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Research Paper

A Plasmid-Encoded VEGF siRNA Reduces Glioblastoma Angiogenesis and Its Combination with Interleukin-4 Blocks Tumor Growth in a Xenograft Mouse Model

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KEY WORDS

VEGF, angiogenesis, mIL4, siRNA, retroviral vector, glioblastoma, PEI, intra-tumor injection

ABBREVIATIONS

mIL4	mouse interleukin 4
PEI	polyethylenimine
SCID	severe combined immunodeficient
siRNA	small interfering RNA
VEGF	vascular endothelial growth factor

ACKNOWLEDGEMENTS

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ABSTRACT

Angiogenesis is required for the development and biologic progression of glioblastoma multiform (GBM), which is the most malignant infiltrative astrocytoma. Vascular endothelial growth factor (VEGF) plays a predominant role in the increased vascularity and endothelial cell proliferation in GBMs driven by the expression of pro-angiogenic cytokines. In this study, we employed a vector-encoded VEGF siRNA to impair VEGF secretion from U87 human glioblastoma cells. The direct intra-tumor injection of a siRNA-encoding plasmid complexed with linear polyethylenimine (PEI) efficiently reduced the vascularization of treated tumors in xenografts established in SCID mice by subcutaneous inoculation of U87 cells, but was not able to reduce tumor growth. We then sought to strengthen the in vivo action of our siRNA by coupling it to a well known direct antiangiogenic agent, mouse interleukin 4 (mIL4). We infected U87 cells with a retroviral vector coexpressing the VEGF siRNA and mIL4 and produced stable cell lines that we used for an in vivo experiment of subcutaneous injection in SCID mice. In this setting, the concomitant expression of mIL4 and siRNA totally abolished the growth of subcutaneous tumors. These results suggest that our retroviral vector might be employed as a potential tool in future antiangiogenic gene therapy trials for glioblastoma.

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INTRODUCTION

Angiogenesis plays an essential role in the malignancy of solid tumors and it is generally accepted that tumor growth is strictly dependent on the capacity to acquire a blood supply. As a consequence, much effort has been directed towards the development of antiangiogenic agents able to disrupt this process. Vascular endothelial growth factor (VEGF) has been shown to play a key role in tumor angiogenesis[1] and, thus, represents an ideal target for an antiangiogenic therapy for most solid tumors. Glioblastoma multiform (GBM) is both the most common glial tumor and the most lethal, with a mean post-operative survival of less than two years[2] and is widely documented that the degree of neovascularization is directly correlated with the degree of malignancy of glioblastomas.[3] Among known angiogenic factors, glioblastomas and glioblastoma cell lines have been shown to overexpress VEGF.[4] RNA interference (RNAi) is a widely conserved RNA-mediated post-transcriptional gene silencing mechanism triggered by double stranded RNA.[5] This natural phenomenon has been developed into a powerful biotechnology tool to specifically inhibit mammalian gene expression[6] by delivering small interfering RNAs (siRNAs) into target cells. Recently, we[7] and others[8-10] have described the use of siRNAs to inhibit VEGF expression in several solid tumor models as a possible antiangiogenic gene-therapy approach. In the present study we demonstrate that the vector-based transduction of U87 glioblastoma cells with a retroviral vector encoding for a VEGF siRNA with interleukin 4 (mIL4) is a strong antitumor factor performing both immuno-modulating and direct antiangiogenic action[11,12] and blocks tumor growth in a xenograft mouse model.

MATERIALS AND METHODS

Short hairpin RNA (shRNA) plasmid constructs

Plasmid vectors. pSuper[13] was a kind gift of Prof. Marta Izquierdo, Universidad Autonoma de Madrid, Spain. Self-complementary inverted repeat sequences, spaced by a 9 base "loop" region, were synthesized as single strand oligonucleotides, phosphorylated in vitro and subsequently annealed by heating to 89°C for 10 minutes and cooled down to 69°C in 30 minutes, and then to 65°C for 10 minutes, in 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP, 25µg/ml BSA, 10%

