

Adenovirus-mediated Soluble FLT-1 Gene Therapy for Ovarian Carcinoma¹

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ABSTRACT

Purpose: We hypothesized that adenovirus-mediated soluble fms-like tyrosine kinase receptor (sFLT-1) gene therapy can inhibit the ovarian tumor growth and increase survival of mice in the context of ovarian carcinoma.

Experimental Design: We constructed an infectivity-enhanced recombinant adenovirus (AdRGDGFPsFLT-1) expressing soluble FLT-1 and green fluorescent protein (GFP). An adenovirus AdRGDGFP expressing GFP alone was used as control. The functional validation of adenovirus-mediated sFLT-1 was determined by an *in vitro* human umbilical vein endothelial cell proliferation inhibition assay. To evaluate the therapeutic potential of adenovirus-expressed sFLT-1 to inhibit the growth of ovarian tumors and to increase the survival duration of mice with ovarian tumors, two tumor models were used. First, SKOV3.ip1 ovarian carcinoma cells were infected *ex vivo* with either AdRGDGFPsFLT-1 or AdRGDGFP or uninfected and then inoculated s.c. into BALB/c nude mice, and tumor growth was monitored. Second, SKOV3.ip1 cells were inoculated i.p. into CB17 SCID mice and then treated with two doses of either AdRGDGFPsFLT-1 or AdRGDGFP or with PBS on days 1 and 14 after inoculation of cells, and the survival duration was monitored.

Results: Treatment with adenovirus-expressed sFLT-1 significantly inhibited the proliferation of human umbilical vein endothelial cells. The s.c. tumor nodules in mice derived from cells infected with AdRGDGFPsFLT-1 were significantly smaller than those infected with either AdRGDGFP or uninfected. In addition, i.p. administration of the AdRGDGFPsFLT-1 resulted in a significant increase in the survival times of mice compared with AdRGDGFP- or PBS-treated mice.

Conclusions: Our results suggest that adenovirus-mediated sFLT-1 gene therapy can effectively inhibit ovarian tumor growth and increase survival in a murine model of ovarian carcinoma.

INTRODUCTION

Ovarian cancer is the leading cause of death among gynecological cancers in the United States (1–4). In this regard, an estimated 23,100 new cases and 14,000 deaths in the United States were forecasted for the year 2000 (4). Of note, this mortality rate has remained largely unchanged for the last decade. Thus, recent advances in surgical and conventional treatment modalities have failed to impact the survival rate for this disease in a significant manner (5, 6). On this basis, it is clear that novel therapeutic approaches for carcinoma of the ovary are warranted.

One recent novel interventional strategy for cancer is based upon interruption of tumor neoangiogenesis (7–13). The recognition that tumor growth and progression are critically linked to new vessel formation (14, 15) has led to therapeutic approaches directed toward abrogation of this process (7, 16). In this regard, a number of lines of evidence have linked ovarian cancer progression to the process of neoangiogenesis (17–20). Specifically, ovarian cancer tumor cells have been shown to overexpress proangiogenic growth factors linked to neoangiogenesis, such as VEGF (17, 18, 21–26).³ In addition, elevated serum levels of VEGF in patients with cancer of the ovary have been associated with increased tumor growth, ascites fluid accumulation (17, 21, 27–30), metastases, poor prognosis, and shorter survival periods (20, 21, 25, 26, 31, 32). On this basis, application of antiangiogenesis strategies in the context of cancer of the ovary would appear warranted.

Critical to the process of tumor neoangiogenesis is the expression of key proangiogenic growth factors and their receptors. In this regard, the major proangiogenic effects of VEGF are mediated through the endothelium-specific VEGF receptors,

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³ The abbreviations used are: VEGF, vascular endothelial growth factor; MOI, multiplicity of infection; PFU, plaque-forming units; HUVEC, human umbilical vein endothelial cell; DMEM:F12, DMEM:Ham's F-12; nt, nucleotide; GFP, green fluorescent protein; CMV, cytomegalovirus.

Flt-1 and Flk-1/KDR (33–36). This recognition has led to strategies targeting this key axis of proangiogenesis. The intervention to block VEGF action has been accomplished by a variety of methods including antibody-based approaches directed against VEGF or its cognate receptors (8, 37–44). Direct delivery of naturally occurring antiangiogenesis agents, such as angiostatin (10, 45), endostatin (11, 13), and thrombospondin-1 (46, 47), has been endeavored as well. The demonstration of efficacy with such agents in the context of model systems has led to the development of human clinical trials that are currently evaluating these agents (7).

As an additional method to interrupt the key neoangiogenesis axis of tumors, gene therapy approaches have been developed. In this regard, production and application of a variety of the naturally occurring antiangiogenic agents has been problematic (48, 49). This recognition has led to the exploitation of gene transfer technology as a means to achieve the expression of such factors *in vivo* at a magnitude and/or for durations compatible with therapeutic effects (50, 51). Studies using gene transfer for neoangiogenesis interruption have included *ex vivo* delivery of thrombospondin-1 in breast cancer cells (52), VEGF antisense oligonucleotides delivery in a primary glioblastoma model in nude mice (53), *FLK-1* mutant receptor delivery by retroviral vectors in various primary tumor models in mice (8, 54), soluble platelet factor 4 by adenoviral and retroviral delivery in primary glioma models in nude mice (55), angiostatin delivery by adenovirus and retrovirus in primary glioma models in nude mice (56), murine endostatin delivery by adenovirus in a s.c. and pulmonary micrometastases model in nude mice (57), cationic liposome-mediated *sFLT-1* delivery to suppress peritoneal metastasis of human gastric cell lines (58), adenovirus-mediated *sFLT-1* delivery to inhibit s.c. tumors and lung and liver metastasis of murine colon cancer cell lines (9), and adenovirus-mediated delivery of truncated *FLT-1* to suppress tumor growth of human lung cancer cell lines (59). We have also used a genetic approach for expression of *sFLT-1* that is an endogenously expressed, potent VEGF antagonist and has shown therapeutic effect in the context of s.c. lung metastasis and intracranial tumor models in mice (60). In aggregate, these studies have established the concept that gene therapy may offer a technical means to realize the potential benefits of antiangiogenesis approaches.

