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Delivery of Soluble VEGF Receptor 1 (sFlt1) by Gene Electrotransfer as a New Antiangiogenic Cancer Therapy

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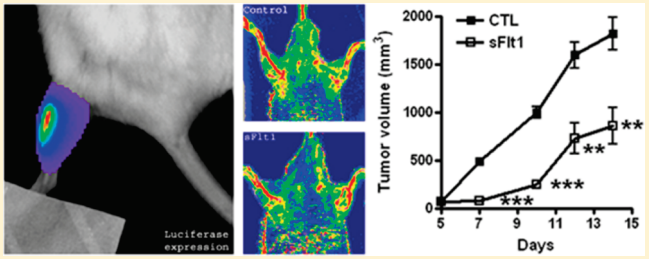
ABSTRACT: Since tumor growth is highly dependent on the formation of new blood vessels, angiogenesis inhibitors have become important players in anticancer treatments. Although less cytotoxic than conventional chemotherapy, most of the available antiangiogenic agents may provoke severe adverse effects which can limit their use. The design of new antiangiogenic strategies therefore requires integrating an early evaluation of possible interference with quiescent endothelial cells and nontumor angiogenesis. Here, we describe such a novel antiangiogenic approach based on the in vivo delivery by gene electrotransfer of a negative regulator of angiogenesis, namely, sFlt1. We found that this soluble variant of the vascular endothelial growth factor receptor 1 (Flt1, also known as VEGFR1), which acts as a VEGF trap, differentially influences tumor and postischemic hind limb angiogenesis in mice. sFlt1 gene electrotransfer in tibial cranial muscle leads to high sFlt1 protein expression and secretion, leading to a significant delay in the growth of syngeneic tumors but not altering the revascularization of ischemic peripheral tissue. The higher sensitivity of tumor-bearing animals toward sFlt1 trapping effects (vs ischemia-recovering animals) might be explained by a distinct pattern of VEGF release, as shown by VEGF measurements in plasma and tissue. In conclusion, our data support sFlt1 gene electrotransfer as a novel and safe modality to target VEGF-driven tumor angiogenesis and to maintain unaltered the recovery potential of ischemic tissues.



KEYWORDS: angiogenesis, cancer, sFlt1, VEGF, gene electrotransfer

INTRODUCTION

Angiogenesis is a tightly regulated process controlled by both inducers and inhibitors of endothelial cell proliferation and migration. The disruption of this balance in favor of proangiogenic signals causes the so-called "angiogenic switch", which supports the development of new blood vessels in tumors.[1] Given the high dependence of tumors toward blood vessels and the very restricted occurrence of angiogenesis in healthy adults, considerable efforts were undertaken to identify angiogenesis inhibitors that could be used in cancer therapy.[2,3] Many of these molecules specifically target the vascular endothelial growth factor (VEGF) signaling pathway which regulates several steps of the angiogenesis process, including endothelial cell proliferation, migration and organization in precapillary network.

The mammalian VEGF family consists of five glycoproteins referred to as VEGF-A, -B, -C, and -D and placenta growth factor (PlGF).[4] VEGFA (commonly named VEGF) is the best characterized member of this family and is expressed as various isoforms, due to alternative splicing. Three structurally similar type III receptor tyrosine kinases exist for the VEGF ligands: FLT1, FLK1 and FLT4 (also known as VEGFR1, VEGFR2 and VEGFR3, respectively). FLK1 appears as the key mediator of VEGF-induced angiogenesis. Because of a weak tyrosine kinase activity and a higher affinity than FLK1 for VEGF,[5] FLT1 is generally considered as a decoy receptor reducing signaling through FLK1. Direct FLT1 signaling is also documented, in particular in response to PlGF (which does not bind FLK1).[4] By alternative splicing, FLT1 encodes a soluble variant (known as sFLT1), which can bind VEGF and PlGF (with a lesser affinity). Modalities aiming to block FLT1 (with antibodies or peptides) or to administer sFLT1-like traps were reported to have antitumor effects.[6−9] The former were documented to be particularly suited to target the numerous tumor cell types and stromal cells which do express FLT1 whereas the latter are reported to block angiogenesis because of VEGF sequestration and interference with FLK signaling. It should be emphasized that the first class of inhibitors (ie, those blocking FLT1), by preventing the binding of VEGFA to sFlt1, could increase the level of free VEGFA, which in turn may become available to activate FLK1 and stimulate (rather than inhibit) angiogenesis.[10]

Many antiangiogenic strategies consist of protein-based therapies, the best example being the humanized anti-VEGF antibody bevacizumab. Although therapeutic proteins are widely used as effective medical treatments, this approach still suffers from inherent problems. The high-cost manufacturing process, the requirement of multiple injections to maintain therapeutic concentrations, and the

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absence of guarantee that proteins will reach the target tissue represent different pitfalls that can be easily overcome by gene therapy. Here, we describe a novel antiangiogenic strategy consisting of the in vivo delivery of a plasmid encoding sFlt1 (the murine homologue of sFLT1) to foster the expression and consequent sustained release of functional sFlt1 protein in the bloodstream of tumor-bearing mice. We used a nonviral gene delivery method based on plasmid injection into the skeletal muscle and subsequent application of electric pulses to promote DNA transfer.[11,12] We found that sFlt1 gene electrotransfer led to a significant inhibition of solid tumor growth but did not alter postischemic angiogenesis, validating sFlt1 gene electrotransfer as an efficient and safe mode of delivery and expression of this secreted protein to target angiogenesis in tumors.

