Antagonism of VEGF by Genetically Engineered Dendritic Cells Is Essential to Induce Antitumor Immunity against Malignant Ascites

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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 VEGF-A/vascular permeability factor and its decoy receptor, soluble fms-like tyrosine kinase receptor-1 (sFLT-1), was a major cause of MA 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.

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 showed the significant prolongation of the 50% survival of malignant effusion from 1.6 to 3.5 months by a combination of chemotherapy and OK-432, a “nonspecific” 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 × 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 by the standard therapy.

Dendritic cells (DC) are unique antigen-presenting cells that can stimulate innate and 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 limited efficacy (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 (6) and fusion (F)-gene-deleted nontransmissible (7–9) recombinant Sendai viruses (rSeV). SeV, a member of the family Paramyxoviridae, has 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; refs. 13, 14); therefore, we have been able to show that DCs activated by rSeVs (DC-rSeV) induced highly efficient antitumor immunity against various tumors (7–9).
Role of VEGF in DC-Based Immunotherapy

On the basis of aforementioned 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 important, we here simply determined that the imbalance of the protein accumulation of VEGF-A/vascular permeability factor (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. These cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin, and streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. Growth and morphology of both lines were 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) was used to activate DCs as a positive control. ELISA was done according to the manufacturer’s instructions, using commercially available Quantikine Immunoassay systems for murine and human VEGF-A, murine and human sFLT-1, mIL-1β, mTNF-α, mIL-6, and JE/m MCP-1 (R&D Systems, Inc.).

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 collection and analyses of ascitis and platelet-poor plasma from human MA patients

Real-time quantitative reverse-transcriptase 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), according to the manufacturer’s instructions for TaqMan methods (15). The oligonucleotide sequences of PCR primers and TaqMan probes are as follows: murine VEGF: forward 5'-TGTAGGA-3', reverse 5'-TCTACATCTGCTGTCG-3', and probe 5'-FAM-CAGCACATCATGCAAGCAGGCCA-TAMRA-3'; murine FLT-1 (extracellular domain): forward 5'-CATTGAAAACGTGAAACCTCAGATCT-3', reverse 5'-CTGCTGCCACGGGATAG-3', and probe 5'-FAM-CCCGTGTCCCTCG-CTTCCAAAGCCCTAMRA-3'; and murine FLT-1 (intracellular domain): forward 5'-GGGAAAGAGTTCTCTGCTGTCT-3', reverse 5'-GACCGGATATAGGTGAACTCATAGAT-3', and probe 5'-FAM-ACCCCCAGACTACAATCTCGTGTTG-TAMRA-3'.

The expression level of the target genes was standardized by the glyceraldehydes-3-phosphate dehydrogenase 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) with CellQuest software (BD Biosciences Japan). Dead cells were excluded by staining with propidium iodide. Data analysis was done using FlowJo 4.5 software (Tree Star, Inc.).

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 of the following plasmids: pSeV⁺/GFP (green fluorescent protein) or pSeV⁺/df-hsFLT-1 (16), pGEM-NP, pGEM-P, and pGEM-L in Superfect reagent (Qiagen). The transfected cells were maintained for 3 hours and then were washed and incubated for 60 hours in minimal essential medium containing araC. The cells were collected and lysed, and the lysate solution was incubated on the stably expressing LLC-MK2 cells in a 24-well plate. Twenty-four hours later, the cells were washed and incubated in MEM containing araC and trypsin. Virus yield is expressed in cell infection units, as previously described (7–9). Human cDNA of soluble flt-1, 2,064-bp encoding 6 of 7 extracellular domains (17), was amplified by PCR with primer set containing Not1 site from total RNA of human umbilical vein endothelial cell. The amplified cDNA was subcloned into the Not1 site of template plasmid pSeV⁺/df. The cDNA sequence was completely identical to the published human soluble flt-1. Expression of hsFLT-1 from rSeV/df-hsFLT-1 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, interleukin (IL)-3, and IL-6 for 3 weeks, followed by DC differentiation culture with granulocyte-macrophage colony–stimulating factor 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) 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 (10⁵) were injected intraperitoneally, and DCs were also injected as defined in protocols in each experiment. The body weight and survival were examined daily.

