sFlt-1 Gene Therapy of Follicular Thyroid Carcinoma

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Tumor progression largely depends on blood supply and neovessel formation, and angiogenesis is emerging as a promising target for cancer therapy. Vascular endothelial growth factor (VEGF), a major proangiogenic molecule, stimulates angiogenesis via promoting endothelial proliferation, survival and migration. VEGF has been found to be up-regulated in various types of tumors and to be associated with tumor progression and poor prognosis. Inhibition of VEGF or its signaling pathway has been shown to suppress tumor angiogenesis and tumor growth. In the present study, we tested the antiangiogenic and antitumor effects of soluble VEGF receptor-1 (soluble Flt (sFlt)-1) on the growth of follicular thyroid carcinoma (FTC). We constructed a 293 embryonic kidney cell line (293-Flt1–3d) that expresses sFlt-1, which is composed of the first three extracellular domains of Flt-1. The 293-Flt1–3d cells inhibited the in vitro growth of human umbilical vein endothelial cells in a paracrine manner. The in vivo antitumor and antiangiogenic activities of the 293-Flt1–3d cells were tested. When 293-Flt1–3d cells were inoculated at a site remote to the FTC-133 tumor transplant, the growth of FTC-133 tumors was inhibited by 70.37%, as compared with the control treatment with 293 cells expressing control gene LacZ. Immunohistochemical analysis of microvessel densities in treated tumors demonstrated that 293-Flt1–3d cells robustly suppressed intratumoral angiogenesis. Our data suggest that a mammalian cell-mediated approach could effectively deliver sFlt-1 gene therapy and inhibit tumor angiogenesis and tumor growth. (Endocrinology 145: 817–822, 2004)

Although follicular thyroid carcinoma (FTC) is usually associated with an optimistic prognosis, effective management of invasive and metastatic FTC has yet to be achieved. Chemotherapy and radiotherapy might benefit some of the patients with metastatic FTC but do not represent a cure. Novel approaches are needed for improved FTC treatment.

Angiogenesis, a process through which new blood vessels develop, is essential to the growth and metastasis of many tumor types (1). Antiangiogenic therapy, therefore, is emerging as a novel and potentially promising anti-cancer strategy. The development of tumor angiogenesis is believed to be dependent on the net balance between the actions of angiogenesis promoters and inhibitors. Proangiogenic factors have been found to be up-regulated in tumors, and such up-regulation has been linked to poor prognosis of disease progression (2). Vascular endothelial growth factor (VEGF), a potent proangiogenic factor, plays an important role as a mitogen as well as a survival factor of vascular endothelial cells, stimulating and maintaining neovascularization in a variety of tumor types (3, 4). VEGF exerts its biological effects on endothelial cells by binding to its cell surface receptors. Receptors identified to bind VEGF include Flt-1 (also known as VEGF receptor-1, i.e. VEGFR-1) and Flk-1 (also known as kinase domain receptor (KDR) or VEGFR-2). Both Flt-1 and Flk-1 belong to the class III receptor-type tyrosine kinase (RTK) receptor family, consisting of seven Ig-like domains in the extracellular portion, a transmembrane region (TM), and an intracellular tyrosine kinase region (3). Binding of VEGF to the receptors induces tyrosine phosphorylation of the intracellular domain, leading to activation of intracellular signaling and subsequent phenotypic changes, such as endothelial survival, proliferation, and migration. It is believed that, in most cases, the biological functions of VEGF defined thus far are mostly mediated by the Flk-1 receptor (6). Although several lines of evidence suggest that binding of VEGF to Flt-1 negatively regulates angiogenesis (7), the precise mechanisms responsible for these effects and their biological significance remain unclarified.

An increasing body of evidence suggests VEGF as a promoter of tumor development and progression. An association between high-level VEGF and poor prognosis of various cancers has been reported (2). Expression of VEGF has been shown in cultured differentiated thyroid carcinoma (DTC, including FTC) cells as well as in clinical DTC samples (8–11). Previous studies demonstrated that the expression of VEGF in DTC might be related to disease prognosis (12–14) and that down-modulation of VEGF inhibited DTC growth in vivo (15, 16). Serum VEGF level was found to be elevated in metastatic DTC patients (11). These data have provided a rational basis for novel anti-DTC approaches targeting the VEGF/VEGFR signaling pathway.

