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Decline of T cell-related immune functions in cancer patients and an attempt to restore them through infusion of activated autologous T cells

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ABSTRACT

We developed a scoring system that can combine several immunological parameters and express the immune status of individuals as a simple numeral. T cell immune score was obtained by using 5 T cell-related parameters: number of T cells, ratio of $CD4^+$T cells to $CD8^+$T cells, number of naïve T cells, ratio of naïve T cells to memory T cells, and T cell proliferative index (TCPI). TCPI was calculated by using number of T cells and their proliferative activity. We assessed T cell immune score in 103 patients with colorectal cancer and 51 healthy age-matched controls.

The results were as follows: (1) T cell-immune score of patients in stages I–IV before surgery was significantly decreased as compared with controls. (2) The number of regulatory T cells in patients in stages I–IV gradually increased with disease progression. (3) T cell immune score was strongly suppressed after surgery, but were recovered to the initial level within a month. (4) Furthermore, restoration of immunological function was attempted in cancer patients by infusion of activated autologous T cells. The effectiveness was confirmed by an increase of TCPI in many cancer patients.

1. Introduction

A strong link exists between advanced age and an increased incidence of cancer (Ershler and Longo, 1997; Denduluri and Ershler, 2004). Many possible factors underlie this close link, among which the relationship between immunologic defects and the increased incidence of cancer associated with aging needs to be explored.

The concept of cancer immunosurveillance was first proposed by Burnet (1970). For many years since then, however, no convincing evidence has been reported in support of immunosurveillance system for spontaneous carcinogenesis in humans as well as animal models.

More recently, however, researchers have been able to use many types of genetically manipulated mice, and a large amount of data has suggested the involvement of both the innate and adaptive immune systems in cancer immunosurveillance. Ablations of NKT, γδT cells, NK cells, or αβ T cells, and IFNγ or IL12 all lead to increased susceptibility of the host to tumors (Dunn et al., 2002).

Nonetheless, it must be noted that the immune system can also promote tumor growth (Prehn, 1970). In fact, tumor growth and metastasis can be significantly more pronounced in young mice with high immune capacity than in old mice with depressed immune capacity (Hirayama et al., 1984, 1993). A similar age-related difference was also observed in the metastatic patterns of gastric cancer in human autopsy cases (Esaki et al., 1990).

Ageing of the immune system is well documented in animal models as well as humans. A large amount of data suggests that T cell-dependent immunity is the most susceptible to ageing (Makinodan and Kay, 1980; Hirokawa et al., 2006; Linton and Dorshkind, 2004; Deng et al., 2004). An extension in ageing population in many countries means that the population number of elderly people is continuously increasing. Autopsy examinations have revealed that the direct causes of death of these elderly people are a preponderance of infection as well as vascular disorders of the heart and brain, and cancer (Hirokawa et al., 2006).

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It is important to note that infection is the major cause of death in the elderly people. Moreover, more than 20% of cancer patients die of infection due to immunologic defects.

The severity of such immunologic defects is variable, with inter-individual differences. Thus, it is important to assess the severity of immunologic defects in patients suffering from various diseases, particularly for determining the treatment process.

The immune system comprises various functions and consists of many types of cells that perform various functions, and it is difficult to select immunological parameters that are suitable for the assessment of immune functions in healthy people and patients suffering from various diseases.

Here in this paper, we propose a scoring method for immunological parameters. By using this scoring method, several different parameters can be combined as a group and processed statistically. We assessed T cell-related immunological parameters of healthy people and patients with colorectal cancer, and confirmed a statistically significant decline in immune functions of cancer patients. In addition, we employed this method to assess the immunological improvement in cancer patients after infusion of activated autologous T cells.

2. Materials and methods

2.1. Assessment of immune parameters

2.1.1. Peripheral blood mononuclear cells

Two milliliters of blood was drawn into a tube with EDTA for hematological analysis and 8 ml of blood was drawn into a cell preparation tube (BD vacutainer, 362761) for the collection of mononuclear cells.

