

## Antagonism of VEGF by genetically engineered dendritic cells is essential to induce antitumor immunity against malignant ascites

Sugiyama, Masahiko

R and D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University | Department of Surgery and Science, Kyushu University

Takeji, Yoshihiro

Department of Surgery and Science, Kyushu University

Tsujitani, Shunichi

Department of Surgery and Science, Kyushu University

Harada, Yui

R and D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University

他

<https://hdl.handle.net/2324/25486>

---

出版情報 : Molecular Cancer Therapeutics. 10 (3), pp.540-549, 2011-03-01. American Association for Cancer Research

バージョン :

権利関係 : (C) 2011 American Association for Cancer Research



**MCT-10-0479R1 (revised manuscript)**

**Manuscript Category: Research Article**

**Title:**

**Antagonism of VEGF by genetically engineered dendritic cells is essential to induce antitumor immunity against malignant ascitis**

**Authors:**

Masahiko Sugiyama<sup>1,2</sup>, Yoshihiro Kakeji<sup>2</sup>, Yui Harada<sup>1</sup>, Mitsuho Onimaru<sup>3</sup>,  
Kumi Yoshida<sup>1</sup>, Sakura Tanaka<sup>4</sup>, Yasunori Emi<sup>2</sup>, Masaru Morita<sup>2</sup>, Yosuke Morodomi<sup>1,2</sup>,  
Mamoru Hasegawa<sup>5</sup>, Yoshihiko Maehara<sup>2</sup>, and Yoshikazu Yonemitsu<sup>1</sup>

**Affiliations:**

<sup>1</sup>R&D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

<sup>2</sup>Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

<sup>3</sup>Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

<sup>4</sup>Division of Pathophysiology and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

<sup>5</sup>DNAVEC Corporation, Tsukuba, Ibaraki 305-0856, Japan

**Key Words:**

Malignant ascitis, VEGF-A, sFLT-1, recombinant Sendai virus, dendritic cells

**Abbreviations:**

VEGF-A/VPF: vascular endothelial growth factor-A/vascular permeability factor, MA: malignant ascitis, sFLT-1: soluble *fms*-like tyrosine kinase receptor-1, DC: dendritic cells, RIG-I: retinoic acid-inducible gene-I, rSeV: recombinant Sendai virus, PPP: platelet poor plasma, GFP: green fluorescent protein, MEM: modified Eagles' medium, FLT-3: *fms*-like tyrosine kinase receptor-3, ELISA: enzyme-linked immuno-sorbent assay, RT-PCR: reverse transcription polymerase chain reaction

**Short Running Head: *Role of VEGF in DC-based immunotherapy***

**Footnotes:**

This work was supported in part by the Japanese Ministry of Education, Culture, Sports, Science, and Technology (to YY:No.21390364, and to YM: No.21249072).

**All correspondence and reprint request to:**

**Yoshikazu Yonemitsu, M.D., Ph.D.**

R&D Laboratory for Innovative Biotherapeutics,  
Graduate School of Pharmaceutical Sciences, Kyushu University  
Rm 505 Collaborative Research Station II  
3-1-1 Maidashi, Higashi-ku  
Fukuoka 812-8582, Japan  
Phone/Fax: +81-92-642-4777  
E-mail: [yonemitsu@med.kyushu-u.ac.jp](mailto:yonemitsu@med.kyushu-u.ac.jp)

**Disclosure:**

Dr. Yonemitsu is a member of the Scientific Advisory Board of DनावेC Corporation.

### Abstract

Malignant ascitis (MA) is a highly intractable and immunotherapy-resistant state of advanced gastrointestinal and ovarian cancers. Using a murine model of MA with CT26 colon cancer cells, we here determined that the imbalance between the vascular endothelial growth factor-A/vascular permeability factor (VEGF-A/VPF) and its decoy receptor, soluble *fms*-like tyrosine kinase receptor-1 (sFLT-1), was a major cause of MA's resistance to dendritic cell (DC)-based immunotherapy. We found that the ratio of VEGF-A/sFLT-1 was increased not only in murine but also in human MA, and F-gene-deleted recombinant Sendai virus (rSeV/dF)-mediated secretion of human sFLT-1 by DCs augmented not only the activity of DCs themselves but also dramatically improved the survival of tumor-bearing animals associated with enhanced CTL activity and its infiltration to peritoneal tumors. These findings were not seen in immunodeficient mice, indicating that a VEGF-A/sFLT-1 imbalance is critical for determining the antitumor immune response by DC-vaccination therapy against MA.

(148 words)

## Introduction

Malignant ascitis (MA) is defined by the National Cancer Institute as ‘a condition in which fluid containing cancer cells collects in the abdomen’, and represents an advanced state of ovarian, pancreaticobiliary, or gastrointestinal cancer (1). Because MA has been highly resistant to the current therapeutics, there is a lack of randomized controlled trials identifying optimal therapy and therefore evidence-based therapeutic guidelines have not been established.

Some institutions have attempted immunotherapies as a possible alternative to treat MA. Actually, an early trial demonstrated the significant prolongation of the 50% survival of malignant effusion from 1.6 months to 3.5 months by a combination of chemotherapy and OK-432, a ‘non-specific’ immunostimulatory agent containing bacterial components (2). A recent study also significantly reduced the accumulation of ascitis and tumor cell numbers via enhanced antitumor immunity induced by catumaxomab, a trifunctional anti-EpCAM x anti-CD3 antibody (3). These results suggest the possible utility of ‘immunotherapy’ against MA; however, the clinical outcome of immunotherapy has been far from that required to the standard therapy.