Several anti-VEGF approaches have been preclinically or clinically tested, including anti-VEGF or anti-VEGFR-2 antibodies, VEGFR-2 selective tyrosine kinase inhibitors, VEGF...
antisense, and soluble receptors of VEGFR (VEGFR-SRs). VEGFR-SRs are molecules consisting of the extracellular VEGF-binding domains of a VEGFR but lacking the TM and the intracellular kinase domains. VEGFR-SRs are able to sequestrate VEGF from receptor stimulation, thereby attenuating or blocking the downstream signaling transduction that leads to angiogenic responses (17). Previously, it has been found that VEGF binds Flt-1 at an affinity 7–10 times higher than its binding with KDR (18–21). Several studies found that adenovirus- or plasmid-mediated gene transfer of various forms of soluble Flt (sFlt)-1 inhibited tumor angiogenesis and growth (22–27). In this study, we constructed an sFlt-1 gene that codes for the 1–3 ectodomains of Flt-1 and established a human embryonic kidney cell line 293 that permanently expresses sFlt-1. The engineered 293 cells were then used as a gene transfer vehicle to treat mice inoculated with human FTC cells. Our data demonstrated that the growth of FTC tumors was inhibited in mice transplanted with sFlt-1-expressing 293 cells at a remote site. Further angiogenesis analysis showed that intratumoral vascularization was suppressed in these sFlt-1-treated tumors. These results lend support to the use of human sFlt-1 in anticancer therapy.

Materials and Methods

Cell culture

Human embryonic kidney cells 293 line (ATCC) were cultured in DMEM supplemented with l-glutamine (12.5 mg/liter), 10% fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), and Fungizone (250 mg/liter) (Invitrogen). Cell line FTA133 was originally isolated from the primary FTC of a patient by Goretzki (28) and kindly given by Dr. Richard Mulligan (Harvard Medical School, Boston, MA) and used as amphotropic packaging cells for recombinant retrovirus (29). Both FTA-133 and CRIP cells were grown in DMEM containing 10% FBS. Human umbilical vein endothelial cells (HUVEC) (Clonetech, Walkersville, MD) were maintained in the EBM-2 medium (Clonetec) supplemented with EGM-2 Singlequots (Clonetecs). All cells were cultured at 37 C and 5% CO2. The same culture condition was used when HUVEC were cultured in the Transwell dual-chamber system.

Cloning of the first three extracellular domains (Flt1–3d) of VEGFR Flt-1

Total cellular RNA was isolated from the HUVEC, using the RNAzol reagent, according to a standard method following the manufacturer’s instruction (Biotecx Laboratories, Houston, TX). The isolated RNA was subjected to RT-PCR using the One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s manual. The upstream sense primer (5’-GGGCTCACTCATGTCAGC-3’ and the downstream antisense primer (5’-GTTCACATGGTGAATGC-3’) were designed to amplify the coding sequence for the Flt-1-3d regions based on information retrieved from the GenBank (accession no. X51602 and Ref. 30). The RT-PCR product was then ligated to the T-Easy plasmid vector (Promega, Madison, WI), following manufacturer’s instruction. The resultant clones (pT-Easy-Flt1–3d) were verified by nucleotide sequencing.

Construction of a retroviral vector expressing Flt1–3d

The Flt1–3d gene was PCR-subcloned from pT-Easy-Flt1–3d into a retroviral vector. The PCR amplification was performed using the high-fidelity Deep Vent DNA polymerase according to the instruction provided by the manufacturer (New England Biolabs, Beverly, MA). The sequences of PCR primers are as follows: 5’-GGGCTCACTCATGTCAGC-3’ (sense) and 5’-GGGATCTCATATATGCTGGAGG-3’ (antisense). ThePCR-amplified product was digested with EcoRI and BamHI restriction enzymes and ligated to the larger fragment of EcoRI and BamHI-digested plasmid pFB-Neo-LacZ, a retroviral vector plasmid (Stratagene, Cedar Creek, CA). After sequence verification, the resultant plasmid was named pFB-Neo-Flt1–3d. This plasmid was transfected to retroviral packaging cell line CRIP (a generous gift from Dr. Richard Mulligan, Harvard Medical School, Boston, MA) using the Lipofectamine 2000 reagent (Invitrogen, Frderick, MD) by following the manufacturer’s manual. Transfected CRIP cells resistant to 800 µg/ml G418 were then selected, and the transfected cell was designated CRIP-Flt1–3d, which should persistently produce recombint retroviral particles expressing Flt1–3d. The plasmid pFB-Neo-LacZ was also transfected into CRIP cells as a transfection control (CRIP-LacZ).