MATERIALS AND METHODS

Plasmids and pDNA Preparation. The luciferase-encoding plasmid (pGL3) was obtained from Promega (Madison, WI, USA). The 7.7 kb expression vector encoding soluble Flt1 was prepared as follows. The insert of pBLAST45-mFlt1(s7) vector (Invivogen, San Diego, CA, USA) was cut out with *Nco*I and *Nhe*I and amplified using the following sets of sense and antisense primers: 5′ primer, 5′-ACCATGGTCAGCTGCTGGGA-3′; 3′ primer, 5′-CTACACGGCCCCCTTCTG-3′ (Invitrogen, Carlsbad, CA, USA). The product was cloned into the expression plasmid pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA, USA), which contains the cytomegalovirus (CMV) immediate early promoter/enhancer. Note that the antisense primer was designed with a stop codon, so the PCR product is expressed as a native protein. The identity and orientation of the resulting construct were further confirmed by DNA sequencing. Plasmids were prepared using Endo-Free Qiagen Gigaprep kit, according to the manufacturer's protocol (Qiagen, Hilden, Germany). Their quality was assessed by the ratio of light absorption (260 nm/280 nm) and by 1% agarose gel electrophoresis before and after digestion with corresponding restriction enzymes. All plasmids were stored at −20 °C until use.

Cell Culture. Human embryonic kidney (HEK) 293 cells were routinely cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% serum and transfected in serum-free medium with the FuGene 6 reagent (Roche Applied Bioscience, Mannheim, Germany) according to the manufacturer's instructions. Cells and supernatants were collected 48 h later and after lysis and precipitation, respectively, were processed through Western blotting as described below.

Animals. Male NMRI mice of 8 weeks old were used for every experiment (Université catholique de Louvain, Brussels). Before any manipulation, mice were anesthetized with a mixture of ketamine 50 mg/mL (Ketalar, Pfizer, Brussels, Belgium) and xylazine 23.3 mg/mL (Sigma, Bornem, Belgium). These procedures were approved by the local authorities according to national animal care regulations.

Plasmid Injection and Electrotransfer. The sFlt1-encoding plasmid (30 μL in 0.9% saline) was injected (approximate injection time of 5 s) into the mouse tibial cranial muscle of the posterior left leg using a Hamilton syringe (Hamilton Company, Reno, NV, USA) with a 30-gauge needle. For all experiments, conductive gel was used to ensure electrical contact with the skin (EKO-GEL, ultrasound transmission gel, Egna, Italy). The pulses were delivered by a Cliniporator system (Cliniporator, IGEA, Carpi, Italy) using two parallel, stainless-steel plate electrodes separated by 4 mm (length, 20 mm; width, 9 mm; depth, 1 mm; IGEA, Carpi, Italy). The leg was placed between electrodes and 8 square-wave electric pulses (200 V/cm. 20 ms. 2 Hz) were delivered.[13] A luciferase-encoding plasmid was administered through the same protocol in order to validate the electrotransfer procedure; injection of a single dose of luciferin (150 mg/kg, ip) at different time intervals allowed in vivo detection of bioluminescence on an IVIS50 imaging system (Xenogen, Hopkinton, MA), as previously described.[14] For the validation study, the dose of pDNA injected into the muscle was 1 μg. For the antiangiogenic study, the dose injected into the muscle was 40 μg.

Tibial Cranial Muscle Homogenates and Immunoblotting. At the indicated time points, mice were euthanized and tibial cranial muscles were surgically removed, frozen and stored at −80 °C. Frozen muscles were ground in a mortar and pestle under liquid nitrogen. Homogenates were then prepared in RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate and 1% sodium deoxycholate), in the presence of inhibitors of proteases (Protease Inhibitor Cocktail, Sigma). The homogenates were further sonicated on ice two times at 100 W for 15 s with a Labsonic U sonicator (B Braun Biotech International, Melsungen, Germany) and centrifuged at 14 000*g* for 10 min. Supernatants were collected and kept at −80 °C before analysis. Equal amounts of proteins (40 μg) were subjected to SDS−PAGE (7% separating gel) followed by electroblot to nitrocellulose membranes from GE Healthcare (Piscataway, NJ, USA). The membranes were blocked 1 h in TBS buffer (20 mM Tris, pH 7.4, 150 mM NaCl) containing 5% powdered milk protein followed by an overnight incubation with diluted antibodies in a fresh solution of powdered milk protein (1%, w/v, Bio-Rad Laboratories, Hercules, CA, USA) in TBS buffer. The membranes were washed five times with TBS containing 0.1% Tween-20 (Sigma, Bornem, Belgium) and incubated for 60 min with a dilution of secondary antibody coupled to horseradish peroxidase. Goat anti-mouse VEGFR1 was purchased from R&D systems (Minneapolis, MN, USA), and mouse monoclonal anti-Hsp90α/β was from Santa Cruz Biotechnology (F-8) (Santa Cruz, CA, USA). They were used at 1/500 and 1/2000, respectively. Peroxidase-conjugated rabbit anti-goat antibody and rabbit anti-mouse polyclonal antibody were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA) and DakoCytomation (Glostrup, Denmark), respectively. When appropriate, bands obtained via Western blot analysis were quantified by using ImageJ software (http://rsb.info.nih.gov/ij/).[15]

Coimmunoprecipitation of VEGF and sFlt-1 from Mouse Sera. Sera were first incubated for 1 h at room temperature with 2 mL of protein G Sepharose (Invitrogen, Carlsbad, CA, USA) for preclearing. After centrifugation (12000*g* for 5 min), the supernatants were collected and incubated with anti-VEGF antibodies (0.2 mg/mL, polyclonal goat IgG, R&D systems, Minneapolis, MN, USA) and incubated for 1 h at room temperature. The antibody/antigen complexes were then precipitated by incubation with protein G Sepharose (Invitrogen, Carlsbad, CA, USA) and centrifugation as described above. The pellets were washed twice in PBS and incubated for 5 min at 95 °C in Laemmli buffer before gel loading. For immunoblotting, anti-VEGFR1 antibody (0.2 mg/mL, polyclonal goat IgG, R&D systems, Minneapolis, MN, USA) was used.