Dendritic cells (DCs) are unique antigen-presenting cells that can stimulate innate as well as acquired immune responses against pathogens and cancers. Over the last decade, there has been much anticipation about the potential for DC-based immunotherapy as a new therapeutic modality for cancers; however, it has been reported that clinical outcome has been limited efficacies have been reported (4). To provide a possible solution, we recently proposed a new concept, ‘immunostimulatory virotherapy’ (5, 6), using a new DC-activating modality, the replication-competent<sup>6</sup> as

well as fusion (F)-gene-deleted nontransmissible (7-9) recombinant Sendai viruses (rSeVs). SeV, a member of the family *Paramyxoviridae*, have a nonsegmented negative-strand RNA genome and shows a broad spectrum of gene transfer (10-12), including DCs (7-9). Importantly, rSeVs lead DCs to highly activated/mature state via a DExD/H-box RNA helicase, retinoic acid-inducible gene-I (RIG-I) (13, 14); therefore, we have able to demonstrate that DCs activated by rSeVs (DC-rSeV) induced highly efficient antitumor immunity against various tumors (7-9).

Based on above reasons, we here attempted the therapeutic efficacy of DC-rSeV against a murine model of MA using CT26 colon cancer cells. Of interest, we here found that murine MA was still resistant to DC-based immunotherapy; and importantly, we here simply determined that the imbalance of the protein accumulation of vascular endothelial growth factor-A/vascular permeability factor (VEGF-A/VPF) and its endogenous decoy receptor, soluble *fms*-like tyrosine kinase-1 (sFLT-1) was a key to reduce the efficacy of DC-based immunotherapy.

## Materials and Methods

### *Cells, reagents, and ELISA*

CT26 murine colon cancer cells and fibrosarcoma MethA cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). These cell lines were maintained in RPMI1640 medium supplemented with 10% FBS, penicillin, and streptomycin in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Growth and morphology of both lines was observed and noted to be consistent with original descriptions of the lines, and no mycoplasma contamination was confirmed by PCR method; however, no further genetic characterization was done. Lipopolysaccharide (LPS; Sigma-Aldrich, Tokyo, Japan) was used to activate DCs as a positive control. ELISA was performed according to the manufacturer's instructions using commercially available Quantikine Immunoassay systems for murine and human VEGF-A, murine and human sFLT-1, mIL-1 $\beta$ , mTNF- $\alpha$ , mIL-6, and JE/mMCP-1 (R&D Systems, Inc., Minneapolis, MN).

### *Ascitis and platelet poor plasma from human MA patients*

The collection and analyses of ascitis and platelet poor plasma (PPP) from 6 subjects with MA (4: gastric cancer, 1: colon cancer, 1: esophageal cancer) were done with written informed consent under the approval of the Institutional Review Board of Kyushu University. The collected ascitis was centrifuged and the supernatant was subjected to ELISA. One subject with MA of gastric cancer that exhibited chylous ascites was excluded from analysis (neither VEGF-A nor hsFLT-1 was detected in the supernatant in this case).

### *Quantitative real-time RT-PCR*

Real-time monitoring of the amplification of target genes and quantification of gene expression levels were done by a Sequence Detection System, model 7000 (Applied Biosystems, Tokyo, Japan), according to the manufacturer's instructions for Taq-Man methods (15). The oligonucleotide sequences of PCR primers and TaqMan probes are as follows; murine VEGF: forward=5'-GCAGGCTGCTGTAACGATGAA-3', reverse=5'-TCACATCTGCTGTGCTGTAGGA-3', and probe= 5'-FAM-CATGCAGATCAT-GCGGATCAAACCTC -TAMRA-3'; hsFLT-1: forward=5'-TCTCACAGGATCTAG-TTCAGGTTCA-3', reverse=5'-TTACTCACCATTTTCAGGCAAAGAC-3', and probe=

5'-FAM-CAGCACATCATGCAAGCAGGCCA-TAMRA-3'; murine FLT-1 (extracellular domain): forward=5'-CATTGTAAACGTGAAACCTCAGATCT-3', reverse= 5'-CTGCTGCCCAGCGGATAG-3', and probe= 5'-FAM-CCGTGTCCTCG-CTTCCAAGCCC-TAMRA-3'; murine FLT-1 (intracellular domain): forward= 5'-GGGAAAGGAGTCCTGCTGTTCT-3', reverse= 5'-GAGCGGAATAGGTGTAAA-CTCATAGAT-3', and probe= 5'-FAM-ACCCCCAGACTACAACCTCCGTGGTGT-TAMRA-3'. The expression level of the target genes was standardized by the GAPDH level in each sample and was expressed as fold increase over the control level.

### ***Flow cytometric analysis***

The cells reacted with appropriate antibodies were analyzed using FACS Calibur (Becton–Dickinson, Tokyo, Japan) with CellQuest software (BD Biosciences Japan, Tokyo, Japan). Dead cells were excluded by staining with propidium iodide. Data analysis was performed using FlowJo 4.5 software (Tree Star, Inc., San Carlos, CA).

