Gene Transfer of a Soluble Receptor of VEGF Inhibits the Growth of Experimental Eyelid Malignant Melanoma

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PURPOSE. To determine the effect of adenovirus-mediated gene transfer of a soluble receptor of vascular endothelial growth factor (VEGF) on the growth of experimental eyelid malignant melanoma.

METHODS. An adenovirus vector encoding a soluble VEGF receptor/flt-1 (Adflt-ExR) was constructed. The bovine retinal endothelial cells (ECs) were incubated in a culture medium of 293E1 cells infected by means of an adenovirus vector or uninfected (control), which contained human recombinant VEGF, and the [3H]thymidine uptake was tested. The experimental eyelid malignant melanoma was induced by the injection of B16 melanoma cells (4 × 10⁶ cells) into the right upper eyelid of BALB/c nu/nu mice, and the size of the tumor was recorded for 3 weeks after tumor cell injection. The effect of Adflt-ExR was examined in three ways. Model 1: B16 cells were infected by Adflt-ExR beforehand (at a multiplicity of infection [MOI] of 10) and injected into the eyelid. Model 2: Adflt-ExR was injected into pre-established B16 cell–induced eyelid malignant melanoma. Model 3: Adflt-ExR was injected into the femoral muscle of mice before B16 cell injection into the eyelid, and the remote effect was evaluated. An adenovirus vector bearing the LacZ gene (AdLacZ) or phosphate-buffered saline was used as a control. The amount of VEGF and the flt-ExR protein was measured by sandwich enzyme-linked immunosorbent assay (ELISA). Vascularization was evaluated by counting the number and the size of the vessels.

RESULTS. The supernatant of Adflt-ExR-transfected cells clearly inhibited VEGF-induced bovine retinal EC proliferation in vitro. In models 1 and 2, the tumor growth in Adflt-ExR–treated mice was significantly lower than that of controls (P < 0.05). In model 3, no significant difference was found (P = 0.14). The molar ratio of VEGF/flt-ExR protein was clearly low in the tumors of Adflt-ExR–treated mice in models 1 and 2 (P < 0.01) but not in model 3 (P > 0.05). In vessel density, the tumors in Adflt-ExR–treated mice had fewer vessels than tumors in control animals in models 1 and 2 (P < 0.05).

CONCLUSIONS. Adenovirus-mediated gene transfer of a soluble form of VEGF receptor (flt-1) gene inhibited the growth of the experimental eyelid malignant melanoma. This method may be useful as an antiangiogenic therapy for eyelid malignant melanoma. (Invest Ophthalmol Vis Sci. 2000;41: 2395–2403)

Although primary melanoma of the eyelid skin accounts for only 1% of all eyelid tumors, the results of treatment have not been satisfactory. The survival of patients with malignant melanoma is related to the depth of invasion. Patients with tumor invasion of more than 1.5 mm have a 5-year survival rate of only 50% to 60%. There is no curative therapy for the late phase of this disease. Despite pharmacologic developments in cancer treatment, melanoma is intrinsically resistant to most antitumor drugs, the cancer may recur after surgical resection, and postsurgical plastic reconstruction is difficult. Furthermore, ozone depletion worldwide is likely to increase incidence of this type of tumor at a rapid rate. As a result, the establishment of therapy for this type of tumor is extremely desirable.

It has been widely known that the growth of tumors that become larger than 1 to 2 mm³ is critically dependent on angiogenesis in the host, which supplies nutrients and growth factors. Thus, antiangiogenesis is a logical choice for cancer therapy. The direct inhibitors of endothelial cells, angiotatin and endostatin, and the indirect inhibition targeting mediators of tumor angiogenesis (e.g., basic fibroblast growth factor,
epidermal growth factor, and vascular endothelial growth factor (VEGF) have been used in experimental cancer therapy. VEGF is an endothelial cell-specific mitogen and an angiogenesis inducer released by a variety of tumor cells, including melanoma cells, and is also known as a key mediator of tumor angiogenesis. The amount of expression in tumors has been found to be related to tumor growth rate, and tumor microvessel density, and the possibility of tumor metastasis. VEGF-mediated angiogenesis is induced by binding of VEGF to the endothelial cell receptors flt-1 and flk-1/kinase. Therefore, the soluble flt-1 protein would be expected to neutralize VEGF and to inhibit tumor angiogenesis and tumor growth.