Tumor Implantation. Male NMRI mice received an im injection of 10^6 syngeneic transplantable liver tumor (TLT) hepatocarcinoma

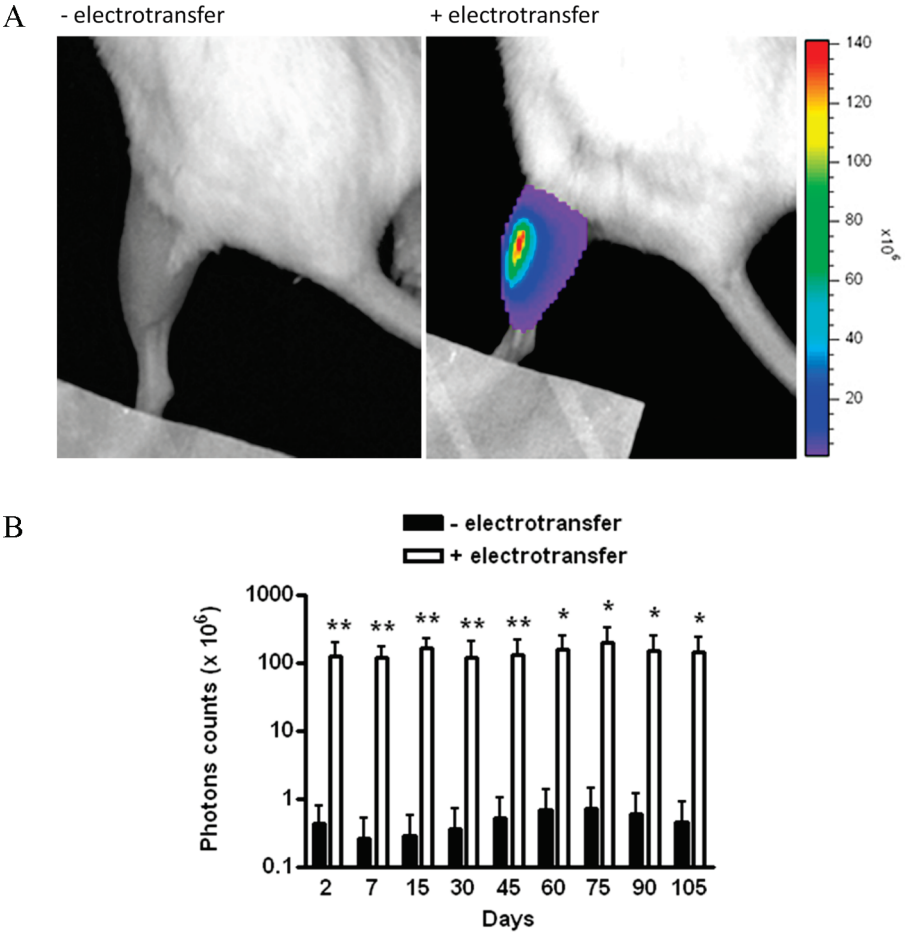

Figure 1. Validation of the muscle electrotransfer technique. (A) Typical light emission from tibial cranial muscle 7 days after the electrotransfer of 1 μg of a luciferase-encoding plasmid. The observation was performed 15 min after ip injection of luciferin (150 mg/kg). Luminescence levels are represented using a false-color scale (see vertical bar). (B) Bar graph represents the extent of luminescence detected from mice (n = 6) injected with 1 μg of a luciferase-encoding plasmid into the tibial cranial muscle with or without electric pulses; note the Y axis log scale; *P < 0.05, **P < 0.01.

cells in the posterior right leg in the vicinity of the saphenous arteriole, as previously described.[16,17] The tumor diameters were tracked with an electronic caliper.

Ischemic Hind Limb Reperfusion Assay. Mice were anesthetized and, after local fur removal using a depilatory cream (Veet, Reckitt Benckiser, Slough, U.K.), were placed on a heating pad (37 °C) to minimize temperature variations. Then, mice underwent a double femoral artery and vein ligation to allow the removal of 2 mm vessel segments under a stereoscopic microscope while innervation was carefully preserved. Blood flow in the ligated and control contralateral legs was measured with a Laser Doppler perfusion imager (LDI, Moor Instruments Ltd., Axminster, Devon, U.K.), as previously described.[18] Briefly, the imager scans a low power laser beam in a raster pattern over skin. Moving blood in the microvasculature causes a Doppler frequency shift of the scattered laser light, which is photodetected and then processed to build a color coded map of blood flow. Perfusion was normalized for the limb surface analyzed.