In the current study, we have applied our gene therapy-based *sFLT-1* approach to the context of carcinoma of the ovary. We have used an adenoviral vector for i.p. delivery of *sFLT-1* and demonstrated therapeutic effect in a murine model system of ovarian carcinoma.

MATERIALS AND METHODS

Cell Lines. SKOV3.ip1 human ovarian carcinoma cells (Dr. Janet Price, M. D. Andersen Cancer Center, Houston, TX) and embryonic kidney cells 293 (American Type Culture Collection, Rockville, MD) were maintained in complete medium composed of DMEM:F12 (Cellgro; Mediatech, Washington D.C.) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 5 mM glutamine (Mediatech), and 1% penicillin and streptomycin (Cellgro; Mediatech). HUVECs were obtained from the American Type Culture Collection and maintained in EBM-2

(Clonetics, Walkersville, MD). All of the cells were grown at 37°C in a humidified atmosphere of 5% CO₂.

Cloning of *sFLT-1* cDNA. The *sFLT-1* cDNA was obtained by PCR amplification of human placenta Quick-Clone cDNA (Clontech Laboratories, Inc., Palo Alto, CA). Quick-Clone cDNA template (1ng), 5' primer flt-1 (nt 243–260; 5'-GCTCACCATGGTCAGCTA-3'; National Center for Biotechnology Information accession no. UO1134) and 3' primer flt-2 (nt 2409–2428; 5'-CTGCTATCATCTCCGAAGCTC-3'), both at a final concentration of 0.4 μM, polymerase enzyme, deoxynucleotide triphosphates, and buffer from the Advantage2 PCR kit (Clontech Laboratories, Inc.) were mixed according to the manufacturer's recommendations and incubated first at 94°C for 1 min followed by 30 cycles of 94°C for 45 s, 58°C for 2 min, and 72°C for 4 min.

The resulting PCR product of 2.2 kb was subcloned into PCR 2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Presence of the *sFLT-1* cDNA was confirmed by restriction digestion analysis (*EcoRI*, *NdeI*, and *XbaI*) and DNA sequencing.

***In Vitro* Transcription and Translation.** The *sFLT-1* cDNA fragment was excised from the PCR 2.1-TOPO TA plasmid with *EcoRI* digestion, subcloned into pcDNA3 vector (Invitrogen) at the *EcoRI* restriction site, and designated the plasmid as pMsFLT-1. The orientation of *sFLT-1* was determined by restriction digestion analysis. *In vitro* transcription and translation was performed with the TNT T3 coupled wheat germ extract system (Promega, Madison, WI) according to the manufacturer's instructions.

Generation of Recombinant Adenovirus-expressing *sFLT-1*. Replication-deficient adenovirus with a double expression cassette containing the GFP and *sFLT-1* cDNA (inserted into the E1-deleted region), each driven by the CMV promoter followed by a polyadenylation signal, was constructed as follows. First, a shuttle vector encoding *sFLT-1* was cloned by excision of *sFLT-1* cDNA from pMsFLT-1 by *KpnI* and *EcoRV* digestion and subcloning into the corresponding sites (*KpnI* and *EcoRV* restriction sites) of the pAdTrack-CMV shuttle vector (61) and was designated as pMP912. This shuttle vector was then linearized with *PacI* and *PmeI* and used for homologous DNA recombination with *ClaI*-linearized pVK503C containing the adenovirus genome (62) in the *Escherichia coli* strain BJ5183 as described previously (63). The plasmid obtained as a result of this recombination contains the genome of AdRGDGFsFLT-1 and was designated as pMP906. Insertion of the transgene in the plasmid was confirmed by PCR analysis and restriction mapping with *EcoRI* digestion.

Recombinant adenovirus AdRGDGFsFLT-1-expressing *sFLT-1* was generated by transfection of 293 cells with *PacI*-digested pMP906 by a method described previously (62, 63) and confirmed by PCR analysis and restriction mapping with *EcoRI* digestion. The virus titer was determined by plaque formation assay using 293 cells, and the titer was expressed in PFU/ml. The control virus AdRGDGFP was constructed similarly except that it lacked the *sFLT-1* cDNA. The expression of GFP was determined *in vitro* by observing green fluorescence of infected cells using a fluorescent imaging microscope.