2.1.2. Flow cytometric analysis

Flow cytometric analyses were performed using a combination of monoclonal antibodies with two or three colors; CD3-RD1/CD20-FITC (T cells and B cells), CD4-FITC/CD8-RD1/CD45RA-ECD (CD4$^+$T cells, CD8$^+$T cells, and naive T cells), CD4-FITC/CD8-RD1/CD45RO-ECD (memory T cells), CD4-FITC/CD8-RD1/CD28-PC5 (CD8$^+$ CD28$^+$T cells), CD56-PE/CD16-FITC (NK cells), CD3-ECD/CD4-FITC/CD25-RD1 (regulatory T cells).

2.1.3. Proliferative response of T cells

The proliferative response of T cells to anti-CD3 mAb was assessed according to the MTS method (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega)).

2.1.4. T cells proliferation index (TCPI)

TCPI was calculated by using the following equation:

$$\text{TCPI} = \text{T cell proliferative activity} \times \left(\frac{\text{T cell number}}{1000}\right).$$

In this equation, T cell proliferative activity was obtained as optical density (OD) ranging between 0.95 and 2.0 by the MTS method mentioned above. TCPI and age showed a significant correlation: TCPI = −0.0174 × (age) + 2.5348 (Fig. 1).

2.1.5. Scoring and grading of immunological parameters (Utsuyama et al., 2007; Hirokawa et al., 2007).

Each value of immunological parameters falls within a range specified in a database obtained from approximately 400 healthy people ranging in age from 20 to 100 years. Each parameter is scored into 3 grades based on its value. In particular, values in the range of cumulative frequency less than 10% of values observed for healthy subject are scored 1, which indicates a low immunity level; those between 10 and 40% are scored 2, which indicate a moderate immunity level; and those 40 or higher are scored 3, which indicates a sufficiently high immunity level. Since higher score of CD4$^+$T cells to CD8$^+$T cells ratios (CD4/CD8 ratios) are frequently observed in extremely aged people and patients suffering from diseases, values greater than 80% of the cumulative frequency are scored 2, which indicates a moderate immunity level.

In the present study, we selected five immunological parameters related to T cell functions and scored them. A total sum of five T cell-related parameters is referred to as the T cell immune score. These include the number of T cells per mm^3, ratio of CD4$^+$T cells to CD8$^+$T cells (CD4/CD8 ratio), number of naïve T cells per mm^3, ratio of naïve T cells to memory T cells (N/M ratio), and TCPI. Then, the T cell immune score was classified into five grades; grade V represents the sufficiently high level of immunity (score, 15); grade IV is the safety zone (score, 14–13); grade III is the observation zone (score, 12–10); grade II is the warning zone (score, 9–7) and grade I is the critical zone (score, 6–5).

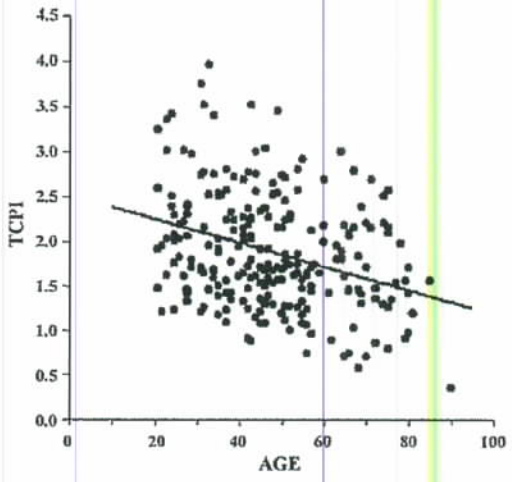

Fig. 1. Distribution of TCPI (T cell proliferation index) in healthy people (approximately 300 males and females) ranging in age between 20 and 90. $y = 0.011x + 2.469$; $R = 0.224$; $P < 0.0001$.