To date, there has been a clear limitation in cancer surgical therapy and chemotherapy. Gene therapy is expected to provide an alternative method for treating cancers. For example, the retrovirus-mediated gene transfer of herpes virus thymidine kinase and the adenovirus-mediated gene transfer of wild-type p53 have been tried for the treatment of malignant melanoma.

Recently, adenovirus-mediated in vivo regional delivery of a soluble form of the extracellular domain of the flt-1 gene was reported to inhibit regional murine colon carcinoma. However, the detailed effect of this treatment on tumor vascularity has not been studied, and no attempts have been made to date to treat periocular malignant tumor by antiangiogenic gene therapy. Antiangiogenic therapy seems to be ideal for periocular tumors, because surgical treatment is not always suitable for some intra- or extraocular tumors. In the present study, we examined the effect of the adenovirus-mediated soluble VEGF receptor flt-1 gene transfer on tumor growth in experimental eyelid malignant melanoma with an investigation into its possible mechanisms.

**MATERIALS AND METHODS**

**Cell Cultures**

Murine melanoma B16 cells and Y79 human retinoblastoma cells (obtained from American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) and Dulbecco’s modified Eagle’s medium (DMEM, Gibco), respectively. Bovine retinal endothelial cells (ECs) and pericytes (PCs) were isolated by a method described previously and maintained in DMEM. RPMI and DMEM were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), penicillin G (100 IU/ml), and streptomycin sulfate (50 mg/ml).

**Animals**

Eight-week-old male BALB/c nu/nu mice (Kyudo, Fukuoka, Japan) were used in all experiments. All animals were treated in a humane manner and were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Adenovirus Vectors**

The replication-deficient E1 and E3 recombinant adenovirus vectors were used in this study. LacZ gene was placed in the presence of a CA promoter that was composed of a cytomegalovirus enhancer and chicken β-actin promoter. AdLacZ-expressing bacterial β-galactosidase was purified by ultracentrifugation through a CsCl2 gradient, followed by extensive dialysis. The cDNA of human immunoglobulin Fc component was placed in the presence of flt-1 cDNA that had been placed in the presence of a CA promoter (Adflt-ExR). The cells infected with Adflt-ExR secreted the protein (flt-ExR) composed of the secreted form of the human VEGF/flt-1 receptor fused with the Fc component of immunoglobulin. Fc component was used as a tag protein. The titer (expressed as plaque forming units [PFU] per milliliter) of each virus stock was assessed by a plaque formation assay using 293E1 cells. B16 cells in monolayers were washed with serum-free RPMI twice and infected with Adflt-ExR or AdLacZ at a multiplicity of infection (MOI) of 20 for 90 minutes. To confirm the gene transfection, B16 cells infected with AdLacZ were stained with X-gal solution.

**In Vitro Effect of Adflt-ExR on VEGF-Induced Endothelial Cell Proliferation**

The 293E1 cells were infected with 10 MOI of either of adenovirus vector (AdLacZ or Adflt-ExR) or remained uninfected (control). After the culture medium was removed, the 293E1 cells were then washed two times with phosphate-buffered saline (PBS) and incubated with serum-free DMEM for 48 hours. The culture medium was then collected and the cell debris removed by centrifugation (500g, 10 minutes). The culture medium in which Adflt-ExR-transfected 293E1 cells (Adflt-ExR medium), AdLacZ-transfected 293E1 cells (AdLacZ medium), and nontransfected 293E1 cells were incubated (control medium) were used. Confluent cultured ECs were cultured in DMEM containing 5% FBS for 24 hours. The medium was then exchanged with the previously corrected medium, which contained 0.01 to 10 ng/ml human recombinant VEGF165 (R&D Systems, Minneapolis, MN) and 3% FBS, and the ECs were incubated for 18 hours. ECs were then pulsed using [3H]thymidine (0.5 mCi/well; Amersham, Arlington Heights, IL) for 6 hours. To determine the inhibitory effect of the supernatant of adenovirus-infected cells on another stimulant, FBS, the [3H]thymidine uptake study was also tested using the supernatant of various 293E1 cells, with or without 10% FBS.

**In Vivo Model**

The inhibitory effect of Adflt-ExR on tumor growth was studied. To see the efficacy of different delivery routes of an adenoviral vector on tumor growth inhibition, the following three models were used.

