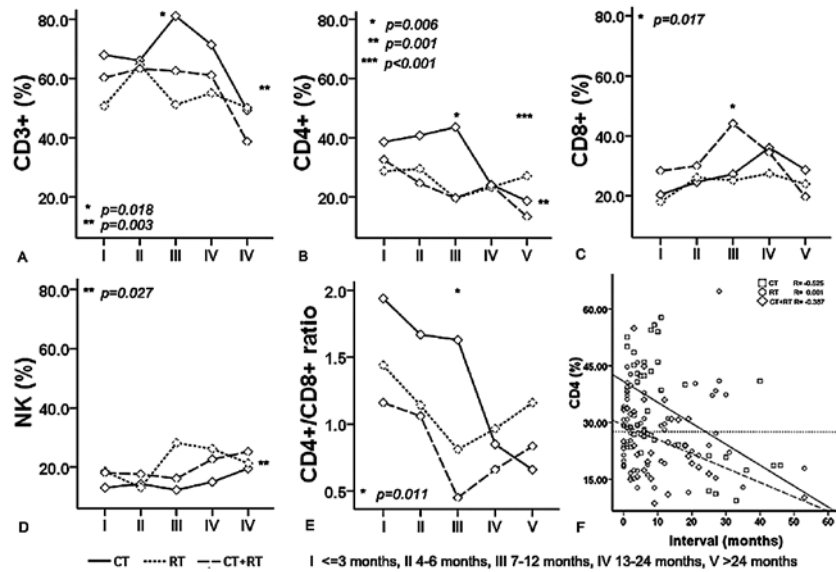
Figure 1. Combined influence of adjuvant therapy and interval after sugery on the percentages of CD3+ (A), CD4+ (B), CD8+ (C) and NK (D) lymphocyte subsets and the CD4/CD8 ratio (E) in the peripheral circulation of ESCC patients. Univariate analysis revealed that the interaction of the type of adjuvant therapy and interval was correlated only with the percentage of CD4+ (P<0.001). The time duration between surgery and a blood collection for this study was variable, ranging from 0 to 53 months, classified by five groups (I, ≤3 months; II, 4-6 months; III, 7-12 months; IV, 13-24 months; V, >24 months). The percentage of CD4+ T cells in an individual sample at different time points relative to surgery is shown (F). As indicated by the regression lines, the percentage of CD4+ T cells decreased with interval in the CT and CT+RT groups throughout the five years after surgery (R=-0.525 and -0.387, respectively). However, the percentage of CD4+ in the RT group was negatively correlated with interval after surgery (R=0.001). CT, chemotherapy; RT, radiotherapy; NK, natural killer cells; *Significant between interval groups on the percentages of CD3+, CD4+ and CD8+ T cells and CD4/CD8 ratio; **Significant between adjuvant therapy groups on the percentages of CD3+, CD4+ and NK cells; ***Interaction of adjuvant therapy and interval on the percentages of CD4+ T cells. P, by univariate analysis.
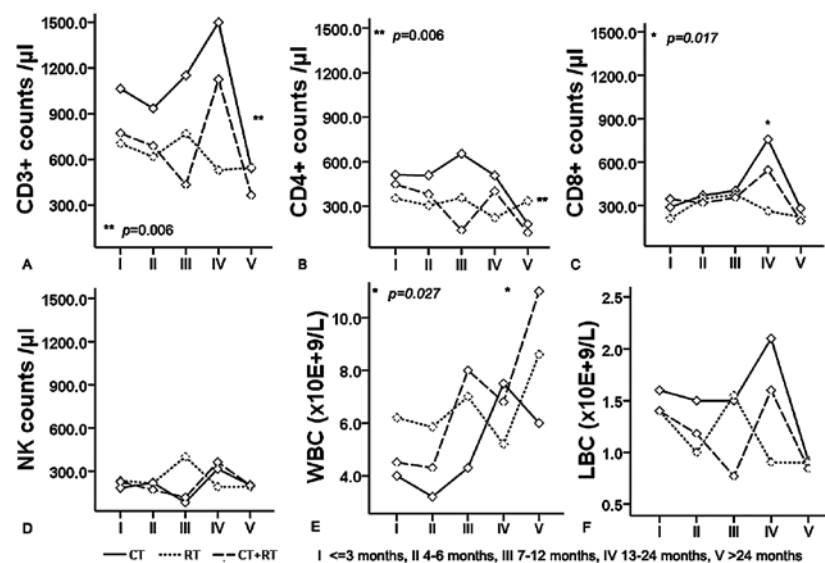

