Immunosuppression involving increased myeloid-derived suppressor cell levels, systemic inflammation and hypoalbuminemia are present in patients with anaplastic thyroid cancer

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Abstract. Anaplastic thyroid carcinoma (ATC) is one of the most aggressive neoplasms in humans and myeloid-derived suppressor cells (MDSCs) contribute to the negative regulation of immune responses in the context of cancer and inflammation. In order to investigate the pathophysiology of thyroid cancer, peripheral blood mononuclear cells (PBMCs) were obtained from 49 patients with thyroid cancer, 18 patients with non-cancerous thyroid diseases and 22 healthy volunteers. The MDSC levels were found to be higher in patients with any type of thyroid cancer (P<0.05), patients with ATC (P<0.001) and patients with medullary thyroid carcinoma (P<0.05), when compared to patients with non-cancerous thyroid diseases. The MDSC levels were also higher in patients with stage III-IV thyroid cancer compared to those in patients with non-cancerous thyroid diseases (P<0.05). The stimulation index (SI) of phytohemagglutinin (PHA)-induced lymphocyte blastogenesis was significantly lower, the C-reactive protein (CRP) levels were significantly higher and the serum albumin levels were significantly lower in patients with ATC compared to those in patients with non-cancerous thyroid diseases. The SI was significantly lower in stage III and IV thyroid cancer compared to that in non-cancerous thyroid disease (P<0.05). Furthermore, the CRP levels were higher and the concentration of albumin was lower in stage IV thyroid cancer compared to those in non-cancerous thyroid disease (P<0.05). Patients with thyroid carcinoma were then classified into one of two groups according to a %PBMC of MDSC cut-off level of 1.578, which was the average %PBMC of MDSC of patients with any type of thyroid carcinoma. In patients with higher MDSC levels, the production of CRP and interleukin (IL)-10 was significantly higher (P<0.05) and the albumin levels were significantly lower (P<0.05) compared to those in patients with lower MDSC levels. These data indicate that MDSCs are increased in patients with ATC. Furthermore, these patients exhibited suppression of cell-mediated immune responses, chronic inflammation and nutritional impairment.

Introduction

Thyroid tumors represent 1% of all neoplasms and differentiated thyroid tumors (e.g., papillary and follicular types) account for 80-85% of all thyroid tumors (1-4). The majority of cases of differentiated thyroid tumors are associated with favorable outcomes. Anaplastic thyroid carcinoma (ATC) accounts for 5-15% of primary malignant thyroid tumors (5). The pronounced differences in the biological behavior of the various histological types of thyroid carcinoma are well known. In contrast to papillary and follicular thyroid carcinomas, ATC is one of the most aggressive neoplasms in humans. ATC is usually rapidly fatal, with a mean survival of 6 months after diagnosis (6,7). Since ATC is rare, there has not been a sufficient number of available cases to investigate, in order to obtain a better understanding of the natural history of this type of tumor and the factors that may affect the response to treatment and survival.

Numerous cancer immunotherapies that were developed in experimental animals have been investigated in clinical trials. Although some exhibited a moderate clinical efficacy,

the majority were not effective (8,9). Recent studies identified cells of myeloid origin that are potent suppressors of tumor immunity and represent a significant impediment to cancer immunotherapy (8,9). Suppressive myeloid cells were previously described in patients with cancer (10,11), although their functional significance in the immune system has only recently been evaluated. Accumulating evidence (12-15) suggests that a population of cells with suppressive activity, referred to as myeloid-derived suppressor cells (MDSCs), may contribute to the negative regulation of immune responses during cancer and other diseases. We previously characterized circulating levels of MDSCs in patients with various types of malignant diseases, including patients with thyroid cancer (16).

Interleukin (IL)-10 is a potent immunosuppressive cytokine that is produced primarily by Th2 cells, macrophages and activated B cells. This cytokine has a wide range of biological activities and possesses immunosuppressive, anti-inflammatory and immunomodulatory properties that promote the regulation of a variety of immune cell differentiation and proliferation events (17). IL-10 is also an immunosuppressive effector cytokine produced by MDSCs (10,11).

Tumor development and growth occurs as a result of interactions between the tumor and host immune/inflammatory cells, with chronic inflammation being crucial in cancer development and progression. The laboratory markers of systemic inflammatory response and nutritional status, including C-reactive protein (CRP) and neutrophil/lymphocyte ratio, have been investigated as prognostic markers, with the evidence supporting their use being optimally demonstrated in surgical patients (18,19).