2.2. Cancer patients

We recruited 103 patients with colorectal cancer for the present study. Table 1 shows the progression stage, number of cases (sex ratio), average age ± S.E. and level of total protein in serum (g/dl). The control group included 51 cases of age-matched healthy people. These patients with colon cancer were examined to see the relationship between immunological status and progression stage of cancer. In addition, 14 patients with advanced cancer (Table 2) were examined to see the effect of the infusion of activated autologous T cells on the immune system. These 14 patients had been treated with either operation, chemotherapy, radiation, or combination of them and they hoped some beneficial result by the immunological intervention.

2.3. Preparation and infusion of activated autologous T cells

Activated autologous T cells were prepared by culturing mononuclear cells culture medium RPMI-1640 with immobilized anti-CD3 mAb in the presence of recombinant IL-2 (rIL-2) in a 175-cm^2 flask for 5 days, and then transferred to a 175 or 225-cm^2 flask without anti-CD3mAb in the presence of rIL-2 for 2 days. The cells were then cultured in culture medium AIM-V with rIL-2 for 7 days. Autologous serum was used for all the culturing processes at a concentration of 1–10%, depending on the proliferative conditions.

2.4. Ethical approval

This study was conducted in compliance with Declaration of Helsinki and applicable national laws and regulation, and was approved as no. 320 by the Ethics Committee of Tokyo Medical and Dental University. Written informed consent was obtained from all subjects.

2.5. Statistics

All statistical analyses were performed using StatView software. Statistical significance was defined as $p < 0.05$.

Table 1
Colorectal cancer cases and age-matched controls

Progression stage	Number (M/F ratio)	Age ± S.E.	TP (g/dl)
Stage 0	10 (M = 6, F = 4)	63.4 ± 4.0	7.33 ± 0.13
Stage I	18 (M = 14, F = 4)	62.4 ± 1.0	7.25 ± 0.17
Stage II	27 (M = 17, F = 10)	67.4 ± 2.1	7.14 ± 0.12
Stage III	30 (M = 20, F = 10)	65.1 ± 2.0	7.07 ± 0.11
Stage IV	18 (M = 11, F = 7)	63.4 ± 2.4	7.08 ± 0.14
Control	51 (M = 24, F = 26)	63.2 ± 2.0	NA

TP: total protein in serum; NA: not assessed. Standard level of TP in healthy control: 6.7–8.3 g/dl. Statistically no significant difference was observed in the level of TP among groups.

Table 2
Effect of the infusion of activated autologous T cells on TCPI levels in cancer patients

	Cases	TCPI before infusion	TCPI after infusion
1	Tongue cancer	0.48	1.42
2	Esophageal cancer-1	0.66	1.05
3	Esophageal cancer-2	0.23	0.44
4	Lung cancer-1	1.18	1.5
5	Lung cancer-2	1.13	1.69
6	Lung cancer-3	0.36	0.46
7	Lung cancer-4	0.53	0.53
8	Gastric cancer	1.70	1.82
9	Pancreatic cancer-1	1.66	1.97
10	Pancreatic cancer-2	1.49	2.36
11	Pancreatic cancer-3	0.53	0.35
12	Colon cancer	1.48	4.11
13	Appendiceal cancer	0.96	0.66
14	Ovarian cancer	1.91	2.55
	Average ± S.E.	1.02±0.15	1.49±0.28
	Healthy control	1.70±0.11	
	Colonic cancer Stages I-IV	1.21±0.06	

TCPI was observed to improve 11 out of 14 cases as indicated by grey boxes.

