

The Contribution of Vascular Endothelial Growth Factor to the Induction of Regulatory T-Cells in Malignant Effusions

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Abstract. *It has been suggested that immunosuppressive cytokines such as transforming growth factor β (TGF- β) and interleukin 10 play an important role in the induction and/or maintenance of regulatory T-cells (Tregs) in patients with cancer. In the present study, whether or not vascular endothelial growth factor (VEGF) contributes to the induction and/or maintenance of Tregs was examined, because of experience with a patient in whom a positive correlation between VEGF concentration and the percentage of Tregs (% Tregs) among the total CD4⁺ T-cells in the pleural effusion was found during dendritic cell activated lymphocyte therapy. CD4⁺CD25^{high} T-cells were estimated as Tregs in the present study. In an in vitro experimental system, VEGF-containing malignant effusions increased the % Tregs in autologous peripheral blood mononuclear cells (PBMCs), which could be suppressed by the addition of a humanized monoclonal anti-VEGF antibody (bevacizumab [Avastin]). When VEGF-producing hepatic carcinoma cells were mix-cultured with PBMCs, the % Tregs increased and this increase was also suppressed by the addition of bevacizumab. Whether or not bevacizumab can affect the % Tregs of PBMCs in patients with colon cancer was also*

examined. Three out of four patients showed a significant decrease of the % Tregs after intravenous injection of bevacizumab. Interestingly, the expression of VEGF receptor-2 (VEGFR-2) was higher in Tregs than in other CD4⁺ T-cells. Taken together, the data presented here indicate a contribution of VEGF to induction and/or maintenance of Tregs in patients with cancer.

It has been reported that several immunosuppressive cytokines, including transforming growth factor- β (TGF- β), IL-10 (interleukin-10) and vascular endothelial growth factor (VEGF) are detected in the tumor microenvironment (1-7) and that they act as an obstacle against antitumor immunity (8-10). Various types of tumor can secrete a large quantity of VEGF and this ability is often associated with a poor prognosis (11-14). VEGF is known to play a crucial role in tumor angiogenesis. On the other hand, VEGF is also known to play an important role in promoting and sustaining the nonresponsiveness of the immune system to growing tumors (15-18). Thus VEGF may act not only as a promoting factor in tumor growth, but also as a suppressing factor in anti-tumor immunity. Therefore, a recombinant humanized monoclonal IgG1 antibody that binds to and inhibits the biological activity of human VEGF has been approved for the first-line treatment of patients with metastatic colorectal cancer (19).

Recently, a unique CD4⁺ T-cell population, which is designated regulatory T-cells (Tregs) has been identified. Tregs have been shown to contribute to the prevention of autoimmune disorders by controlling the activity of autoreactive T-cells (20-22). Tregs are increased in the peripheral blood and cancer tissues in several types of advanced cancer (23-28) and these increases in Tregs may play critical roles in immune tolerance of carcinomas (29), since most tumor-associated antigens are self-antigens. Thus, several clinicians are now evaluating the depletion of Tregs in an attempt to improve the therapeutic effect of tumor

Abbreviations: IL-10, Interleukin-10; TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor; Tregs, regulatory T-cells; Foxp3, forkhead box protein 3; FACS, fluorescence-activated cell sorting; DC, dendritic cells; DAK, dendritic cell-activated lymphocytes; PBMCs, peripheral blood mononuclear cells.

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Table I. Profiles of cancer patients.

