Suppression of Tumor Angiogenesis and Growth by Gene Transfer of a Soluble Form of Vascular Endothelial Growth Factor Receptor into a Remote Organ

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ABSTRACT

Antiangiogenic therapy shows promise as a strategy for cancer treatment. We constructed an adenovirus (AdVEGF-ExR) expressing the entire extracellular domain of the human vascular endothelial growth factor (VEGF) receptor (flt-1) fused to the Fc portion of human IgG. The soluble receptor secreted from AdVEGF-ExR-infected cells bound to VEGF and inhibited VEGF-induced DNA synthesis in endothelial cells. When human lung cancer cell line H157, which produces not only VEGF but also fibroblast growth factor 2 and interleukin 8 at substantial levels, was infected with AdVEGF-ExR, cell growth in vitro was not affected. However, when H157 cells infected with AdVEGF-ExR were injected s.c. into nude mice, tumor formation stopped on the 10th day after reaching a certain size (about 100 mm³), and tumor size declined gradually thereafter. When AdVEGF-ExR was injected into skeletal muscle and uninfected H157 cells were injected s.c., the soluble receptor was detectable in the circulating blood for 3 weeks, tumor growth ceased after 10 days, and tumor size declined thereafter. Histological examination revealed that intratumor angiogenesis was markedly suppressed, and apoptosis was enhanced. Using the same experimental protocol, a significant suppression of tumor growth was also seen in four of five other lung cancer cell lines, some of which secreted VEGF at nominal levels, at least under normoxic conditions in vitro. Our results demonstrate that adenovirus-mediated expression of a soluble VEGF receptor in a remote organ could inhibit tumor angiogenesis and enhance apoptosis and thereby suppress tumor growth in vivo. Adenovirus-mediated overexpression of a soluble VEGF receptor in a remote organ may have the potential to be a feasible and effective strategy for cancer treatment.

INTRODUCTION

Angiogenesis is required for various physiological and pathophysiological events, including tumor development and metastasis (1, 2). Clinical studies have shown that the density of intratumoral microvessels correlates well with the grade of invasiveness, the frequency of metastasis, and clinical prognosis in many types of cancer, including bronchogenic carcinoma (3–5). For tumor angiogenesis, angiogenic growth factors such as VEGF, FGF, and IL-8 need to be produced in order to support the continued growth of the tumor and sequester VEGF from receptors on the target cells, thus achieving an effective suppression of tumor growth. In addition, the soluble receptor may form a heterodimeric complex with a wild-type VEGF receptor and function as a dominant negative receptor (23, 24). These animal studies lend further support to the idea that VEGF plays a critical role in tumor angiogenesis, and they indicate the potential of anti-VEGF treatment as a means of tumor suppression. However, the above-mentioned methods require either a substantial amount of protein or a direct insertion of the molecules into cancer cells.

In this study, we investigated whether tumor growth could be efficiently suppressed by a soluble form of VEGF receptor (flt-1; fused to Fc portion of human IgG) expressed in a remote organ by adenovirus-mediated gene transfer. Gene transfer using an adenovirus can induce a high-level expression of the transferred gene for a substantial period of time, even with a single application. The soluble receptor should be secreted from infected cells into the blood stream and should reach most, if not all, sites of angiogenesis within the tumor and sequester VEGF from receptors on the target cells, thus achieving an effective suppression of tumor growth. In addition, the soluble receptor may form a heterodimeric complex with a wild-type VEGF receptor and function as a dominant negative receptor (23, 24). It has been reported recently that either a direct transfection of a plasmid encoding a soluble VEGF receptor into tumor cells (25) or a regional expression of a soluble receptor near tumor sites (or within the tumor) by adenovirus-mediated gene transfer (26) suppresses tumor growth in vivo. However, in the latter study, systemic delivery of the soluble receptor failed to suppress tumor growth (26). Theoretically, the soluble receptor should be effective in suppressing tumor growth in a remote area. In the present study, using several cancer cell lines, we investigate whether adenovirus-mediated expression of the soluble receptor can effectively suppress tumor angiogenesis and tumor growth in a remote area, which is an important clinical question.