Figure 2. Combined influence of adjuvant therapy and interval after sugery on the absolute counts of CD3+ (A), CD4+ (B), CD8+ (C) and NK (D) lymphocyte subsets and WBCs (E), LBCs (F) in the peripheral circulation of ESCC patients. *Significant between interval groups on the absolute counts of CD8+ and WBCs; **Significant between adjuvant therapy groups on the absolute counts of CD3+ and CD4+. I, ≤3 months; II, 4-6 months; III, 7-12 months; IV, 13-24 months; V, 24 months. CT, chemotherapy; RT, radiotherapy; NK, natural killer cells; LBC, lymphocyte blood cells; WBC, white blood cells. P, by univariate analysis.

Discussion

The loss of lymphocytes contributing to immunosuppression by decreasing the number of available immune cells is a well-known phenomenon. Neoadjuvant therapy for esophageal cancer causes significant disturbances in host cellular immunity with reduced T, NK and B cell counts (9). Although

the role of the immune response in controlling tumor growth and cancer recurrence is controversial, there is evidence in esophageal cancer suggesting that T cells have a beneficial prognostic impact (14).

We assessed the cellular immune response of 73 patients with ESCC who were treated with postoperative adjuvant therapy. The implications of the changes in the absolute and

Table V. The total WBC and lymphocyte subset counts during various times after surgery in ESCC.

	I (n=52) ≤3 months	II (n=23) 4-6 months	III (n=24) 7-12 months	IV (n=18) 13-24 months	V (n=22) >24 months	P-value[a]
CD3+ (cells/μl)	747.0[e] (495.0-1048.7)	793.2[g] (546.3-998.6)	912.9[h] (557.1-1209.0)	1039.4[i] (554.0-1357.0)	543.6 (301.3-804.4)	0.070
CD4+ (cells/μl)	430.4[e] (286.5-509.3)	351.7[g] (281.3-644.3)	406.3[h] (194.2-721.0)	347.3[i] (230.5-633.8)	246.3 (160.2-335.1)	0.015
CD8+ (cells/μl)	261.2[c,d] (172.4-465.9)	336.3 (270.0-443.7)	376.1[h] (316.7-524.9)	493.8[i] (297.8-757.9)	254.3 (137.1-426.5)	0.030
NK (cells/μl)	225.2 (106.4-391.3)	214.8 (114.8-301.5)	228.8 (82.1-462.7)	323.3[i] (198.8-515.9)	197.8 (111.8-317.4)	0.360
WBCs (x10^9/l)	5.4[b,e] (4.2-7.1)	4.2[f,g] (3.2-6.0)	5.5[h] (4.2-8.2)	6.3 (4.7-9.6)	7.3 (5.6-10.7)	0.005
LBCs (x10^9/l)	1.4[e] (1.0-1.7)	1.3 (0.9-1.8)	1.5[h] (0.9-1.9)	1.6[i] (1.0-2.1)	0.9 (0.8-1.5)	0.070

Median values are shown with the 25th and 75th percentiles in parentheses. [a]Kruskall-Wallis test (three or more group differences). [b]I vs. II; [c]I vs. III; [d]I vs. IV; [e]I vs. V; [f]II vs. III; [g]II vs. V; [h]III vs. V; [i]IV vs. V; P<0.05, by using the Mann-Whitney U test (two group differences). WBCs, white blood cells; NK, natural killer cells; LBCs, lymphocyte blood cells.

relative proportions of lymphocyte phenotypes may be significant. Patients undergoing dose-intensive chemotherapy and/or radiotherapy may have both qualitative and quantitative deficiencies in cellular immunity. In our study, we found that the percentages of each T lymphocyte subset (CD3+, CD4+, CD8+ and NK) were associated with the types of postoperative adjuvant therapy. Significant differences were noted in the absolute counts of CD3+, CD4+, CD8+ and WBCs among adjuvant therapy groups. In addition, the absolute count of WBCs was increased in the RT group when compared with that in the CT and CT+RT groups, while the absolute counts of CD3+, CD4+ and CD8+ were relatively lower. This implies that RT alone has a profound effect on the absolute counts of CD3+, CD4+ and CD8+, compared with the CT and CT+RT groups.