### ***Construction and recovery of rSeVs***

rSeVs were constructed as described previously (7-9). In brief, LLC-MK2 cells were transfected with a plasmid mixture containing each plasmid: pSeV+18/dF-GFP or pSeV+18/dF-hsFLT-1 (16), pGEM-NP, pGEM-P, and pGEM-L in Superfect reagent (Qiagen, Tokyo, Japan). The transfected cells were maintained for 3 h, then were washed and incubated for 60 h in MEM containing araC. The cells were collected and lysed, and the lysate solution was incubated on the stably F-expressing LLC-MK2 cells in a 24-well plate. Twenty-four hours later, cells were washed and incubated in MEM containing araC and trypsin. Virus yield is expressed in cell infection units (CIU), as previously described (7-9). Human cDNA of soluble *flt-1*, 2,064 bp encoding six of seven extracellular domains (17) was amplified by PCR with primer set containing *NotI* site from total RNA of HUVEC. The amplified cDNA was subcloned into the *NotI* site of template plasmid pSeV+18/dF. The cDNA sequence was completely identical to the published human soluble *flt-1*. Expression of hsFLT-1 from rSeV/dF-hsFLT1 was determined by specific ELISA.

### ***Preparation and log-scale expansion of murine bone marrow-derived DCs***



The DCs used in this study were subjected to the ‘log-scale expansion’ procedure by the floating culture method under the ‘FS36’ cocktail of containing FLT-3 ligand, stem cell factor, IL-3, and IL-6 for 3 weeks, followed by DC-differentiation culture with GM-CSF and IL-4 for 7 days, as previously described (18). Expanded immature DCs were stocked at -80°C until they were used in experiments.

### ***Animal studies***

Female 8-week-old Balb/c mice and *nu/nu* mice were obtained from KBT Oriental Co., Ltd. (Charles River grade, Tosu, Saga, Japan), and were kept under specific pathogen-free and humane conditions. The animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee and by the Biosafety Committee for Recombinant DNA experiments of Kyushu University. These experiments were also done in accordance with recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government. For the MA murine model, CT26 cells ( $10^5$ ) were injected intraperitoneally, and DCs were also injected as defined protocols in each experiment. The body weight and survival were examined daily.

### ***<sup>51</sup>Cr release assay***

For CTL assay, as previously described (7-9), splenocytes from tumor-bearing mice on day 21 (7 days after 3 treatments) were obtained and erythrocytes were depleted. The effector cells were cocultured with irradiated (100 Gy) tumor cells as the stimulator. Three days later, 30 IU/ml murine rIL-2 was added to the medium. After 5 days, the cultured cells were collected and used as effector cells. Target cells were labeled with 100 mCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (Amersham Biosciences, Buckinghamshire, UK) for 90 min at 37 °C. The labeled target cells were incubated with the effector cells for 4 hours at 37 °C in 96-well plates in 200 µl medium at various effector (E)/target (T) ratios. The radioactivity of the supernatants was counted using a gamma counter. The maximum or spontaneous release was defined as counts from samples incubated with 2% Triton X-100 or medium alone, respectively. Cytolytic activity was calculated using the following formula: percentage of specific <sup>51</sup>Cr release = (experimental release - spontaneous release) x 100/ (maximum release - spontaneous release). Assays were performed in duplicated wells.

***Immunohistochemistry***

Peritoneally disseminated tumor nodules were obtained from CT26 MA mice after weekly treatment with each DC for 3 weeks. Freshly excised tumor tissues were embedded in OCT compound, and the cryostat sections were subjected to immunochemical examinations with monoclonal antibodies specific to CD4 (L3T4; eBioScience, San Diego, CA), CD8 (Ly-2; eBioScience), or pan-NK CD49b ( $\alpha$ 2-integrin, eBioScience) as primary antibodies, as previously described<sup>7</sup>. Three randomly selected and totally viable tumor areas were scanned as TIF images under an optical measure-assisted microscope at x200. The mean number of positive cells was used for the data of each tumor.

***Statistical analysis***

All data were expressed as means  $\pm$  SD. The data were examined statistically using one-way ANOVA and Scheffe's adjustment. When the number of evaluated groups was small, the data were subjected to the Mann-Whitney U test. The survival curves were determined using Kaplan-Meier's method, and the log-rank test was used for comparison. A probability value of  $P < 0.05$  was considered statistically significant. Statistical analyses were determined using StatView software.

## Results

### **CT26-induced MA is highly resistant to immunostimulatory virotherapy using rSeV/dF**

To seek the major factor(s) involved in the pathophysiology of MA to hinder the efficacy of immunotherapy, we here used a well-established MA model induced by peritoneal dissemination of CT26 murine colon cancer cells and virally activated dendritic cell-based immunotherapy, the so-called ‘immunostimulatory virotherapy’, using F-gene-deleted nontransmissible recombinant Sendai virus (DC-rSeV/dF). This recently developed concept has shown dramatic enhancement of antitumor immunity (5-9).

Figure 1a shows the efficacies of various regimens of intraperitoneal injections of DC-rSeV/dF-GFP without any tumor antigen (once or twice per week for 3 or 6 weeks). Although significant prolongation of the survival of tumor-bearing immunocompetent syngeneic balb/c mice, the outcome was unsatisfactory. In addition, our preliminary experiment demonstrated that MA mice treated by similar regimen did not show the significant elevation of CTL activity (data not shown), suggesting that there might be one or more immunosuppressive factors in the tumor-disseminated peritoneal cavity.