Western Blot Analysis. Conditioned media was generated according to a modified protocol (9) by infecting

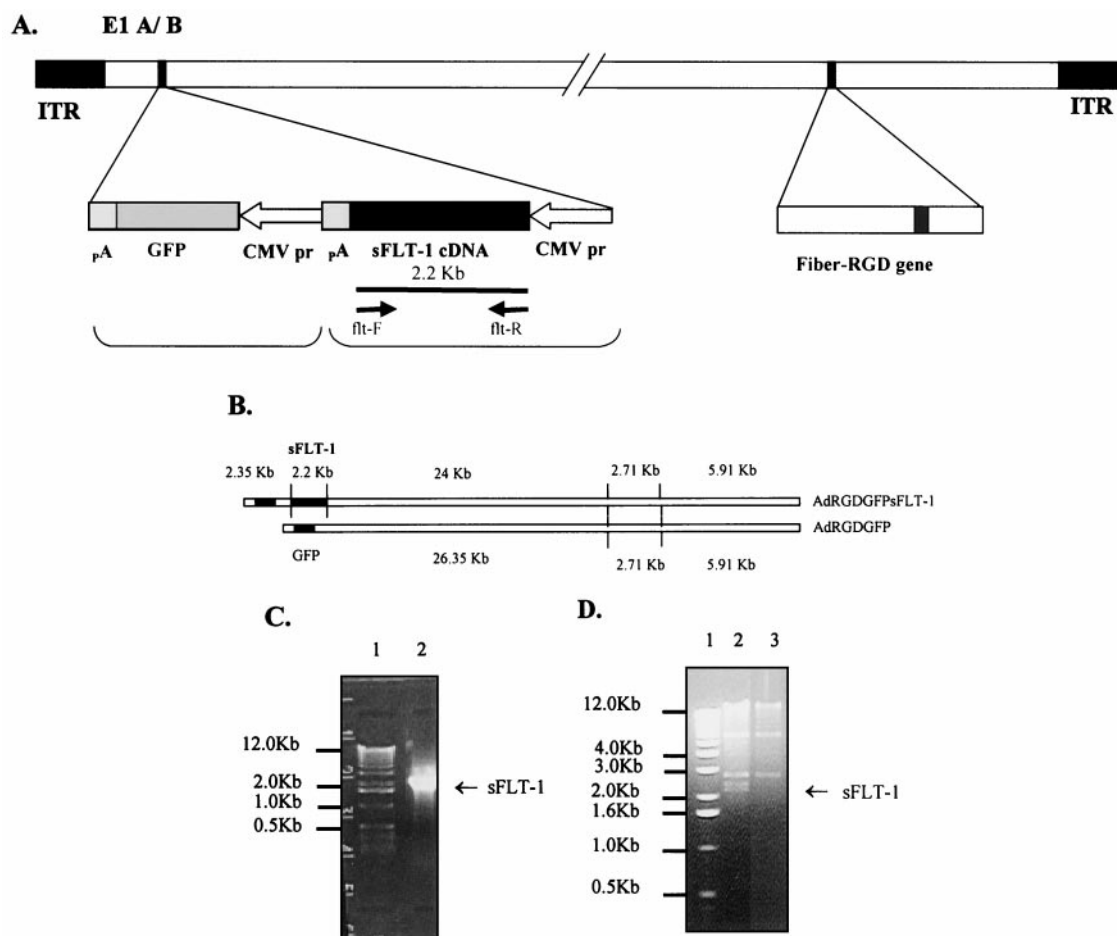


Fig. 1 Construction of recombinant Arg-Gly-Asp adenovirus vector encoding sFLT-1. **A**, map of recombinant adenovirus vector. A double expression cassette inserted into the E1A/E1B-deleted region contains the GFP and sFLT-1 cDNA, each driven by a CMV promoter (CMV pr), followed by a polyadenylation signal (pA). The region of the modified fiber gene encoding Arg-Gly-Asp 4C peptide within the HI loop of the fiber knob is illustrated as well. Also indicated is the site of PCR primers designed for specific amplification of sFLT-1 cDNA. **B**, comparison of *EcoRI* restriction digestion map of AdRGDGFPsFLT-1 and control AdRGDGFP. **C**, PCR of sFLT-1 using the adenoviral genome as template, flt-F (nt 240–260) and flt-R (nt 2409–2428) as forward and reverse primers, respectively. Lane 1, M_r marker; Lane 2, PCR product (sFLT-1) of AdRGDGFPsFLT-1. **D**, restriction digestion of AdRGDGFPsFLT-1 with *EcoRI*. Lane 1, M_r marker; Lane 2, *EcoRI*-digested AdRGDGFPsFLT-1 showing presence of 2.2-kb sFLT-1 cDNA; Lane 3, *EcoRI*-digested control adenovirus vector AdRGDGFP showing absence of 2.2-kb sFLT-1 fragment.

SKOV3.ip1 cells at a MOI of 100 of either AdRGDGFPsFLT-1 or AdRGDGFP for 2 h. The infection media was removed. Monolayers were carefully washed with PBS, and then serum-free DMEM:F12 media was added. The resultant conditioned media was collected after 72 h, centrifuged at $2000 \times g$ for 15 min, filtered (0.22- μ m; Corning, Corning, NY), and frozen (-70°C) until use. Conditioned media was concentrated $5\times$ using centriplus concentrators (Amicon, Inc., Beverly, MA), and 1 ml of this media was mixed with 50 μ l of 50% slurry of heparin-Sepharose CL-6B beads (Pharmacia) in PBS (to allow binding of sFLT-1) and incubated overnight with rocking at 4°C . Beads were harvested by centrifugation, washed three times with PBS, and eluted sFLT-1 by boiling in 20 μ l of SDS/PAGE sample buffer (60). Eluted samples and recombinant human Flt-Fc protein (positive control; R&D Systems, Inc., Minneapolis, MN) were electrophoretically separated on a 9% SDS/PAGE gel, transferred to polyvinylidene difluoride membrane (Bio-Rad), and analyzed by Western blotting with mono-

clonal anti-FLT-1 receptor antibody (V4262; Sigma Chemical Co.) as primary antibody and goat antimouse IgG (A2554; Sigma Chemical Co.) as secondary antibody.