3. Results

3.1. Immunological analyses of peripheral blood of patients with colorectal cancer

One hundred and three patients with colorectal cancer were selected at the outpatient clinic of colorectal surgery at the Tokyo Medical & Dental University. Blood samples were obtained from the patients before the treatment by surgery and chemotherapy. All the cancer patients examined underwent surgical resection of the colorectal cancer. The excised cancer tumors were pathologically examined and classified into five classes, i.e., stages 0–IV, based on the progression stage of the cancer. Stage 0 indicates that the cancer still remains in the mucosal layer without invasion. Stages I–IV indicate that the cancer invades into the submucosal layer (I), muscular layer (II), subserosal layer (III) or metastasizes to other organs (IV). These data were compared with those of the healthy, age-matched controls.

3.1.1. The numbers of white blood cell and T cells, and TCPI

The number of white blood cells (WBCs), including granulocytes and lymphocytes, showed a trend of increase in the cancer patients in progression stages I–IV as compared with healthy controls (Fig. 2a). Conversely, the number of T cells was significantly lower in the cancer patients in progression stages I and II than in the healthy controls (Fig. 2b). TCPI reflecting proliferative capacity of T cells was also significantly lower in the cancer patients in progression stages I–IV than in the healthy controls (Fig. 2c).

3.1.2. T cell subpopulations (Fig. 3)

The number of $CD4^+$ T cells was significantly lower in the cancer patients in progression stages II and III than in the healthy controls (Fig. 3a). However, no significant change was observed in the number of $CD8^+$ T cells (Fig. 3b) and the $CD4^/CD8$ ratio (Fig. 3c) between the healthy controls and cancer patients.

$CD4^+$T cells can be classified into two subpopulations, naïve T cells (mostly $CD4^+CD45RA^+$) and memory T cells (mostly $CD4^+CD45RO^+$). The significant decrease was observed in the number of naïve T cells in the cancer patients in progression stages I–IV compared to the controls (Fig. 3d). No significant difference was observed in the number of memory T cells (Fig. 3e) and in the ratio of naïve T cells to memory T cells (N/M ratio) (Fig. 3f) between the healthy controls and cancer patients. Levels of most T cell-related parameters in the cancer patients in progression stage 0 did not show a statistically significant difference as compared with those of the healthy controls and the cancer patients in progression stages I–IV; this was probably because the number of study subjects was insufficient for achieving statistical significance. However, the level of $CD4^+$ T cells was significantly lower in cancer patients at progression stage 0 than in the healthy controls. The finding suggests that the immune system plays a role in the development of cancer even in the mucosal layer.

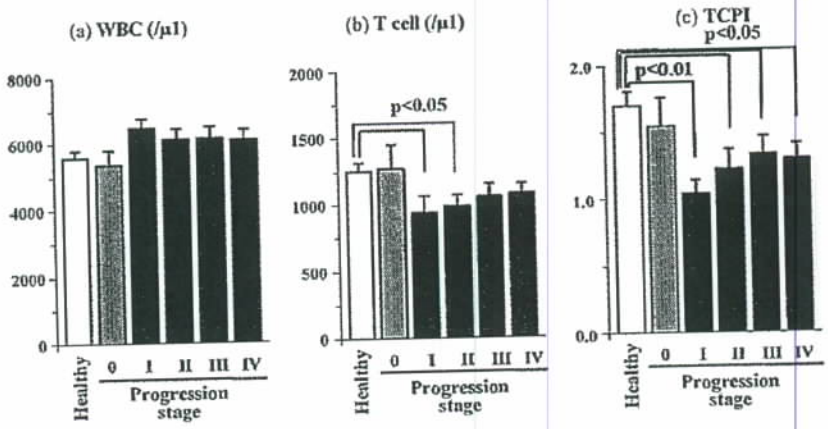

Fig. 2. Comparison of (a) WBC number, (b) T cell number and (c) TCPI among healthy people and cancer patients in progression stages 0–IV.