Transduction of 293 cells and confirmation of Flt1–3d expression

Supernatants taken from the cultured CRIP-Flt1-3d cells and CRIP-LacZ cells were used to infect 293 cells by incubation at 37 C and 5% CO2 for 4 h, respectively. Subsequently, the infection medium was replaced with DMEM with 10% FBS, and G418 was added to the culture at a final concentration of 800 µg/ml. To confirm the expression of Flt1–3d by 293 cells, cellular RNA was isolated from the 293 cells transduced with the Flt1–3d gene, treated with RNase-free DNase (Invitrogen), and then subjected to RT-PCR using the cloning primers described above (sense: 5’-GGGCTCACTCATGTCAGC-3’; antisense: 5’-GTTCACATGGTGAATGC-3’). RT-PCR was performed using the One-Step RT-PCR kit (Invitrogen) according to the manufacturer’s instruction. The Flt1–3d-transduced 293 cell was named 293-Flt1–3d. The 293 cells transduced with the LacZ gene (293-LacZ) were used as a transduction control in subsequent experiments. Expression of Flt1–3d was also verified with the ELISA method using a commercial kit (Research Diagnostics, Inc., Flanders, NJ) according to the instruction provided by the manufacturer. Before the ELISA experiment, parental 293 cells, 293-LacZ cells, and 293-Flt1–3d cells were planted in a 96-well plate at a density of 3000 cells/well, and supernatants were taken at 12, 24, and 48 h, respectively, and subjected to ELISA detection. sFlt-1 concentrations were calculated according to the standard curves, and all data points were calculated as mean concentration of three-cell culture wells ± so.

Assessment of the in vitro growth of HUVEC

The inhibitory effect of Flt1–3d on HUVEC proliferation was evaluated through a dual-chamber cell coculture system using the Transwell cell culture inserts with pore size of 0.45 µm, which allows free diffusion of macromolecules, such as Flt1–3d (31–33) (BD Labware, Franklin Lakes, NJ). Briefly, HUVEC were cultured in a 12-well plate at a density of 2 × 105 cells/well. The 293-Flt1–3d cells, 293-LacZ cells, and parental 293 cells were inoculated on the culture membrane of the Transwell inserts at a density of 2 × 104 insert. Subsequently, the Transwell inserts were placed in the HUVEC-containing culture wells in the 12-well plate. After incubation at 37 C and 5% CO2, HUVEC were then trypsinized, stained with Trypan Blue dye, and counted with a hemocytometer under microscope. The cell count at each time point was determined as the mean of triplicate wells.

In vivo animal studies

To test the effect of 293-Flt1–3d cells on the growth of FTC xenograft, 4- to 6-wk-old female nude mice (Taconic, Germantown, NY) were divided into two groups, with five mice in each group. All mice were inoculated sc with 1 × 106 human FTC-133 at a dorsal flank site. At the same time, mice in the control group and in the therapeutic group were also inoculated sc with 2 × 106 293-LacZ cells or 2 × 106 293-Flt1–3d cells at the other dorsal flank, respectively. The growth of tumor nodules was monitored, and the tumor volumes were measured with a caliper and calculated as length × width2/2, according to a method previously described (34). Each data point was presented as mean volume ± se. Mice were killed when tumors reached 2.0 cm in diameter, per the protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and the tumors were resected.
Immunohistochemistry analysis for microvessel formation

Tumor specimens were fixed and frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). Five-micrometer cryosections were cut and stained with hematoxylin and eosin for histopathological analysis. To analyze the microvessel formation in tumors, sections were stained with anti-CD31 monoclonal antibody (Dako Corp., Carpinteria, CA) and subsequently with the avidin biotin complex (ABC) method. Positively stained vascular endothelial cells (brown) were visualized and imaged using a Magnifire digital camera (Olympus, Melville, NY) attached to an Olympus Provis microscope. Any endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered a single, countable microvessel. The microvessel densities (MVDs) were determined according to methods described previously (35–37). Briefly, regions of highest MVD (“hot spot” regions) were scanned at low magnification (×40–100) and counted at higher magnification (×200). Three such hot-spot fields were counted in each tumor section, and the mean MVD value was recorded.