HUVECs Proliferation Inhibition Assay. To demonstrate that the sFLT-1 in the conditioned media would suppress VEGF-driven proliferation of endothelial cells, conditioned media was obtained from SKOV3.ip1 cells infected with AdRGDGFPsFLT-1, AdRGDGFP, or PBS (uninfected). HUVECs were grown in 12-well plates at 37°C in EBM-2 media (Clonetics). At 80% confluency, EBM-2 media was removed, cells layers were washed with PBS, and 1.0 ml of ($5\times$ concentrated) conditioned media (Triplicates) was added. After 15 min, VEGF at 10 ng/ml was added to each well and incubated at 37°C . After 72 h, the cells were trypsinized, and the number of viable cells was counted using a trypan blue assay.

s.c. Growth of Adenovirus-infected Ovarian Tumor Cells in BALB/c Mice. SKOV3.ip1 ovarian cancer cells were infected *in vitro* with (MOI = 100) AdRGDGFPsFLT1, AdRG-

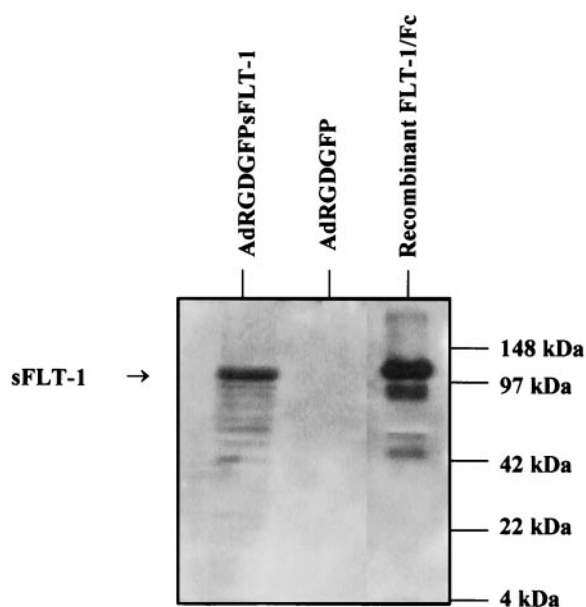


Fig. 2 Secretion of sFLT-1 mediated by a recombinant adenovirus AdRGDGFPsFLT-1. SKOV3.ip1 cells were infected with AdRGDGFPsFLT-1 or an irrelevant control virus, AdRGDGF, at an MOI of 100. After 2 h of infection, the cells were washed with PBS, and serum-free media was added. After 72 h, the supernatant (conditioned media) was collected, and secretion of sFLT-1 (M_r 110,000) was confirmed by Western blot of conditioned media using mouse monoclonal anti-FLT-1 antibody. Recombinant human FLT-1/Fc chimera protein (M_r 123,000) was used as a positive control. kDa shown in thousands.

DGFP, or PBS (uninfected) for 2 h. The infection media was replaced with fresh culture media, DMEM:F12 containing 10% FCS. After 24 h, infected cells were trypsinized, resuspended in HBSS, and injected s.c. in the flanks and shoulders of 4–6-week-old female BALB/c nude mice [Charles River Laboratories, Hartford, CT]. A total of 10 nodules in three animals ($n = 3$)/group were injected, each nodule containing 4×10^6 SKOV3.ip1 cells, and tumor growth was monitored by measuring with calipers every 3 days for 20 days. Tumor volume was calculated according to the formula: volume = $L \times W \times H$, where L = length, W = width, and H = height.

Regional Antitumor Effects of Adenovirus-mediated Secretion of sFLT-1. CB17 SCID mice (Harlan Sprague Dawley, Chicago, IL; $n = 13$ /group) were injected with 4×10^6 SKOV3.ip1 cells i.p. on day 0. Animals were then injected i.p. with 1×10^9 PFU (in 500 μ l of HBSS) of AdRGDGFPsFLT-1, AdRGDGF, or PBS on day 1. A second dose of 1×10^8 PFU was administered on day 14, and survival duration was monitored.

Statistical Analysis. The SE of the s.c. tumor size was calculated. The P to determine the significant difference between s.c. tumor sizes of different groups was calculated by paired t test.

Survival estimates were calculated for each group (uninfected, AdRGDGF, and AdRGDGFPsFLT-1) using the log-rank test for testing equality of survival duration for the uninfected, AdRGDGF, and AdRGDGFPsFLT-1 groups. The log-rank test and Kaplan-Meier survival analysis were performed to deter-