### **Murine as well as human MA exhibits the imbalance of VEGF-A/sFLT-1 ratio in ascitis**

We therefore hypothesized that vascular endothelial growth factor-A/vascular permeability factor (VEGF-A/VPF) might hinder the DC-mediated antitumor response, because tumor-secreting VEGF-A/VPF was first found in MA (19, 20) and showed

immunosuppressive activity on DCs (21). As shown in Fig. 1b, the specific ELISA demonstrated that mVEGF-A/VPF was markedly accumulated in the ascitis but not in platelet-poor plasma (PPP; excluding VEGF-A encapsulated into the  $\alpha$ -granules of platelets) from mice associated with peritoneal CT26 tumor cell dissemination (n=11, 20 days after tumor inoculation). Murine soluble *fms*-like tyrosine kinase-1 (msFLT1), a high-level protein content of a soluble ‘decoy’ receptor for VEGF-A/VPF, was seen in the PPP of tumor-free control mice (mean=1,116 $\pm$ 339 pg/ml, n=4), and an increased level was also detected in the PPP of tumor-bearing mice (2,933 $\pm$ 897 pg/ml, n=11, mVEGF-A/msFLT1 ratio=0.05 $\pm$ 0.02, day 21 after tumor inoculation). In contrast, the expression level of msFLT-1 was significantly lower than that of VEGF-A in the ascitis of MA mice (mVEGF-A/msFLT1 ratio=2.91 $\pm$ 1.97, day 21 after tumor inoculation). This MA animal model did not show any systemic edema (assessed by the dry/wet weight ratio of the hind limb; data not shown), thus suggesting that the increase in the mVEGF-A/msFLT1 ratio would be crucial for inducing ascitis as well as peripheral edema; a very low level of circulating VEGF in PPP was detected in healthy mice. These findings seen in murine ascitis were also representative in human subjects with MA (n=5), as shown in Fig. 1c. Inversely, a dramatically low level of hsFLT1 in PPP (mean=29.1 pg/ml, mean of healthy mouse control=48.2 pg/ml). All these patients showed moderate to severe edema on their extremities, thus supporting the concept that an imbalance of VEGF-A/msFLT1 might be a determinant of MA and systemic edema.

#### **Antagonism of VEGF-A/VPF activity by exogenous human sFLT-1 gene augments the therapeutic outcome of DC-rSeV/dF**

These results led us to perform further study, using DC-rSeV/dF-expressing

hsFLT1 for neutralizing VEGF-A/VPF activity. We here used human sFLT-1 transgene to distinguish the endogenous and transgene expression, and it has been already demonstrated that hsFLT-1 was sufficiently active to neutralize murine VEGF (16, 22-24). Actually, DC-rSeV/dF-hsFLT1 dramatically improved the survival of tumor-bearing mice (Fig. 1d, left) and inhibited the increase of their body weight (Fig. 1d, right). Further studies related to the treatment parameters, repeat administration (Fig. 2a), and the number of DCs per dose (Fig. 2b) using expanded murine DCs (12) demonstrated that ‘once per week for 6 weeks and  $10^6$  DCs per dose’ was the optimized condition for efficient control of CT26-MA in the use of DC-rSeV/dF-hsFLT1. RT-PCR analyses revealed no significant expression of other possible ligands for FLT-1, VEGF-B, VEGF-C, and PlGF from CT26 cells (data not shown). In addition, our preliminary study demonstrated that similar treatment regimen via intravenous injection route could not show any improvement of survival of MA mice (data not shown), suggesting that intraperitoneal route may be prefer to show the therapeutic efficacy.

#### **Functional expression of exogenous hsFLT-1 gene expression from DC-rSeV/dF for the degradation of endogenous and exogenous VEGF-A/VPF**

Next, we assessed the effects of rSeV/dF-hsFLT1 on DC functions, and LPS and rSeV/dF-GFP were used as controls. Naïve murine DCs, as well as DCs stimulated by LPS or rSeV/dF, secreted considerable amounts of mVEGF-A/VPF into culture medium; the secretion was dramatically reduced by expression of hsFLT1 without any change in msFLT1 (Fig. 3a, upper 3 graphs). The addition of excess exogenous mVEGF-A/VPF protein (4,000 pg/ml) resulted in the marked reductions in mVEGF as well as murine and human sFLT1s in the use of rSeV/dF-hsFLT1 (Fig. 3a, lower 3

graphs). This suggests the degradation and/or loss of immunoreactivity of mVEGF by complexed with hsFLT-1, indicating that recombinant gene expression of hsFLT1 is functionally active.

To further examine the transcriptional regulation of mVEGF and mFLT-1, we next assessed the intracellular mRNA levels in the same condition as Fig. 3a. We here examined mFLT-1 mRNA by Taq-Man probes targeting both intracellular and extracellular domains to distinguish whether the protein expression of sFLT-1 might be regulated by both transcriptional (17) and post-translational (25) mechanisms. As results, neither addition of excess mVEGF nor infection of rSeV/dF-hsFLT1 contributed to the significant increase of mRNA of endogenous mVEGF as well as intracellular and extracellular mFLT-1 (Fig. 3b). In contrast, as it has been well known in several cells, LPS stimulated mVEGF mRNA in DCs (Fig. 3b, upper left graph), that might be reasonable for the similar increase obtained in Figure 3a at protein level. LPS has been shown to downregulate *flt-1* gene expression (26) as well as sFLT-1 (27) in other situation, DC-specific unknown mechanisms for LPS-induced downregulation of *flt-1* mRNA may be involved.