VEGF Measurements. For plasma VEGF measurements, blood samples were collected every four days through retro-orbital punction in EDTA-containing tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) before centrifugation for 20 min at 2000g to collect plasma and further storage at −20 °C. For tissue VEGF measurements, tumor and muscle homogenates were obtained as described above. Mouse VEGF was determined using a quantitative sandwich enzyme immunoassay technique (R&D systems, Minneapolis, MN, USA). Briefly, a polyclonal antibody specific for mouse VEGF-A (164 and 120 amino acid residue forms) precoated onto microplate (R&D systems, Minneapolis, MN, USA) binds VEGF present in the mouse plasma. After washing, an anti-VEGF antibody conjugated to horseradish peroxidase is added to the wells and sample values are determined from a standard curve (built by using purified recombinant mouse VEGF).

Statistical Analysis. Results are presented as mean values, and the error bars represent the standard error of the mean. For statistical comparison of results at a given time point, data were analyzed by either unpaired Student's t test or the Mann−Whitney test, which was applied to the data in Figure 1B, which do not have a normal distribution.

RESULTS

Validation of the Muscle Electrotransfer Technique. The efficacy of our electrotransfer system was first assessed. We used a luciferase-encoding plasmid that was injected im into the mouse tibial cranial muscle. Electric pulses were then immediately delivered through external electrodes placed at each side of the leg. Two days later, injection of luciferin led to the detection of

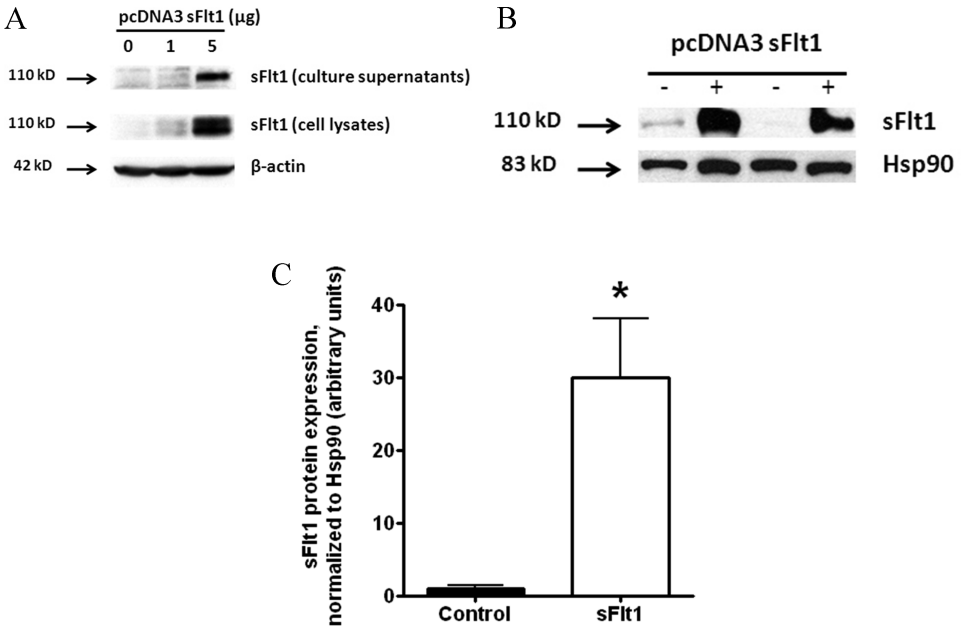

Figure 2. In vitro and in vivo expression of sFlt1. (A) Representative sFlt1 immunoblotting experiments from HEK-293 cells transfected (with FuGene 6) with 0, 1, or 5 μg of an expression vector encoding soluble Flt1 (pcDNA3-sFlt1). Cells and their corresponding supernatants were collected 48 h after transfection; beta-actin expression is shown as a control of gel loading for cell lysates. (B) Representative sFlt1 immunoblotting experiments from mouse tibial cranial muscles, 48 h after electrotransfer with or without 40 μg of pcDNA3-sFlt1 plasmid. Two mice in each group are shown. (C) Bar graph represents the extent of sFlt1 protein expression, normalized to that of Hsp90, in corresponding conditions, $^*P < 0.05$, $n = 6$.

luciferase activity at the site of pDNA injection, as documented in Figure 1A. A high and constant expression of luciferase was observed for up to one hundred days (Figure 1B).

After this period of time, luciferase expression slowly decreased to reach one-tenth of its initial value at day 200 (data not shown). Animals injected with the luciferase plasmid but not exposed to local electric pulses exhibited a low luciferase activity (Figure 1B).

In Vitro and in Vivo Expression of sFlt1. We first examined whether the mouse soluble Flt1 receptor could be detected both intra- and extracellularly after transfection of HEK-293 cells with a sFlt1-expressing vector. We found that a dose-dependent release of sFlt1 in the cell culture supernatant mirrored the intracellular expression of the recombinant protein (Figure 2A). Note that two bands were observed in cell lysates, most likely reflecting different glycosylation status. We then examined the extent of sFlt1 expression in mice after in vivo electrotransfer of the same plasmid. Immunoblotting performed on muscle protein extracts confirmed a high expression of sFlt1 in electrotransferred mice when compared with control animals (Figure 2B). Quantification of the immunoblot data revealed a 30-fold increase in sFlt1 receptor expression in electrotransferred animals (Figure 2C).