MATERIALS AND METHODS

Preparation of Adenoviral Vectors. Replication-defective E1– and E3– adenoviral vectors expressing either the entire ectodomain of the human VEGF mRNA in many human tumors (9, 10), and VEGF mRNA has been found to be much more abundant in cancer cells than in endothelium, suggesting that cancer cells themselves generate VEGF and induce angiogenesis through a paracrine loop (11). It is known that hypoxia is a strong inducer of the transcription of both VEGF (12) and its receptor (13). In fact, VEGF mRNA can be detected in ischemic tumor cells located close to the central necrotic area (12). This suggests that hypoxia within the microenvironment of a rapidly growing tumor can enhance VEGF gene expression and thus induce angiogenesis. This, in turn, will support the continued growth of the tumor. In addition to these clinical studies, tumor suppression has been achieved in animal experiments by inhibiting VEGF or its receptor, which was achieved using: (a) neutralizing antibodies to VEGF (14–17); (b) a blocking antibody to VEGF receptor (18); (c) antisense oligonucleotides against VEGF (19); (d) an antisense VEGF expression plasmid (20); (e) a VEGF-diphtheria toxin conjugate (21); (f) a truncated VEGF receptor that inhibits the functioning of the wild-type receptor in a dominant negative fashion (22); and (g) a soluble form of VEGF receptor (23). These animal studies lend further support to the idea that VEGF plays a critical role in tumor angiogenesis, and they indicate the potential of anti-VEGF treatment as a means of tumor suppression. However, the above-mentioned methods require either a substantial amount of protein or a direct insertion of the molecules into cancer cells.
receptor (flt-1) fused to the Fc portion of human IgG (AdVEGF-ExR) or bacterial β-galactosidase (AdLacZ; Refs. 27 and 28) were prepared as described previously (27–29). A CA promoter comprising a cytomegalovirus enhancer and a chicken β-actin promoter (30) was used for expression. The titer of the virus stock was assessed by a plaque formation assay using 293 cells, and the titer was expressed in pfu.

**Cell Culture.** The following human lung cancer cell lines were used: (a) NCI-H157, NCI-H460, NCI-H1299, NCI-H322, NCI-H522, and NCI-H538 (generously provided by Dr. A. F. Gazdar, University of Texas Southwestern Medical Center, Dallas, TX); (b) EBC1, PC9, A549, LK2, and N417 (obtained from the Health Science Research Resources Bank, Tokyo, Japan); and (c) QG56, QG98, and QG95 (from the National Kyushu Cancer Center, Fukuoka, Japan). The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) in a humidified incubator with 5% CO2 at 37°C. Bovine vascular RECs and COS cells were cultured in DMEM with 10% fetal bovine serum. In vitro gene transfer into cells was carried out by incubation with the adenoviral vector in serum-free medium [DMEM 1640 containing 0.05% BSA, 1 μg/ml insulin, 5 μg/ml transferrin, and 25 mmol/liter HEPES (pH 7.4)] for 2 h at room temperature with gentle agitation, as described previously (31, 32).

**Measurement of VEGF and FGF-2 in Culture Media.** Confluent cancer cells were cultured for 24 h, and then the medium was collected. After centrifugation, the supernatant was stored at −80°C until the assay. The VEGF protein in the culture media was determined using an ELISA kit (Immunotech Biological Laboratories, Tokyo, Japan) according to the manufacturer’s instructions. Each of the values given here is the mean of triplicate determination with respect to standardized cell numbers. FGF-2 was also determined using an ELISA kit (Amersham International, Buckinghamshire, United Kingdom).