Most patients with solid malignancies are exempt from diseases primarily affecting the lymphoid organs possibly interfering with immune reconstitution. Therefore, both short- and long-term alterations of the lymphocyte subsets may have relevant clinical impact on the immune competence of patients with adjuvant therapy (21). As most peripheral lymphocyte subsets shared a significant difference in the rate of recovery, we found that the percentages of CD3+, CD4+ and CD8+ were significantly different among the interval groups. Notably, the CD4/CD8 ratio exhibited a decreasing trend as the interval prolonged. Decreased CD4/CD8 ratios have been found to be associated with a high tumor load, which was correlated with poor survival (14). We found significant differences in the absolute counts of CD4+, CD8+ and WBCs among the interval groups. All of the absolute counts of each lymphocyte subset (CD3+, CD4+, CD8+ and NK) and LBCs, except for WBCs, were relatively lower in the >24 month group than those of the other groups. These findings provide an overall image of lymphocyte subset homeostasis in ESCC patients with adjuvant therapy and suggest a new scheme for the interpretation of immunodeficiency derived from chemo- and/or radiotherapy after surgery. In general, a significant time effect for ESCC patients following adjuvant therapy is reflected.

While most of the focus in cancer immunology is on CD8+ cytotoxic T lymphocyte responses, recent evidence indicates that CD4+ T lymphocyte cells play an important role in the modulation of immune responses by enhancement and suppression of CD8+ CTL responses (22,23). Successful immunity to cancer will therefore require activation of tumor-specific CD4+ T cells (24,25). In our study, we found that the impaired immunity was profound; particularly CD4+ T helper cells exhibited a decreasing trend during a long interval, leading to a prolonged T-cell imbalance after surgery. Univariate analysis revealed that the interaction of the type of adjuvant therapy and interval was correlated only with the percentage of CD4+ T cells. This relationship appeared to result mainly from an impaired response in the percentage of CD4+ T cells with a long interval after surgery in the CT or CT+RT patients, rather a steady regulation throughout interval in the RT alone patients. Variations in circulating CD4+ cell subsets may account for the alterations in CD4+ activity of peripheral blood lymphocytes. CD4+ recovery was delayed for more than one year following dose-intensive chemotherapy in an adult population (26). An unfavorable prognosis can be expected when CD4+ T helper cell functions progressively fail to mediate protective antitumor immunity (14). Therefore, the

percentages of CD4+ can be used as an indicator of patient cellular immunity after surgery in ESCC.

NK cells are effector lymphocytes of the innate immune system that control several types of tumors and microbial infections by limiting their spread and subsequent tissue damage. NK cells are components of the innate immune system capable of lysing target cells without prior sensitization (27). We found no significant differences in the absolute counts of the NK subset among adjuvant therapy groups, and no significant difference in the percentages and absolute counts of NK cells among interval groups. Meanwhile, the interaction of adjuvant therapy and interval displayed no correlation with the percentages and absolute counts of NK. However, contrary to previous reports (28), the number of NK cells did not impair a response in our study. This implies that NK cells tranquilize and participate in antitumor immunity in patients with ESCC.

In conclusion, it may be important to analyze significant disturbances of host cellular immunity induced by postoperative adjuvant therapy in esophageal cancer patients. This adjuvant therapy caused impaired T and NK cells, reduced CD4+ T cells, leading to prolonged T-cell imbalance after surgery. These findings suggest that an alteration in the immune function in patients with adjuvant therapy may account for these differences. An adjuvant therapy-induced lymphocyte imbalance may play a significant role in ESCC after surgery. The main limitation of this study is the absence of sequential data for detailed analysis of trends in such a long interval considered after surgery. Nevertheless, improving the understanding of the relations between treatment, immune system and course of cancer may be relevant in order to develop more effective treatment options for patients with ESCC.

Acknowledgements

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