⁵¹Cr 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 Na₂⁵¹CrO₄ (Amersham Biosciences) for 90 minutes 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 γ-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 ⁵¹Cr release = (experimental release – spontaneous release) × 100/ (maximum release – spontaneous release). Assays were done in triplicate 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 (optimum cutting temperature) compound, and the cryostat sections were subjected to immunochemical examinations with monoclonal antibodies specific to CD4 (L3T4; eBioScience), CD8 (Ly-2; eBioScience), or pan-NK CD49b (α2-integrin; eBioScience) as primary antibodies, as previously described (7). Three randomly selected and totally viable tumor areas were scanned as TIF images under an optical microscope-assisted microscope at 200× magnification. The mean number of positive cells was used for the data of each tumor.

Statistical analysis

All data were expressed as means ± SD. The data were examined statistically, using 1-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 the Kaplan–Meier 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 intraperitoneal dissemination of CT26 murine colon cancer cells and virally activated DC-based immunotherapy, the so-called “immunostimulatory virotherapy,” using Fgene-deleted nontransmissible recombinant Sendai virus (DC-rSeV/dF). This recently developed concept has shown dramatic enhancement in 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 showed that MA mice treated by similar regimen did not show the significant elevation in CTL activity (data not shown), suggesting that there might be 1 or more immunosuppressive factors in the tumor-disseminated peritoneal cavity.

Murine and human MA exhibits the imbalance of VEGF-A/sFLT-1 ratio in ascits

We therefore hypothesized that 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 Figure 1B, the specific ELISA showed that mVEGF-A/VPF was markedly accumulated in the ascitis of MA mice (mVEGF-A/msFLT-1 ratio ¼ 2.91 ± 0.05, 11, 20 days after tumor inoculation). Murine soluble fms-like tyrosine kinase-1 (msFLT-1), 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 ¼ 1,116 ± 339 pg/mL; n ¼ 4), and an increased level was also detected in the PPP of tumor-bearing mice (2,933 ± 897 pg/mL; n ¼ 11, mVEGF-A/msFLT-1 ratio ¼ 0.05 ± 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/msFLT-1 ratio ¼ 2.91 ± 1.97, day 21 after tumor inoculation). This MA animal model
The 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/ msFLT-1 ratio would be crucial for inducing ascitis and peripheral edema; a very low level of circulating VEGF in PPP was detected in healthy mice.
findings seen in murine ascitis were also representative in human subjects with MA \((n = 5)\), as shown in Figure 1C. Inversely, a dramatically low level of hsFLT-1 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/msFLT-1 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 carry out further study, using DC-rSeV/dF-expressing hsFLT-1 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 shown that hsFLT-1 was sufficiently active to neutralize murine VEGF (16, 22–24). Actually, DC-rSeV/dF-hsFLT-1 dramatically improved the survival of tumor-bearing mice (Fig. 1D, left) and inhibited the increase in 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), showed that “once per week for 6 weeks and 10^6 DCs per dose” was the optimized condition for efficient control of CT26-MA with the use of DC-rSeV/dF-hsFLT-1. Reverse transcriptase PCR analyses revealed no significant expression of other possible ligands for FLT-1, VEGF-B, VEGF-C, and PlGF (placental growth factor) from CT26 cells (data not shown). In addition, our preliminary study showed 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 preferred 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-hsFLT-1 on DC functions, and LPS and rSeV/dF-GFP were used as controls. Naive murine DCs, and 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 hsFLT-1 without any change in msFLT-1 (Fig. 3A, top 3 graphs). The addition of excess exogenous mVEGF-A/VPF protein (4,000 pg/mL) resulted in the marked reductions in mVEGF and murine and human sFLT-1s with the use of rSeV/dF-hsFLT-1 (Fig. 3A, bottom 3 graphs). This suggests the degradation and/or loss of immunoreactivity of mVEGF when complexed with hsFLT-1, indicating that recombinant gene expression of hsFLT-1 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 that given in Figure 3A. We here examined mFLT-1 mRNA by TaqMan probes targeting both intracellular and extracellular domains to distinguish whether the protein expression of sFLT-1 might be regulated by both transcriptional (17) and posttranslational (25) mechanisms. As a result, neither addition of excess mVEGF nor infection of rSeV/dF-hsFLT-1 contributed to the significant increase in mRNA of endogenous mVEGF and 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, top left graph), which might
be reasonable for the similar increase in protein levels as in Figure 3A. LPS has been shown to downregulate flt-1 gene expression (26) and exogenous VEGF-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. Top, note that msFLT-1 was detected in all DCs and hsFLT-1 was found only in rSeV/dF-hsFLT-1. A dramatic reduction in endogenous mVEGF-A was seen only in the medium of DC-rSeV/dF-hsFLT-1. Bottom, 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-hsFLT-1 contributed to the significant increase in mRNA of endogenous mVEGF and intracellular and extracellular mFLT-1. In contrast, LPS stimulated mVEGF mRNA in DCs (top left). 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.