DMSO. This results in the formation of dsDNA molecules with the respective sticky restriction site ends. Successful annealing was checked by agarose gel electrophoresis, and the double stranded molecules were cloned into BglII-HindIII sites of pSuper, thus obtaining plasmids pSuper-si175 and pSuper-si92, respectively. Correct inserts of transformants were confirmed by sequencing. The oligonucleotides encoding the VEGF siRNA175, targeting nucleotide 175 of human VEGF mRNA coding sequence, were as follows:

si175fw
5'gatccccTGGACATCTTCCAGGAGTAttcaagagaTACTCCTGGAAGATGTCCAtttttggaaa3';
si175rev
5'agcttttccaaaaaTGGACATCTTCCAGGAGTAtctcttgaaTACTCCTGGAAGATGTCCAggg3'.

Upper case letters represent siRNA nucleotides specifically annealing to VEGF mRNA.

SiRNA92, targeting rat, but not human VEGF mRNA, was used as a control. The corresponding DNA oligonucleotides used for cloning siRNA92 into pSuper were as follows:

si92fw
5'gatccccCAGAAGGGGAGCAGAAAGCttcaagagaGCTTTCTGCTCCCCTTCTGtttttggaaa3';
si92rev
5'agcttttccaaaaaCAGAAGGGGAGCAGAAAGCtctcttgaaGCTTTCTGCTCCCCTTCTGggg3'.

Retroviral vectors. To produce "double copy" retroviral vectors encoding siRNA175 or siRNA92, retroviral vector pBabepuro[14] (a kind gift of Prof. M. Izquierdo, Universidad Autonoma de Madrid, Spain) was used; the complete coding cassettes harboring the H1 promoter and shRNA coding sequences were excised EcoRI-HindIII from pSuper-si175 and from pSuper-si92. The obtained fragments were then blunt-ended by Klenow fill-in and cloned into the blunt-ended unique NheI site of pBabepuro, located in the 3'LTR region. The plasmids obtained were named pBabepuro-si175 and pBabepuro-si92, respectively. pBabepuro-mIL4 vector was constructed by cloning mouse IL4 cDNA, excised from plasmid pBluescriptSK(-)-mIL4 (a kind gift of Prof. G. Finocchiaro, Istituto Neurologico Besta, Milano, Italy), into the EcoRI site of pBabepuro. The vector pBabepuro-mIL4-si175, coexpressing mIL4 and siRNA175, was prepared by cloning the H1promoter-shRNA175 cassette into the blunt-ended NheI site of pBabepuro-mIL4.

Cell culture and transfections. The human glioma cell line U-87 MG was obtained from American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum, 100U/ml of penicillin G sodium, 100 μg/ml of streptomycin-sulphate in a humidified atmosphere containing 5% CO_2 at 37°C. All media and serum were purchased from Invitrogen s.r.l.

Transfections were performed with Lipofectamine-2000 reagent (Invitrogen) using 4 μg of plasmid DNA in Optimem (Invitrogen), as recommended by the manufacturer. For transient transfections, 4 μg of shRNA-encoding plasmids were cotransfected with a 1:4 relative amount of a reporter plasmid, pEGFP-C3 (Clontech), in order to monitor transfection efficiency. Cells were analyzed by fluorescence microscopy 48 hours after transfection to calculate the transfection efficiency. For each well, the cell number in four random microscopic fields was counted and transfection efficiency for each sample was estimated as the mean value of green fluorescent protein (GFP) expressing cells over 100 cells per field. A reproducible efficiency of approximately 40% was estimated as detected by fluorescence microscope analysis of green fluorescent, positive cells. Cells were counted and conditioned media were collected and subjected to a short centrifugation (10 min 1000xg) to eliminate cellular debris 48 hours after transfection; supernatants were stored at -80°C and subsequently used for ELISA measurement.