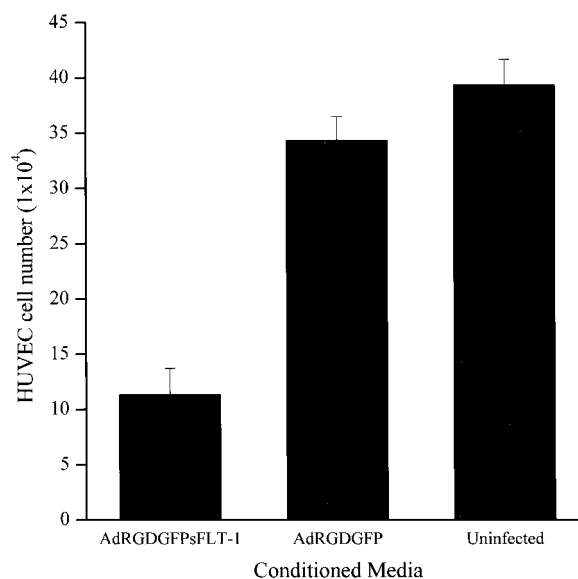


Fig. 3 Secreted sFLT-1 obtained after infection with AdRGDGFPsFLT-1 inhibits proliferation of HUVECs. Conditioned media obtained from SKOV3.ip1 cells infected with AdRGDGFPsFLT-1, AdRGDGF, or PBS (uninfected) was added to the HUVECs in 12-well plates. After 15 min, VEGF at 10 ng/ml was added to each well. After 72 h, the cells were trypsinized, and the number of viable cells was counted using trypan blue assay. Results indicate mean and SD of experiments performed in triplicates.

mine the significant difference in survival between the uninfected, AdRGDGF control, and AdRGDGFPsFLT-1 groups. The median and mean survival duration of the uninfected group, AdRGDGF control group, and the treatment group was calculated as well.

RESULTS

Cloning and Validation of sFLT-1 cDNA. The sFLT-1 cDNA was obtained by PCR amplification from human placenta cDNA and was subcloned into a PCR 2.1-TOPO TA cloning vector. Restriction digestion mapping and DNA sequencing of this vector confirmed the presence of sFLT-1 cDNA. To confirm the expression of sFLT-1, the cDNA was subcloned into pcDNA3 vector, designated as pMsFLT-1. Transcription/translational analysis confirmed the expression of sFLT-1 cDNA product from pMsFLT-1 plasmid (data not shown).

Construction of Recombinant Adenovirus-expressing GFP and sFLT-1. As a first step in constructing an adenovirus, the sFLT-1 cDNA was excised from pMsFLT-1 and subcloned into the pAdTrack-CMV shuttle vector generating pMP912. Recombinant adenovirus AdRGDGFPsFLT-1 was then generated through cotransfection of the adenovirus shuttle vector (pMP912) containing *GFP* and *sFLT-1* cDNA in a double expression cassette with an E1A/B-deleted adenoviral backbone vector (pVK503C; Fig. 1A). Similarly, the control virus AdRGDGF expressing GFP alone was constructed. Presence of *sFLT-1* in the recombinant adenovirus was confirmed by PCR (Fig. 1B); PCR with irrelevant virus did not yield any band (data not shown).

Table 1 Effect of sFLT-1 on growth of s.c. tumors^a

Treatment	Average tumor size (mm ³) ± SD					
	Day 7	Day 9	Day 12	Day 15	Day 17	Day 20
AdRGDGFPsFLT-1	12.92 ± 3.81	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
AdRGDGFP	187.45 ± 19.09	278.43 ± 51.5	344.64 ± 80.449	405.54 ± 134.41	410.63 ± 139.7	570.63 ± 261.27
Uninfected	209.71 ± 25.43	329.7 ± 57.33	404 ± 125.73	448.34 ± 146.11	569.58 ± 193	658.83 ± 212.096

^a $n = 10$ s.c. tumor nodules/treatment group; 4×10^6 SKOV3.ip1 cells/nodule; uninfected vs. AdRGDGFPsFLT-1 ($P = 0.0126$); AdRGDGFP vs. AdRGDGFPsFLT-1 ($P = 0.05$).

Restriction digestion of AdRGDGFPsFLT-1 virus with *EcoRI* resulted in 2.2-, 2.35-, 2.71-, 5.91-, and 24-kb fragments, whereas digestion of control virus AdRGDGFP with *EcoRI* resulted in 2.71-, 5.91-, and 26.35-kb fragments (Fig. 1D), as predicted from the restriction digestion map (Fig. 1C). Comparison of the *EcoRI* restriction-digested fragments of AdRGDGFPsFLT-1 and AdRGDGFP indicates that AdRGDGFPsFLT-1 *EcoRI* digestion yielded two additional fragments of sizes 2.2 kb and 2.35 kb because of the presence of two *EcoRI* sites in the insert flanking sFLT-1 (Fig. 1, C and D). One of the two additional fragments (2.2 kb) is sFLT-1 cDNA, thus confirming the insertion of the transgene.

To determine the expression/secretion of sFLT-1 *in vitro*, human ovarian cancer cells (SKOV3.ip1) were infected with 100 MOI of AdRGDGFPsFLT-1 or AdRGDGFP. The serum-free conditioned media was collected, centrifuged, filtered through 0.22- μ m filters, and concentrated 5 \times . This conditioned media and Flt/Fc recombinant protein (M_r 123,000; positive control) were subjected to Western blotting analysis. The results showed that sFLT-1 (identified by anti-FLT-1 monoclonal antibody) was present in AdRGDGFPsFLT-1 conditioned media but not in AdRGDGFP-infected condition media (Fig. 2), thus confirming the adenovirus-mediated secretion of soluble FLT-1. The sFLT-1 identified by Western blot is approximately M_r 110,000. This is in agreement with the previous findings (64). The additional bands seen below the Flt/Fc M_r 123,000 band as well as sFLT-1 M_r 110,000 band may have resulted from proteolysis. Similar bands were also noted in an article published previously (65). The green fluorescence of infected cells as detected by fluorescence microscopy confirmed the expression of GFP, which was used to track expression of sFLT-1 *in vitro* (data not shown).