Case No.	Age (years)	Gender	Effusion	Primary site	Site of metastasis	Operation	Previous therapy	Immunotherapy
1	50	F	Ascites	Stomach	Pt.	+	Chemotherapy	DAK
2	52	M	PE	Stomach	Pt.	+	Chemotherapy	DAK
3	60	M	Ascites	Stomach	Pt.	+	Chemotherapy	DAK
4	55	F	Ascites	Colon	Lung, liver, Pl	+	Chemotherapy	DC+DAK
5	44	M	Ascites	Pancreas	Liver	–	Chemotherapy	DAK
6	55	F	Ascites	Pancreas	Pt	–	Chemo+Radiation	DAK
7	59	M	Ascites	Pancreas	Liver	–	Chemotherapy	DAK
8	65	F	Ascites	Pancreas	Pt	+	Chemo+Radiation	DC+DAK
9	69	F	Ascites	Pancreas	Pt	+	Chemotherapy	DAK
10	73	M	Ascites	Pancreas	Liver, Pt	–	Chemotherapy	DAK
11	54	M	Ascites	Bile duct	Liver	–	Chemotherapy	none
12	62	F	PE	Lung	Bone	–	Chemotherapy	DC+DAK
13	79	M	PE	Lung	Brain, liver, bone, LN	+	Chemotherapy	DAK
14	48	F	PE	Breast	Lung, liver, Pl	+	Chemotherapy	DAK
15	65	F	Ascites	Breast	Liver, bone, Pt	+	Chemotherapy	DAK
16	70	F	PE	Uterus	Pl, Pt	+	Chemotherapy	DAK
17	53	F	Ascites	Ovary	Pt	+	Chemotherapy	DAK
18	64	F	Ascites	Ovary	Pt	+	Chemotherapy	DAK

PE: Pleural effusion; Pt: peritoneum; Pl: pleural membrane; LN: lymph nodes; DC: dendritic cell vaccine; DAK: DC-activated lymphocytes.

immunotherapies such as monocyte-derived dendritic cell (DC)-based vaccine therapy and activated lymphocyte infusion therapy. In fact, several reports have shown that depletion of Tregs results in increased immune responses towards tumors in animal tumor models (30-36). Interestingly, the contribution of immunosuppressive cytokines such as TGF- β and IL-10 to the induction or maintenance of Tregs has been reported previously (9, 37).

In the present study, whether or not VEGF could participate in the induction or maintenance of Tregs in patients with cancer was examined.

Materials and Methods

Cells. VEGF-producing human hepatocellular carcinoma cells, HepG2, were maintained at 37°C under a humidified atmosphere of 5% CO₂ and 95% air in RPMI-1640 medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin; Meijiseika, Tokyo, Japan) (38).

Patients and therapeutic protocols. Eighteen patients with malignant effusions and multiple metastases were studied according to a protocol which was approved by the Kyushu University Ethics Committee, Japan. The patient profiles are shown in Table I. Seventeen out of the 18 patients received immunotherapy consisting of an intravenous injection of 1-10×10⁸ tumor-pulsed DC-activated lymphocytes (DAK), in addition some patients also received a subcutaneous injection of 2-30×10⁶ mature DCs loaded with necrotic autologous tumor cells (DC vaccine) into the left supra-clavicular area every 2 or 4 weeks (39). In principle, this immunotherapy was continued for as long as possible in the outpatient clinic.

Table II. Profiles of patients who received bevacizumab.

Case no.	Age (years)	Gender	Primary site	Site of metastasis	Operation	Serious complication
1	73	M	Colon	Lung, liver	+	None
2	61	F	Colon	Lung, LN	+	None
3	68	M	Colon	Liver, LN	+	None
4	72	F	Colon	Liver, LN	+	None

LN: Lymph nodes.

Preparation of DAK and DC vaccine. Autologous tumor-pulsed DCs (DC vaccine) were prepared as described previously (39). Briefly, immature DCs were prepared from peripheral blood mononuclear cells (PBMCs) using recombinant human granulocyte/monocyte colony-stimulating factor (GM-CSF; 200 ng/ml; Novartis Pharma, Basel, Switzerland) and recombinant human IL-4 (500 U/ml; Ono, Tokyo, Japan) for 7 days. Tumor cells obtained from tumor masses or malignant effusions were lysed by five freeze-thaw cycles (necrotic tumor cells). The immature DCs were incubated with the necrotic tumor cells overnight and then cultured for 2 days with tumor-pulsed DCs in medium containing tumor necrosis factor- α (TNF- α ; 1000 U/ml; R and D Systems Inc., Minneapolis, MN, USA) and prostaglandin E₂ (PGE₂; 1 µg/ml; Sigma, St. Louis, MO, USA).