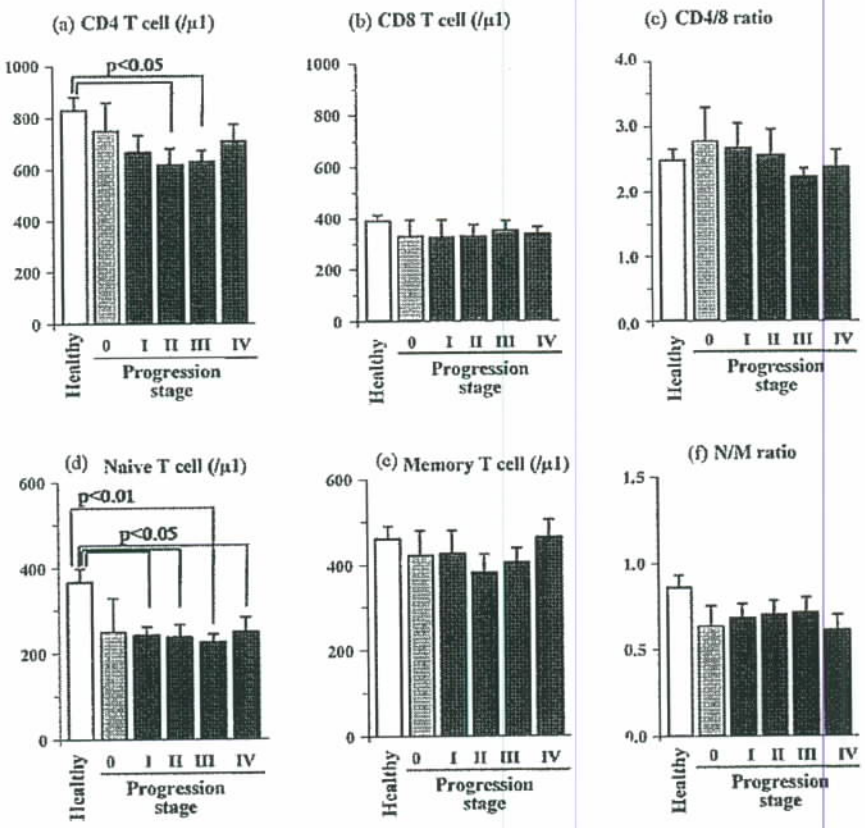

Fig. 3. Comparison of (a) $CD4^+$ T cell number, (b) $CD8^+$ T cell number, (c) CD4/CD8 ratio, (d) naïve T cell number, (e) memory T cell number and (f) N/M ratio among healthy people and cancer patients in progression stages 0–IV.

3.1.3. Regulatory T cells, B cells and NK cells

The number of $CD4^+CD25^+$ T cells, so-called regulatory T cells, increased with the advance of progression stage of the cancer, and a significant increase was observed in cancer patients in stages II and III (Fig. 4a). These data are interesting because regulatory T cells are known to suppress the activity of killer T cells against cancer.

No significant difference was observed in the number of B cells (Fig. 4b) and NK cells (Fig. 4c) between the cancer patients and the healthy controls.

3.1.4. Scoring and grading of immunological vigor

A significant decrease was observed in the T cell immune score (Fig. 4d) as well as the grade (Fig. 4e) between the cancer patients in progression stages 0–IV and the healthy controls. It is interesting to note that the levels of both T cell immune score and the grade were significantly lower in the cancer patients in progression stage 0 than in the healthy control. This suggests again that some functions of T cells are related with development of tumors that are still in the mucosal layer.

Fig. 5 shows the distribution of T cell immune score and TCPI against age in all cancer patients and controls. Regression lines of the former were observed in the lower level than those of the controls in both T cell immune score and TCPI. Statistically significant differences were observed in TCPI, indicating a definite decline in T cell immunity in the cancer patients.

3.1.5. Effect of surgical operation on TCPI

Surgical operation, the resection of tumor from the colon or rectum, greatly influenced immunological condition of patients. For instance, TCPI declined to 50–60% of the initial level, when assessed 1 day after the operation. However, the level quickly recovered to 80–100% of the initial level within a month (data not shown).

