Intravenous Delivery of Adenovirus-mediated Soluble FLT-1 Results in Liver Toxicity

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ABSTRACT

Purpose: Vascular endothelial growth factor (VEGF) is a potent angiogenic agent and plays a major role in tumor growth and metastases. We have previously reported the locoregional (i.p.) delivery of adenovirus-mediated antiangiogenic soluble FLT-1 (sFLT-1; a naturally encoded potent VEGF antagonist) gene therapy to inhibit VEGF action in a murine ovarian carcinoma model. This study was predicated on the fact that systemic delivery of sFLT-1 might allow an approach for therapy of disseminated tumor. The purpose of this study is to test the effects of i.v. delivered, adenovirus-mediated sFLT-1 on the survival duration in a murine ovarian tumor model and to evaluate the safety of i.v.-delivered versus i.p.-delivered adenovirus-mediated sFLT-1 in non-tumor-bearing mice.

Experimental Design: To determine the effects of i.v.-administered adenovirus-mediated sFLT-1 on survival duration of mice bearing i.p. human ovarian tumors, an E1A/B-deleted, (replication-deficient) infectivity-enhanced recombinant adenovirus AdRGDGFPsFLT-1 encoding cDNA for both sFLT-1 and GFP (green fluorescent protein), a control adenovirus AdRGDGFP encoding GFP alone, or PBS was delivered i.v. The therapeutic effect of sFLT-1 was evaluated by survival duration of the mice. Furthermore, the safety of i.v.- or i.p.-delivered adenovirus-mediated sFLT-1 was evaluated by administering AdRGDGFPsFLT-1, AdRGDGFP, or PBS either i.v. or i.p. into non-tumor-bearing mice. Adenovirus-mediated gene expression was determined by determining GFP expression using fluorescent microscopy and by assessing sFLT-1 expression in liver, lungs, spleen, and kidneys by immunohistochemistry using anti-FLT-1 monoclonal antibody. Systemic levels of sFLT-1 were evaluated by ELISA and the toxicity was evaluated by histopathology.

Results: The i.v. delivery of AdRGDGFPsFLT-1 in the ovarian tumor model resulted in a shorter duration of survival of the mice as compared with the control group. Furthermore, in the safety evaluation experiment, i.v. administration of AdRGDGFPsFLT-1 in non-tumor-bearing mice principally localized to the liver. This localization lead to sFLT-1 overexpression, mainly in the liver, resulting in hemorrhage and tissue toxicity. However, i.p. delivery of AdRGDGFPsFLT-1 did not localize principally to the liver, leading to negligible expression of sFLT-1, and no intrahepatic hemorrhage or toxicity was observed. The i.v. delivery of the control virus AdRGDGFP also principally localized to the liver, leading to GFP expression mainly in the liver. However, neither hemorrhage nor morphological cytotoxicity was observed. i.p. delivery of AdRGDGFP resulted in ectopic localization to the liver with very little GFP expression and no toxicity. These results suggest that overexpression of sFLT-1 in the liver as a result of i.v. delivery is hepatotoxic.

Conclusions: Our results suggest that i.v. delivery of the sFLT-1 gene via replication-deficient, infectivity-enhanced recombinant adenoviral vectors will result in overexpression of sFLT-1 in the liver leading to unacceptable hepatotoxicity. Tumor-specific targeting of the vectors and tumor-specific expression strategies should be used to ensure a clinically useful antiangiogenesis gene therapy.