sFlt1 Gene Therapy Delays Tumor Growth. Since electrotransfer achieved high levels of sFlt1 in vivo, we then looked at the putative antiangiogenic properties of such gene therapy in a solid tumor model. To evaluate the potential of the sFlt1 strategy as a preventive and therapeutic strategy, we injected TLT carcinoma cells in the posterior right leg, either one day before or three days after sFlt1 gene electrotransfer. Importantly, to avoid a direct influence of electrotransfer on the tumor bed and to evaluate the influence of secreted sFlt1 receptor, the sFlt1 plasmid was always electrotransferred in the contralateral leg (vs the tumor-bearing leg). We found that when electrotransfer was carried out one day after tumor cell injection, a 2-day tumor growth delay was observed (Figure 3A). The inhibitory effect of sFlt1 expression was more pronounced when the plasmid was electrotransferred three days before tumor cell injection (Figure 3B). Finally, we performed sFlt1/VEGF coimmunoprecipitation experiments from mouse blood samples to document that muscle electrotransfer of sFlt1 led to the secretion of the protein in vivo. This experiment indicated that significant amounts of sFlt1 were present in the bloodstream of electrotransferred animals and that this recombinant sFlt1 was functionally active since associated with VEGF (Figure 3C).

sFlt1 Gene Administration Does Not Impair Postischemic Neovascularization. Since the clinical use of antiangiogenic strategies has been reported to cause adverse effects, the putative impact of sFlt1 gene transfer on nontumor angiogenesis was assessed in an ischemic hind limb reperfusion model. In this model, the removal of 2 mm femoral artery/vein segments leads to a dramatic reduction in hind limb perfusion. Postischemic angiogenesis then progressively restores the blood flow in the operated leg, as measured by laser Doppler imaging in control mice (Figures 4A).

Interestingly, we found that sFlt1 gene electrotransfer did not impair neovascularization in the ischemic hind limb as shown by the similar time course of blood flow restoration between control and electrotransferred mice (Figures 4B).

Plasma VEGF Is Higher in Tumor-Bearing Mice than in Ischemic Animals. To further understand the differential influence of sFlt1 receptor expression on tumoral vs postischemic angiogenesis, we determined VEGF concentrations in the plasma of the animals as well as in the ischemic muscle or the tumor. A distinct pattern was observed: tumors gave rise to elevated plasma VEGF concentrations whereas tissue VEGF was higher in the ischemic muscle than in the tumors (Figure 5A and 5B).

While the plasma VEGF concentration peaked at 254 pg/mL in tumor-bearing mice and then slightly decreased to plateau

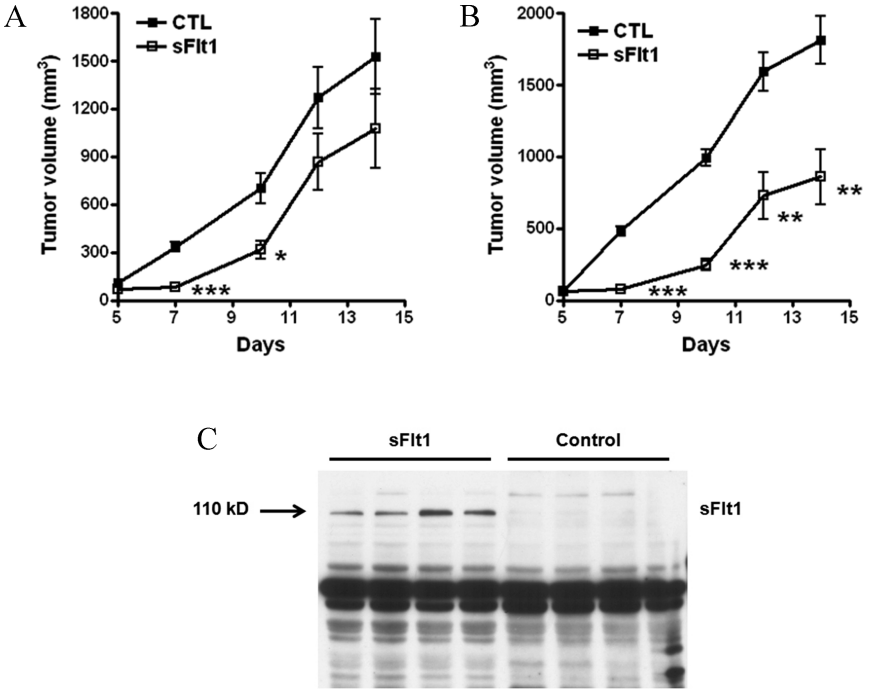

Figure 3. sFlt1 gene therapy delays tumor growth. Graphs represent the kinetics of tumor growth in mice electrotransferred with 40 μg of pcDNA3-sFlt1 plasmid (sFlt1) or saline only (CTL), either one day after (A) or three days before (B) tumor implantation; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 8$. Note that tumor cell injection and plasmid electrotransfer were performed in different legs of the animals to exclude any interference on the tumor bed. (C) Coimmunoprecipitation of VEGF and sFlt1. Sera were collected at day 15 after tumor implantation and immunoprecipitated with anti-VEGF antibodies. The precipitates were run on SDS−PAGE and immunoblotted with anti-sFlt1 antibody; lower size bands reflect i.a. the presence of IgG used in the immunoprecipitation. Four mice from sFlt1- and saline-electrotransferred groups are shown.

around 150 pg/mL, plasma VEGF concentration never exceeded 60 pg/mL in ischemic animals (Figure 5A). In the ischemic tissue, VEGF concentration rapidly peaked at 52 pg/mg before slowly decreasing back to basal levels (Figure 5B). In tumors, VEGF concentrations progressively increased with the tumor burden to reach 20 pg/mL and then remained quite stable from day 5 to day 15 (around 15−20 pg/mL).