**Measurement of VEGF-ExR Protein in Mouse Serum.** The amount of soluble VEGF-ExR, which was tagged with the Fc portion of human IgG, in the serum of mice was measured by an ELISA using an anti-human IgG antibody, as described previously (33). Mice were injected with a single injection of AdVEGF-ExR (5 × 105 pfu) in the femoral muscle were sacrificed at 3, 5, 7, 11, 14, or 21 days after the injection. 

**[3H]Thymidine Uptake in RECs.** COS cells infected at MOI 10 with either AdLacZ or AdVEGF-ExR or left uninfected were incubated with serum-free DMEM for 48 h. The medium was then collected, and the cell debris was removed by centrifugation (500 × g, 10 min). Confluent bovine RECs (1 × 106 cells/well) were incubated with serum-free DMEM for 24 h. The medium was then exchanged for the supernatant prepared from COS cells. The supernatants were supplemented with 0.01% BSA and various concentrations of rhVEGF (0.1–100 ng/ml). Human IgG (1.5 mg/ml) was added into medium and the amount was exchanged for the supernatant prepared from COS cells. The medium was then exchanged for the supernatant prepared from COS cells. RECs were cultured for 24 h, the medium was collected. After centrifugation, the supernatant was stored at −20°C until the assay. The VEGF protein in the culture media was determined using an ELISA kit (Amersham International, Buckinghamshire, United Kingdom).

**Cell Proliferation Assay.** Cell proliferation was monitored spectrophotometrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO), as described previously (32).

**Tumor Formation in Nude Mice.** All animals were treated using protocols approved by the animal care committees of Kyushu University. The experiment was carried out under both the Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese government. Lung cancer cells (5 × 106) were injected s.c. into the dorsal skin of nude mice, and tumorigenesis was monitored for 4 weeks. When tumour formation was seen, tumour volume was calculated according to the formula a2 × b, where a and b are the smallest and largest diameters, respectively (32). Tumor size was measured twice a week for 1 month. The mice were observed for 120 days to examine the survival kinetics. Human IgG isolated from normal human serum (DAKO, Carpinteria, CA) was injected into some mice. VEGF produced by cancer cells in vivo was detected by immuno-staining with polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Student’s t test was used to compare tumor volumes, with P < 0.05 being considered significant.

**In Vivo Angiogenesis Assay.** Growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA) containing 100 pg rhVEGF (in 200 μl) was injected into the s.c. space of mice. The mice were then i.m. injected with either AdVEGF-ExR or AdLacZ at a dose of 5 × 106 pfu. Seven days later, the gel plugs were resected, fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with H&E. Some sections were subjected to immunostaining with an antibody recognizing factor VIII antigen (DAKO, Glostrup, Denmark), a biotinylated rabbit antihuman IgG antibody (Nichirei, Tokyo, Japan), peroxidase-labeled streptavidin, and diaminobenzidine, as described previously (28).

**Detection and Quantification of Apoptosis.** Apoptosis among cancer cells in tumor specimens was detected by a DNA nick end-labeling method (using an in situ Apoptosis Detection Kit; TAKARA, Tokyo, Japan) according to the manufacturer’s instructions. The sections were counterstained with hematoxylin. Apoptotic cells were counted under a light microscope (×200 magnification) in five randomly chosen fields, and the apoptosis index was calculated as a percentage of all cancer cells in these fields.

**RESULTS**

**VEGF and FGF-2 Production by Lung Cancer Cell Lines.** We first examined whether cancer cells secrete angiogenic growth factors. We prepared 14 cancer cell lines derived from human lung cancer and measured the levels of VEGF and FGF-2 in the culture medium by ELISA because these are both considered major angiogenic growth factors. Some cancer cells secreted VEGF and FGF-2 at significant levels, although the amounts in the medium differed considerably among the cell lines examined (Fig. 1).