The aim of the present study was to characterize MDSC levels in normal volunteers and patients with thyroid cancer and to investigate the association between MDSC levels and immunosuppression, chronic inflammation and nutrition.

Materials and methods

Samples. Blood samples were collected from 49 patients with thyroid cancer, including 38 patients with papillary, 6 with anaplastic, 3 with medullary and 2 with follicular carcinoma. Samples were also collected from 18 patients with benign thyroid diseases [e.g., adenomatous goiter, follicular adenoma and hyperthyroidism (Basedow's disease)] and from 22 healthy volunteers of similar age and gender distribution. Patients who received treatment (e.g., surgery, chemotherapy or palliative care) and were followed up at the Department of Organ Regulatory Surgery at Fukushima Medical University between January, 2011 and January, 2013 were enrolled in this study. The patients were aged 18-90 years and had histologically confirmed thyroid cancer. Of the 49 patients, 8 had stage I, 1 had stage II, 6 had stage III and 34 had stage IV disease. All patients were newly diagnosed and the blood samples were collected prior to the initiation of any treatment. Peripheral blood mononuclear cells (PBMCs) were separated on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). The isolated PBMCs were washed twice with RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and were maintained frozen at -80°C in a freezing medium (BLC-1; Juji-Field Co., Ltd, Tokyo, Japan) until use.

This study was approved by the Ethics Committee of Fukushima Medical University (1095) and written informed consent was obtained from all the patients and healthy volunteers prior to enrollment.

Flow cytometry. The cells were labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-cyanin 5.1 (PC5). The antibodies used included those directed against FITC-conjugated CD14 (Abcam, Cambridge, UK), PE-conjugated CD11b (Beckman Coulter, Marseille, France) and PC5-conjugated CD33 (Beckman Coulter), diluted in phosphate-buffered saline (PBS) to 10 and 50 μg/ml. The cells were incubated with the antibodies for 20 min at 4°C and were then washed with PBS. Data acquisition and analysis were performed using a FACSAria II flow cytometer (BD Biosciences, Mountain View, CA, USA), accompanied by FlowJo software (TreeStar Inc., Ashland, OR, USA). The typical expression patterns are shown in Fig. 1.

Cytokine production by PBMCs. Approximately 10^6 PBMCs were incubated in 1 ml of RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (Gibco BRL, St. Louis, MO, USA) and 100 μg/ml phytohemagglutinin (PHA; Sigma, Rockville, MD, USA) in 5% CO_2 at 37°C for 24 h. The aliquots of these supernatants were then frozen and kept at -80°C until later use. The supernatant samples were subsequently thawed and used for measurement of the IL-10 concentration using enzyme-linked immunosorbent assay (ELISA) test kits (R&D Systems, Minneapolis, MN, USA). Each sample was only used once after thawing.

Lymphocyte proliferation assay. Lymphocyte proliferation assays were performed using PBMCs suspended in RPMI-1640 medium (Wako Pure Chemical Industries) containing 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA). Following the addition of 10 μg/ml PHA into the PBMC culture wells that were kept at 37°C in a 5% CO_2 atmosphere, PHA mitogenesis was observed for 80 h. ^{3}H-thymidine (Japan Radioisotope Association, Tokyo, Japan) was added to the wells for the last 8 h of incubation. The cells were harvested and ^{3}H-thymidine incorporation was determined using a liquid scintillation counter (Perkin-Elmer Inc., Waltham, MA, USA) and expressed as counts per minute (cpm). The stimulation index (SI) was obtained by calculating total cpm/control cpm. PBMCs that had not been subjected to PHA addition were used as controls.

Markers of nutritional status and chronic inflammation. To evaluate the nutritional status of the subjects, the serum concentrations of albumin (determined by nephelometry) were assessed. CRP was used as an indicator of inflammation.

Statistical analysis. The differences between the groups were determined by Student's t-tests. $P<0.05$ was considered to indicate a statistically significant difference. Inadequate amounts of blood were obtained from some patients; in these cases, certain measurements were not possible.

