Targeted Systemic Radiotherapy with scVEGF/^{177}Lu Leads to Sustained Disruption of the Tumor Vasculature and Intratumoral Apoptosis

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Tumor vessels abundantly express receptors for vascular endothelial growth factor (VEGF), despite treatment with conventional or antiangiogenic drugs. We wished to determine whether the high levels of VEGF receptor (VEGFR) within the tumor vasculature could be leveraged for intracellular delivery of therapeutically significant doses of scVEGF/^{177}Lu, a novel radiopharmaceutical based on a recombinant single-chain (sc) derivative of VEGF, in orthotopic breast cancer models. **Methods:** scVEGF-PEG (polyethylene gycol)-DOTA conjugates containing 2.0-, 3.4-, or 5.0-kDa PEG linkers site-specifically conjugated to a cysteine-containing tag (Cys-tag) in scVEGF were radiolabeled with ^{177}Lu (scVEGF/^{177}Lu) for in vivo studies. Human MDA231luc and mouse 4T1luc cell lines were injected orthotopically to establish breast carcinoma tumors in immunodeficient and immunocompetent hosts, respectively. The effects of scVEGF/^{177}Lu were defined by analysis of changes in tumor growth and immunohistochemical staining for the endothelial markers CD31 and VEGFR-2 and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining for intratumoral apoptosis. **Results:** Biodistribution assays and dosimetric calculations established that scVEGF/^{177}Lu with a 3.4-kDa PEG linker delivered the highest dose of radiation to tumors (69.9 cGy/MBq/g of tissue) and the lowest dose to the kidneys (33.3 cGy/MBq/organ). Total doses below 40 MBq/mouse of scVEGF/^{177}Lu did not affect renal function, and 3 divided doses of 6.3 MBq/mouse or a bolus dose of 18.9 MBq/mouse induced only transient lymphopenia and weight loss (<10% baseline weight). In mice with orthotopic mammary breast carcinoma, intravenous injections of well-tolerated bolus and fractionated doses of scVEGF/^{177}Lu in the range from 6.3 to 18.9 MBq/mouse (25–76 MBq/m^2) resulted in dose-dependent tumor growth inhibition. Immunohistochemical analysis of tumors at 4–5 wk after single injections of scVEGF/^{177}Lu indicated dose-dependent regression of tumor vasculature and widespread intratumoral apoptosis. A single dose of 7.4 MBq/mouse of scVEGF/^{177}Lu given before a course of bevacizumab or sunitinib treatment enhanced the antiangiogenic effects of both drugs. **Conclusion:** Selective targeting of VEGFR in tumor vasculature with well-tolerated doses of scVEGF/^{177}Lu is effective in orthotopic breast cancer models. As high levels of VEGFR expression in the tumor vasculature are a common feature in a variety of cancers, targeting tumor angiogenesis with scVEGF/^{177}Lu warrants further exploration.

Key Words: VEGF receptors; ^{177}Lu; targeted radiotherapy; angiogenesis; breast cancer

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Vascular endothelial growth factor (VEGF) and its receptors (VEGFR) play a central role in the development and maintenance of the tumor vasculature (*1*). Because of the central role of VEGF or VEGFR signal transduction pathway in tumor angiogenesis, it was hoped that treatment with anti-VEGF antibodies, traps, or VEGFR tyrosine kinase inhibitors would result in vascular regression and tumor starvation, with minimal side effects (*2,3*). Unfortunately, despite continuous overexpression of VEGFRs, endothelial cells (ECs) of the tumor vasculature develop an evasive resistance to VEGF or VEGFR signaling inhibitors (*4,5*), which supports renewed tumor growth, reducing or eliminating any potential improvement in overall patient survival. Thus, to achieve a significant increase in the efficacy of antiangiogenic therapy, it is necessary to develop strategies that lead to a sustainable regression of tumor vasculature.

One feasible strategy is to use the abundant expression of easily accessible VEGFR in tumor vasculature for targeted intracellular delivery of therapeutic radionuclides. We have previously exploited VEGFR overexpression in tumor vasculature for selective SPECT and PET of these receptors (*6,7*) and their response to antiangiogenic therapy (*8,9*), using scVEGF, an engineered single-chain (sc) form of VEGF (*6–10*) for the intracellular delivery of ^{64}Cu, ^{68}Ga, and^{99m}Tc. In the current study, we chose ^{177}Lu as our radiotherapeutic isotope because of its relatively mild β-emission (~1.5-mm maximum penetration in soft tissue) capable of inducing bystander cytotoxic effects in tumors but not in surrounding host tissue (*11*), its logistically convenient intermediate half-life (6.7 d), and its commercial availability under good manufacturing practice conditions for possible future clinical development.