Generation of virus particles and infection of target cells. The pantropic packaging cell line GP293 (Clontech) was grown in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin-sulphate in a humidified atmosphere containing 5% CO_2 at 37°C. The cells were transfected in 100 mm dishes by Lipofectamine-2000 reagent using 10 μg of the specific pBabepuro-based plasmid and 1 μg of pVSV-G (Clontech). The supernatant was collected 48 hours post-transfection, then filtered through 0.45 μm syringe filter and stored at -80°C or directly used to infect target U87 cells. For infection, U87 cells were plated the day before infection at 100.000 cells per 60 mm dish, and the next day virus supernatant was added with polybrene 8 μg/ml for six hours. Then supernatant was replaced and selection was started after 48 hours with puromycin 2 μg/ml.

RNA extraction and RT-PCR analysis. RNA was isolated from cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, Total RNA (3 μg) was reverse-transcribed in 25μl final volume, in the presence of 100 pmoles random hexaprimers (pdN6) for 5' at 70°C followed by 60' at 37°C, using MMoLV Reverse Transcriptase (Promega), and following the manufacturer's recommendations. Then, a PCR reaction was carried out on 10 μl of RT reaction using 3 U Taq DNA polymerase (Eppendorf) in a total volume of 50 μl. Amplification was performed for 35 cycles (1' denaturing at 94°C, 1' annealing at 55°C, and 30'' extension at 72°C) in a thermal cycler (Hybaid). Primers specific for mIL4 were: forward 5'-AGGATTTGTTAGCATCTCTTG-3', reverse 5'-TTCATGGTGCAGCTTATCGAT-3' and primers for β-actin: forward 5'-GGCCCAGAGCAAGAGAGGTATCC-3', reverse 5'-AGCCACGATTTCCCTCTCAGC-3'. The expected amplified products were 414 bp for mIL4, and 417 bp for β-actin. Amplification results were analyzed by 1.5% agarose gel electrophoresis and visualized under UV illumination after staining with ethidium bromide.

Northern blot analysis. Total RNA (25 μg) was separated on a 12% denaturing urea/polyacrylamide gel. The RNA was then transferred to Hybond N^+ nylon membrane by electroblotting. Probes (si175: 5'TGGACATCTTCCAGGAGTA3'; U6 snrna: 5'GGAACGCTTCACAATTTGCG3') were generated by T4 polynucleotide kinase (New England Biolabs) mediated end-labeling of DNA oligonucleotides with (γ^{32}P) ATP.

ELISA measurement of VEGF secretion. To quantitate the secreted VEGF in conditioned media of transfected cells, human VEGF ELISA was performed using human VEGF Duo Set ELISA kit (R&D Systems), following the manufacturer' s protocol using 100 μl of sample, in triplicate. Assays were repeated at least twice. VEGF concentration was normalized by dividing for the total number of cells or by the total amount of proteins, expressed as μg of total proteins and measured by a standard Bradford assay (Sigma-Aldrich).

Plasmid/polyethylenimine formulations. Linear polyethylenimine (PEI) (25 kDa, Polysciences Inc., Warrington, PA 18976) 100 mM in nitrogen residues was prepared in 20 mM Hepes—5% glucose, pH 7.00. For 50 μg of plasmid DNA, 20 μl of PEI were used, corresponding to a N/P ratio of 13.5. A PEI/plasmid DNA mixture was prepared as previously described[15] by slowly adding DNA to the PEI solution and gently pipetting to thoroughly mix. The solution was then allowed to incubate at room temperature for 15 minutes before use.

In vivo tumor xenograft models. In DNA therapy experiments, to establish U87 tumor xenografts in mice, 5-week-old B-17 SCID mice (Harlan Italy S.R.L.) were injected subcutaneously (s.c.) in the right flank with 1.0×10^6 U87 cells in 100 μl of PBS. When all mice had developed a clearly palpable tumor, mice were randomized for DNA/PEI therapy in five experimental groups (n = 5). DNA/PEI formulations containing 50 μg DNA were directly injected into tumors every other day in a final volume of 60 or 70 μl. Two groups of control mice received a solution containing PEI without DNA or medium alone, respectively.

In experiments involving preinfected U87 cell lines, single cell suspensions (1.0×10^6 cells) were inoculated s.c. into the right flank in 100μl of PBS. Four experimental groups were used (for each group, n = 5), plus one control group where animals were injected with w.t. U87 cells.