Interestingly, addition of excess VEGF modestly upregulated mRNA level of hsFLT-1 transgene (Fig. 3c), suggesting that VEGF might alter the transcription of rSeV expression system, similar to basic fibroblast growth factor (Yonemitsu Y, et al., unpublished data).

### **Recovery of immunostimulatory function of DC-rSeV/dF, that is disturbed by VEGF-A/VPF, by exogenous hsFLT-1 gene expression**

Figure 4a shows the effect of rSeV/dF-hsFLT1 on the expression of typical

co-stimulatory molecules. While rSeV/dF-GFP infection did not affect DC expression of the typical co-stimulatory molecules, DC-rSeV/dF-hsFLT1 showed significant increases in positive cell numbers of, at least, CD40, CD83, and CD86. Furthermore, modest (mIL-1 $\beta$ ) or marked (mIL-6 and JE/mMCP-1) restoration of proinflammatory cytokine expression was observed in DC-rSeV/dF-hsFLT1 (Fig. 4b), indicating that VEGF-A/VPF derived from DCs was a significant and autocrine/paracrine-negative regulator for immunostimulatory functions of DCs.

**Antitumor immunity, rather than antiangiogenesis, is the major mechanism of therapeutic effect of DC-rSeV/dF against MA**

Finally, we assessed whether or not the mechanism underlying DC-rSeV/dF-hsFLT1's anti-MA effect depended on either 'antiangiogenesis' or 'antitumor immunity'. We first examined the effect of DC-rSeV/dF-hsFLT1 without any tumor antigen on CT26 intraperitoneal tumors using balb/c *nu/nu* mice. As indicated in Fig. 5a, the survival benefit of DC-rSeV/dF-hsFLT1 was not seen in immunodeficient *nu/nu* mice at all. Since an immunohistochemical study of peritoneal tumor nodules using PECAM-1 to assess tumor angiogenesis did not show a significant difference in the numbers of microvessels per viable tumor area (data not shown), the therapeutic effect of DC-rSeV/dF-hsFLT1 was highly suspected as the immune-mediated mechanism.

We thus assessed the immune-related parameters. First, the splenocytes obtained from immunocompetent mice on day 21 after intraperitoneal inoculation of CT26 tumor cells were subjected to a cytotoxic T-lymphocyte (CTL) assay. As shown in Fig. 5b, a significant increase in specific lytic activity against CT26 cells was observed under

repeated administration of DC-rSeV/dF-hsFLT1 (days 0, 7, and 14), while no such effect was found in any of the groups against syngeneic MethA tumor cells.

Another set of experiments for assessing infiltrating cell subsets to tumor nodules on day 21 revealed that DC-rSeV/dF-hsFLT1 therapy increased the numbers of infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes, but not NK cells into tumor nodules (Fig. 6a).

Using all 10 animals that survived more than 100 days due to the intraperitoneal treatment of DC-rSeV/dF-hsFLT1 that were demonstrated in Fig. 2a and b ( $10^6$  cells per dose, once per week for 6 weeks), we assessed the establishment of CT26 tumor-specific long-lasting protective immunity. In this case,  $1 \times 10^5$  cells of CT26 or MethA were inoculated intradermally on the right or left abdominal wall, respectively (Fig. 6b). While all of the control naïve animals developed both tumors, all 10 survivors after treatment of DC-rSeV/dF-hsFLT1 rejected only CT26 and not MethA, indicating the induction of tumor-specific long-lasting protective immunity against CT26.



## Discussion

VPF activity of VEGF-A/VPF was first identified over 20 years ago from cancer cells that also induced ascitis fluid (19, 20). Although a number of studies have been published to show that VEGF-A/VPF was highly accumulated in the ascitis of human and animal subjects, its pathophysiological role has not been well defined. To our knowledge, only one published report has demonstrated that the VEGF-A/sFLT-1 ratio was increased in ascitis of patients with ovarian cancers (28); however, its biological significance has not been assessed. Therefore, the significance of our current study is its demonstration that the increase in the VEGF-A/sFLT-1 ratio presumably determines not only ascitis fluid correction but also antitumor immune response induced by DCs.

At the initial stage of this study, we first confirmed that CT26 MA model was highly resistant to DC-rSeV/dF-mediated ‘immunostimulatory virotherapy’, that was previously shown to induce efficient antitumor immunity against multiple dermal tumors and lung metastasis (6-9). Therefore, we hypothesized that MA might contain strong immunosuppressive substances. We first measured the expression level of transforming growth factor- $\beta$  (TGF- $\beta$ ) and interleukin-10 (IL-10), well-known typical suppressors for Th1 response; however, no significant increase in ascitis as well as PPP of CT26 MA mice was found in comparison with those of control mice (data not shown). We thus next focused on the role of VEGF-A/VPF and its potent autocrine upstream regulator, platelet-derived growth factor-AA (PDGF-AA) (29-31). Of interest, accumulation of VEGF-A/VPF, but not of PDGF-AA, in MA was found (data not shown); therefore, elevated level of VEGF-A/VPF might be due to the unregulated expression of VEGF-A/VPF from cancer cells.

Interestingly, we here found that baseline expression of sFLT-1 was much higher

in both ascitis and PPP of mice than of human subjects; this finding well explains the possible reason why the edema of extremities, a typical symptom of human subjects, is not seen in mouse model of MA. These findings thus suggest that the strategy regarding VEGF-A/VPF antagonism is likely more important in human MA than murine models.