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We report here that scVEGF/^{177}Lu given at well-tolerated doses induced sustained disruption of the tumor vasculature, widespread intratumoral apoptosis, and inhibition of tumor growth in orthotopic breast cancer models. Furthermore, the lowest effective dose of scVEGF/^{177}Lu given before treatment with the widely used anti-VEGF antibody bevacizumab (Avastin; Genentech/Roche) or the oral tyrosine kinase inhibitor sunitinib (Sutent; Pfizer) enhanced tumor growth inhibition. Our results suggest that systemic radiotherapy with scVEGF/^{177}Lu could be a useful pretreatment adjuvant, particularly for the treatment of tumors for which treatment options are severely limited.

MATERIALS AND METHODS

Primary Tumor Models

The animal protocols were approved by the Stanford University Institutional Animal Care and Use Committee. Orthotopic 4T1luc breast tumors in 5- to 8-wk-old female BALB/c mice and MDA231luc breast tumors in 5- to 8-wk-old ICR (Institute of Cancer Research, Fox Chase)–severe combined immune-deficient (SCID) mice (ICR and C.B-17-SCID background; Taconic) were generated as described (*6,8*). Tumor volume was calculated as $V = 0.52 \times A^2 \times B$, where A is the short axis and B is the long axis of a tumor. Tumor doubling time (Td) was calculated assuming an exponential pattern of tumor growth. Treatment with scVEGF/^{177}Lu injected via the infraorbital venous sinus, or drug therapy, was initiated 2–3 wk after tumor cell implantation, when tumors reached 80–250 mm^3.

Assessment of Systemic Radiotoxicity in Normal Mice

The maximally tolerated dose of scVEGF/^{177}Lu was defined as the highest dose in which there were no deaths, signs of infection or distress, or a weight loss greater than 20% of baseline over an 8-wk period of observation. Maximally tolerated dose was assessed with groups of 10 adult male 5- to 8-wk-old Swiss Webster mice as previously described (*12*). In addition, blood tests of bone marrow and renal function, including white blood cell with manual differential and platelet counts or blood urea nitrogen (BUN) and creatinine, respectively, were required to normalize by week 8 after initiation of radiotherapy.

Preparation of scVEGF/^{177}Lu

^{177}Lu was obtained from Perkin-Elmer at a specific activity of 888–1,100 GBq/mg (24–30 Ci/mg) in 50 mM HCl. The optimized protocol for DOTA chelation of ^{177}Lu (*13*) was used, with minor modifications for radiolabeling of scVEGF-PEG-DOTA conjugates prepared as described previously (*6,10*). The optimal protein-to-^{177}Lu molar ratio was within a 1.2–2.0 range, with the final protein concentration not lower than 0.5 mg/mL. Radiolabeling was performed in 100 mM NaAc buffer, pH 5.0, for 20 min at 55° C, followed by addition of ethylenediaminetetraacetic acid to a final concentration of 1 mM. The functional activity of the scVEGF is not affected by these loading conditions (*6,7,10*). scVEGF/^{177}Lu was purified on NAP-5 columns in 50 mM NaAc, pH 5.8. Radiochemical purity, determined by radio–thin-layer chromatography with Tec-Control dark green chromatography strips (Biodex) developed in 0.1 M NH_4Ac, 50 mM ethylenediaminetetraacetic acid, pH 6.0, was repeatedly greater than 96%, with a specific activity of 2.2–2.9 MBq/μg of protein.

Serial Biodistribution Assay

Tracer (7.4 MBq) was injected via the infraorbital venous sinus into tumor-bearing mice, and groups of 10–11 mice were sacrificed at 1, 4, 24, 96, or 168 h after injection. Organs and tissues were removed, rinsed in phosphate-buffered saline, weighed, and counted in a γ-well counter (1470 Wizard γ-Counter; Perkin-Elmer) along with 3 samples of standard activity (1/100 of an injected dose in a 1-mL volume of water) at energy levels and windows for ^{177}Lu. Uptake of radioactivity per organ and per remaining carcasses for individual mice was then used to calculate the average percentage injected dose per gram of tissue (%ID/g).

Dosimetry of scVEGF/^{177}Lu in Tumor-Bearing Mice

Time dependence of biodistribution was used for dosimetric calculations of β-doses in mice, using the MIRD format (*14*). To calculate the residency time, for each organ or tissue, time-dependent changes in biodistribution expressed as %ID/g versus time in hours were integrated via the trapezoidal method in the first 24–48 h, followed by exponential integration when the down slope of the curve stabilized. To obtain total absorbed dose, the residency times for each organ or tissue were multiplied by published S values of ^{177}Lu in rats (*15*) and were then expressed as centigrays per megabecquerel of injected activity per organ or per gram of tumor tissue. Because of the relatively low-energy ^{177}Lu decay (~1.5-mm penetration), it was assumed that there was no cross-radiation between organs to simplify the calculation of absorbed dose.