3.2. Effect of the infusion of activated autologous T cells in patients with advanced cancer

Together, the data so far presented above have indicated that a decline in T cell-related immunological parameters was detectable in untreated colon cancer patients.

In the next step, we examined cancer patients in the advanced stage; patients with tongue cancer, esophageal cancer (2 cases), lung cancer (4 cases), gastric cancer, pancreatic cancer (3 cases), colon cancer, appendical cancer and ovarian cancer (Table 2). All the patients were in the advanced stages of cancer, with multiple metastases, and they underwent an infusion of autologous activated T cells (so-called LAK cells).

The activated autologous T cells prepared in the present study comprised T cells (99%), and the CD4/CD8 ratio was approximately 2–3. The proportion of NK cells was less than 1% in the activated T cells.

The number of cells per infusion was approximately 5×10^9, and the infusion was repeated 5–6 times over 10 weeks. Various parameters were examined before and after the infusion of activated autologous T cells.

Fig. 6 shows the immunological parameters before and after the infusion of activated autologous T cells in an advanced cancer patient. Most of the immunological parameters that were

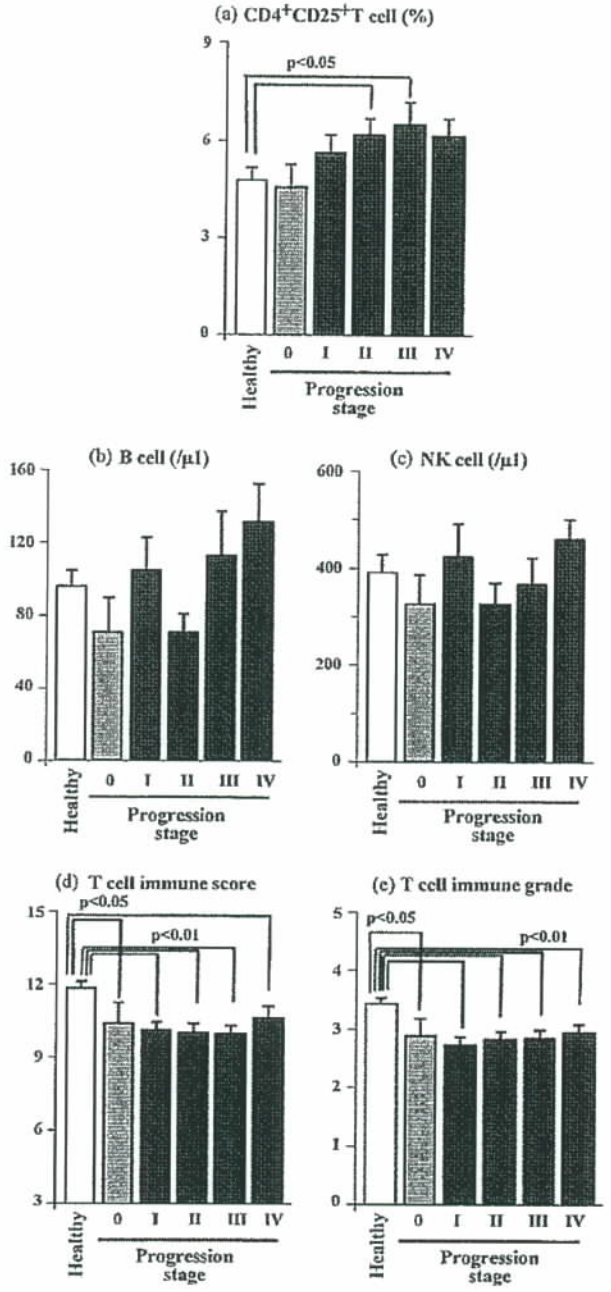

Fig. 4. Comparison of percentage of $CD4^+CD25^+$ T cells (a), B cell number (b), NK cell number (c), T cell immune score (d) and T cell immune grade (e), among healthy people and cancer patients in progression stages 0–IV.

examined improved after the infusion, except for the ratio of T cell subpopulations such as the ratio of $CD4^+$ T cells to $CD8^+$T cells (CD4/CD8 ratio) and that of naïve T cells to memory T cells (N/M ratio).