The capacity to induce tumor formation in vivo was also investi-
soluble VEGF receptor (flt-1) into COS cells was expressed as a secretory fusion protein (Mr 130,000) with human IgG. Western blotting analysis showed that the soluble VEGF receptor secreted from AdVEGF-ExR-infected COS cells binds to rhVEGF (data not shown). We confirmed that this soluble VEGF receptor secreted from AdVEGF-ExR-infected COS cells inhibited VEGF-induced DNA synthesis in RECs (Fig. 2). We examined whether the soluble VEGF receptor affects cancer cell growth. The growth of H157 cells infected with AdVEGF-ExR (MOI 10) or left uninfected was s.c. injected into nude mice, and tumor formation was monitored macroscopically for 4 weeks. The tumor increased gradually in size until it reached 200 mm³ in volume (1–10 days) and then began to grow rapidly (Fig. 4). In contrast, when H157 cells infected with AdVEGF-ExR (MOI 10) were implanted s.c., the tumor stopped growing around day 10 after inoculation and actually decreased in size thereafter (Fig. 4). Macroscopically, the tumors composed of AdVEGF-ExR-infected H157 cells looked as red in color as the control tumors until day 7, but the former turned white.

VEGF-induced Cellular Response but not Cancer Cell Growth in vivo. We constructed an adenovirus (AdVEGF-ExR) expressing the entire extracellular domain of the human VEGF receptor (flt-1) fused to the Fc portion of human IgG. Western blotting analysis showed that a soluble VEGF receptor of Mr 130,000 was indeed secreted into the culture medium from AdVEGF-ExR-infected COS cells (data not shown). We confirmed that this soluble VEGF receptor secreted from AdVEGF-ExR-infected cells binds to rhVEGF (data not shown). We examined whether the soluble VEGF receptor could inhibit the action of VEGF. DNA synthesis in response to rhVEGF in RECs was measured by [³H]thymidine incorporation. In the medium prepared from the AdVEGF-ExR-infected COS cells, VEGF-induced DNA synthesis in RECs was significantly suppressed, but it was not affected in the medium from AdLacZ-infected cells or from uninfected COS cells with a considerable amount of human IgG (1.5 mg/ml; Fig. 2).

H157 cells were infected with either AdVEGF-ExR or AdLacZ at MOI 20 or left uninfected, and cell growth was monitored daily. No significant difference in cell growth was found among these cells (Fig. 3). The results demonstrate that neither AdVEGF-ExR infection nor the soluble VEGF receptor affects cancer cell growth. The growth of other cell lines used in this study was also unchanged after AdVEGF-ExR infection (data not shown).

AdVEGF-ExR-infected Cancer Cells Did Not Form Substantial Tumors in vivo. H157 cancer cells that had been infected with AdLacZ (MOI 10) or left uninfected were s.c. injected into nude mice, and tumor formation was monitored macroscopically for 4 weeks. The tumor increased gradually in size until it reached 200 mm³ in volume (1–10 days) and then began to grow rapidly (Fig. 4). In contrast, when H157 cells infected with AdVEGF-ExR (MOI 10) were implanted s.c., the tumor stopped growing around day 10 after inoculation and actually decreased in size thereafter (Fig. 4). Macroscopically, the tumors composed of AdVEGF-ExR-infected H157 cells looked as red in color as the control tumors until day 7, but the former turned white.

Fig. 2. Inhibition of VEGF-induced DNA synthesis by the soluble VEGF receptor in bovine RECs. Serum-free medium was obtained from cultures of COS cells that had been infected with either AdVEGF-ExR or AdLacZ or left uninfected. Confluent quiescent RECs were stimulated with rhVEGF for 24 h in the above-mentioned medium. Human IgG (1.5 mg/ml) was added to medium prepared from uninfected COS cells. [³H]Thymidine incorporation was measured, and the means ± SD (n = 4) are shown.