INTRODUCTION

Angiogenesis plays a critical role in neoplastic processes and is essential for tumor progression and metastatic spread of solid tumors (1–3). Among many factors regulating angiogenesis, VEGF is the most potent (4–6). The major proangiogenic effects of VEGF are mediated through the endothelium-specific receptors FLT-1 and FLK-1/KDR (7–12). This recognition has led to strategies targeting this key axis of proangiogenesis. The intervention to block VEGF action has been accomplished by a...
Liver Toxicity of sFLT-1 Gene Therapy

The delivery of the tumor model and evaluated the systemic toxicity because of i.v. delivery of adenovirus-mediated sFLT-1 in a murine ovarian carcinoma model (30). These findings predicated that i.v. delivery of sFLT-1 gene therapy might allow an approach for the therapy of disseminated tumor. However, i.v. delivery of adenovirus-mediated sFLT-1 gene therapy might interfere with the vascular-"nology of normal organs because VEGF is not only a regulator of angiogenesis of but it also acts as a survival factor for endothelial cells both in vitro and in vivo (31–36). On this basis, several investigators have speculated that systemic overexpression of sFLT-1 may lead to systemic toxicity (37, 38). To date, the potential side effects of systemic (i.v.) angiogenic (sFLT-1) gene therapy on normal tissues and physiological angiogenesis are not known.

To that end, we determined the effect of systemic (i.v.) delivery of adenovirus-mediated sFLT-1 in a murine ovarian tumor model and evaluated the systemic toxicity because of i.v. delivery of the sFLT-1 gene by a replication deficient, recombinant adenovirus AdRGDGFPsFLT-1, encoding cDNA for sFLT-1 and GFP, and its validation was reported in our previous study (30). Generation of a control adenovirus AdRGDGFP, expressing GFP alone was also reported previously (30).

Determination of the Effects of i.v.-administered Adenovirus-mediated sFLT-1 in a SCID Mouse Model of Ovarian Carcinoma. CB17 SCID mice (Harlan Sprague Dawley, Chicago, IL; n = 10/group) were given injections of 4 × 10^6 SKOV3.ip1 cells i.p. on day 0. Mice were then given, via the tail vein, i.v. injections of 1 × 10^9 pfu (in 500 μl of HBSS) of AdRGDGFPsFLT-1, AdRGDGFP, or PBS on day 2, and survival duration was monitored. i.p. tumor size was noted at the time of necropsy. For histopathological evaluation, on day 21 postinjection, animals were euthanized; and the liver, lungs, spleen, and kidneys were collected and formalin fixed, and paraffin-embedded sections were prepared from these organs. Slides were then H&E-stained by standard techniques (see below, Histopathological Analysis and IHC).

Determination of Adenovirus-mediated GFP Expression in Vivo. CB17 SCID mice (Harlan Sprague Dawley, Chicago, IL; n = 10/group) were given injections of 4 × 10^6 SKOV3.ip1 cells i.p. on day 0. Mice were given injections i.v., via the tail vein, of 1 × 10^9 pfu (in 500 μl of HBSS) of AdRGDGFPsFLT-1, AdRGDGFP, or PBS on day 2. For adenovirus-mediated GFP expression evaluation, liver, lung, spleen, and kidney were collected on day 21 postinjection and were quickly frozen in Tissue-Tek OCT compound (EM Science). Thin sections of frozen samples were cut on a cryostat and were observed under fluorescence microscope to determine GFP expression.

Evaluation of Toxicity Attributable to Systemic Delivery of sFLT-1. CB17 SCID mice (Harlan Sprague Dawley, Chicago, IL; n = 10/group) were given injections of either i.v. (by tail vein) or i.p. 1 × 10^9 pfu (in 200 μl of HBSS) of AdRGDGFPsFLT-1, AdRGDGFP, or PBS. The animals were then sacrificed on day 21 post-viral-injection, and the harvested liver, lungs, kidneys, and spleen were obtained to perform histopathological evaluation for toxicity. These tissue samples were also used for IHC to determine sFLT-1 expression.