For preparation of the DAKs, non-adherent cells from each patient's PBMCs were cultured with tumor-pulsed DCs for 1 week in Hy-medium containing 175 JRU/ml of human recombinant IL-2 (Nipro, Tokyo, Japan).

Bevacizumab (Avastin)-based therapy. Four patients with advanced colon cancer with multiple metastases received bevacizumab (5 mg/kg) every 2 weeks by intravenous injection (Table II).

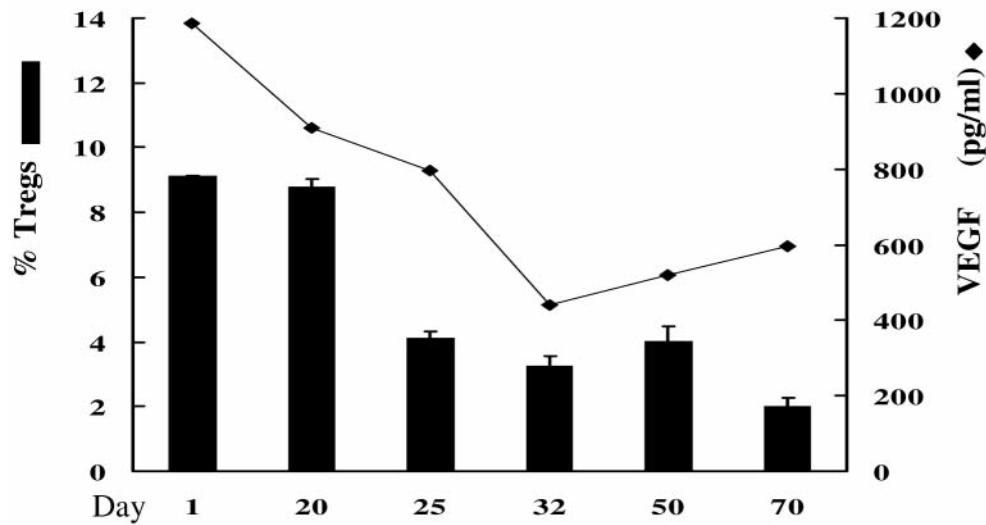


Figure 1. Kinetic study of % Tregs and VEGF concentration in malignant effusion collected from a patient with ovarian cancer during DAK therapy.

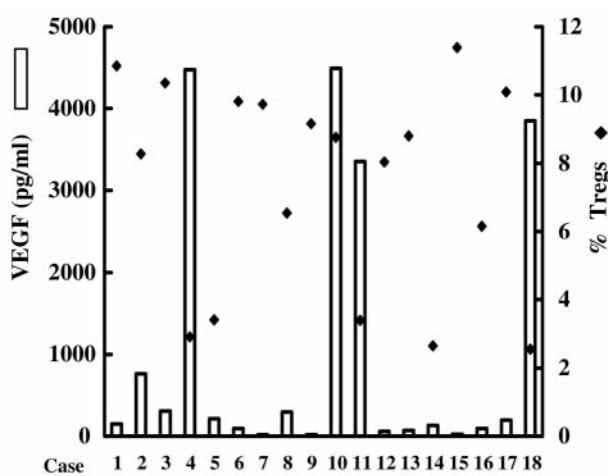


Figure 2. The relationship between % Tregs and VEGF concentration in malignant effusions. % Tregs of EDMCs and VEGF concentration in malignant effusions collected from the 18 cancer patients, as listed in Table 1.