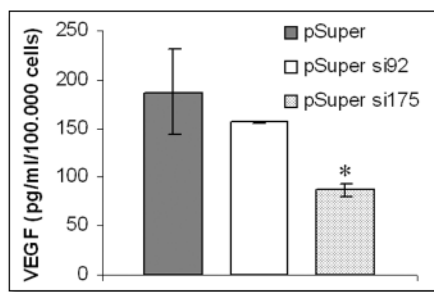

Figure 1. Knock-down of VEGF expression in transiently transfected U87 cells. Cultured supernatants of U87 cells transfected with the indicated plasmids were collected after 48 hours from transfection and secreted VEGF content was quantitated by ELISA. Data are representative of three individual experiments, and VEGF concentration (± st.dev.) is shown as pg/ml/ 100.000 cells. *p = 0.0286.

Tumor volume was determined by external measurements by a caliper and calculated as $V = L \times l^2 \times 0.5$, where L and l represent the larger and the smaller tumor diameter. In all cases, the observations were ended for ethical reasons when tumor volume became large compared with animal size and started impairing animal motility. At this time, animals were sacrificed and tumors were immediately frozen in liquid nitrogen or fixed for immunohistochemistry. Animal care was provided according to the institutional guidelines.

Immunohistochemical staining for CD31 expression. Rat monoclonal antibody against mouse CD31/PECAM-1 (clone Mec13.3; BD Pharmingen) was used at a concentration of 2.5 μg/ml. Secondary biotinylated anti-rat IgG (Vector, BA4001) were used at 1:200 dilution. For immunohistochemical analysis, tumors were harvested on ice, washed in PBS, embedded in OCT and stored at -80°C. All material was sectioned at 10 μm. Slides were fixed in cold methanol 5 min at -20°C and air dried. Sections were hydrated in PBS and then incubated in 0.05% H_2O_2 in PBS 20 min at room temperature in order to extinguish endogenous peroxidase activity. Following antigen retrieval pretreatment with 30 μg/ml proteinase K in 0.2M Tris-HCl, pH 7.2, for 30 min at 37°C, slides were incubated with 0.5% Blocking Reagent (NEN) for 30 min at room temperature, and left in primary antibody overnight at 4°C. As a control for non-specific binding of the secondary antibody, the anti-CD31 antibody was replaced by rat IgG in parallel slides. After incubation with a secondary biotinylated anti-rat antibody, staining was revealed using the Tyramide Amplification System (TSA-Indirect Kit, NEN Life Sciences). Slides were counterstained with Carazzi's hematoxylin for 15 seconds, dehydrated and mounted in Permount mounting medium. Images were taken using a Zeiss Axioplan2 microscope.

Differences in vascularity (number of PECAM-1-positive structures per microscopic field) were determined for each section. A total of three sections for each tumor, from two tumors per experimental group, were analyzed.

Statistical analysis. The results are presented as mean ± SD. Statistical analysis was performed using a Student's t-test or a Mann-Whitney test (Jandel Scientific), and all differences with $p < 0.05$ were considered significant.

RESULTS

Vector-based expression of VEGF siRNA175 reduces expression of VEGF in human glioblastoma cells in vitro. In order to obtain a molecular tool to inhibit VEGF expression in glioblastoma cells, we designed two distinct siRNAs targeting VEGF mRNA. Of these, siRNA175 is predictably able to cut human VEGF 165 and 121 mRNAs at 175 nucleotides from the start codon, and also all rat and mouse VEGF mRNA isoforms. Because siRNA92 is species-specific it was designed to cut specifically all rat VEGF mRNA isoforms, but not human nor mouse, at 92 nt from the AUG. For its characteristics, the plasmid encoding siRNA92 (pSuper-si92) was used as a negative control siRNA in our experimental setting, involving only glioblastoma cells of human origin.

We cloned DNA fragments encoding for our shRNAs into vector pSuper,[13] under the control of RNA polymerase III H1 promoter. The transcribed RNA is predicted to fold back to form a short hairpin RNA (shRNA), further cleaved by Dicer[16] to form the active siRNA actually cleaving the target mRNA.