A recent interesting study demonstrated that monocyte-derived human mature DCs activated by TNF- $\alpha$  or soluble CD40L with IFN- $\gamma$ , but not immature ones, expressed considerable amount of sFLT-1, and showed antiangiogenic potential, significantly inhibiting tumor growth of immunodeficient mice (32). Similar positive function of DC-derived sFLT-1 against VEGF-A/VPF was also reported by other groups (33), suggesting that mature DCs might be resistant to VEGF-A/VPF-mediated functional suppression. However, as previously demonstrated, maturation of DCs are dramatically inhibited by VEGF (21, 34), and we here showed that DC-rSeV/dF-GFP could show only a modest effect on the survival of MA model of immune-competent mice (Fig. 1a). To explain these findings, we have to hypothesize that mature/activated DCs can be reversible to immature/quiescent state and quantitative balance of VEGF/sFLT-1 in circumstance of DCs may determine their mature/immature conditions. This notion seems very reasonable, because addition of excess mVEGF resulted in the consumption of sFLT-1, as shown in Figure 3a. Since huge amount of VEGF-A/VPF has been shown to be concentrated in the ascitis, it may be possible that the amount of sFLT-1 expressed by functionally mature and activated DCs by cytokines as well as viruses was not sufficient to neutralize the VEGF-A/VPF in ascitis.

In summary, we here clearly demonstrated that the imbalance between VEGF-A/VPF and its soluble decoy receptor, sFLT-1, would determine the resistance of MA against DC-based immunotherapy, and the correction of this ratio by gene transfer

of hsFLT-1 into DCs dramatically augmented not only DC function itself but also tumor-specific immune response. To our best knowledge, this is the first report demonstrating an effective treatment regimen against a murine model of MA. Therefore, this new concept, ‘targeting VEGF-A/VPF activity during intraperitoneal DC vaccination’, would be a significant strategy to treat MA in clinical setting.

### **Acknowledgments**

The authors thank Drs. Akihiro Tagawa, Takumi Kanaya, Hiroshi Ban, and Takashi Hironaka for their excellent technical assistance in rSeV vector construction and large-scale production. Ms. Chie Arimatsu and Ms. Ryoko Nakamura assisted in the animal experiments.

## References

1. Chung M, Kozuch P. Treatment of malignant ascitis. *Curr Treat Options Oncol* 2008;9:215-33.
2. Nio Y, Nagami H, Tamura K, et al. Multi-institutional randomized clinical study on the comparative effects of intracavitary chemotherapy alone versus immunotherapy alone versus immunochemotherapy for malignant effusion. *Br J Cancer* 1999;80:775-85.
3. Burges A, Wimberger P, Kümper C, et al. Effective relief of malignant ascites in patients with advanced ovarian cancer by a trifunctional anti-EpCAM x anti-CD3 antibody: a phase I/II study. *Clin Cancer Res* 2007;13:3899-905.
4. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 2004;10:909-15.
5. Yonemitsu Y, Ueda Y, Kinoh H, et al. Immunostimulatory virotherapy using recombinant Sendai virus as a new cancer therapeutic regimen. *Front Biosci* 2008;13:4953-9.
6. Shibata S, Okano S, Yonemitsu Y, et al. Induction of efficient antitumor immunity using dendritic cells activated by recombinant Sendai virus and its modulation by exogenous IFN- $\beta$  gene. *J Immunol* 2006;177:3564-76.
7. Yoneyama Y, Ueda Y, Akutsu Y, et al. Development of immunostimulatory virotherapy using non-transmissible Sendai virus-activated dendritic cells. *Biochem Biophys Res Commun* 2007;355:129-35.
8. Tatsuta K, Tanaka S, Tajiri T, et al. Complete elimination of established neuroblastoma by synergistic action of  $\gamma$ -irradiation and DCs treated with rSeV expressing interferon- $\beta$  gene. *Gene Ther* 2009;16:240-51.
9. Komaru A, Ueda Y, Furuya A, et al. Sustained and NK/CD4<sup>+</sup> T cell-dependent efficient prevention of lung metastasis induced by dendritic cells harboring recombinant Sendai virus. *J Immunol* 2009;183:4211-9.
10. Yonemitsu Y, Kitson C, Ferrari S, et al. Efficient gene transfer to airway epithelium using recombinant Sendai virus. *Nat Biotechnol* 2000;18: 970-3.
11. Nagai Y. Paramyxovirus replication and pathogenesis. Reverse genetics transforms understanding. *Rev Med Virol* 1999;9:83-99.
12. Markwell MA, Svennerholm L, Paulson JC. Specific gangliosides function as host cell receptors for Sendai virus. *Proc Natl Acad Sci USA* 1981;78:5406-10.
13. Kawai T, Takahashi K, Sato S, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005;6:981-8.
14. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006;441(7089):101-5.