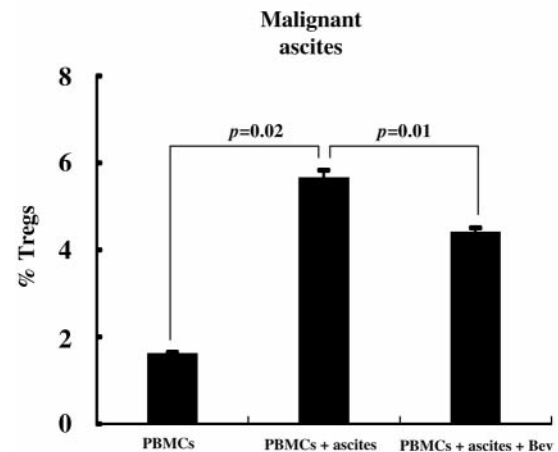


Figure 3. The induction of Tregs in PBMCs by malignant effusion and its suppression by bevacizumab. PBMCs collected from a patient with cholangio-carcinoma were cultured for 7 days with autologous patient-derived ascitic fluid (VEGF: 3353 pg/ml). Bev: bevacizumab.

Flow cytometric analysis. The malignant effusion-derived mononuclear cells (EDMCs) and the PBMCs were stained with anti-CD25, anti-CD4, anti-CD8 (BD Biosciences, Tokyo, Japan) or anti-human VEGF receptor-2 (VEGFR-2; R and D Systems). Intracellular staining of forkhead box protein 3 (FOXP3) was conducted using a PE-conjugated anti-human FOXP3 Staining Set (clone PCH101; e-Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. Two- or three-color flow cytometry was performed using fluorescence-activated cell sorting (FACS) Calibur™ (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). CD25^{high} cells or FOXP3⁺ cells after gating of CD4⁺ lymphocytes were evaluated as Tregs (25, 28, 40). The percentage of Tregs among the total CD4⁺ T-cells was represented as % Tregs.

Transwell experiments. PBMCs (1×10^6 cells) from a healthy volunteer and HepG2 cells (5×10^5 cells) were cultured separately using polycarbonate 24-well transwell inserts (TR; 0.4 μ M) (Becton Dickinson Labware, NJ, USA). The cells were suspended in RPMI-1640 containing 1% albumin and 2% human serum with or without bevacizumab (10 μ g/ml) and cultured for 7 days.

ELISA. The culture supernatants or malignant effusions were collected and the concentrations of VEGF were measured by an ELISA kit (Biosource International, Inc, Camarillo, CA, USA).

Statistical analysis. Fisher's exact probability test was used for the statistical analyses. The data were analyzed with a SAS statistical