We first assayed our shRNA-encoding construct in vitro to determine its efficiency to knock down VEGF expression in transient transfections of glioblastoma cells. U87 glioblastoma cells were transfected with pSuper-si175 and pSuper-si92 and VEGF expression was measured at 48 h after transfection by detecting the secretion of this growth factor in the conditioned media of transfected cells. A marked knock-down effect (-54%) was registered when pSuper-si175 was employed, while transfection with pSuper-si92 did not affect VEGF secretion, which was comparable to that of empty-vector transfected cells (Fig. 1). A similar reduction of VEGF production was obtained with pSuper-si175 also in C6 rat glioblastoma cells (-60%) (data not shown), thus validating the predicted cross-species activity of siRNA175. As a further confirmation of the predicted species-specificity of our VEGF siRNAs, in C6 rat cells, pSuper-si92 did work and reduced VEGF production, even if to a lesser extent than pSuper-si175 (-30%) (data not shown).

Intratumoral injection of the plasmid vector encoding VEGF siRNA175 strongly reduces tumor vascularization in vivo. Our in vitro data suggested that the decrease in VEGF secretion obtained by using plasmid encoded siRNA175 might be exploited also in an in vivo model of tumor growth and angiogenesis. To determine whether inhibition of VEGF secretion affected human glioblastoma angiogenesis in vivo, we induced U87 tumors in SCID mice, and treated mice with established tumors by direct intra-tumor injection of pSuper vectors, in the presence of polyethylenimine (PEI), a well known enhancer of transducing efficiency of naked DNA in vitro and in vivo.[17] As shown in Figure 2, treatments with pSuper-si175 were effective in reducing tumor angiogenesis, as measured by anti-CD31 immunohistochemical staining, while pSuper-si92 did not affect tumor vascular density. Tumors treated with the empty vector pSuper were thoroughly vascularized and undistinguishable from untreated or PEI-treated ones (data not shown). We counted 320 ± 119.53 vessels in pSuper-treated tumors versus 66 ± 22.59 vessels in pSuper-si175-treated ones; the vessels found in pSuper-si92 tumor section were 116.5 ± 37.5, but we are aware that this is an underestimation, since the sample underwent a partial deterioration that impaired the labeling in some areas.

However, injection of pSuper-si175 complexed with PEI, even if active against vascularization, was not able to reduce tumor growth (Fig. 2). We also tried to improve our DNA/PEI complex injection schedule by augmenting the number of injections (up to six times in two weeks) and consequently the total amount of injected DNA, reaching 300 μg divided into six injections, but we did not obtain better results in terms of tumor growth (data not shown).

Construction of retroviral vectors encoding siRNA175 alone or in combination with the soluble antiangiogenic factor mIL4: Stable infection of U87 cells and reduction of VEGF secretion from infected cells. We wanted to embed our VEGF siRNA in a delivery vector able to efficiently transduce target cells, in vitro and in vivo. To date, the best characterized and most widely used stable expression methods for the delivery of siRNAs into mammalian cells are those based on retroviral vectors, integrating into the host cells genome. Thus, we cloned the entire shRNA175 transcription cassette under the control of H1 promoter into the 3'LTR of the retroviral vector pBabepuro. This specific location in the 3'LTR allows the cloned region to be duplicated during reverse transcription, with one of the duplicated copies being located in the 5'LTR of the integrated provirus, and outside the possible negative interfering effects of the strong LTR promoter.[18,19] The vector pBabepuro-si175 was used in vitro to infect U87 cells and then compared to the control vectors pBabepuro and pBabepuro-si92 that contained the inactive siRNA92. As shown in Figure 3A, siRNA175 was expressed in infected U87 cells after several passages of selection (lanes 2 and 3).