Results

PBMCs obtained from 49 patients with thyroid cancer, 18 with non-cancerous thyroid diseases and 22 healthy volunteers were investigated in this study. The concentrations of MDSCs
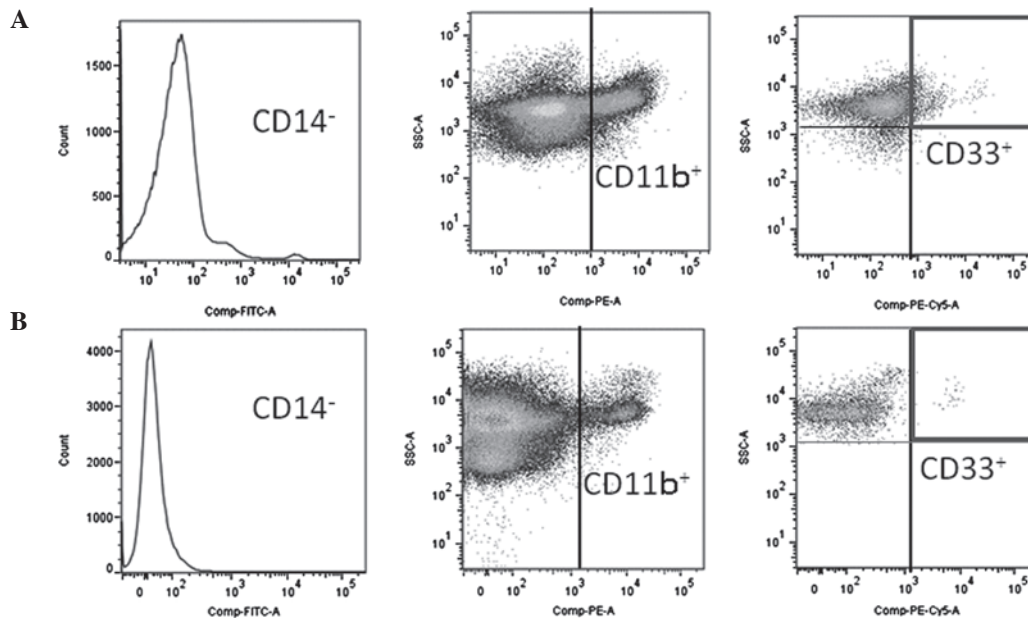
Figure 1. Immunophenotyping of myeloid-derived suppressor cells by flow cytometry. The cells were labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-cyanin 5.1 (PC5). The antibodies used included those targeting FITC-conjugated CD14, PE-conjugated CD11b and PC5-conjugated CD33. (A) Patients with anaplastic thyroid cancer and (B) healthy volunteers.
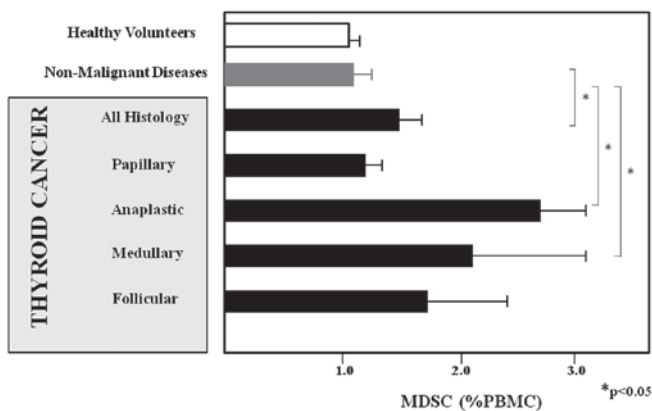

Figure 2. Circulating levels of myeloid-derived suppressor cells (MDSCs) in patients with thyroid cancer according to histology. Peripheral blood mononuclear cells (PBMCs) from 49 patients with thyroid cancer, 18 patients with non-cancerous thyroid diseases and 22 healthy volunteers were collected and tested in this study. The concentrations of MDSCs were significantly higher in patients with any type of thyroid cancer (1.578 ± 0.177, $P<0.05$), anaplastic thyroid carcinoma (2.676 ± 0.544, $P<0.001$), or medullary thyroid carcinoma (2.063 ± 1.028, $P<0.05$) compared to healthy volunteers (1.047 ± 0.120 %PBMC). No difference in the MDSC concentration was observed when comparing patients with papillary thyroid carcinoma (1.025 ± 0.109), patients with non-cancerous diseases (1.188 ± 0.161 %PBMC) and healthy volunteers.
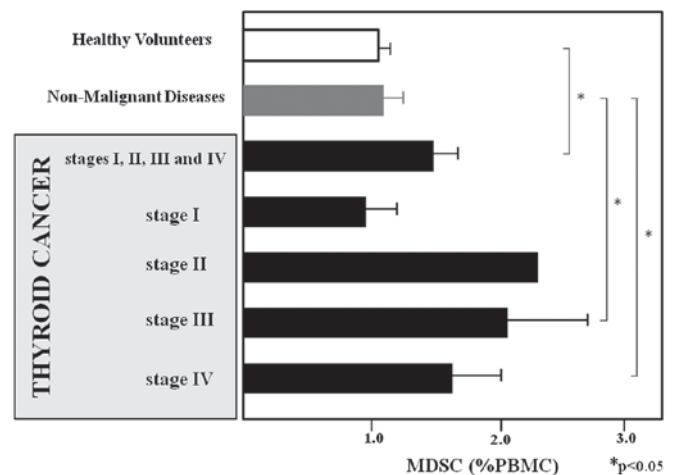