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

Figure 4A shows the effect of rSeV/dF-hsFLT-1 on the expression of typical costimulatory molecules. Although rSeV/dF-GFP infection did not affect DC expression of the typical costimulatory molecules, DC-rSeV/dF-hsFLT-1 showed significant increases in positive cell numbers of, at least, CD40, CD83, and CD86. Furthermore, modest (mIL-1β) or marked (mIL-6 and JE/mMCP-1) restoration of proinflammatory cytokine expression was observed in DC-rSeV/dF-hsFLT-1 (Fig. 4B), indicating that VEGF-A/VPF derived from DCs was a significant and autocrine/paracrine-negative regulator of 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-hsFLT-1’s anti-MA effect...
We first examined the effect of DC-rSeV/dF-hsFLT-1 without any tumor antigen on CT26 intra-peritoneal tumors, using Balb/c nu/nu mice. As indicated in Figure 5A, the survival benefit of DC-rSeV/dF-hsFLT-1 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-hsFLT-1 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 CTL assay. As shown in Figure 5B, a significant increase in specific lytic activity against CT26 cells was observed under repeated administration of DC-rSeV/dF-hsFLT-1 (days 0, 7, and 14), whereas 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-hsFLT-1 therapy increased the numbers of
infiltrating CD4+ and CD8+ T lymphocytes, but not natural killer (NK) cells, into tumor nodules (Fig. 6A).

Using all 10 animals that survived more than 100 days as a result of the intraperitoneal treatment of DC-rSeV/dF-hsFLT-1 that are showed in Figure 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, 1/10^5 cells of CT26 or MethA were inoculated intradermally on the right or left abdominal wall, respectively (Fig. 6B). Although all of the control naive animals developed both tumors, all 10 survivors after treatment of DC-rSeV/dF-hsFLT-1 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 more than 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 pathophysiologic role has not been well defined. To our knowledge, only one published report has shown 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 the 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,” which 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 TGF-β and IL-10, well-known typical suppressors for Th1 (helper T cells) response; however, no significant increase in ascitis and PPP of CT26 MA mice was found in comparison with that 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; refs. 29–31). Of interest, accumulation of VEGF-A/VPF, but not of PDGF-AA, in MA was found (data not shown); therefore, elevated levels of VEGF-A/VPF might be due to the unregulated expression of VEGF-A/VPF from cancer cells.

Interestingly, we here show that baseline expression of sFLT-1 was much higher in both ascitis and PPP of mice than in 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 in murine models.

A recent interesting study showed that monocyte-derived human mature DCs activated by TNF-α or soluble CD40L with IFN-γ, 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 shown, maturation of DCs is 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 immunocompetent mice (Fig. 1A). To explain these findings, we have to hypothesize that mature/activated DCs by cytokines and viruses was not sufficient to neutralize the VEGF-A/VPF in ascitis.

In summary, we here clearly showed 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 the best of our knowledge, this is the first report showing 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.

Disclosure of Potential Conflicts of Interest

Y. Yonemitsu is a member of the Scientific Advisory Board of DNA-VEC Corporation.

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References

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