Fig. 3. AdVEGF-ExR infection did not affect cancer cell growth in vivo. H157 cells were infected at MOI 20 with AdVEGF-ExR or left uninfected, and cell growth was monitored daily. The means ± SD (n = 4) are shown.
Because AdVEGF-ExR infection did not affect cancer cell growth in vitro (Fig. 3), the inhibitory effect on tumor formation of AdVEGF-ExR infection was presumably attained via indirect mechanisms, such as an inhibition of tumor angiogenesis. It should be noted that whereas the tumor decreased in size quite markedly, it did not disappear completely. No significant differences in tumor growth or macroscopic appearance were seen between tumors induced using uninfected or AdLacZ-infected H157 cells. Some mice were injected with a large amount of human IgG (10 mg), but no significant differences were observed regarding tumor growth and appearance (data not shown).

After i.m. injection of AdVEGF-ExR, the Soluble VEGF Receptor in the Circulating Blood Inhibits VEGF-induced Angiogenesis in Vivo. Next, we investigated whether a soluble VEGF receptor expressed in muscle inhibits VEGF-induced angiogenesis in a remote area. After an i.m. injection of AdVEGF-ExR (5 × 10⁸ pfu), we quantified the soluble VEGF receptor in the circulating blood using an ELISA. Considerable amounts of the soluble receptor (nanomolar order) were detectable in the blood (Fig. 5). The serum level of the soluble receptor peaked on day 7 after the injection and declined gradually thereafter. However, it was still detectable on day 21 (n = 4).

When Matrigel (a gel of basement membrane proteins) containing rhVEGF (100 pg) was inserted into the s.c. space of a nude mouse, a proliferation of endothelial cells (positively immunostained with an antibody against factor VIII antigen; data not shown) was observed in the gel plug within 7 days (Fig. 6A). However, virtually no endothelial cells were found in the rhVEGF-Matrigel when AdVEGF-ExR (5 × 10⁸ pfu) was injected into the skeletal muscle (Fig. 6B). This inhibitory effect depended on the balance between the amount of rhVEGF in the gel and the titer of AdVEGF-ExR in the muscle: the greater the amount of rhVEGF mixed in the gel, the weaker the inhibition (data not shown). These results demonstrate that the soluble VEGF receptor produced by AdVEGF-ExR-infected cells can suppress the action of VEGF in a remote area in vivo.

Expression of the Soluble VEGF Receptor in a Remote Organ Suppresses Tumor Angiogenesis and Tumor Growth in Vivo. We next investigated whether expression of the soluble VEGF receptor in a remote organ could suppress tumor formation. To this end, we injected either AdVEGF-ExR, AdLacZ (5 × 10⁸ pfu), or saline into the femoral muscle in nude mice, and uninfected H157 cells were injected s.c. into the same animals. As shown in Fig. 7A, in a mouse injected with AdVEGF-ExR, the tumor began decreasing in size 10 days after the inoculation and had become only a white trace by day 21 (but it did not disappear completely). In contrast, a large reddish tumor was formed in a H157-inoculated mouse in which either AdLacZ or saline was injected into the muscle. No inhibitory effect was seen when 10 mg of human IgG were injected into the muscle once per week for 4 weeks. Representative photographs (AdLacZ versus AdVEGF-ExR) are shown (Fig. 7G). A similar inhibitory effect was seen in mice inoculated with EBC1 cells (Fig. 7B).

Three weeks after the inoculation, the tumors were subjected to a histological examination. The tumors in the control mice (injected with either AdLacZ or saline) were full of cancer cells (Fig. 8A), and the tumor stroma contained many blood vessels (Fig. 8B; confirmed by immunostaining with an anti-factor VIII antibody). In contrast, the tumors in mice given a single injection of AdVEGF-ExR into the muscle showed extensive necrosis with infiltrated neutrophils but virtually no living cancer cells (Fig. 8C).
The mice that had been inoculated with cancer cells were observed for 4 months. Of those mice injected with AdLacZ or saline, all 12 (6 in each group) died between 60 and 75 days after the inoculation. In contrast, the mice injected with AdVEGF-ExR (n = 6) were all alive 120 days after the inoculation, and no regrowth of the tumor was observed.