Functional Characterization of Adenovirus-secreted sFLT-1 *in Vitro*. The functional validation of adenovirus-secreted sFLT-1 was determined by HUVEC inhibition assay. The concentrated conditioned media obtained from AdRGDGFPsFLT-1- or AdRGDGFP-infected cells was applied to the HUVECs grown in 12-well plates, followed by stimulation with VEGF. Conditioned media from AdRGDGFPsFLT-1 cells inhibited HUVEC proliferation by 60% compared with conditioned media from AdRGDGFP-infected cells and 72% compared with conditioned media from uninfected cells (Fig. 3), thus confirming the adenovirus-mediated secretion of functionally active soluble FLT-1. In contrast, HUVECs proliferated significantly in conditioned media from uninfected cells because of unimpeded stimulation from VEGF, resulting in cells packing tighter than confluent monolayers, thus resulting in more than

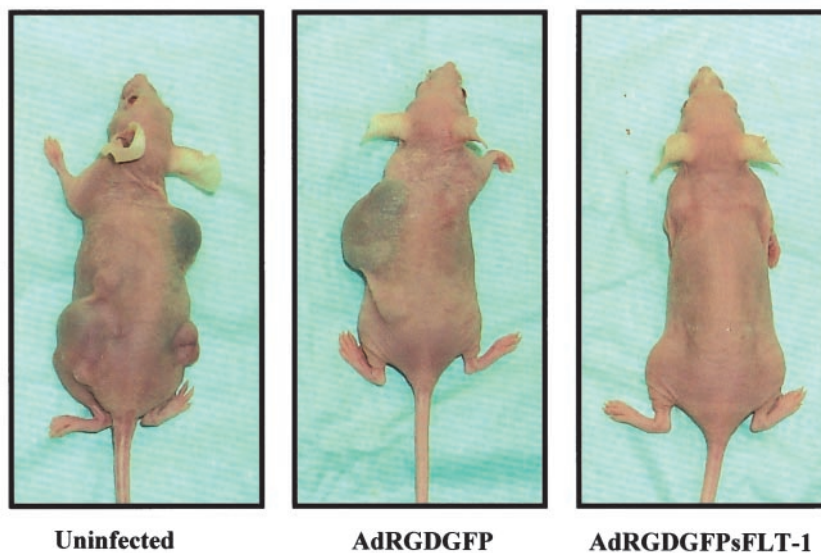
three times the cells than in AdRGDGFPsFLT-1-infected conditioned media.

Effect of sFLT-1 on Implantation and Growth of s.c. Tumors. SKOV3.ip1 cells infected with AdRGDGFPsFLT-1, AdRGDGFP, or uninfected cells showed identical growth rates *in vitro* (data not shown). In contrast, AdRGDGFP-infected or uninfected tumor cells implanted s.c. in flanks and shoulders of BALB/c nude mice formed significantly large tumors compared with AdRGDGFPsFLT-1-infected tumor cells (uninfected versus AdRGDGFPsFLT-1, $P = 0.0126$; AdRGDGFP versus AdRGDGFPsFLT-1, $P = 0.05$; Table 1). In fact, the AdRGDGFPsFLT-1-infected tumor size was almost negligible by day 20 after implantation (Fig. 4, A and B). These results suggest that *in situ* secretion of sFLT-1 can abrogate tumor implantation and suppress tumor growth. The suppression of *in vivo* tumor growth but not of *in vitro* tumor cell proliferation suggests that the growth inhibition was attributable to sFLT-1 expression and not attributable to a direct effect of the transgene expression on cellular growth kinetics.

Adenovirus-mediated Secretion of sFLT-1 Significantly Increases Duration of Survival in Mice with i.p. Ovarian Tumors. To determine the effect of adenovirus-mediated delivery of sFLT-1 on the survival duration of CB17 SCID mice implanted with ovarian tumors cells, we injected 4×10^6 SKOV3.ip1 cells i.p. on day 0 and then delivered two doses of either AdRGDGFP and/or AdRGDGFPsFLT-1 viruses or PBS also i.p. on days 1 and 14, and the duration of survival was monitored. Statistical analysis was performed on survival data of uninfected, AdRGDGFP, and AdRGDGFPsFLT-1 groups.

The median survival of the uninfected group and the AdRGDGFP group was 41 days, whereas the median survival of the treatment group was 53 days (Fig. 5). The mean survival of the treatment group was higher than that of the uninfected control group, 51 and 41 days, respectively. These results suggest that mice injected with AdRGDGFPsFLT-1 survived significantly longer compared with control group mice (AdRGDGFP or PBS). The log-rank test for testing equality of survival for the uninfected, AdRGDGFP, and AdRGDGFPsFLT-1 groups had a P of 0.0020, thus indicating that there was a significant difference in survival duration between the control group, AdRGDGFP, and the treatment group, AdRGDGFPsFLT-1 ($P = 0.0017$). Similar testing revealed that there was a significant difference in survival between the uninfected control group and AdRGDGFPsFLT-1 ($P = 0.0056$). These results suggest that mice injected with AdRGDGFPsFLT-1 survived significantly longer compared with control group mice (AdRGDGFP or PBS).

A.



B.