15. Tanii M, Yonemitsu Y, Fujii T, et al. Diabetic microangiopathy in ischemic limb is a disease of disturbance of the platelet-derived growth factor-BB/protein kinase C axis but not of impaired expression of angiogenic factors. *Circ Res* 2006;98:55-62.
16. Murakami Y, Ikeda Y, Yonemitsu Y, et al. Inhibition of choroidal neovascularization via brief subretinal exposure to a newly developed lentiviral vector pseudotyped with Sendai viral envelope proteins. *Hum Gene Ther* 2010;21: 199-209.
17. Kendall RL, Thomas KA. Inhibition of vascular endothelial growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci USA* 1993;90:10705-9.
18. Harada Y, Ueda Y, Kinoh H, et al. Cytokine-based log-scale expansion of functional murine dendritic cells. *PLoS ONE* 2009;4:e6674.
19. Senger DR, Galli SJ, Dvorak AM, et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219(4587):983-5.
20. Senger DR, Perruzzi CA, Feder J, et al. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* 1986;46:5629-32.
21. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* 1996;2:1096-103.
22. Kong HL, Hecht D, Song W, et al. Regional suppression of tumor growth by *in vivo* transfer of cDNA encoding a secreted form of the extracellular domain of the *flt-1* vascular endothelial growth factor receptor. *Hum Gene Ther* 1998;9:823-33.
23. Afuwape AO, Feldman M, Paleolog EM. Adenoviral delivery of soluble VEGF receptor 1 (sFlt-1) abrogates disease activity in murine collagen-induced arthritis. *Gene Ther* 2003;10:1950-60.
24. Ku CH, White KE, Dei Cas A, et al. Inducible overexpression of sFLT-1 in podocytes ameliorates glomerulopathy in diabetic mice. *Diabetes* 2008;57:2824-33.
25. Rahimi N, Golde TE, Meyer RD. Identification of ligand-induced proteolytic cleavage and ectodomain shedding of VEGFR-1/FLT1 in leukemic cancer cells. *Cancer Res* 2009;69:2607-14.
26. Saban MR, Hellmich H, Nguyen NB, et al. Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation. *Physiol Genomics*. 2001;5:147-60.
27. Lee MC, Wei SC, Tsai-Wu JJ, et al. Novel PKC signaling is required for LPS-induced soluble Flt-1 expression in macrophages. *J Leukoc Biol* 2008;84:835-41
28. Artini PG, Ruggiero M, Monteleone P, et al. Vascular endothelial growth factor and its soluble receptor in benign and malignant ovarian tumors. *Biomed Pharmacother* 2008;62:373-7.

29. Tsutsumi N, Yonemitsu Y, Shikada et al. Essential role of PDGFR $\alpha$ -p70S6K signaling in mesenchymal cells during therapeutic and tumor angiogenesis in vivo: role of PDGFR $\alpha$  during angiogenesis. *Circ Res* 2004;94:1186-94.
30. Shikada Y, Yonemitsu Y, Koga T, et al. Platelet-derived growth factor-AA is an essential and autocrine regulator of vascular endothelial growth factor expression in non-small cell lung carcinomas. *Cancer Res* 2005;65:7241-8.
31. Dong J, Grunstein J, Tejada M, et al. VEGF-null cells require PDGFR $\alpha$  signaling-mediated stromal fibroblast recruitment for tumorigenesis. *EMBO J* 2004;23:2800-10.
32. Kishuku M, Nishioka Y, Abe S, et al. Expression of soluble vascular endothelial growth factor receptor-1 in human monocyte-derived mature dendritic cells contributes to their antiangiogenic property. *J Immunol* 2009;183:8176-85.
33. Xie Y, Fan J, Chen J, et al. A novel function for dendritic cell: clearance of VEGF via VEGF receptor-1. *Biochem Biophys Res Commun* 2009;380:243-8.
34. Oyama T, Ran S, Ishida T, et al. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. *J Immunol* 1998;160:1224-32.

### Legends for Figures

#### Figure 1.

#### **Role of VEGF-A/VPF and its soluble decoy receptor sFLT-1 on virally activated DC-based immunotherapy to treat murine model of malignant ascitis (MA).**

\* $P < 0.01$  and # $P < 0.05$ .

**a.** Survival curve of mice with MA treated by various regimens with DC-rSeV/dF-GFP without pulsation of tumor antigen. On day 0,  $10^5$  cells of CT26 were inoculated intraperitoneally, and DC therapy was started at that time via various regimens (once or twice per week for 3 or 6 weeks). All regimens were significantly effective at prolonging the survival of tumorbearing mice (\* $P < 0.01$  vs. buffer-treated control), but the therapeutic effects were rather modest.

**b.** Protein concentrations of mVEGF-A/VPF and msFLT-1 (left graph) and the ratio of mVEGF-A/msFLT-1 (right graph) in ascitis or circulation (PPP: platelet-poor plasma) of MA mice (n=11). Ascitis and PPP of tumor-bearing mice were collected 21 days after tumor inoculation and subjected to specific ELISA.

**c.** Protein concentrations of hVEGF-A/VPF and hsFLT-1 (left graph) and the ratio of hVEGF-A/hsFLT-1 (right graph) in ascitis or the circulation of patients with MA (n=5). Ascitis and PPP were collected after written informed consent was obtained, and were subjected to specific ELISA.

**d.** Augmentation of therapeutic effect (left: survival, and right: increase in body weight) via intraperitoneal injection of virally activated DCs ( $10^6$  cells per dose) expressing hsFLT-1 (DC-rSeV/dF-hsFLT1) on the CT26 MA model. DCs were administered weekly for 3 weeks. Note that DCs activated by LPS, a well-known stimulator of DC function, showed modest improvement on the survival, but not at all on the suppression of body weight. In contrast, virally activated DCs, DC-rSeV/dF-GFP, demonstrated a pronounced effect on both survival and the increase in body weight, and exogenous expression of hsFLT-1 strongly augmented the effects.