We examined the effect of the infusion of activated autologous T cells in 14 cases and found that the most pronounced improvement was observed in TCPI. The values varied across cases and were unsuitable for statistical analysis; each case is represented individually in Table 2. After the infusion of activated autologous T cells, an improvement in TCPI was observed in 11 out of 14 cases. The average value of TCPI before the infusion was 1.02, which is apparently lower than that observed in colonic cancer stages I–IV (1.21). After the infusion, the average TCPI was increased to 1.49, although it was still definitely lower than that of healthy controls (1.70). It was not obvious whether the infusion of activated autologous T cells was effective in reducing tumor size, but most of the patients told to physicians at the time of interview that in general, they experienced an improvement in their health status after the infusion.

4. Discussion

We proposed a new immunological parameter calculated by combination of number and proliferative activity of T cells, which was referred to as TCPI. TCPI significantly decreased in the cancer patients in progression stages I–IV. In addition, we developed a new concept of T cell immune score and grade, that are calculated using five immunological parameters; the number of T cells/mm^3, the number of naïve T cells/mm^3, the ratio of $CD4^+$ T cells to $CD8^+$T cells (CD4/CD8 ratio), the ratio of naïve T cells to memory T cells (N/M ratio) and TCPI. Both T cell immune score and grade clearly decreased in the cancer patients in progression stages 0–IV compared with the healthy controls. The decline in TCPI and T cell immune score/grade could be mainly ascribed to the effect of cancer on the immune system of the host.

Since the cancer cells still remain in the mucosal layer in progression stage 0, they may not influence the whole immune system. However, the presence of a significant decrease in T cell immune score/grade suggests that the immune system may play a role in the biologic behavior of tumor cells that are still in the mucosal layer.

In this respect, it is important to note the concept of cancer immunosurveillance. This concept was first proposed by Burnet (Burnet, 1964), and since then, many researchers have attempted to obtain convincing evidences to prove this concept, by mainly using nude mice (Stutman, 1974; Rygaard and Povlsen, 1974). Nude mice, however, were not ideal animal models to prove cancer immunosurveillance, because they contain extrathymic T cells and NK cells. Meanwhile, newly developed Rag-$2^{-/-}$ mice enabled researchers to perform unequivocal carcinogenesis experiments (Shankaran et al., 2001).

The immunostimulation theory shed light on an alternative aspect of the immune system, suggesting that it might promote the growth of tumors (Prehn, 1970). In this respect, we examined the biological behavior of tumor cells in aged mice and elderly people with depressed immune capacities. We found that tumor growth and metastasis were significantly more pronounced in young mice with high immune capacity than in old mice with depressed immune capacity (Hirayama et al., 1984, 1993) and that a similar age-related difference was also observed in human autopsy cases (Esaki et al., 1990).

With regards to humans, studies of immunosuppressed transplant recipients revealed a remarkably increased risk of not only selected malignancies, but also cancers with no known viral etiology (Nakachi et al., 2004).

The existence of functional cancer immunosurveillance has been widely accepted in recent times. However, it has also become clear that the immune system can facilitate tumor progression by sculpting the immunogenic phenotype of tumors as they develop (Dunn et al., 2002).