Under the same protocol as the one used in H157 cells, we tested the growth-inhibitory effects of AdVEGF-ExR using other cell lines (PC9, QG56, N417, and H460). As shown in Fig. 7, C–E, partial but significant inhibitory effects were seen in mice inoculated with PC9, QG56, and N417 cells. However, no significant suppression of tumor growth was seen in mice inoculated with H460 cells (Fig. 7F), which do not produce VEGF or FGF (Fig. 1) but secrete IL-8 at a considerable level (34). These data suggest that a soluble VEGF receptor suppresses the growth of more than one type of tumor and that it may be effective against many types of tumors.

Apoptosis in Tumors Was Enhanced by Inhibition of Angiogenesis. To obtain an insight into the mechanisms underlying the inhibitory effect of the soluble VEGF receptor on tumor growth, we analyzed the H157 tumors histologically, focusing on the incidence of apoptosis. First, we analyzed tumors at day 5 after the inoculation, a time at which tumor cells do not require angiogenesis for their growth (preangiogenic stage). Although the central area of the tumor was already necrotic, the majority of cancer cells were alive. No blood vessels were seen. The nutritional support for the cancer cells was presumably obtained by simple diffusion from the adjacent host tissues. Interestingly, TUNEL staining revealed the presence of many apoptotic cancer cells in the intermediate zone between the central necrotic area and the peripheral viable area adjacent to the host tissue (Fig. 9A). As shown in Fig. 9C, the cytoplasm of all cancer cells stained positive for VEGF. In the day 21 tumor (postangiogenic stage), new vessel formation was observed (compare Fig. 8A, B), and only a few cells were apoptotic (Fig. 9B). In contrast, tumors from the AdVEGF-ExR-treated mice showed extensive necrosis and apoptosis at day 21 after the inoculation (Fig. 8C). To evaluate apoptosis in a semiquantitative manner, we counted the apoptotic cells by microscopy. The apoptosis index of the day 5 tumor was 10 times that of the day 21 tumor (Fig. 9D). These results suggest that apoptosis
may occur in cancer cells in an ischemic environment, that tumor angiogenesis rescues cancer cells from apoptosis, and that the soluble VEGF receptor may suppress tumor angiogenesis, thereby maintaining a high rate of apoptosis among cancer cells and inhibiting tumor growth.

**DISCUSSION**

We previously transferred antiproliferative molecules such as wild-type p53 and p21WAF1/Sdi1/Cip1 into cancer cells using adenoviral vectors and tested their anticancer effects both in vitro and in vivo. Although cell growth in vitro was completely inhibited by these molecules as long as the cancer cells were susceptible to adenoviral vectors (32), tumor growth in vivo was only partially suppressed. In practice, it would be extremely difficult to infect all cancer cells within a tumor even through a direct intratumor injection of the vector. We have found that diffusion of the adenovirus is physically blocked by the tumor stroma, leading to the conclusion that transferring antigrowth molecules directly into cancer cells may not be a practical strategy for cancer gene therapy unless a specialized vector, such as a self-replicating adenovirus (35), is used. Moreover, this kind of approach may not be effective for the prevention of metastasis. We have therefore been seeking a different approach as an alternative to the direct growth inhibition of cancer cells.