Autoradiography was performed as previously described (*8*) and analyzed using ImageJ software available from the National Institutes of Health.

Immunohistochemical Staining

Cryosections (20 μm) were stained as described previously (*7,8*). For quantitative analysis of avascular areas, color images were acquired with a microscope objective (×4) and exported into Photoshop (Adobe). The whole image was covered with the lattice of 100 × 100 μm squares, and the fraction of squares without immunostaining for either CD31 or VEGFR-2 was determined.

Statistical analysis was performed with Number Cruncher Statistical Software (version 2007; NCSS), which includes routines to test datasets for the validity of assumptions of normal distribution and equal variance. Data that met these assumptions were analyzed by 1-way or 2-way ANOVA, and post hoc comparisons were performed with a Dunnett 1-sided multiple-comparison test. The P values of individual pairwise comparisons were calculated from the F distribution. Statistical comparisons of data that were not normally distributed were made by the distribution-free Kruskal–Wallis multiple-comparison Z-value test (Dunn test). Linear regression analysis was applied to estimate radiopharmaceutical dose effects on tumor doubling times.

RESULTS

Biodistribution and Dosimetry of scVEGF/^{177}Lu for Linker Optimization

To select the radiopharmaceutical with the most favorable characteristics, we synthesized scVEGF-PEG-DOTA conjugates with 2.0-, 3.4-, and 5.0-kDa PEG linkers, loaded each with ^{177}Lu, and analyzed biodistribution. Dosimetric calculations based on biodistribution in a 4T1luc tumor model showed that the presence of the smallest 2.0-kDa PEG linker in scVEGF/^{177}Lu resulted in the lowest

nontarget dose to the marrow, spleen, liver, and kidneys, as compared with the 3.4- and 5.0-kDa PEG linkers; however, the 2.0-kDa PEG linker also had a greater than 3-fold decrease in tumor dose (Table 1). On the other hand, a 5.0-kDa PEG linker in scVEGF/^{177}Lu, compared with a 3.4-kDa PEG linker, did not improve delivery of radiation to tumor and resulted in higher nontarget doses to the bone marrow, spleen, and liver (Table 1). We therefore selected scVEGF/^{177}Lu with the 3.4-kDa linker for biodistribution and dosimetry calculations in MDA231luc tumor–bearing mice (Table 1). Interestingly, delivery of radiation to tumor and kidneys in MDA231luc tumor–bearing SCID/Ncr (National Institutes of Health, Cancer Research) mice was similar to that of 4T1luc tumor–bearing immunocompetent BALB/c mice but with a far lower dose to the marrow and spleen, which may be related to the relative lack of VEGFR-expressing immune cells in SCID mice, as compared with BALB/c mice. Taken together, dosimetry calculations indicated that among the tested variants, scVEGF/^{177}Lu with a 3.4-kDa PEG linker provided the best combination of maximal target and minimal nontarget delivery of radiation and therefore was selected for further studies; its biodistribution in 4T1luc tumor–bearing mice is shown in Table 2.

Tumor Uptake of scVEGF/^{177}Lu

In both 4T1luc and MDA231luc models, tumor uptake of scVEGF/^{177}Lu was 2–3 %ID/g at 4 h after injection (Table 2, for 4T1luc tumors), as expected for radiopharmaceuticals designed to selectively target only ECs, not tumor cells (quantitative analyses are given in the "Discussion" section). In agreement with data obtained with scVEGF-based SPECT, PET, and near-infrared fluorophore tracers in short-term imaging experiments (*6–10*), the distribution of radioactivity in tumor cryosections was remarkably heterogeneous for both models, with an average 3- to 4-fold higher activity within the so-called tumor angiogenic rim (Fig. 1, for MDA231luc tumors). Interestingly, heterogeneous distribution of radioactivity persisted for at least 1 wk after injection of scVEGF/^{177}Lu (Supplemental Fig. 1, for 4T1luc tumors; supplemental materials are available online only at http://jnm.snmjournals.org).

Safety of scVEGF/^{177}Lu

The use of scVEGF raises concerns about its potential proangiogenic and tumor growth–stimulating activity. However, we found that scVEGF-PEG-DOTA did not stimulate tumor growth of 4T1luc tumors, whether given at early or late stages of tumor progression (Figs. 2A and 2B). Cumulative doses of scVEGF-PEG-DOTA used in these experiments were 10- to 20-fold higher than those used in therapeutic experiments.