**Model 1: Subcutaneous Allograft of Infected Tumor Cells**

B16 cells (4 x 10^6 cells in 50 μl RPMI medium) infected by either Adflt-ExR or AdLacZ at an MOI of 20 for 90 minutes were implanted subcutaneously in the eyelid of each mouse.

**Model 2: Treatment of Pre-established Primary Subcutaneous Tumors**

A total of 4 x 10^6 B16 cells were implanted subcutaneously in the eyelids of mice. Seven days later, tumors that reached 6 mm^3 in volume received an intratumoral injection of Adflt-ExR (5 x 10^8 PFU/tumor) or AdLacZ (5 x 10^8 PFU/tumor).

**Model 3: Adenovirus Infection Distant from the Tumor (remote effect)**

Adflt-ExR or AdLacZ (5 x 10^9 PFU in 0.2 ml) was injected intramuscularly in the thighs of mice. Three days later, 4 x 10^6 B16 cells were implanted in the eyelid. In our preliminary study, we used a single injection; however, this treatment had no effect on the formation of remote tumors.
Each adeno viral vector injection was repeated every 7 days. Tumors were measured once a week. Three weeks after tumor cell injection, the mice were killed, and tumors were extracted. The tumor volume was calculated by the formula (longer diameter) × (shorter diameter)³/², as reported previously.¹⁷

**VEGF Expression of B16 Cells In Vitro and In Vivo**

The amount of protein was assessed by the Coomassie plus protein assay using bovine serum albumin (BSA; Sigma, St. Louis, MO) as a standard, and the amount of VEGF was measured by sandwich enzyme linked immunosorbent assay (ELISA; Quantikine M; R&D Systems). For assessment of VEGF in vivo, the tumors (n = 3, for each model) were resected, soaked in lysis buffer (1 M Tris, 3 M NaCl, 20% Triton X-100, 0.1 M EDTA, and 1 mM phenylmethylsulfonyl fluoride) and homogenized (Kontes homogenizer, Vineland, NJ). The tissues were centrifuged (14,500 g, 15 minutes, 4°C), and the amount of VEGF was measured. For assessment of VEGF in vitro, B16 cells (4 × 10⁶ cells in 60-mm dishes) were incubated in 1.5 ml DMEM for 48 hours. The conditioned medium was then collected.¹⁷,3²,3³ Cell lysate was also extracted with lysis buffer, and the amount of VEGF in cell medium and cell lysate was measured. Cultured bovine retinal ECs, PCs, and Y79 cells also were used as controls.

**Assessment of Tumor Vascularity**

Three weeks after B16 cell injection, the tumors (n = 5, for each model) were resected and fixed by 4% paraformaldehyde (PFA). Ten randomly selected PFA-fixed and paraffin-embedded sections per tumor were stained by periodic acid–Schiff (PAS) (PFA). Ten randomly selected PFA-fixed and paraffin-embedded sections (×200) in each section.³⁵ At the same time, the number of large vessels, defined as larger than 50 µm in diameter in the shorter axis, were counted in each section. The sections were randomly selected by one examiner (SS), the evaluations were performed by two masked observers (TS and HY), and the scores were analyzed.

**Histologic Detection of VEGF and Flt-ExR Protein in Tumor Tissues**

The tumor sections were immunohistochemically stained with rabbit polyclonal antibodies for VEGF (Santa Cruz Biotechnology, Santa Cruz, CA). Because a soluble VEGF receptor produced by Adflt-ExR–transfected cells (flt-ExR protein) has a human IgG-Fc, its distribution was visualized by immunohistochemical staining using rabbit polyclonal anti-human IgG-Fc (CH₂ lesion) antibodies (Dakopatts, Gostrup, Denmark). Our preliminary study showed that this antibody did not cross react with mouse immunoglobulins immunohistochemically (data not shown).