In this study, in an attempt to inhibit tumor angiogenesis, we transferred a soluble VEGF receptor (flt-1) either directly into cancer cells or to a remote organ. Most tumors are thought to share common angiogenic factors (2), thus a successful antiangiogenic molecule may be effective against a wide range of tumors. Two major receptors for VEGF, flt-1 and flk-1 (or KDR), have been identified (36–38). Because flt-1 has the highest affinity for VEGF (at least for VEGF165), with a $K_d$ that, at approximately $10^{-12}$ M, is 7–10 times higher than that of flk-1 (24, 37–39), we used a soluble flt-1 receptor in our study. We confirmed that the soluble flt-1 produced from the AdVEGF-ExR-infected cells binds to VEGF (data not shown) and inhibits its action both in vitro (VEGF-induced DNA synthesis in endothelial cells; Fig. 2) and in vivo (VEGF-induced angiogenesis; Fig. 4). Although we did not confirm this ourselves, it has been reported that a VEGF receptor (flt-1) almost identical to ours shows the same high affinity for VEGF as the wild-type receptor (24, 40). When we injected AdVEGF-ExR (5 x $10^8$ pfu) into muscle, around 450 ng/ml soluble receptor was detectable in the circulating blood. This amount would seem to be in excess of the VEGF present in tumors, as judged by: (a) a rough estimate of the capacity for VEGF production shown by cancer cells in vitro (Fig. 1); (b) histological examination of the tumor on day 5 after inoculation (Fig. 9A); and (c) an assumption that the doubling time of cancer cells is 24 h in vivo, although it is 48 h in vitro. The
number of cancer cells present on day 5 after injection would be $5 \times 10^6 \times 2^4 \times 0.1 (8 \times 10^6)$ because roughly 10% of the cancer cells seem to have survived in the tumor (Fig. 9A). This number of cells (i.e., $8 \times 10^6$) would secrete 480 pm of VEGF in 24 h. On the basis of this and their molecular sizes (VEGF, $M_r$ 38,000; the soluble receptor, $M_r$ 130,000), the amount of VEGF-ExR would be over 5000 pm/24 h, which is about 10 times the amount of VEGF (on a molar basis), leading to the notion that it would neutralize VEGF completely. In addition to the sequestering effect of the soluble receptor, both soluble $\text{flt-1}$ (40) and $\text{flk-1}$ (23) can form a heteromeric complex with their wild-type receptor [soluble $\text{flt-1}$ can also form a complex with wild-type $\text{flk-1}$ (24)] and inhibit VEGF signaling as a dominant negative receptor. If this is true in animals, then the soluble receptor should achieve inhibition of VEGF signaling even in the presence of a saturating concentration of VEGF, provided that the amount of the soluble receptor supplied is high in comparison with that of the wild-type receptor (41). AdVEGF-ExR-infected cancer cells did not form substantial tumors in vivo (Fig. 4), and not a single mouse died of cancer during the observation period (4 months). This result is comparable with that achieved in a study in which cancer cells were stably transfected with a plasmid encoding soluble $\text{flt-1}$ and then injected into mice (25).

The most important finding in our study was that tumor formation was almost completely suppressed when the soluble receptor was expressed not within the tumor or even close to the tumor, but in a remote organ (by injection of AdVEGF-ExR into skeletal muscle). An inhibitory effect on tumor formation using a similar method has recently been reported using two murine cancer cell lines (42) and systemic i.v. injection of an adenovirus expressing a soluble form of Tie2, an endothelial-specific receptor with tyrosine kinase, which is known to be involved in angiogenesis. Our results and their results (42) demonstrate the effectiveness and usefulness of a strategy involving adenovirus-mediated gene transfer of a soluble receptor for an angiogenic growth factor into a remote organ or the systemic blood, respectively. With a view to future clinical application, the fact that the expression vector does not have to be applied directly into the tumor or even close to the tumor should be of considerable interest in the field. In contrast to our findings, Kong et al. (26) have reported that only regional application of an adenoviral vector expressing a soluble VEGF receptor shows an inhibitory effect and that systemic application does not suppress tumor formation. At present, we do not know the reason for this difference in results between our study and their study. Possible explanations include the different model systems used (our study used human cell lines in nude mice; their study used mouse cell lines in BALB/c mice) or the tagging of the Fc portion of IgG at the COOH-terminal of the receptor (our soluble receptor; no such fusion was used in their study). Provided that a sufficient amount of the soluble receptor was detectable in the blood, we believe that it
should inhibit angiogenesis even in a remote area. In their study (26), the amount of soluble receptor protein that appeared was not measured, and it is not known whether the amount differed between systemic and regional application. Additional studies need to be done because the issue of whether or not the soluble receptor can effectively inhibit cancer located in a remote area is very important clinically.