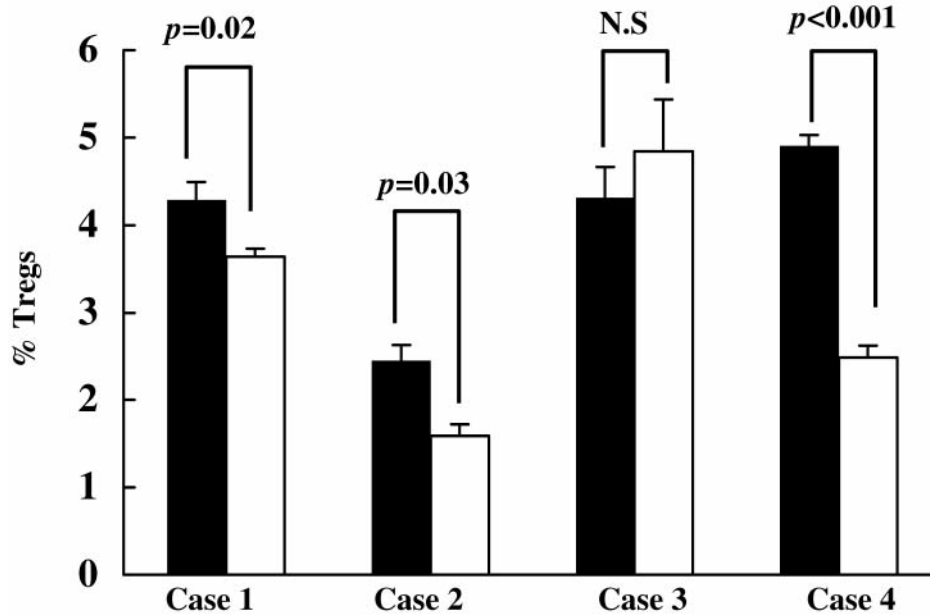


Figure 4. Decrease of % Tregs in colon cancer patients by bevacizumab. % Tregs in PBMCs before (solid column) and after (open column) the intravenous injection of bevacizumab in four patients with colon cancer, as shown in Table II.

software package (Abacus Concepts, Berkeley, CA, USA). Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Case report. A 70-year-old female presented with stage IV ovarian cancer with malignant pleural effusion. She had received prior second-line chemotherapy and was evaluated as having progressive disease at the time of entry into the immunotherapy. Before therapy, the % Tregs of the EDMCs and VEGF concentration in pleural effusion were 9.14% and 1,186.0 pg/ml, respectively. During DAK therapy, the change in % Tregs roughly paralleled the change in VEGF concentration (Figure 1).

Relationship between % Tregs and VEGF concentration in malignant effusions. Since an association between Tregs and VEGF was suggested, the % Tregs of EDMCs and VEGF concentration was measured in the malignant effusions collected from the 18 patients (Table I). Unfortunately, there was no significant correlation between the % Tregs and the VEGF concentration (Figure 2).

Induction of Tregs in PBMCs by malignant effusion and its suppression by bevacizumab. When PBMCs collected from a patient with cholangio-carcinoma were cultured for 7 days with the patient-derived ascitic fluid (VEGF: 3,353 pg/ml), the % Tregs in the PBMCs increased, and its increase was

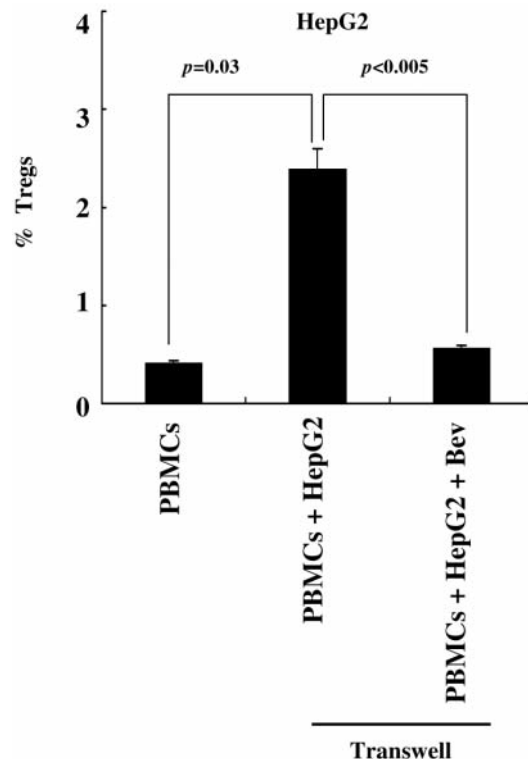


Figure 5. The induction of Tregs in PBMCs by HepG2 cells, and its suppression by bevacizumab. PBMCs from a healthy volunteer and HepG2 cells which secrete a large a quantity of VEGF (VEGF: 2,036 pg/ml). PBMCs were cultured separately using a transwell culture system.