#### Figure 2.

#### **Optimization of treatment regimen of DC-rSeV/dF-hsFLT1. \* $P < 0.01$ .**

**a.** Survival curve indicating that repeated treatments contribute to the therapeutic effect of DC-rSeV/dF-hsFLT1 on the CT26 MA model.



**b.** Survival curve indicating that  $10^6$  cell per dose is critical to the therapeutic effect of DC-rSeV/dF-hsFLT1 on the CT26 MA model.

**Figure 3.**

**Functional expression of exogenous hsFLT-1 gene expression from DC-rSeV/dF for the degradation of endogenous and exogenous VEGF-A/VPF. \* $P < 0.01$**

**a.** Degradation of endogenous (upper 3 graphs) and exogenous (lower 3 graphs) mVEGF-A/VPF. Twenty-four hours after stimulation with LPS or rSeV/dF with GFP or hsFLT-1, the culture medium was subjected to specific ELISA. Upper panels: Note that msFLT-1 was detected in all DCs and hsFLT-1 was found only in rSeV/dF-hsFLT1. A dramatic reduction of endogenous mVEGF-A was seen only in the medium of DC-rSeV/dF-hsFLT1. Lower panels: Degradation of exogenously added mVEGF-A/VPF protein. Marked reductions not only of mVEGF-A/VPF, but also of murine and human sFLT-1 were found, probably due to the degradation of mVEGF-A/sFLT-1 complexes. Each group contains  $n=3$ .

**b.** Neither addition of excess mVEGF nor infection of rSeV/dF-hsFLT1 contributed to the significant increase of mRNA of endogenous mVEGF as well as intracellular and extracellular mFLT-1. In contrast, LPS stimulated mVEGF mRNA in DCs (upper left graph). Each group contains  $n=4$ .

**c.** A modest enhancement of rSeV/dF-mediated hsFLT-1 transgene expression induced by recombinant mVEGF-A/VPF. Each group contains  $n=4$ .

**Figure 4.**

**Exogenous expression of hsFLT-1 augments the immunostimulatory function of virally activated DCs (DC-rSeV/dF). \* $P < 0.01$ , ND: not detected**

**a.** Exogenous hsFLT-1 expression for antagonism of endogenous mVEGF/VPF augments the expression of co-stimulatory molecules of DCs. Two days after stimulation by each stimulant, the expressions of co-stimulatory molecules were assessed by FACS analyses. (left) A typical FACS profile, positive cell ratio (%) and mean fluorescent intensity (MFI), of CD86. LPS-stimulated DCs were used as a positive control. Note that rSeV/dF-GFP infection showed a modest increase of both positive cell ratio and MFI, and the use of rSeV/dF-hsFLT1 enhanced the levels of these

parameters. The data were representative in three independent experiments. CD40 and CD83, but not CD80, showed similar results (data not shown) (right). The use of rSeV/dF-hsFLT1 to antagonize endogenous mVEGF/VPF augments the positive cell ratio of co-stimulatory molecules. Each group contains n=3.

**b.** Effect of exogenous expression of hsFLT-1 to antagonize endogenous mVEGF/VPF on the expression of typical proinflammatory cytokines/chemokines from DCs. Note that the use of rSeV/dF-hsFLT1 to antagonize endogenous mVEGF/VPF dramatically augments the expression of mIL-6 and JE/mMCP-1. Each group contains n=3.

### **Figure 5.**

#### **Therapeutic outcome of DC-rSeV/dF-hsFLT1 against CT26 MA model is immune-mediated mechanism**

**a.** The therapeutic effect of intraperitoneal injection of DC-rSeV/dF-hsFLT1 requires an acquired immune response. The optimized therapeutic regimen, determined in Fig. 2, was done on balb/c background immunocompetent or immunodeficient *nu/nu* mice with the CT26 MA model. Survival was analyzed by the log-rank test. Note that no therapeutic effect was seen in *nu/nu* mice at all.

**b.** CT26-specific CTL activity. Naïve mice and third-party tumor cells (MethA: mouse fibrosarcoma) were used as negative controls. Splenocytes were obtained from CT26 MA mice after weekly treatment with each DC for 3 weeks and were then subjected to <sup>51</sup>Cr release assay. Note that no specific lysis activity against MethA was found at all (left graph). Strong lytic activity against CT26 was found only on the splenocytes from mice treated with DC-rSeV/dF-hsFLT1 (right graph).

### **Figure 6.**

#### **Antagonism of VEGF-A/VPF activity dramatically augments the tumor-specific immune response induced by DC-based intraperitoneal immunotherapy without antigen loading on murine model of MA. \*P<0.01**

**a.** DC-rSeV/dF-hsFLT1 promotes intratumor infiltration of CD4- and CD8-positive T-lymphocytes, but not NK cells.

**b.** Establishment of CT26-specific antitumor immunity induced by intraperitoneal injection of DC-rSeV/dF-hsFLT1 assessed by the second challenge test. 10<sup>5</sup> cells of CT26 (right abdominal wall) and MethA (left abdominal wall) were implanted

intradermally to the naïve mice (n=10) or long-term survivors (>100 days, obtained in Fig. 2, n=10), and tumor formation was assessed on day 35. Note that naïve mice accepted both tumors, whereas all immunized mice formed only MethA tumors and not CT26 at all.