**ELISA for Flt-ExR Protein**

The sera of mice injected intramuscularly with either Adflt-ExR or AdLacZ was collected at 3, 7, 10, and 14 days after injection (n = 3, for each day). The flt-ExR concentration was determined by ELISA, as described previously with some modification.³⁵ Rabbit polyclonal anti-human IgG (100 µl, 5 mg/l) in 50 mM NaHCO₃, was placed on a 96-well microtiter plate (Costar, Cambridge, MA) and incubated overnight at 4°C. After the plate was coated with 3% skim milk in PBS, the samples were applied (100 ml/well) and incubated at 25°C for 2 hours. Peroxidase-conjugated anti-human IgG (Fc fragment: 100 µl, 5 mg/l) was added and incubated for 2 hours at 25°C. Then peroxidase substrate (100 µl) was added with a reaction time of 5 minutes. The absorption was measured at 450 nm using a multiscan spectrophotometer. These two anti-IgG antibodies used in this study did not cross react with any mouse immunoglobulins (data not shown). The flt-ExR protein concentration in the soluble protein extracted from the tumors (n = 3, for each model) was also measured by sandwich ELISA.

**Statistical Analysis**

The tumor size was analyzed using the Wilcoxon rank-sum test. P < 0.05 was considered to be statistically significant.

**RESULTS**

**In Vitro Effect of Adflt-ExR**

A soluble receptor fused with Fc (flt-ExR protein) was secreted by Adflt-ExR–transfected 293E1 cells. The concentration in the culture medium was 1.78 ± 0.21 picomoles/10⁶ cells per 24 hours. No flt-ExR protein was found in the culture medium of 293E1 cells, with or without AdLacZ infection. The [³H]thymidine uptake of the ECs incubated with AdLacZ-treated medium or untreated medium was clearly stimulated by VEGF in a dose-dependent manner. In contrast, the [³H]thymidine uptake of the ECs incubated with Adflt-ExR–treated medium did not increase after the administration of VEGF (Fig. 1A). Because this effect could have been caused by the nonspecific effect of Adflt-ExR–treated medium, ECs were also incubated in the medium with or without 10% FCS. These same inhibitory effects were not observed in ECs stimulated with 10% FCS (Fig. 1B). The results indicate that the inhibitory effect of Adflt-ExR on EC proliferation was specific to the stimulation by VEGF, but not by FCS.

**VEGF Expression of B16 Melanoma Cell In Vitro**

The concentrations of VEGF protein in cell medium and cell lysate were measured by ELISA. Compared with the cell medium of ECs (0.042 pg/ml · h), PCs (0.052 pg/ml · h), Y79 cells (3.3 pg/ml · h), B16 cells (100 pg/ml · h). In the cell lysate, a similar result was seen (PCs: 4.0 pg/mg lysate, B16 cells: 41.7 pg/mg lysate; Table 1).

**In Vivo Effect of Adflt-ExR Injection**

The tumors were measured once a week, and tumor volume was calculated according to the formula shown in the Methods section. Because there were no differences in the tumor growth between PBS- or AdLacZ-treated mice in each model, AdLacZ-treated mice were used as control subjects (data not shown). Also, tumor growth was dependent on the viability of B16 cells; the actual tumor size varied in each set of experiments. Therefore, three experiments were performed (n = 7 in each experiment). Similar growth patterns were observed in each group. Representative results are shown in Figure 2, and the percentage of tumor size in Adflt-ExR/AdLacZ of all treated animals on the day of tumor excision is shown in Table 2.
Model 1: Growth of Subcutaneous Allograft of Infected Tumor Cells. B16 cells infected with Adflt-ExR or AdLacZ, or uninfected cells exhibited the same growth rates in vitro (data not shown). The tumor emerged in all mice (7/7) injected with AdLacZ-infected B16 cells, whereas it appeared in 42% (3/7) of mice injected with Adflt-ExR. The tumors induced by AdLacZ-infected cells grew more rapidly than the tumors with Adflt-ExR–infected cells (P < 0.01; Fig. 2A).

Model 2: Effect of Intratumoral Adflt-ExR Infection of Pre-established Primary Subcutaneous Tumors. There was no difference in size of the tumors in mice that received intratumoral injection of Adflt-ExR or AdLacZ. However, after 12 days, the tumor size in mice with Adflt-ExR injection was significantly smaller than in those with AdLacZ injection (P < 0.05; Fig. 2B).

Model 3: Effect of Adflt-ExR Remote Infection in Mice. The tumor was observed in all mice throughout the experimental period. The tumor size in mice with Adflt-ExR injection seemed smaller than those of the other two groups; however, there was no statistically significant difference (P = 0.14; Fig. 2C).