Based on our previous findings that the antiangiogenic action of siRNA175 per se might not be sufficient to impair glioblastoma tumor growth in vivo, we designed a vector where the expression of siRNA175 is flanked by that of a "direct" antiangiogenic factor, mouse interleukin 4

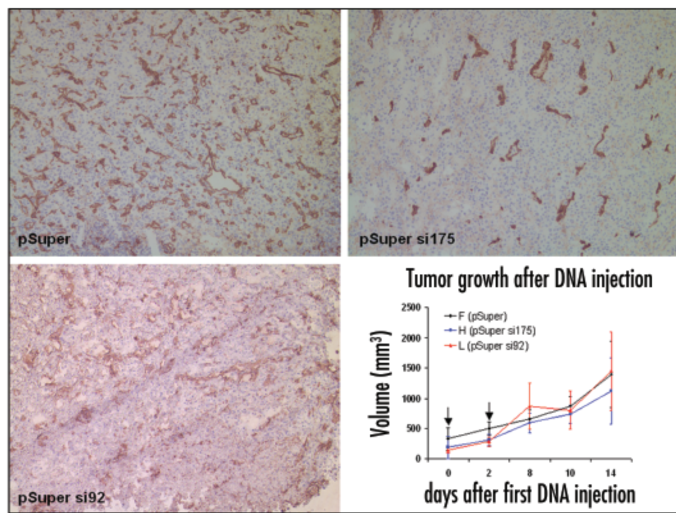

Figure 2. Effects of intra-tumor injection of a plasmid-based VEGF siRNA on tumor growth and on tumor vessel density in SCID mice. Vessel density was assessed using immunohistochemistry for CD31. Representative sections of tumors treated with empty vector pSuper, or anti-VEGF pSuper si175, or control plasmid pSuper si92, are shown. All sections are counterstained with hematoxylin. The graph shows subcutaneous tumor growth of treated tumors after plasmid DNA injection. All mice received a s.c. injection of U87 cells in one flank; when all of them had developed a clearly palpable tumor, mice were randomized into three groups (n = 5) and given 2 intratumoral injections of polyethylenimine/plasmid DNA. Tumor size was monitored and mice were euthanized when tumor mass started impairing viability. Arrows indicate DNA injections. Original magnification, × 10.

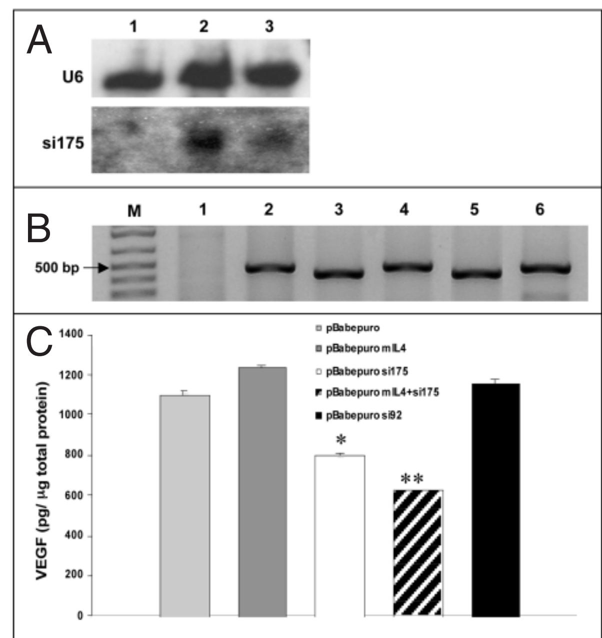

Figure 3. Retroviral infection allows the stable selection of U87 cells expressing VEGF siRNA175 and mIL4. VEGF secretion is permanently reduced in infected cells. (A) Northern blot analysis of U87 cells infected with pBabepuro (lane 1), pBabepuro si175 + mIL4 (lane 2), pBabepuro si175 (lane 3). Small RNA U6 is used as a loading control. (B) RT-PCR analysis of mIL4 expression in infected U87 cells. Lanes 1 and 2: pBabepuro; lane 3 and 4: pBabepuro-mIL4; lanes 5 and 6: pBabepuro si175 + mIL4. Lanes 1, 3 and 5 show the amplified fragment for mIL4, whereas lanes 2, 4 and 6 display the respective β-actin control bands. c) ELISA analysis of VEGF secreted into the conditioned media of infected U87 cells after several passages in selective medium. VEGF concentration is expressed in pg/µg total protein. *p = 0.014; **p = 0.001.