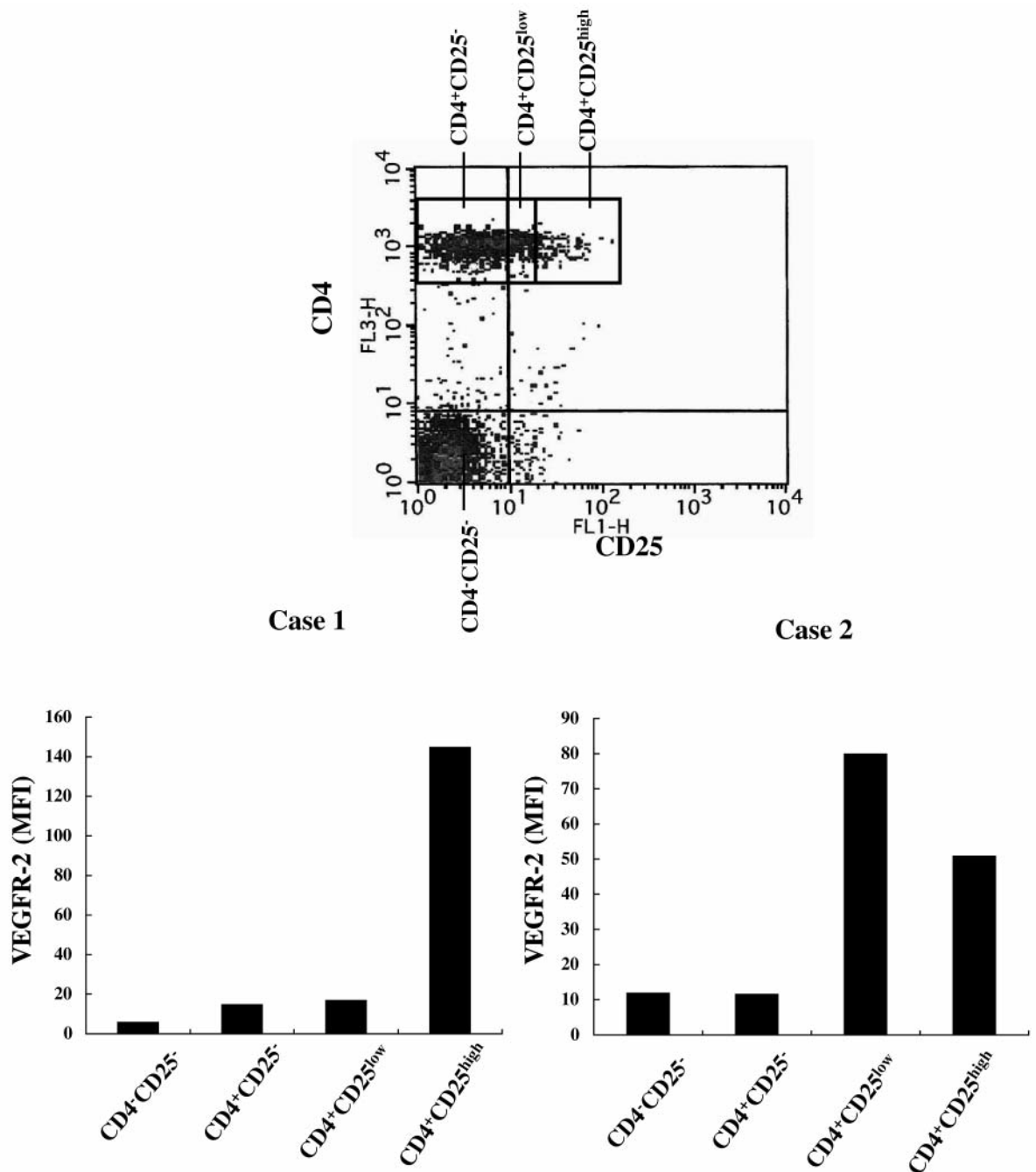


Figure 6. The expression of VEGFR-2 on Tregs. VEGFR-2 expression was examined in gated-lymphocyte populations using three-color flow cytometry analysis in PBMCs collected from two healthy volunteers (Case 1 and Case 2). MFI: mean fluorescent intensity.

significantly suppressed by the addition of bevacizumab at the initiation of culture (Figure 3).