Histopathological Analysis and IHC. Tissue samples of lung, liver, spleen, and kidney were fixed in 10% phosphate-buffered formalin (pH 7.0) and were embedded in paraffin. Paraffin sections (4–6 μm) were cut, mounted on super frost plus slides (Allegience Health Care, IL), and melted at 58°C in an oven for 1 h. Slides were then deparaffinized with xylene and rehydrated using a decreasing ethanol series of baths. Antigen retrieval was carried out by standard microwave techniques at pH 9.0. Subsequently, endogenous peroxidase was quenched using a fresh aqueous solution of 3% hydrogen peroxide. Sections were incubated in goat serum to block nonspecific staining. Sections were then incubated with anti-FLT-1 mouse monoclonal antibody (20 μg/ml; Sigma Chemical Co., St. Louis, MO). After excess primary antibody was washed off from the sections using Tris buffer, the sections were incubated with biotinylated goat antimouse antibody, washed, and incubated with peroxidase-labeled streptavidin (Signet USA-HP) for 20 min each. Subsequently, the sections were washed, and the substrate diaminobenzidine tetrahydrochloride (DAB, which yields a brown reaction product; BioGenex HK153–5K) was

MATERIALS AND METHODS

Cell Lines. SKOV3.ip1 human ovarian carcinoma cells obtained from Dr. Janet Price, M. D. Anderson Cancer Center, Houston, TX, 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). These cells were grown at 37°C in a humidified atmosphere of 5% CO₂.

Viral Vectors and Validation. Generation of E1A/B-deleted, (replication-deficient) infectivity-enhanced recombinant adenovirus AdRGDGFPsFLT-1, encoding cDNA for sFLT-1 and GFP, and its validation was reported in our previous study (30). Generation of a control adenovirus AdRGDGFP, expressing GFP alone was also reported previously (30).
added. Sections were lightly counterstained with hematoxylin. In all cases, negative controls included incubations without primary antibody. The sections were dehydrated through graded alcohols and three xylene baths. Coverslips were then mounted on the slides with permount.

**Determination of Systemic Levels of sFLT-1 by ELISA after i.v. or i.p. Delivery of AdRGDGFPsFLT-1.** Systemic (plasma) levels of adenovirus-mediated sFLT-1 after i.v. or i.p. injection of AdRGDGFPsFLT-1 were determined by using VEGF-R1 ELISA kit (Research Diagnostics Inc., Flankers, NJ), per manufacturer’s recommendations. Briefly, mice (n = 5/group) were given injections, either i.v. or i.p., of 1 x 10⁹ pfu (in 200 μl of HBSS) of AdRGDGFPsFLT-1, AdRGDGFP, or PBS. After 21 days, whole blood was collected from the mice, and plasma was obtained. The 96-microwell plate (provided in the kit), coated with monoclonal anti-sFLT-1 (termed as sVEGF-R1) antibody, was washed with wash buffer. Plasma samples were diluted 1:10 by sample diluent, and 100 μl of diluted sample were added to the microwell plates in duplicate. Similarly, sFLT-1 standards ranging from 0.16 to 10 ng/ml, also diluted in sample diluent, were added to the microwell plates in duplicate. The sample diluent alone was added in blank wells. Biotin-conjugate (50 μl) was then added to all of the wells and incubated at room temperature for 1 h. After washing the wells again for four times, tetramethyl-benzidine substrate solution was added and incubated at room temperature for 20 min. The enzymatic reaction was stopped by adding 100 μl of stop solution to the wells, and absorbance was determined by spectrophotometric readings at 450 nm. The sFLT-1 concentration in the plasma samples was calculated based on the standard curve (created by plotting mean absorbance value against the sFLT-1 standard concentration) using the SoftMax 3.2 microplate data acquisition and analysis software program (Molecular Devices Corporation, Sunnyvalle CA).

**Statistical Analysis.** Survival estimates were calculated for each group (PBS, AdRGDGFP, and AdRGDGFPsFLT-1) using the Kaplan-Meier method. The log-rank test was performed to test for differences in duration of survival between the three groups. The median and mean survival duration of the PBS, AdRGDGFP, and AdRGDGFPsFLT-1 groups was calculated as well. P < 0.05 was considered statistically significant in all of the analyses. These analyses were accomplished using SAS software (version 8.02; SAS Institute, Inc., Cary, NC).