For our strategy against cancer to be effective, the target cancer cells need to depend on VEGF for tumor angiogenesis. However, tumor-associated angiogenesis is known to be promoted by several cytokines or growth factors (including FGF, VEGF, platelet-derived endothelial cell growth factor, and IL-8), and many cancer cell lines produce multiple angiogenic factors. In fact, H157 cells produce FGF-2 (Fig. 1) and IL-8 at substantial levels (34) in addition to VEGF. On the other hand, QC56 and N417 cells do not produce a large amount of VEGF, at least under normoxic conditions (Fig. 1). Nevertheless, anti-VEGF treatment achieved a significant tumor suppression in those cancer cell lines (H157, EBC1, QC56, N417, and PC9) in our study (Fig. 7, A–E): it was effective in five of the six cancer cell lines tested. For QC56 and N417 cells, which form tumors slowly in vitro, an application of AdVEGF-ExR at a later stage (e.g., on day 20) instead of on the day of inoculation (the timing used in the present study) could have achieved much more powerful effects because the production of the soluble receptor might already have fallen below the effective level at a time when it was required to suppress tumor angiogenesis. Our examination of 14 lung cancer cell lines (Fig. 1) suggests that cancer cells producing a substantial amount of VEGF in vitro will form tumors in vivo. Using monoclonal anti-VEGF antibodies, the growth of a broad spectrum of tumor cells can be inhibited both in vitro and in vivo (14–16, 43, 44). These findings suggest that VEGF may play a key role in tumor angiogenesis in a wide variety of cancers. It has not yet been determined whether a single factor plays a critical role in angiogenesis, even when many factors are available, or whether multiple factors act in concert to achieve angiogenesis. Naturally, a combined approach using multiple soluble receptors for a variety of angiogenic factors would be interesting and would probably achieve a potent inhibition in a wide variety of tumors. Indeed, we are currently investigating such an approach using adenoviruses expressing soluble receptors for either FGF-2 or platelet-derived growth factor, in addition to AdVEGF-ExR.

Recently, fragments of plasminogen (45), collagen XVIII (46), and metalloproteinase 2 (47), denoted as angiostatin, endostatin, and PEX, respectively, have been shown to inhibit tumor angiogenesis, to induce apoptosis, and to suppress tumor growth. In addition to studies using a recombinant protein of those molecules (48, 49), an intratumoral injection of an adenovirus expressing a modified angiostatin to be seclerable suppressed angiogenesis, induced apoptosis, and led to a significant arrest of tumor growth (50). The molecular mechanisms underlying the inhibition of angiogenesis (and the induction of an unexpectedly high level of apoptosis) by those fragmented molecules have yet to be elucidated fully. The enormous amount of recombinant protein required for antitumor effects in vivo (49) may raise the concern that those molecules may not inhibit angiogenesis very efficiently. Nevertheless, a direct comparison between those fragmented molecules and molecules more directly involved in angiogenesis would be interesting. Moreover, an approach using a combination of those molecules should be tried.

In summary, our study shows that VEGF is indeed a critical growth factor for tumor angiogenesis, at least in certain forms of cancer, and our results support the idea that adenovirus-mediated overexpression of a soluble VEGF receptor into a remote organ seems to have potential as a feasible and effective method of cancer gene therapy, although further investigations are required, especially on systemic side effects and its effectiveness against a wide range of cancer cells.

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REFERENCES

SOLUBLE FLT-1 SUPPRESSES LUNG TUMOR ANGIOGENESIS AND GROWTH IN VIVO


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