Statistical analysis

For in vitro and in vivo experiments, HUVEC cell counts, tumor volumes, and MVDs were presented as mean ± se. The Student’s t test (two-tailed) was used to examine the statistical significance of the differences on a one-group-vs.-one-group basis. The level of significance was set at P < 0.05.

Expression of Flt1–3d

To evaluate the antiangiogenic and antitumor activities of sFlt-1, we first cloned and expressed the 1–3 extracellular domains of Flt-1 (Flt1–3d) in the 293 cells via a retroviral gene transfer approach. Transcription of the exogenous Flt1–3d in 293 cells was confirmed by RT-PCR. As shown in Fig. 1A, the 293-Flt1–3d cells contained transcript that could be amplified by primers specific for Flt1–3d, as opposed to the negative amplification from the 293-LacZ RNA template. The expression of Flt1–3d was further subjected to ELISA verification. The results (Fig. 1B) showed that the constructed 293-Flt1–3d cells efficiently secret sFlt-1.

Inhibition of HUVEC proliferation by Flt1–3d in vitro

To determine whether the 293 cells transduced by Flt1–3d (293-Flt1–3d cells) were able to inhibit the growth of vascular endothelial cells, we employed a modified Boyden chamber coculture system in this study (31–33). Because the Transwell insert (upper chamber) allows free trafficking of Flt1–3d from the 293-Flt1–3d cells to the HUVEC cultured in the lower chamber, the anti-endothelial effects of Flt1–3d could be examined by monitoring the growth of HUVEC. As shown in Fig. 2, when HUVEC were cocultured with the 293-Flt1–3d, cells demonstrated a significant growth inhibition, as compared with the HUVEC cocultured with the parental 293 cells or with the 293-LacZ control cells. The inhibition rate at the fourth day of coculture reached 73.83%, suggesting that a functional Flt1–3d protein was expressed and secreted by the 293-Flt1–3d cells.

The antitumor and antiangiogenic effects of Flt1–3d in vivo

To test the antitumor and antiangiogenic effects of Flt1–3d in vivo, we employed the human FTC xenograft model in this study. Our previous study has confirmed that sc transplanation of 1 × 10⁶ FTC-133 cells in nude mice results in growth of tumor (36). In the current investigation, the 293-Flt1–3d cells that express Flt1–3d gene or the 293-LacZ control cells were inoculated at a site distal to the FTC-133 tumor cell transplant. Seven to 10 d after tumor transplantation, FTC-133 tumors became palpable in both treatment and control groups (Fig. 3). At the 28th day, the mean size of FTC-133 tumors in the 293-LacZ control group reached approximately 2,000 mm³. However, in mice that were simultaneously inoculated with the 293-Flt1–3d therapeutic cells, the growth of FTC-133 tumors was inhibited by 70.37%, as compared with the control group, giving rise to tumor nodules with a mean volume of approximately 600 mm³ at the experimental endpoint (Fig. 3). These results suggest that sFlt-1 gene therapy is effective in suppressing the growth of xenografted FTC.

To determine whether sFlt-1 gene therapy inhibited intratumoral vessel formation, we assessed the MVD in the tumor nodules resected from Flt1–3d-treated and control mice, respectively, by using endothelial cell-specific CD31 immuno-
staining. A representative immunohistochemical analysis with CD31 antibody is shown in Fig. 4 (A and B). As demonstrated in Fig. 4C, the density of CD31+ vascular endothelial cells was dramatically reduced in tumor nodules resected from mice treated with 293-Flt1–3d, suggesting a significant antiangiogenic activity of sFlt-1 gene therapy against experimental FTC.

**Discussion**

Results presented in this report demonstrate that transfer of sFlt-1 gene effectively inhibits the growth of xenografted human FTC-133 thyroid tumors in immunodeficient mice.