(mIL4). The expression of mIL4, placed under the control of the viral LTR promoter, was demonstrated in U87 cells infected with pBabepuro-mIL4-si175 vector (Fig. 3B, lane 5), as well as in cells infected with pBabepuro-mIL4, coding only mIL4 (Fig. 3B, lane 3). Cells infected with pBabepuro-mIL4-si175 efficiently expressed also siRNA175 (Fig. 3A, lane 2).

The anti-VEGF effects of the stable infection of U87 cells with the retroviral vectors encoding siRNA175 were assayed by measuring VEGF concentration in the conditioned media of established cell lines. Figure 3C shows that siRNA175 plays its anti-VEGF role in U87 cells infected with pBabepuro-si175, and, even more efficiently, in cells infected with pBabepuro-mIL4-si175. Neither the expression of mIL4 alone nor that of control siRNA92 affected VEGF secretion from the infected cells.

The combined expression of siRNA175 and of mIL4 blocks tumor growth in vivo. Next we investigated whether the combination of the direct antiangiogenic factor mIL4 with the indirect agent siRNA175 is effective in a s.c. xenograft mouse model of tumor growth. As clearly shown in Figure 4A, when U87 cells expressing siRNA175 were injected subcutaneously into SCID mice, the growth of the originated tumors was not sensibly different from that of control tumors. However, as previously observed with direct intratumor injection of plasmid DNA, when siRNA175 was delivered by a retroviral vector (pBabepuro-si175), the infected cells generated tumors often less vascularized than control ones, (pBabepuro alone) (Fig. 4B, the direct microvessel count revealed 51 ± 1.7 vessels in the control samples versus 37.7 ± 10.9 in the siRNA175 treated samples). Tumors expressing mIL4 alone grew more slowly than other tumors, confirming the powerful action of this lymphokine.[11] Most notably, when si175 and mIL4 were coexpressed in the same cells, thanks to the infection with pBabepuro-mIL4-si175, no tumor was ever generated in any of the injected mice.

DISCUSSION

Vascular endothelial growth factor (VEGF) is an angiogenic factor that plays a pervasive role in sustaining the growth of many solid tumors,[20] and for this reason, it has been often indicated as a promising target for antitumor therapy.[10,23] Glioblastoma multiform (GBM) is strictly dependent on the formation of an adequate vascular network for its progressive growth, and VEGF was shown to be the prominent angiogenic factor secreted by GBM.[24] The inhibition of VEGF production from GBM or the blockade of the relative intracellular pathway has been tried by several experimental approaches ranging from the use of anti-VEGF antibodies to that of specific inhibitors of VEGF receptors.[25-27] More recently, RNA interference has emerged as the ultimate molecular tool used to knock-down gene expression, and short interfering RNAs (siRNAs) have been designed to inhibit the expression of several genes related to tumor growth.[28,29] Various encouraging results were obtained in several different tumor models when VEGF was targeted either by employing siRNA oligonucleotides or by vector-mediated RNAi.[7-10,30] The data we are now reporting show that transduction of human glioblastoma cells with a plasmid encoding a VEGF siRNA is able to in vitro reduce the concentration of the secreted VEGF and consequently impairs the intravessel density of the originated tumors. This is observed when preestablished subcutaneous tumors are directly injected with the PEI-complexed plasmid vector, and also when a retroviral vector encoding the same siRNA is used to infect glioblastoma cells prior to their subcutaneous injection into SCID mice. However, both treatments are equally ineffective in reducing tumor growth. This picture deeply changes when the expression of the VEGF siRNA is coupled, in the same retroviral