Decrease of % Tregs in patients with colon cancer by bevacizumab. The % Tregs in the PBMCs was measured

before and after the intravenous injection of bevacizumab in four patients with colon cancer (Table II). In three out of the four patients, bevacizumab therapy significantly reduced the % Tregs (Figure 4). No particular adverse reactions occurred during the observation period.

Induction of Tregs in PBMCs by HepG2 cells and its suppression by bevacizumab. To confirm a possibility that VEGF can induce Tregs in PBMCs, PBMCs were cultured separately using a transwell culture system with HepG2 cells, which secrete a large quantity of VEGF. The HepG2 cells increased significantly the % Tregs and bevacizumab significantly reduced the % Tregs to almost the control level (Figure 5).

Expression of VEGFR-2 on Tregs. The expression of VEGF receptor-2 (VEGFR-2) was significantly higher on the Tregs compared with other CD4⁺ T-cells in a healthy volunteer (Case 1, Figure 6, left panel). On the other hand, both CD4⁺CD25^{low} T-cells and Tregs expressed VEGFR-2 in a second healthy volunteer (Case 2, Figure 6, right panel), but the grade of its expression was significantly lower compared with Case 1.

Discussion

In the present study, it was found that VEGF may play an important role in the induction or maintenance of Tregs in a tumor microenvironment. In addition, for the first time it was shown that VEGFR2 is expressed on Tregs in some cases.

As several investigators have indicated (23, 24), the % Tregs was significantly increased in malignant effusions compared with that in PBMCs from healthy volunteers (data not shown). Immunosuppressive cytokines such as TGF- β and IL-10, which contribute to the induction and/or maintenance of Tregs, are also known to increase in malignant effusions. Although these cytokines were detected in most of the malignant effusions examined, there was no significant correlation between the % Tregs and the concentration of these cytokines (data not shown). A patient showing increased % Tregs in EDMCs and VEGF in the malignant effusion was encountered. Interestingly, during DAK therapy, the change in % Tregs paralleled changes in VEGF concentration (Figure 1). We hypothesized that there is some link between Tregs and VEGF which may be supported by the following data. First, when a patient's PBMCs were used as target cells, a malignant effusion containing a large quantity of VEGF increased the % Tregs in the PBMCs, which was inhibited by bevacizumab (Figure 3). Second, VEGF-producing HepG2 cells increased the % Tregs in PBMCs and this increase was also inhibited by the presence of bevacizumab (Figure 5). In addition, the administration of bevacizumab to treat patients with advanced colon cancer significantly reduced the % Tregs in the PBMCs in three out of the four patients (Figure 4). These *in vivo* data may also indirectly support our hypothesis. Interestingly, Li *et al.* (41) reported that the overexpression of VEGF from tumors resulted in increased numbers of Tregs in the tumor. Although these

findings strongly suggest a link between the induction of Tregs and VEGF, there was no significant correlation of the % Tregs in the EDMCs and VEGF concentration in the malignant effusions (Figure 2). In order to address these contradictory results, the expression status of VEGFR on the Tregs was examined. VEGF activity is mediated essentially through two receptors, VEGFR-1 and VEGFR-2 (42, 43) and VEGFR-2 seems to be responsible for the angiogenic effect of VEGF. As expected, VEGFR-2 was expressed on the Tregs of the PBMCs collected from two healthy volunteers (Figure 6). However, the level of expression of VEGFR-2 differed between the two people. These preliminary data suggest that differing levels of VEGFR-2 expression on Tregs in individuals may result in different reactions of the Tregs to VEGF. However, no definite answer was found for the lack of correlation between the % Tregs in the EDMCs and the VEGF concentration in the malignant effusions. Nevertheless, our data suggest a contribution of VEGF to the induction or maintenance of Tregs in the tumor microenvironment. If so, VEGF and VEGFR-2 may be therapeutic targets to improve the efficacy of immunotherapy.

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