From a practical clinical viewpoint, it is important to identify the immune status of individual patients, particularly those with cancers. In the case of patients with depressed immune functions, it would be obligatory to replenish their immune capacity and

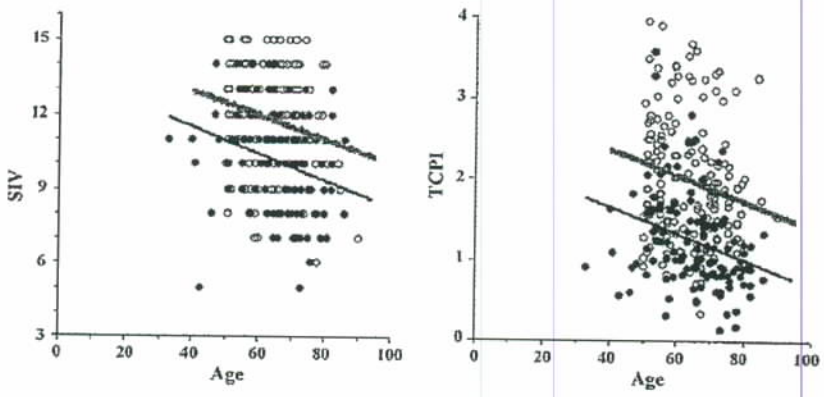

Fig. 5. Distribution of T cell immune score and TCPI against age in 103 cases of cancer patients (open circles) as compared with controls (solid circles). Regression lines of the former (solid line) were observed in the lower level than those of the controls (grey line:) in both T cell immune score (cancer: $y = -0.054x + 13.70$; $R = 0.282$; $P < 0.01$; control: $y = -0.048x + 14.87$; $R = 0.223$ $P < 0.01$) and TCPI (cancer: $y = -0.016x + 2.30$; $R = 0.284$; $P < 0.01$; control: $y = -0.015x + 2.79$; $R = 0.182$; $P < 0.02$). Statistically significant difference was observed in TCPI between cancer patients and controls.

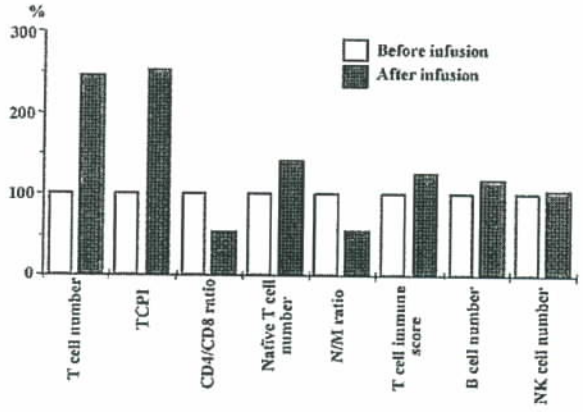

Fig. 6. Immunological restoration in a cancer patient after infusion of activated autologous T cells 6 times for 10 weeks. Open columns, before the infusion. Grey columns, after the infusion.

restore it to the normal level. For this purpose, we should be aware of the precise immune status of cancer patients.

The present study proposed a method for the scoring and grading of immunological parameters (Utsuyama et al., 2007; Hirokawa et al., 2007). By this method, several immunological parameters of different characteristics can be grouped and represented by a numeral. For instance, we demonstrated a T cell immune score that was calculated based on 5 T cell-related immunological parameters. Cancer patients in the early progression stage showed a significant decline in their T cell immune score. There was no change in NK cells, or at least not their numbers in these patients.

TCPI may be useful as a marker of immune status, because it was partly restored in advanced cancer patients by infusion of activated autologous T cells. However, this treatment should be performed very cautiously, because the immune system could facilitate disease progression. Therefore, it is necessary to determine functions of subpopulation of infused cells and delete some of them if necessary.

Immunological intervention by the infusion of activated autologous T cells was still preliminary in the present study. For statistical analysis, at least 30 cases would be necessary for each cancer group. After the infusion of activated autologous T cells, the improvement of TCPI was observed in 11 out of 14 patients, that were in the extremely advanced stage of cancer. Therefore, the similar immunological improvement could be expected not only in cancer patients at earlier progression stages but also in the elderly people with immunological depression.

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