Figure 3. Circulating levels of myeloid-derived suppressor cells (MDSCs) in patients with thyroid cancer according to stage. The MDSC levels of the patients with stage I, II, III and IV thyroid cancer were 0.986 ± 0.235, 2.36, 2.07 ± 0.668 and 1.648 ± 0.242 %PBMC, respectively. The MDSC levels were higher in patients with stage III-IV thyroid cancer compared to those in healthy volunteers ($P<0.05$).

were significantly higher in patients with any type of thyroid cancer (1.578 ± 0.177, $P<0.05$), ATC (2.676 ± 0.544, $P<0.001$), or medullary thyroid carcinoma (2.063 ± 1.028, $P<0.05$) when compared to healthy volunteers (1.047 ± 0.120 %PBMC). By contrast, there was no significant difference in the MDSC levels when comparing patients with papillary thyroid carcinoma (1.025 ± 0.109), non-cancerous thyroid diseases (1.188 ± 0.161 %PBMC) and healthy volunteers (Fig. 2). The levels of MDSCs according to the clinical stage of thyroid cancer are shown in Fig. 3. The MDSC levels of patients

with stage I, II, III and IV thyroid cancer were 0.986 ± 0.235, 2.36, 2.07 ± 0.668 and 1.648 ± 0.242 %PBMC, respectively, and those of patients with stage III-IV disease were significantly higher compared to those of healthy volunteers ($P<0.05$). Data regarding SI, CRP and albumin levels of patients with non-cancerous thyroid diseases and the histology of thyroid cancer are presented in Table I. The SI and CRP levels were significantly higher and the albumin levels were significantly lower in patients with ATC compared to those in patients with non-cancerous thyroid diseases ($P<0.05$). However, there was no significant difference in these parameters when comparing patients with non-cancerous thyroid diseases to patients with papillary, follicular and medullary thyroid carcinomas.

Table I. Stimulation index (SI) and serum concentrations of C-reactive protein (CRP) and albumin in patients with thyroid cancer according to histological type.

Histological type	SI	CRP (mg/dl)	Albumin (mg/dl)
Non-malignant disease (18)	796.6+184.2	0.08+0.03	3.98+0.12
Papillary carcinoma (38)	513.1+78.1	0.64+0.33	3.72+0.10
Anaplastic carcinoma (6)	281.5+212.8[a]	7.51+2.78[a]	2.96+0.56[a]
Medullary carcinoma (3)	450.5+116.8	0.10+0.01	4.13+0.13
Follicular carcinoma (2)	353.3+102.3	0.07+0.02	4.02+0.01

[a]$P<0.05$. In patients with anaplastic thyroid cancer, SI and CRP levels were significantly elevated ($P<0.05$) and albumin levels were significantly reduced compared to those in patients with non-cancerous thyroid diseases ($P<0.05$). No differences in these parameters were observed when comparing patients with papillary, follicular and medullary thyroid carcinomas.

Table II. Stimulation index (SI) and serum concentrations of C-reactive protein (CRP) and albumin in patients with thyroid cancer according to tumor stage.

Tumor stage	SI	CRP (mg/dl)	Albumin (mg/dl)
Non-malignant disease (18)	796.6+184.2	0.08+0.03	3.98+0.12
Stage I (8)	708.3+184.2	0.10+0.05	3.93+0.11
Stage II (1)	700.8	0.05	4.10
Stage III (6)	422.2+132.2[a]	0.08+0.03	3.92+0.18
Stage IV (34)	420.9+55.6[a]	1.91+0.64[b]	3.56+0.12[a]

[a]$P<0.05$, [b]$P<0.01$. Data regarding SI, CRP and albumin in patients with non-cancerous thyroid diseases and in patients with stage I, II, III and IV thyroid carcinomas are presented. The SI was significantly lower in patients with stage III and IV thyroid cancer compared to that in patients with non-cancerous diseases ($P<0.05$). The CRP levels were higher in patients with stage IV thyroid cancer compared to those in patients with non-cancerous thyroid diseases ($P<0.01$). The concentration of albumin was lower in patients with stage IV thyroid cancer compared to that in patients with non-cancerous thyroid diseases ($P<0.05$).

Table III. Comparison of patients according to level of myeloid-derived suppressor cells (MDSCs).