**RESULTS**

**Systemic Delivery of Adenovirus-mediated sFLT-1 Leads to Liver Toxicity and Significantly Decreases Duration of Survival in Mice With i.p. Ovarian Tumors.** To determine the effect of i.v. delivery of adenovirus-mediated sFLT-1 on the survival duration of CB17 SCID mice implanted with human ovarian tumors, we injected 4 x 10⁶ SKOV3.ip1 cells i.p. on day 0 and then i.v. delivered 1 x 10⁹ pfu of AdRGDGFP, AdRGDGFPsFLT-1, or PBS on day 2, and the tumor growth and duration of survival was monitored. Statistical analysis was performed on survival data of PBS, AdRGDGFP, and AdRGDGFPsFLT-1 groups.

The median survival of the PBS group and the AdRGDGFP group was 35 and 36 days, respectively. Whereas the median survival of the AdRGDGFPsFLT-1 treated group was 31 days (Fig. 1) in contrast to our previous study, in which the median survival was 53 days for the AdRGDGFPsFLT-1 versus 41 days for the controls (30). The mean survival of the AdRGDGFPsFLT-1 treatment group was 30 days, which is significantly lower than those of the PBS and AdRGDGFP control group, 36 and 35 days, respectively. These results suggest that mice that were given injections of AdRGDGFPsFLT-1 died significantly earlier compared with the control group mice (AdRGDGFP or PBS). The log-rank test for testing equality of survival for the PBS, AdRGDGFP, and AdRGDGFPsFLT-1 groups had a P of 0.0011, thus indicating that at least one group was different. Similar testing revealed a significant difference in survival between PBS and AdRGDGFPsFLT-1 (P = 0.0232), and AdRGDGFP and AdRGDGFPsFLT-1 (P = 0.0014). These results thus suggest that mice treated with AdRGDGFPsFLT-1 survived a significantly shorter duration compared with the PBS or AdRGDGFP control groups. There was no significant difference between the PBS and AdRGDGFP groups (P = 0.2239). Although the duration of survival was shorter, the tumor sizes in the AdRGDGFPsFLT-1-treated group were significantly smaller than in AdRGDGFP or PBS groups, as determined at the time of necropsy (data not shown).

The histopathological analysis of the livers in the AdRGDGFPsFLT-1-treated revealed severe hemorrhage, massive hepatic necrosis, and residual hepatocyte apoptosis, whereas the control groups did not show similar changes (Fig. 1B). A few inflammatory cells and hyperplastic Kupffer cells were observed in the sinusoids but no central vein or portal tract hemorrhages were observed in the liver of control groups (Fig. 1B). Furthermore, no signs of significant cytotoxicity were observed in other organs (lungs, spleen, and kidneys) sampled in any group (data not shown).

**i.v. Administration of Either AdRGDGFPsFLT-1 or AdRGDGFP Results in Gene Expression Primarily in the Liver.** To determine whether there was adenovirus-mediated gene expression after i.v. delivery of the AdRGDGFPsFLT-1 or AdRGDGFP in liver and other organs, mice were sacrificed 21 days after injection, and the liver, lungs, spleen, and kidneys were harvested, and their tissues were frozen for sectioning. Frozen sections were observed under fluorescence microscopy for GFP expression. Sections of liver obtained from AdRGDGFPsFLT-1- and AdRGDGFP-treated groups showed GFP expression (Fig. 2), whereas, as predicted, the PBS-treated group did not show any GFP expression. Expression of GFP in other organs such as lung, kidney, and spleen was insignificant (data not shown).