### Table 1. Concentration of VEGF in Cell Medium and Cell Lysate

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Medium (pg/ml/h)</th>
<th>Cell Lysate (pg/mg)</th>
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<tr>
<td>Endothelial</td>
<td>0.042</td>
<td>—</td>
</tr>
<tr>
<td>Pericyte</td>
<td>0.052</td>
<td>4</td>
</tr>
<tr>
<td>Y79</td>
<td>3.3</td>
<td>—</td>
</tr>
<tr>
<td>B16</td>
<td>100*</td>
<td>41.7*</td>
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</table>

* Significant difference compared with other types of cells (P < 0.01).
TABLE 2. Percentage of Difference in Tumor Size on the Day of Excision

<table>
<thead>
<tr>
<th></th>
<th>Model 1 (n = 21)</th>
<th>Model 2 (n = 18)</th>
<th>Model 3 (n = 21)</th>
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<td>Difference</td>
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<td></td>
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<tr>
<td>(Adflt-ExR/AdLacZ)</td>
<td>16.5*</td>
<td>35.2†</td>
<td>72.5</td>
</tr>
</tbody>
</table>

* P < 0.01.
† P < 0.05.

Assessment of Tumor Vascularity

To evaluate the level of tumor angiogenesis, the tumor section was stained by PAS staining and the number of vessels counted by light microscopy. As shown in Figure 3A, in all in vivo models, tumors of Adflt-ExR–treated mice were less vascularized, whereas those of AdLacZ-treated mice were highly vascularized. Average scores were Model 1: Adflt-ExR, 53.9 per five high-power fields (5 HPFs) and AdLacZ, 75.5/5 HPFs; Model 2: Adflt-ExR, 37.5/5 HPFs and AdLacZ, 52.5/5 HPFs; Model 3: Adflt-ExR 59.7/5 HPFs and AdLacZ, 63.6/5 HPFs. The vessel density of the Adflt-ExR treated group was significantly less than that of the control group in models 1 and 2 (Model 1: P < 0.05, Model 2: P < 0.01). However, there was no statistically significant difference in model 3 (P = 0.29). In addition, it was evident that the number of larger vessels was higher in control mice (AdLacZ-treated or PBS-treated: 50%–60%) than in Adflt-ExR–treated mice (20%–22%; Fig. 3B; P = 0.02).

Distribution of VEGF andflt-ExR Protein In Vivo

Positive staining for VEGF was found in the sections from Ad FLT-ExR-, AdLacZ- or PBS-treated mice (Fig. 4A). VEGF was mainly localized in the cytoplasm of tumor cells. flt-ExR protein was positively stained in the intra- and extravascular spaces of tumor cells of Adflt-ExR–treated mice (Figs. 4B, 4C, 4D). However, no apparent staining for flt-ExR protein was found in control mice (PBS- or AdLacZ-treated mice; Figs. 4E, 4F, 4G).

Concentration of VEGF andflt-ExR Protein In Vivo

The VEGF concentration of the treated group (Adflt-ExR–infected group) was significantly less than that of the control group in models 1 and 2 (average concentration, Model 1: Adflt-ExR–treated, 4.85 pg/mg of tumor and AdLacZ–treated, 39.3 pg/mg of tumor; and Model 2: Adflt-ExR–treated, 2.58 pg/mg tumor and AdLacZ–treated, 12 pg/mg tumor; Fig. 5A). There was no significant difference in VEGF concentration in tumors in model 3 (average concentration: Adflt-ExR–treated, 24.6 pg/mg tumor and AdLacZ–treated, 30.6 pg/mg tumor; Fig. 5A). In contrast, the intratumoral flt-ExR protein was clearly detectable in Adflt-ExR–treated mice of model 1 or model 2 (Fig. 5B). However, flt-ExR protein was not detectable in tumors of control mice (PBS-treated or AdLacZ–treated mice) in all three models. The molecular weight of flt-ExR protein was 130 kDa by Western blot analysis (data not shown). The molar ratio of VEGF–flt-ExR protein was summarized in Table 3. The molar ratio of flt-ExR protein and VEGF (VEGF–flt-ExR protein) was clearly low in the tumors of Adflt-ExR–treated mice in both models 1 and 2 (Model 1: Adflt-ExR–treated, 0.03; AdLacZ–treated, 6.69; P < 0.01; Model 2: Adflt-ExR–treated, 0.01; AdLacZ–treated, 4.08; P < 0.01). The results showed that the flt-ExR protein was more abundantly present than VEGF in the tumors of Adflt-ExR–treated mice in models 1 and 2, but was not in the tumors of Adflt-ExR–treated mice in model 3 (Adflt-ExR–treated 0.86, AdLacZ–treated 3.55; P > 0.05). To see the remote effect, the serum from Adflt-ExR or AdLacZ intramuscularly injected mice was collected, and the flt-ExR protein concentration was measured. Seven days after Adflt-ExR injection, the serum concentration of flt-ExR protein peaked, and after this point, the concentration decreased gradually (Fig. 5C). In the serum from AdLacZ–injected mice, there was no detectable flt-ExR protein at any time.