DISCUSSION

Angiogenesis is an important hallmark of cancer, promoting tumor progression and metastatic dissemination.[19,20] Any strategy altering tumor angiogenesis has therefore the potential to interfere with tumor growth by reducing nutrient and oxygen delivery to the tumors. Among the angiogenic actors, the VEGF pathway is the most widely acknowledged target because of the multiple implications of this growth factor in new blood vessel formation. Antibodies directed against VEGF and small molecules inhibiting the tyrosine kinase activity of VEGFR2 receptors expressed on endothelial cells are now part of the anticancer drug armamentarium. Although originally considered to be safe, these agents are now recognized to cause a number of adverse effects such as bleeding, disturbed wound healing or hypertension.[21,22] There is therefore a need for new antiangiogenic treatments associated with less collateral damage on the healthy vasculature.

In this study, we examined whether administration of the soluble sFlt1 receptor could represent such an alternative and whether in vivo gene electrotransfer was a feasible strategy to achieve this goal. The data obtained confirmed that sFlt1-encoding DNA delivery into the skeletal muscle leads to significant inhibition of tumor growth in mouse and did not interfere with nontumor, postischemic angiogenesis. Differences in the circulating amounts of VEGF may actually account for the lack of side effects of sFlt1 administration on postischemic neovascularization. We found that although VEGF concentrations rapidly increased in the ischemic tissues soon after the surgical removal of femoral vessels to reach levels 2.5-fold higher than in tumors, the plasma concentration of VEGF remained low in operated mice (vs tumor-bearing mice). The nature of VEGF may account for this difference with high MW VEGF isoforms (ie, VEGF165, VEGF189) diffusing to smaller distance than the low MW isoform (ie, VEGF121). Also PlGF, which is known to be neutralized by sFlt1, could account for differences in the nature of growth factors involved in tumor vs postischemic neovascularization. Whether larger amounts of low MW VEGF isoforms and/or PlGF are produced by tumor cells warrants further exploration. Another obvious issue which should also receive more attention is how circulating VEGF may support tumor angiogenesis and, in particular, whether it may be correlated with a contribution of hematopoietic endothelial progenitor cells to tumor neovascularization. Our results however already pave the way for the optimization of a strategy delivering sFlt1 receptor in order to further exploit the differential dependency on VEGF or, more likely, subtle differences in the profile of VEGF release between tumor and ischemic tissues. Our data actually positions this strategy to an even more attractive rank than other antiangiogenic modalities which aim to target the tumor vasculature and to spare healthy endothelial cells but usually do not integrate the need to avoid interference with beneficial angiogenesis.

A second finding of this study is the demonstration that sFlt1 overexpression through gene electrotransfer technique is an achievable goal. As previously reported,[12,13,23] using a luciferase-encoding

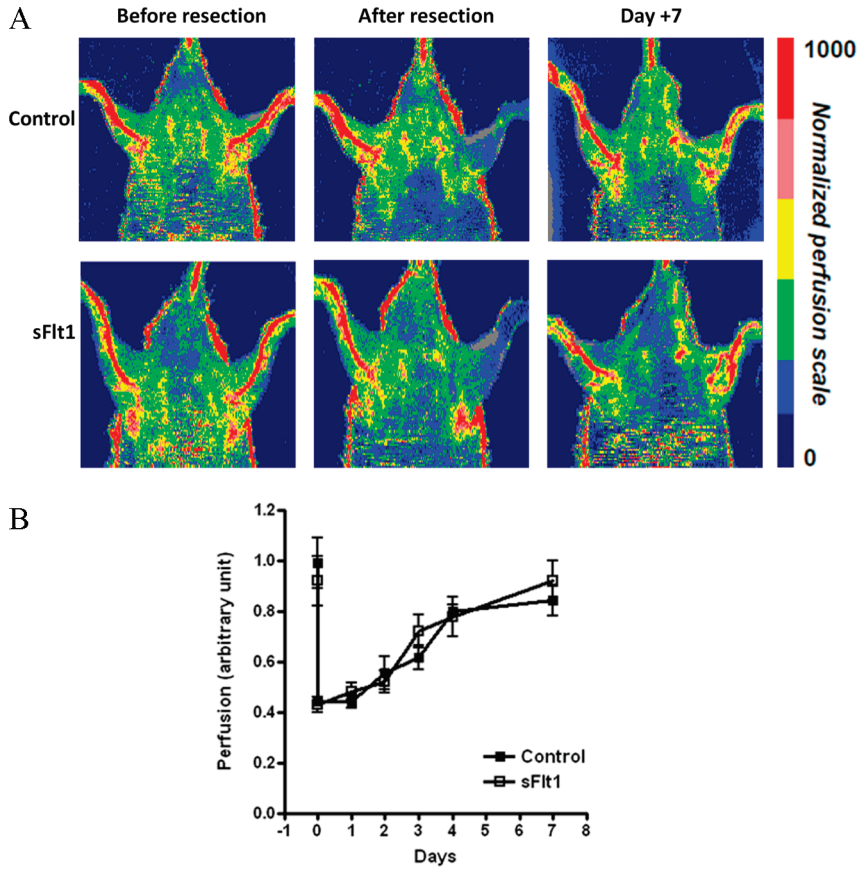

Figure 4. sFlt1 gene administration does not impair postischemic neovascularization. Mice were electrotransferred with 40 μg of pcDNA3-sFlt1 plasmid (sFlt1) or saline only (control), and were operated three days later to remove 2 mm femoral vessel segments, as described in the Materials and Methods section. (A) Typical laser Doppler images of blood flow in posterior hind limbs of mice just before and immediately after the ligature (day 0) as well as at day 7 postsurgery. (B) Graph represents the time course of blood flow restoration in mice treated or not with the sFlt1 plasmid (n = 5).