Several approaches were recently tested for their effectiveness in delivering sFlt-1 gene therapy in vivo. Goldman et al. (22) reported that the growth of tumor cells transfected with sFlt-1-expressing plasmid was inhibited. Another study showed that tumor growth was inhibited when an adeno-virus vector carrying sFlt-1 gene was inoculated near the tumor sites in syngeneic mice (23). Takayama et al. and Kuo et al. reported that remote injection of an adeno-virus expressing human sFlt-1 fused to the IgG Fc fragment completely suppressed human tumor xenografts (24, 38). Similar
adenovirus-mediated approaches were found to be effective against some other tumors, such as melanoma (25). It is of note that some of these studies used an sFlt-1 gene fused to the IgG Fc fragment, which presumably enhances the stability of sFlt-1 protein and provides a tag for immunodetection. Moreover, it was reported that adenovirus-mediated sFlt-1 gene transfer without Fc fusion was highly toxic to mice, leading to animal death, and that this in vivo toxicity seemed to be absent in animals inoculated with sFlt-1/Fc fusion gene (38). Although adenovirus vectors have the advantage of high expression and transduction efficiency, several drawbacks of the system, such as unwanted immune response, have yet to be addressed (for a review, see Ref. 39). Recently, there has been an increased interest in employing mammalian cell-mediated approaches to deliver therapeutic genes (40, 41). By retrovirally engineering 293 cells, we have established a stable cell line (293-Flt1−3d) persistently expressing secretable sFlt-1, as demonstrated by RT-PCR and endothelial cell growth assay. When implanted in athymic mice, the 293-sFlt-1 cells revealed a robust inhibitory effect on the growth of FTC xenografted tumors, leading to a growth inhibition by 70.37% at the experimental endpoint. Immunohistochemistry analysis suggests that the observed antitumor effect is associated with a suppression of tumor vessel formation. Our data showed that systemic delivery of sFlt-1 gene mediated by mammalian cells could be effective in inhibiting tumor angiogenesis and growth. In our study, we constructed the sFlt-1 expression cassette as a nonfusion gene. No visible toxicity, such as loss of weight, change in animal behavior, decreased food or water intake, and premature mortality, was found in animals injected with the 293-sFlt1−3 therapeutic cell. The different toxicity profiles found in the previous study and the present one remain to be explored. Furthermore, whether this mammalian cell-based strategy could be modified to be an ex vivo approach is under investigation in our laboratory.

An sFlt-1 gene was initially identified as an endogenous mRNA species generated by alternative splicing in human endothelial cells. This endogenous sFlt-1 gene codes for six Ig-like extracellular domains of Flt-1 but lacks the seventh extracellular domain, the TM, and the intracellular region (17). A recombinant version of this natural sFlt-1 was shown to inhibit angiogenesis and tumor growth. In addition to the endogenous sFlt-1, an sFlt-1 molecule consisting of the entire ectodomain of Flt-1 was also constructed and tested in previous studies, and it was found that such a construct was able to inhibit tumor angiogenesis and growth (24, 26). Ferrara and associates (42) have identified that the second Ig-like extracellular domain of Flt-1 is a key region for VEGF binding. The first three extracellular domains of murine sFlt-1, when transferred as an Fc-fused gene, were shown to have potent antiangiogenic and antitumor activities (38). A fusion protein, VEGF-Trap, composed of the second Ig domain of Flt-1 and the third Ig domain of KDR also showed significant antiangiogenic efficacy when administered as a recombinant protein in mice (43). The human sFlt-1 molecule constructed in the present study contained the extracellular domains 1–3 of the Flt-1 receptor and was able to effectively block angiogenesis and tumor growth. With a view of future application of sFlt-1 gene therapy, it is of great interest to investigate whether the minimal length required for sFlt-1 to exert its antiangiogenic effects could be further reduced.

Tumor angiogenesis is usually attributed to multiple factors and pathways. In addition, both the endothelial cell and the tumor cell compartments in tumors contribute to tumor angiogenesis. Our previous work has suggested that tumor cells might have the properties of counteracting antiangiogenic agents when they are used as monotherapies, and that such an anti-angiogenic function could be overcome, at least partly, by simultaneously suppressing certain intracellular signaling pathways such as the epidermal growth factor receptor signaling (44, 45). Thus, combinations of different antiangiogenic approaches have more potent therapeutic efficacy than single agents. Antiangiogenic therapy in combination with chemotherapy, radiotherapy, or immunotherapy has also been shown to enhance the overall angiostatic and antitumor effects (37, 44, 46–48). We have previously shown that endostatin recombinant protein, as well as gene therapy, inhibited FTC xenografts (36). It is of interest to further ask whether a combination of strategies attacking different proangiogenic targets, such as a combination of endostatin and sFlt-1, could more effectively treat thyroid cancers. Furthermore, because of the importance of metastasis as a determinant of FTC prognosis, it will be of great interest to study whether antiangiogenic approaches can effectively inhibit FTC metastasis, and this will be a subject of future investigation in the laboratory.

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