Variables	High MDSC levels (n=35)	Low MDSC levels (n=19)	P-value
Age (years)	63.15+2.52	59.57+3.19	0.451
Tumor diameter (mm)	25.16+8.1	13.81+1.96	0.07
MDSC (%PBMC)	2.850+0.353	0.885+0.051	1.43E-09
CRP (mg/dl)	3.351+1.395	0.709+0.359	0.017
Albumin (mg/dl)	3.318+0.185	3.806+0.120	0.028
IL-10 production (μg/ml)	3664.6+767.0	1503.8+508.4	0.021

Patients with thyroid carcinoma were classified in one of two groups based on a %peripheral blood mononuclear cells (PBMCs) of MDSC cut-off level of 1.578, which was the average %PBMC of MDSC of patients with any type of thyroid carcinoma. The size of the thyroid nodule tended to be larger ($P<0.10$) in patients with higher compared to those with lower MDSC levels. In patients with higher MDSC levels, the CRP and IL-10 production were significantly higher ($P<0.05$) and the levels of albumin were significantly lower ($P<0.05$) compared to those in patients with lower MDSC levels.

Data regarding SI, CRP and albumin levels in patients with non-cancerous thyroid diseases according to the clinical stage of thyroid cancer are presented in Table II. The SI was significantly lower in patients with stage III-IV thyroid cancer compared to that in patients with non-cancerous thyroid diseases ($P<0.05$). Furthermore, the CRP and albumin levels were higher in patients with stage IV thyroid cancer compared to those in patients with non-cancerous thyroid diseases ($P<0.05$).

Subsequently, patients with thyroid carcinoma were categorized into one of two groups according to a %PBMC of MDSC cut-off level of 1.578, which was the average %PBMC of MDSC

in patients with any type of thyroid carcinoma. As shown in Table III, the size of the thyroid nodule tended to be larger (P<0.10) in patients with higher MDSC levels. Furthermore, in patients with higher MDSC levels, the production of CRP and IL-10 was significantly higher (P<0.05) and the levels of albumin were significantly lower (P<0.05) compared to those in patients with lower MDSC levels.

Discussion

The aim of this study was to characterize MDSC levels in patients with thyroid cancer, patients with benign thyroid diseases and healthy volunteers. Patients with ATC exhibited significantly elevated levels of circulating MDSCs, SI and CRP and significantly decreased levels of serum albumin compared to patients with non-cancerous thyroid diseases. Furthermore, MDSC levels were higher and SI was lower in patients with stage III and IV thyroid cancer compared to healthy volunteers. The CRP and albumin levels were lower in patients with stage IV thyroid cancer compared to those in patients with non-cancerous thyroid diseases. Additionally, the levels of CRP were significantly higher, the albumin concentration was lower and the production of IL-10 was elevated in patients with higher compared to those with lower MDSC levels. These data suggest that MDSC levels are increased in patients with ATC and advanced thyroid cancer. In addition, these patients exhibited impaired cell-mediated immune responses, chronic inflammation and nutritional impairment.

The ATC tissue produces large amounts of cytokines (e.g., IL-8 and IL-6) and growth factors (e.g., granulocyte colony-stimulating factor) (20-22). The exact mechanism underlying the increased production of immature myeloid cells in cancer patients has not been elucidated. However, the soluble factors produced by the ATC cells may affect the normal pathway of cell differentiation, resulting in the accumulation of MDSCs and increased clinical aggressiveness of ATC. The serum CRP level is a sensitive marker of inflammation and it is found to be increased in response to tissue damage or infection. The CRP level is also considered to be a prognostic factor in cancer patients (23,24). This acute phase reactant is produced primarily in the liver and its expression is upregulated by pro-inflammatory cytokines, such as IL-6 and IL-8 (25,26). The host immune system responds to tumor growth via elevated levels of inflammatory cytokines, which may further increase CRP levels. Hypoalbuminemia, typically observed in patients with cancer cachexia, is frequently associated with chronic inflammation. Furthermore, nutritional impairment associated with inflammation and immune suppression is observed in patients with ATC, which appears to be independent of the clinical aggressiveness of ATC.

The present study demonstrated that MDSC levels were higher in patients with systemic inflammation and hypoalbuminemia. In addition, immune suppression was closely associated with the Th2-dominant condition. Previous studies suggested that the MDSC levels may decrease following administration of chemotherapeutic or certain anti-inflammatory agents (15,27). Therefore, the regulation of MDSC levels using these strategies may facilitate anti-ATC treatment, including immunotherapy. However, further studies are required to verify this hypothesis.

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