FIGURE 3. (A) Tumor vessel density. The number of vessels of the tumor section per 5 high power fields (5 HPFs) was counted. In all three models, tumors of Adflt-ExR–treated mice were poorly vascularized, whereas those of AdLacZ–treated mice were highly vascularized. Vessel density of the treated group (Adflt-ExR–treated group) was significantly less than that of the control group (AdLacZ–treated group; model 1: P < 0.05, model 2: P < 0.01). However, there was no statistically significant difference in model 3 (P = 0.29). (B) The number of vessels that were smaller than 50 μm in diameter per 5 high power fields (boxed bars) were counted. The ratio of large vessels to total vessels was higher in control mice (AdLacZ–treated or PBS–treated: 50%–62%) than in Adflt-ExR–treated mice (20%–22%; P < 0.05).

**DISCUSSION**

Inhibition of Tumor Growth

The present experiments clearly showed that the preinfection or intratumoral injection of an adenovirus vector encoding for...
VEGF-soluble receptor/flt-1 inhibited the growth of eyelid malignant melanoma in mice. The soluble VEGF/flt-1 receptor secreted by Adflt-ExR-infected cells strongly and specifically inhibited the VEGF-induced endothelial cell growth in vitro. In tumors in Adflt-ExR-treated mice, the flt-ExR protein was more abundant than VEGF protein. These results indicate that the inhibition of tumor growth could be mediated by the inhibition of VEGF bioactivity with Adflt-ExR infection, at least in part. The histologic method may not always be an ideal method to evaluate tumor vascularity, because of the bias in sampling and its evaluation. To avoid this error, we randomly selected the samples and evaluated them by masked observers. The histologic study disclosed that the number of tumor vessels in Adflt-ExR-treated mice was significantly less than in control mice. Because VEGF is known to be a potent inducer of vascular endothelial cell proliferation, it is understandable that the number of tumor vessels was less in Adflt-ExR-treated mice. Furthermore, the vessels with larger diameter are less frequently found in Adflt-ExR-treated mice than in control mice. It has recently been found that VEGF dilates the vessel diameter through the pathway dependent on nitric oxide (NO) synthesis and modulates the responses through various vaso-motor stimuli. Therefore, it is feasible that the tumor of Adflt-ExR-treated mice had vessels with smaller diameter and that this effect may also be due to blockade of the VEGF activity. Additionally, VEGF is reported to induce tumor vascularity by maturing the newly formed vessels and inhibiting vascular endothelial cell apoptosis. No data on this effect were recorded in our experiments. VEGF may augment B16 tumor angiogenesis through all these various processes. In Figure 3A, the control levels of total vessel number in the tumor changed among the three models. Although we could not determine the exact reason, there was a variation in each experiment, probably because of tumor cell viability. When

**Figure 4.** Immunohistochemical micrographs of eyelid malignant melanoma in mice. (A) Tumor sections (model 3, AdLacZ-treated) were immunostained for VEGF. The cytoplasm of tumor cells was strongly stained with rabbit polyclonal antibodies for VEGF. Tumor sections (AdLacZ-treated and Adflt-ExR-treated) models 1 and 2 were stained similarly (data not shown). (B through G) The tumor sections from Adflt-ExR-treated mice (model 1: B, model 2: C, model 3: D) and AdLacZ-treated mice (model 1: E, model 2: F, model 3: G) were immunohistochemically stained with rabbit polyclonal anti-human IgG-Fc antibodies. flt-ExR protein was positively stained (red, arrowhead) in the intra- and extravascular spaces of tumor cells of Adflt-ExR-treated mice (B, C, and D). However, no apparent staining was found in control mice (AdLacZ-treated mice, E, F, and G). The pigmented granules originated from the implanted tumor cells (B16). Avidin-biotin complex immunoperoxidase staining method; magnification, (A) ×400; (B through G) ×200.
the experiment was performed with the same batch of cells, we obtained results similar to those presented in Figure 3A. This phenomenon may reflect the varied responses to cancer drug therapy in each patient.