**i.v. Delivery of AdRGDGFPsFLT-1 Leads to Expression of sFLT-1 Primarily in the Liver.** To determine the expression of sFLT-1 in vivo after i.v. delivery of the AdRGDGFPsFLT-1, mice were sacrificed, their liver, lungs, spleen, and kidneys were obtained, and paraffin sections of these organs were stained for expression of sFLT-1 using a monoclonal antibody against FLT-1. The group of mice that were given injections of AdRGDGFPsFLT-1 i.v. showed significant expression of sFLT-1 in the liver (Fig. 3), whereas no expression of sFLT-1 was observed in the liver sections from...
the i.p.-administered AdRGDGFPsFLT-1 group. There was no significant expression of sFLT-1 in the other organs from the AdRGDGFPsFLT-1-treated group (data not shown). As expected, the sFLT-1 expression in the liver or other organs from either the AdRGDGFP or PBS groups was not observed.

High Systemic Levels of sFLT-1 Are Achieved by i.v. Delivery of AdRGDGFPsFLT-1 but not by i.p. Delivery. Systemic levels (plasma levels) of sFLT-1 in AdRGDGFPsFLT-1, AdRGDGFP, or PBS groups, when injected i.v. or i.p. was determined by ELISA. The average sFLT-1 concentration levels in the i.v.-injected AdRGDGFPsFLT-1 group was ~9.6 ng/ml, whereas i.p.-injected AdRGDGFPsFLT-1 was almost negligible (<1 ng/ml; Fig. 4). The concentration of sFLT-1 in the control groups was, similarly, almost zero, as expected. These results suggest that i.v.-injected AdRGDGFPsFLT-1 results in higher systemic levels of sFLT-1 compared with i.p.-administered virus.

Systemic Delivery of Ad-mediated sFLT-1 Leads to Liver Toxicity. To examine the toxicity to the liver because of expression of sFLT-1 after i.v. administration of the AdRGDGFPsFLT-1, mice were sacrificed, liver samples were obtained, and H&E-stained sections were prepared. The histopathological evaluation demonstrated significant hemorrhage, focal necrosis without inflammation, and hepatocyte dropout, along with increased apoptosis with accompanying cell death and rare attempts at regeneration and focal fibrosis (Fig. 3). Lung, spleen, and kidney organs in this group were congested but showed no other significant abnormalities. In the i.p.-deliv-
In the AdRGDGFPsFLT-1 treated group, the liver showed minimal congestion and very rare hepatocyte dropouts; otherwise, liver was normal. Lung, spleen, and kidney were unremarkable in this group. In control groups, a few lymphocytes were seen around portal tracts in liver but other organs were normal. There was no difference observed when control virus or PBS was delivered by either the i.v. or the i.p. route.

**DISCUSSION**

Delivering adenovirus-mediated antiangiogenic gene therapy in a locoregional manner with sFLT-1 may represent a valuable clinical method to inhibit tumor growth. However, here we show for the first time that i.v. delivery of adenovirus-mediated sFLT-1 therapy for disseminated carcinoma induces systemic toxicity and shortens survival in an experimental model. In this regard, several studies have reported the application of sFLT-1 gene therapy to inhibit angiogenesis in order to inhibit tumor growth (25–29). We have recently shown that Ad-mediated sFLT-1 gene therapy is effective in an ovarian tumor model by locoregional delivery (i.p.; Ref. 30). However, for disseminated carcinoma, therapeutic levels of sFLT-1 must be achieved systemically. To achieve this end, i.v. delivery of Ad-mediated sFLT-1 seems a valid approach. On this basis, we tested the therapeutic effect of i.v.-delivered AdRGDGFPsFLT-1 in an ovarian tumor model by implanting SKOV3.ip1 cells i.p. followed by injection of AdRGDGFPsFLT-1, AdRGDGFP, or PBS, 48 h after implantation. The dosage of AdRGDGFPsFLT-1 used in our previous study (1 × 10⁹ pfu) was the same as the dosage used in our previous study (30). However, contrary to our expectations, the AdRGDGFPsFLT-1-treated group survived for a shorter duration than the control groups (Fig. 1A) although the tumor size was significantly smaller than control groups (data not shown). Similar results were reported in a previous study, in which adenovirus-mediated endostatin was administered i.v. to inhibit lung metastases of EOMA cells (39). The i.v. delivery of Ad-mediated endostatin did inhibit lung metastases, but this was associated with lung bleeding, weight loss, and death (39).