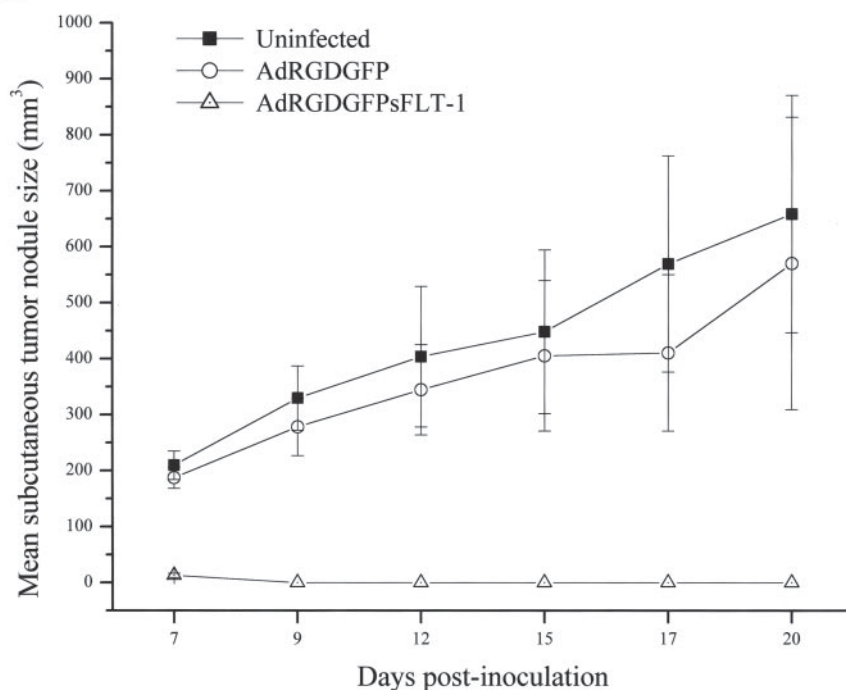


Fig. 4 *In situ* secretion of sFLT-1 inhibits tumorigenesis in murine model. SKOV3.ip1 ovarian cancer cells were infected *in vitro* (MOI = 100) with AdRGDGFPsFLT1, AdRGDGFP, or PBS (uninfected). After 24 h, infected cells were injected s.c. 4×10^6 /nodule, and tumor development and tumor size were monitored. A, tumor nodule size in AdRGDGFPsFLT-1-infected, AdRGDGFP-infected, or uninfected groups of mice at day 20. B, quantitation of tumor sizes of AdRGDGFPsFLT-1, AdRGDGFP, or uninfected groups using three-dimensional measurement analysis.

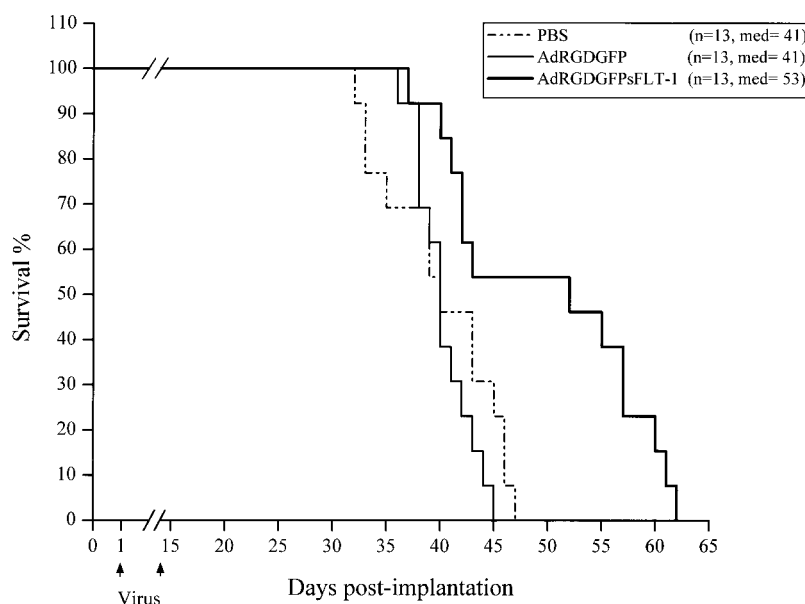
DISCUSSION

Strategies to inhibit neoangiogenesis represent a potentially powerful approach for treatment of neoplastic disease. Furthermore, gene therapy methods may offer direct applications with respect to practical realization of such functional end points. In this regard, we have developed an approach to inhibit tumor neoangiogenesis via abrogation of the VEGF axis with its natural inhibitor, sFLT-1. In the current study, we have applied this therapy in the context of cancer of the ovary. Our studies demonstrate therapeutic gain in the context of adenoviral vector-mediated delivery of sFLT-1 *i.p.*

The therapeutic effect of adenovirus-expressed sFLT-1 is exerted in a paracrine manner by inhibiting angiogenesis. Therefore, the therapeutic effect of AdRGDGFPsFLT-1 does not depend on the quantitative transduction of the tumor cells, as opposed to gene therapy strategies relying on mutation compensation or molecular chemotherapy that may have limited clinical efficacy. On this basis, the ability of AdRGDGFPsFLT-1 to deliver an amplified antitumor effect may help circumvent the current limitations of vectors to accomplish quantitative tumor transduction.