Considering these findings, it seems that gene transfer of Adflt-ExR inhibited tumor angiogenesis through modulating VEGF bioactivity and resulted in B16 tumor growth inhibition. In fact, we have to admit that AdLacZ was not an ideal control group in models 1 and 2. There was no significant difference between model 3 and models 1 and 2. No detectable flt-ExR protein was present in tumors of control mice (AdLacZ-treated mice). The flt-ExR protein concentration of serum from Adflt-ExR or AdLacZ intramuscularly injected mice was measured. Seven days after Adflt-ExR injection, the serum concentration of flt-ExR protein was the highest, and after this point, the concentration decreased gradually. In sera of AdLacZ intramuscularly injected mice, there was no detectable flt-ExR protein at any time.

**Other Angiogenic Factors and Possible Clinical Application**

Our results demonstrate strong VEGF expression by B16 melanoma cells in vitro and in vivo. We did not measure other angiogenic mediators, but VEGF is probably most important in the angiogenesis of B16 cells. In this study, the suppression of tumor growth of experimental malignant melanoma by regional administration of Adflt-ExR by blocking VEGF was successful. In the angiogenesis of one melanoma cell type, bFGF may be involved by increasing the rate of synthesis and secretion of VEGF. Thus, the use of adenovirus, which mediated the suppressive protein of the other angiogenic mediator, such as b-FGF, together with Adflt-ExR may accomplish more effective inhibition of tumor growth. Actually, the treatment used in models 2 and 3 did not eliminate the tumor, and all the animals in these groups died of the tumor in the long run, indicating that supplementation with another treatment, such as an anti-

**TABLE 3. The Molar Ratio of VEGF to flt-ExR Protein in the Tumors**

<table>
<thead>
<tr>
<th>Model</th>
<th>Mice</th>
<th>VEGF (10(^{-15}) mol/mg tumor)</th>
<th>flt-ExR (10(^{-15}) mol/mg tumor)</th>
<th>Molar Ratio VEGF/flt-ExR Protein</th>
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<tr>
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<td>6.69 (P &lt; 0.05)</td>
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<td>0.74</td>
<td>0.86†</td>
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</table>

* Significant difference between AdLacZ-treated and Adflt-ExR-treated (P < 0.01).
† No significant difference between two groups (P > 0.05).
tumor drug, is necessary in clinical use. However, combining the present treatment with such therapies as angiogenesis inhibitors augments the therapeutic effect in malignant melanoma.

Many antitumor drugs have been used to treat cancers, but there are problems in delivery, selectivity for the tumor cell, and drug resistance. Angiogenesis inhibitors that directly target the normal endothelial cells of the tumor (for example, anti-VEGF monoclonal antibody, angiotatin, AGM 1470, and the fli-1 receptor protein) could cause suppression of physiological angiogenesis. The direct injection of these proteins or antibodies can suppress tumor growth. However, the drawback of this therapy is the necessity of frequent administration to obtain a sufficient therapeutic effect. In contrast, a single or occasional injection was sufficient for adenovirus-mediated gene therapy used in this study. Nonetheless, a major weakness of adenovirus vector in clinical gene therapy is its immunogenecity. However, this does not apply to patients with advanced cancer. Their immune systems may not be strong enough to cause a strong immunoreaction that would result in the rejection of the adnoviral gene transfer. Even though immunoreaction occurs in the tumor tissue, this immunoreaction could induce the destruction of the surrounding tumor tissue, resulting in tumor regression. Therefore, an adenovirus vector-mediated gene transfer can be a suitable method for cancer gene therapy.

In conclusion, the tumor growth of experimental eyelid malignant melanoma was suppressed by using adenovirus vector to transfer a cDNA encoding a soluble VEGF receptor fli-1. The present models are of a periorcular malignancy, not an ocular malignancy. Therefore, this method may provide a strong tool for the effective treatment of various periorcular malignant diseases.

References

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