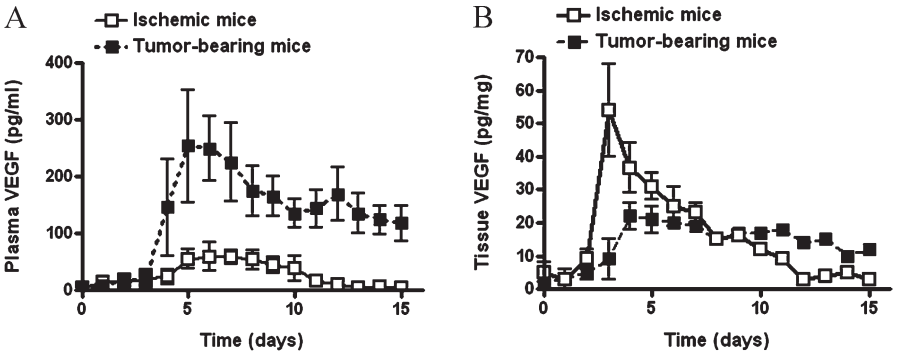

Figure 5. Plasma VEGF is higher in tumor-bearing mice than in ischemic animals. Mice were injected with TLT tumor cells or underwent the removal of 2 mm femoral vessel segments, respectively. Graphs represent the time course of VEGF production as determined (A) in the plasma and (B) in the tumor or ischemic tissues; in A, each point is the average of 4 measurements (16 mice were used, and blood was collected every four days for a given mouse), and in B, each point from days 0−7 represents the average of two mice (i.e., 16 different mice), and each point from days 8−15 was obtained from a single mouse (i.e., 8 different mice).

plasmid, we documented that this technique allowed achievement of a reproducible and long-lasting expression of this reporter gene upon im injection and subsequent application of electric pulses. The inhibitory effects of sFlt1 gene transfer on tumor growth and the VEGF/sFlt1 coimmunoprecipitation from blood samples further validate that the secretion of functional proteins from the electrotransferred tissue is possible. Thus, a similar strategy has been recently described by Tevz et al., using a plasmid encoding interleukin-12, demonstrating the broad application of this approach.[24] However, it is noteworthy that sFlt1 gene electrotransfer had only a short-term effect, leading to a delay in tumor growth but not to tumor eradication. The aggressiveness of our mouse tumor model is very likely to account for this transient therapeutic effect, as supported by several lines of evidence. First, the *mouse* isoform of

sFlt1 was used in our study and the occurrence of an immune response (a classical limitation of gene electrotransfer) may therefore be excluded; the long-term expression of a nonmurine protein such as luciferase also supports the lack of a major immune response against recombinant proteins in our mouse model. Second, our coimmunoprecipitation data indicate that recombinant sFlt1 still interacts with one of its main targets, namely, VEGF, at day 15 postimplantation of tumor cells (Figure 3C). Third, the therapeutic impact of any antiangiogenic treatments, including sFlt1 gene transfer, is *per se* influenced by the rapidly evolving angiogenic status of growing mouse tumors; prominent angiogenesis at early times of tumor growth can for instance be followed by tumor cell adaptation to hypoxia. Of note, with the same tumor model used in this study, using a VEGF receptor tyrosine kinase inhibitor (i.e., SU5416), we got a similar tumor growth delay response despite a daily administration of the drug (not shown).

For the same reasons as underlined here above, the better tumor response observed in our hands when the electrotransfer procedure preceded injection of tumor cells (Figure 3B) could reflect a better match between angiogenic burden and optimal secretion of sFlt1 (which may need a few days to install). Importantly, these data also support the prophylactic potential of sFlt1 gene therapy to combat the metastatic progression of the disease. Indeed, immature angiogenic blood vessels facilitate intravasation of metastatic tumor cells but an angiogenic switch is most often required for micrometastases to exit dormancy (even years after primary tumor eradication).

Taken together, these results suggest that electrotransfer can be a novel and interesting modality of antiangiogenic therapy based on the overexpression of negative regulators of angiogenesis. In that sense, both the low-toxicity profile of this approach and the use of other regulators should be further confirmed by other studies.

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ABBREVIATIONS USED

VEGF, vascular endothelial growth factor; PlGF, placenta growth factor; FLT, fms-related tyrosine kinase; FLK, fetal liver kinase; CMV, cytomegalovirus; HEK, human embryonic kidney; RIPA, radioimmunoprecipitation assay; TBS, tris buffered saline; TLT, transplantable liver tumor; DMEM, Dulbecco's modified Eagle medium; LDI, laser Doppler perfusion imager

REFERENCES

(1) Carmeliet, P. Angiogenesis in life, disease and medicine. *Nature* **2005**, *438*, 932–936.

(2) Loges, S.; Roncal, C.; Carmeliet, P. Development of targeted angiogenic medicine. *J. Thromb. Haemostasis* **2009**, *7*, 21–33.

(3) Feron, O. Targeting the tumor vascular compartment to improve conventional cancer therapy. *Trends Pharmacol. Sci.* **2004**, *25*, 536–542.

(4) Ellis, L.; Hicklin, D. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat. Rev. Cancer* **2008**, *8*, 579–591.

(5) Waltenberger, J.; Claesson-Welsh, L.; Siegbahn, A.; Shibuya, M.; Heldin, C. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.* **1994**, *269*, 26988–26995.