The ovarian tumor model we have used was based on the

Fig. 5 Regional antitumor effects of secreted sFLT-1. Kaplan-Meier survival plot of CB17 SCID mice ($n = 15/\text{group}$) that received injections with 4×10^6 SKOV3.ip1 cells i.p. on day 0. Animals then received injections of 1×10^9 PFU of AdRGDGFPsFLT-1, AdRGDGFP, or PBS i.p. on day 1. A second dose of 1×10^8 PFU was administered on day 14. The key shows the median survival times in days and the number of animals/group (n).



previously established (66, 67) animal model of human ovarian carcinoma in which tumor cells were injected i.p., and survival was used as an end point to evaluate various therapeutics. The survival as an experimental end point represents a stringent orthotopic model for assessment of the therapeutic ability of sFLT-1 in mice with ovarian tumors. In this model, injection of tumor cells i.p. does not produce a single solid tumor. Instead, it results in many small diffuse tumor nodules and bloody ascites. In the current study, we have observed the delayed development of bloody ascites (as noted by external gross examination of mice) in AdRGDGFPsFLT-1-treated group, as compared with AdRGDGFP- or PBS-treated groups (data not shown). This observation may be attributable to the potent anti-VEGF activity of sFLT-1. However, the direct demonstration of the antiangiogenic activity in s.c. tumors was not possible because we did not obtain any tumors in the sFLT-1-treated group because of its highly effective inhibition of tumor growth.

Tumor angiogenesis and its progression depend on the balance between angiogenic and antiangiogenic factors. There are multiple angiogenic factors such as interleukin-8 and basic fibroblast growth factor in addition to VEGF that are associated with ovarian cancer. However, no studies have been performed to this point that evaluated the extent of dependence of ovarian cancer angiogenesis on VEGF relative to the other angiogenic factors. In this regard, in an experimental model, duration of survival depends on the number of tumor cells injected, proangiogenic factors produced by tumors, and antiangiogenic factors expressed by adenoviral vector gene therapy. In our experiments, i.p. injections of tumor cells followed by i.p. administration of AdRGDGFPsFLT-1 resulted in significant prolongation of survival of mice compared with AdRGDGFP- or PBS-treated groups. Because the proangiogenic activity of other factors was not blocked, the survival, although statistically significant, was modest (10–12 days). This suggests that angiogenesis is a complex mechanism; thus, blocking the action of a single potent

angiogenic factor may not result in complete abrogation of angiogenesis.

A variety of previous studies have used sFLT-1 gene expression as an antiangiogenesis gene therapy approach. These interventions have used various methods to effect sFLT-1 gene transfer, including naked plasmid DNA (60) and cationic liposome-formulated plasmid DNA (58), as well as adenoviral-mediated approaches (9). Our delivery system is based upon previous ovarian cancer gene therapy approaches, whereby adenoviral vectors have been used to achieve efficient tumor cell transduction *in situ* (68). On this basis, we used adenoviral vectors for efficient gene delivery of the sFLT-1 gene.

It is noteworthy that sFLT-1 can achieve antitumor effects when administered systemically. In this regard, Kong *et al.* (9) reported that i.v. administration of an adenovirus encoding soluble FLT-1 (Adsflt-1) suppressed tumor growth in liver and spleen but had little effect on lung metastases and s.c. tumor growth. In another study, Takayama *et al.* (59) observed that i.m. administration of adenovirus (AdVEGF-ExR) expressing the Flt-1/Fc (extracellular domain of FLT-1 fused to Fc portion of human IgG) inhibited s.c. tumor growth. Of note, we have found that i.v. administration of AdRGDGFPsFLT-1 is associated with hepatic necrosis and vasculopathy in a murine model (data not shown). This interruption of the VEGF axis in the hepatic context may be deleterious for local vasculature. On this basis, our i.p. delivery schema may offer an advantage *vis à vis* locally inhibiting VEGF action (by sequestration of VEGF and also by heterodimerizing with wild-type receptors FLT-1 and FLK-1/KDR, by sFLT-1), thus decreasing the hepatotoxicity. In our experiments, mice administered with two doses of viral vectors i.p. did not show any hepatotoxicity (data not shown).

The potential for improving the therapeutic index by using gene-based therapy cannot be overemphasized. Moreover, malignant neoplasms confined to the i.p. cavity, such as ovarian and extra-ovarian cancers, can be considered to be captured

within a "container" for extended periods of time. The containment of tumor cells within the peritoneal cavity favors vector concentration, allowing effective *in vivo* gene transfer. Furthermore, this strategy may increase the safety margin, as suggested by the lack of toxicity in human trials using i.p. delivery of adenovirus encoding a gene for the herpes simplex virus thymidine kinase or a gene for a single chain antibody against erbB-2 protein (69, 70). Neither of these trials demonstrated significant untoward toxicity that was thought to be vector related. In addition, the adenoviral vector constructed for the current study is genetically infectivity enhanced by incorporating an Arg-Gly-Asp motif in the HI loop of the viral knob domain for increased transduction of ovarian cancer cell lines and uncultured primary ovarian cancer cells (68, 71). Moreover, in the presence of human ascites, the Arg-Gly-Asp modification may allow partial escape from neutralizing antibodies (71).

Nevertheless, it must be recognized that limiting the amounts of key angiogenesis factors have precluded a full understanding of potential toxicities associated with ectopic or excessive expression of antiangiogenic factors. On this basis, future studies will be directed to understanding the biodistribution of sFLT-1 consequent to i.p. delivery of AdRGDGFPs-FLT-1, as well as temporal and dosimetric aspects of the therapy.

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