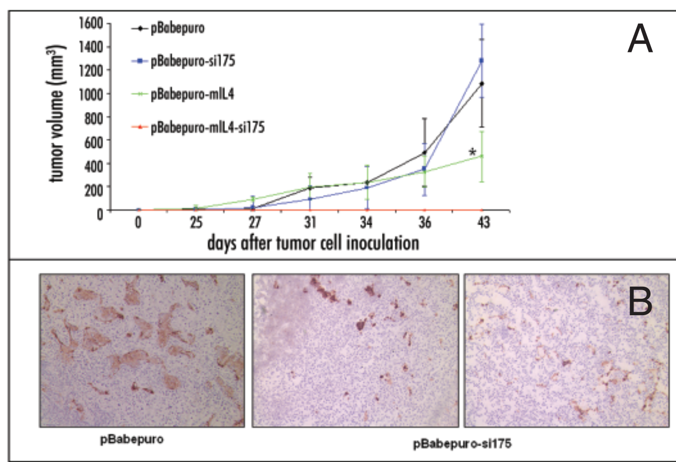

Figure 4. Effects of the stable expression of VEGF siRNA 175 and of mIL4 on subcutaneous U87 tumor growth and vascularization in SCID mice. (A) Tumor growth. Permanently infected U87 cells were subcutaneously injected into the right flank of SCID mice and the originated tumor growth was monitored. No tumors were ever produced in mice inoculated with U87 cells coexpressing si175 + mIL4. The volume values presented are the average (± st. dev.) of every group (n = 5) *p = 0.0206. (B) Immunoistochemistry for CD31. One section is shown from a tumor originated from a control mouse, inoculated with U87 cells infected with empty vector pBabepuro, and two sections are shown from tumors originated from U87 expressing VEGF siRNA175. Original magnification, × 10.

vector, to that of interleukin 4 (IL4), a crucial modulator of the immune system and an active antitumor cytokine.[12] IL4 is known to contrast tumor growth both by creating an immunostimulatory tumor microenvironment and by its own direct antiangiogenic action on endothelial cells.[11] It is very likely that the anti-tumor effect of IL4 we are reporting here is due to its antiangiogenic action rather than to the immunostimulatory effect of this cytokine since our animal model is an immunosuppressed one, the SCID mouse. The combined expression of these two factors leads to the total inhibition of tumor growth. This result is a further evidence in favor of the idea that antiangiogenic therapy can be successfully applied to aggressive tumors, such as GBM, only by using a combination of more than one antiangiogenic factor.[31] In fact, no data have ever been published so far about a successful application of VEGF siRNAs to block glioblastoma tumor growth, even if the same technology efficiently worked against other types of tumors. For example, we used the same approach against prostate carcinoma and obtained a significant reduction of tumor growth[7] which we accomplished also when we employed pSuper-si175 (our unpublished data).

Conversely, by the use of a direct angiogenesis inhibitor, mIL4, in conjunction with siRNA175, acting indirectly by reducing VEGF concentration, a double effect can be achieved that acts both on microvascular endothelial cells and on tumor cells, preventing their cross-talk with the endothelial cell compartment. This bimodal action can presumably explain the observed synergistic effect of the two factors; while siRNA175 specifically targets only VEGF, mIL4 can block angiogenesis by affecting a broader range of pathways involving endothelial cell activation.[32] It has been recently demonstrated, in fact, that IL4 performs its antiangiogenic action by directly interfering with cell cycle progression of endothelial cells by affecting p53, $p21^{Waf1}$, cyclin D1 and cyclin E expression.[33] The coexpression of these two factors by a single retroviral vector provides the opportunity to deliver the "therapeutic" vector directly and specifically where it is needed, which is inside the growing tumor mass. This local, versus a systemic administration is also a guarantee against the undesired side-effects that would on the contrary be produced by a systemic administration of two biologically active molecules, such as the VEGF siRNA and interleukin 4.[34]

In conclusion, we show that the antiangiogenic gene therapy of glioblastoma via a VEGF siRNA may deserve further clinical investigation, provided that this approach is flanked with another antitumor strategy. Even though glioblastoma confirms its aggressive nature which is directly linked to its high resistance to therapy, we believe that our results indicate that this refractory pathology can be effectively addressed by interfering with more than one pathway that leads to its development.

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