The smaller size of the tumors in the AdRGDGFPsFLT-1-treated group suggests that sFLT-1 has a therapeutic effect. Therefore, it should have prolonged the survival duration as observed in our previous study, in which the virus was administered i.p (30). On the contrary, the survival duration was shorter than in the control groups. To determine the cause of the shorter duration of survival, we performed histopathological analyses of major organs known to be associated with systemic toxicity. These studies revealed that the AdRGDGFPsFLT-1-treated liver demonstrated various degrees of hepatic necrosis and accompanying abnormalities, whereas the control groups did not show any such changes. Because no cytotoxicity was observed in the liver of control groups, we hypothesized that the toxicity may have resulted secondary to locoregional overexpression of sFLT-1 in the liver. To test our hypothesis, we confirmed the Ad-mediated sFLT-1 expression in the liver and other organs, by determining the expression of the GFP marker encoded by AdRGDGFPsFLT-1 virus in frozen sections of these organs. We observed intense GFP expression in livers of...
Fig. 3  i.v. delivery, but not i.p. delivery, of AdRGDGFPsFLT-1 leads to expression of sFLT-1 primarily in the liver, resulting in hepatotoxicity in non-tumor-bearing mice. CB17 SCID mice (n = 7/group) were given injections of AdRGDGFPsFLT-1, AdRGDGFP, or PBS i.v. In a different group of mice, AdRGDGFPsFLT-1, AdRGDGFP, or PBS were injected i.p. Livers from i.v.- and i.p.-treated mice were sectioned and stained with H&E and for sFLT-1 expression. A, liver from i.v.-delivered AdRGDGFPsFLT-1 showing high sFLT-1 expression. B, liver from i.v.-delivered AdRGDGFPsFLT-1, showing liver toxicity (hemorrhage and congestion, focal necrosis with minimal inflammation, and hepatocyte dropout, increased apoptosis. C, liver from i.p.-delivered AdRGDGFPsFLT-1, showing negligible sFLT-1 expression. D, liver from i.p.-delivered AdRGDGFPsFLT-1 is histologically unremarkable, with no toxicity. E and G, livers from i.v.- and i.p.-delivered AdRGDGFP virus, respectively, again without sFLT-1 expression. F and H, livers from i.v.- and i.p.-delivered AdRGDGFP virus, respectively, are normal without any significant histopathological abnormalities.
routes of injections were warranted. Furthermore, a safety eval-
compare the safety of AdRGDGFPsFLT-1 therapy by different
AdRGDGFPsFLT-1 virus. Therefore, studies to evaluate and
of the difference in the route of administration of the
The difference in the toxicities elicited may, thus, be because
observe any toxicity based on gross examination of organs.
the same dose (1 × 10⁸ pfu) of AdRGDGFPsFLT-1, AdRGDGFP, or PBS, via either i.v. or i.p. routes of administration, and blood was collected on day 21 postinjec-
tion. Plasma was obtained from whole blood samples, and sFLT-1 levels were quantified by ELISA. The means ± SD from each group of mice are shown. Systemic sFLT-1 levels are almost zero (<1 ng/ml) in all of the groups except the i.v.-delivered AdRGDGFPsFLT-1 group, which is ~9.6 ng/ml. These results suggest that i.v. administration of AdRGDGFPsFLT-1 virus results in higher systemic levels of sFLT-1 and for a longer duration of time.