(6) Goldman, C.; Kendall, R.; Cabrera, G.; Soroceanu, L.; Heike, Y.; Gillespie, G.; Siegal, G.; Mao, X.; Bett, A.; Huckle, W.; Thomas, K.; Curiel, D. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8795–8800.

(7) Holash, J.; Davis, S.; Papadopoulos, N.; Croll, S.; Ho, L.; Russell, M.; Boland, P.; Leidich, R.; Hylton, D.; Burova, E.; Ioffe, E.; Huang, T.; Radziejewski, C.; Bailey, K.; Fandl, J.; Daly, T.; Wiegand, S.; Yancopoulos, G.; Rudge, J. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11393–11398.

(8) Taylor, A. P.; Goldenberg, D. M. Role of placenta growth factor in malignancy and evidence that an antagonistic PlGF/Flt-1 peptide inhibits the growth and metastasis of human breast cancer xenografts. *Mol. Cancer Ther.* **2007**, *6*, 524–531.

(9) Wu, Y.; Zhong, Z.; Huber, J.; Bassi, R.; Finnerty, B.; Corcoran, E.; Li, H.; Navarro, E.; Balderes, P.; Jimenez, X.; Koo, H.; Mangalampalli, V. R.; Ludwig, D. L.; Tonra, J. R.; Hicklin, D. J. Anti-vascular endothelial growth factor receptor-1 antagonist antibody as a therapeutic agent for cancer. *Clin. Cancer Res.* **2006**, *12*, 6573–6584.

(10) Fischer, C.; Mazzone, M.; Jonckx, B.; Carmeliet, P. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat. Rev. Cancer* **2008**, *8*, 942–956.

(11) Aihara, H.; Miyazaki, J. Gene transfer into muscle by electroporation in vivo. *Nat. Biotechnol.* **1998**, *16*, 867–870.

(12) Mir, L.; Bureau, M.; Gehl, J.; Rangara, R.; Rouy, D.; Caillaud, J.; Delaere, P.; Branellec, D.; Schwartz, B.; Scherman, D. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4262–4267.

(13) Bloquel, C.; Fabre, E.; Bureau, M.; Scherman, D. Plasmid DNA electrotransfer for intracellular and secreted proteins expression: new methodological developments and applications. *J. Gene Med.* **2004**, *6* (Suppl. 1), S11–23.

(14) Dewever, J.; Frerart, F.; Bouzin, C.; Baudelet, C.; Ansiaux, R.; Sonveaux, P.; Gallez, B.; Dessy, C.; Feron, O. Caveolin-1 is critical for the maturation of tumor blood vessels through the regulation of both endothelial tube formation and mural cell recruitment. *Am. J. Pathol.* **2007**, *171*, 1619–1628.

(15) Abramoff, M.; Magelhaes, P.; Ram, S. Image Processing with ImageJ. *Biophotonics Int.* **2004**, *11*, 36–42.

(16) Sonveaux, P.; Dessy, C.; Brouet, A.; Jordan, B.; Grégoire, V.; Gallez, B.; Balligand, J.; Feron, O. Modulation of the tumor vasculature functionality by ionizing radiation accounts for tumor radiosensitization and promotes gene delivery. *FASEB J.* **2002**, *16*, 1979–1981.

(17) Sonveaux, P.; Végran, F.; Schroeder, T.; Wergin, M.; Verrax, J.; Rabbani, Z.; De Saedeleer, C.; Kennedy, K.; Diepart, C.; Jordan, B.; Kelley, M.; Gallez, B.; Wahl, M.; Feron, O.; Dewhirst, M. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J. Clin. Invest.* **2008**, *118*, 3930–3942.

(18) Sonveaux, P.; Martinive, P.; DeWever, J.; Batova, Z.; Daneau, G.; Pelat, M.; Ghisdal, P.; Grégoire, V.; Dessy, C.; Balligand, J.; Feron, O. Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. *Circ. Res.* **2004**, *95*, 154–161.

(19) Hanahan, D.; Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **1996**, *86*, 353–364.

(20) Hanahan, D.; Weinberg, R. The hallmarks of cancer. *Cell* **2000**, *100*, 57–70.

(21) Verheul, H.; Pinedo, H. Possible molecular mechanisms involved in the toxicity of angiogenesis inhibition. *Nat. Rev. Cancer* **2007**, *7*, 475–485.

(22) Lee, S.; Chen, T.; Barber, C.; Jordan, M.; Murdock, J.; Desai, S.; Ferrara, N.; Nagy, A.; Roos, K.; Iruela-Arispe, M. Autocrine VEGF signaling is required for vascular homeostasis. *Cell* **2007**, *130*, 691–703.

(23) Andre, F. M.; Gehl, J.; Sersa, G.; Preat, V.; Hojman, P.; Eriksen, J.; Golzio, M.; Cemazar, M.; Pavselj, N.; Rols, M. P.; Miklavcic, D.; Neumann, E.; Teissie, J.; Mir, L. M. Efficiency of high- and low-voltage pulse combinations for gene electrotransfer in muscle, liver, tumor, and skin. *Hum. Gene Ther.* **2008**, *19*, 1261–1271.

(24) Tevz, G.; Kranjc, S.; Cemazar, M.; Kamensek, U.; Coer, A.; Krzan, M.; Vidic, S.; Pavlin, D.; Sersa, G. Controlled systemic release of interleukin-12 after gene electrotransfer to muscle for cancer gene therapy alone or in combination with ionizing radiation in murine sarcomas. *J. Gene Med.* **2009**, *11*, 1125–1137.