![Graph showing sFLT-1 concentration in ng/ml](Image)

**Fig. 4** Systemic levels of sFLT-1 as determined by ELISA are high after i.v. administration of AdRGDGFPsFLT-1 compared with i.p. delivery. CB17 SCID mice (n = 5/group) were given injections of 1 × 10⁹ pfu of AdRGDGFPsFLT-1, AdRGDGFP, or PBS, via either i.v. or i.p. routes of administration, and blood was collected on day 21 postinjec-
tion. Plasma was obtained from whole blood samples, and sFLT-1 levels were quantified by ELISA. The means ± SD from each group of mice are shown. Systemic sFLT-1 levels are almost zero (<1 ng/ml) in all of the groups except the i.v.-delivered AdRGDGFPsFLT-1 group, which is ~9.6 ng/ml. These results suggest that i.v. administration of AdRGDGFPsFLT-1 virus results in higher systemic levels of sFLT-1 and for a longer duration of time.

both the AdRGDGFPsFLT-1 and the AdRGDGFP groups. Other organs in both of these groups showed very faint GFP expression. Because no morphological toxicity was observed in livers of the AdRGDGFP-treated group, even though high GFP expression was present, GFP-related toxicity was effectively ruled out. All of these results taken together suggest that the toxicity observed in the Ad-mediated sFLT-1 group was sFLT-1 related. However, in our previous study where we have shown a therapeutic effect in an ovarian tumor model by i.p. delivery of the same dose (1 × 10⁹ pfu) of AdRGDGFPsFLT-1, we did not observe any toxicity based on gross examination of organs. The difference in the toxicities elicited may, thus, be because of the difference in the route of administration of the AdRGDGFPsFLT-1 virus. Therefore, studies to evaluate and compare the safety of AdRGDGFPsFLT-1 therapy by different routes of injections were warranted. Furthermore, a safety evaluation of AdRGDGFPsFLT-1 therapy has to be performed in non-tumor-bearing mice to rule out potential toxicity because of factors generated by the tumor. To address this issue, we have evaluated the sFLT-1 toxicity in non-tumor-bearing mice by injecting AdRGDGFPsFLT-1, AdRGDGFP, or PBS, either by i.v. or i.p. route. After 21 days, we sacrificed the animals, harvested their organs, evaluated the toxicity by histopathological analysis, and confirmed the sFLT-1 gene expression in the organs by IHC and plasma levels of sFLT-1 by ELISA.

In the i.v.-delivered AdRGDGFPsFLT-1-treated group, we observed hemorrhages in the liver, focal necrosis with minimal inflammation, and hepatocyte dropout, increased apoptosis, and rare areas of regeneration. Consistent with the toxicity, high sFLT-1 expression was observed in the liver. Whereas in the i.p.-delivered AdRGDGFPsFLT-1 group, no significant cyto-
toxicity was observed, except for minimal congestion and very rare single-cell dropout. The lungs, spleen, and kidneys were unremarkable in this group, and sFLT-1 expression was not observed in either the liver or the other organs of this group. These results suggest that the toxicity is either vector related or sFLT-1 related. Because no toxicity was observed (a few lymphocytes were seen around the portal tracts in liver) when the control virus (AdRGDGFP) was delivered either by i.v. or i.p route, it can be concluded that toxicity is not specifically vector related but rather is the result of sFLT-1 expression.

In i.v.-delivered AdRGDGFPsFLT-1, even on day 21 postinjection, high systemic levels of sFLT-1 (9.6 ng/ml) were observed compared with the i.p.-delivered AdRGDGFPsFLT-1 group, in which the sFLT-1 was almost zero. These results suggest that i.v.-injected AdRGDGFPsFLT-1 results in higher systemic levels of sFLT-1 and may persist for a longer time. Higher systemic levels and a longer duration of expression obtained by i.v. delivery of AdRGDGFPsFLT-1 may be the reasons contributing to hepatotoxicity.

i.v.-delivered adenovirus principally localizes to liver (40, 41). Therefore, the toxicity that we observed in the i.v.-delivered AdRGDGFPsFLT-1 group is, at least in part, caused by localization of the AdRGDGFPsFLT-1 primarily to the liver, leading to locoregional overexpression of sFLT-1. On the basis of this, we can conclude that overexpressed AdRGDGFPsFLT-1 leads to cytotoxicity in the liver resulting in early mortality. The lack of toxicity in i.p.-delivered AdRGDGFPsFLT-1 may be because i.p.-delivered virus does not principally localize to liver. Al-
though, ectopic localization to the liver takes place because of i.p. delivery, the amount of gene expression in the liver because of ectopic localization is insignificant compared with i.v.-deliv-
ered adenovirus-mediated gene expression. These results suggest that the route of injection of AdRGDGFPsFLT-1 makes a significant difference in eliciting toxicity even though the administered dose is the same.

A previous study (25) has reported that the i.m. injection of 5 × 10⁸ pfu of Ad-sFLT-1 (AdVEGF-ExR) has a therapeutic effect in s.c. tumors, but no comment was offered on systemic toxicity. Even if no toxicity was observed, this could be because of many variations in the experimental design. One key variable in that study was the route of administration of virus. The authors injected virus i.m. not i.v. Because i.m.-injected virus does not principally localize to liver, the hepatotoxicity may not have been observed. Furthermore, the virus dose that they administered was 5 × 10⁸ pfu, which is one log less than the
In the present study, we have administered adenovirus-mediated human sFLT-1 in a murine model. This issue might raise a question as to whether or not human sFLT-1 can sequester murine VEGF and/or heterodimerize with murine FLT-1 and KDR/Flik-1. It has been previously reported that the mouse sFLT-1 has 85.5% identity with human sFLT-1 and it binds to recombinant human VEGF. In addition, it cross-reacts with monoclonal antibody against human FLT-1. These findings led us to the conclusion that human sFLT-1 can sequester murine VEGF and may heterodimerize with the murine membrane spanning FLT-1 thus blocking the action of VEGF (48). However, additional studies aimed at confirming whether the toxicity observed was caused by excessive VEGF chelation or by heterodimerization with murine membrane-spanning FLT-1, or both, or whether it was caused by some as-yet-unidentified mechanism are warranted.

Because VEGF is a major angiogenic agent and several studies have reported that the delivery of VEGF to myocardium enhances the angiogenesis in diseases such as ischemic heart disease, it seems reasonable to attempt to expand these studies as a therapeutic option. However, the administration of VEGF has resulted in abnormally leaky vessels and hemorrhagia (49, 50) and has accelerated plaque formation in atherosclerotic disease (51). Furthermore, a recent report summarized the lower-extremity edema-inducing effect of VEGF when it was administered to patients with lower-extremity ischemia (52). In addition, the i.v. injection of adenovirus-mediated VEGF in mice resulted in system-wide increases in intravascular permeability and in multiorgan edema, leading to the death of a high percentage of animals (53). These findings and the results of our present study suggest that either local or systemic overexpression of VEGF, or its antagonists, in enhancing or inhibiting angiogenesis in pathological conditions, needs to be handled with caution. To circumvent the toxicity because of systemic delivery of Ad-mediated sFLT-1, there is a need for specific expression in tumors. This can be achieved by two strategies. First, by specifically targeting the adenovirus vector to the tumor and its vasculature (transductional targeting), and second, by expression of the transgene under the control of tumor-selective promoters (transcriptional targeting) that are “liver-off and tumor-on.” To this end, our laboratory has reported tumor-specific gene expression under the control of the cyclooxygenase-2 (COX-2) promoter, which has the liver-off and tumor-on property. Its use significantly mitigates the hepatotoxicity (54). These two strategies to express adenovirus-mediated sFLT-1 specifically in tumors may permit systemic treatment of disseminated tumors without resulting in systemic toxicity.

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