To assess radiotoxicity of scVEGF/^{177}Lu, normal adult male Swiss Webster mice were injected with radiopharmaceutical, in a dose range of 18.5–82 MBq/mouse, given either as a bolus or as 3 fractionated doses. No significant changes in BUN or creatinine levels were observed at a dose of 37 MBq/mouse (or less) at 8 wk after the start of radiotherapy (Fig. 2C). Mice maintained normal body weight at a total dose of 18.5 MBq of scVEGF/^{177}Lu/mouse, with a maximal weight loss of less than 10% of baseline at weeks 1–3 after administration of scVEGF/^{177}Lu, but this loss was regained and exceeded by approximately 10% at 11 wk (data not shown). Higher doses, either divided or given as a bolus, induced transient weight loss at weeks 1–3 approaching 20% of baseline and were considered nontolerable (data not shown). Hematologic assays of bolus doses (18.5, 37, or 74 MBq/mouse) demonstrated a transient fall in white blood cell count (comprising mostly a loss in lymphocytes) at weeks 1–3, which recovered to baseline by week 8 (Fig. 2D). Divided weekly dosing demonstrated a similar pattern for the lowest dose tested (6.3 MBq/mouse × 3), as compared with bolus; however, divided dosing induced a slower decline and a faster rebound of white blood cell count. Higher doses (12.2 or 25.9 MBq/mouse × 3) induced a prolonged lymphopenia without recovery by week 8. Hemocrit,

TABLE 1
Dosimetry of scVEGF/^{177}Lu Containing 2.0-, 3.4-, and 5.0-kDa PEG Linkers

	PEG length			
	4T1luc tumor			
Organ	2.0 kDa	3.4 kDa	5.0 kDa	MDA231luc tumor, 3.4 kDa
Heart	0.2	0.7	0.7	1.3
Lungs	0.3	0.8	0.7	0.5
Liver	0.9	1.8	2.4	5.3
Spleen	3.4	9.2	15.9	5.0
Kidney	13.1	33.0	32.5	39.7
Muscle	0.1	0.2	0.2	0.4
Tumor (/g)	17.6	69.9	61.6	58.7
Bone	1.8	4.2	29.5	4.0
Marrow	0.5	1.7	3.9	0.4

Data are cGy/MBq of injected activity.

TABLE 2
Biodistribution of scVEGF/^{177}Lu Containing 3.4-kDa PEG Linker in 4T1luc Tumor–Bearing BALB/c Mice

Organ	4 h	26 h	96 h	168 h
Blood	0.31 ± 0.042	0.11 ± 0.013	0.04 ± 0.004	0.06 ± 0.009
Heart	2.26 ± 0.12	1.42 ± 0.15	0.66 ± 0.074	0.15 ± 0.007
Lungs	5.58 ± 0.45	2.43 ± 0.46	1.45 ± 0.11	0.08 ± 0.006
Liver	7.18 ± 1.28	5.34 ± 0.96	2.71 ± 0.29	1.09 ± 0.28
Spleen	5.84 ± 0.82	4.71 ± 0.22	1.95 ± 0.33	4.03 ± 0.57
Kidney	58.3 ± 6.5	51.8 ± 5.1	29.8 ± 3.6	5.20 ± 3.9
Stomach	2.9 ± 0.13	1.7 ± 0.14	0.60 ± 0.08	0.20 ± 0.08
Small bowel	2.20 ± 0.62	1.30 ± 0.24	0.40 ± 0.04	0.10 ± 0.015
Large bowel	0.80 ± 0.26	0.80 ± 0.16	0.20 ± 0.06	0.20 ± 0.053
Muscle	0.50 ± 0.093	0.40 ± 0.026	0.20 ± 0.018	0.10 ± 0.02
Bone	1.30 ± 0.37	1.0 ± 0.18	1.0 ± 0.13	0.5 ± 0.18
Marrow	2.6 ± 0.25	2.0 ± 0.14	1.9 ± 0.061	0.40 ± 0.15
Tumor	2.32 ± 0.14	1.55 ± 0.1	0.76 ± 0.062	0.53 ± 0.10

Data are mean ± SD and expressed as %ID/g.

red blood cell indices and counts, and platelet counts remained within normal limits for all doses and schedules (data not shown).

Systemic Radiotherapy with scVEGF/^{177}Lu

A single injection of scVEGF/^{177}Lu at a dose of 18.9 MBq/mouse (2,835 MBq/m^2) inhibited growth of orthotopic MDA231luc tumors (Fig. 3A), reaching statistical significance at 1 wk after scVEGF/^{177}Lu injection ($P < 0.01$), and was sustained ($P < 0.0001$) for at least 4–5 wk. To analyze the effects of single injections of different doses of scVEGF/^{177}Lu on MDA231luc tumors, tumor growth in individual mice was approximated as exponential, and the characteristic tumor volume doubling time (Td) was calculated for each tumor. Regression analysis indicated that tumor doubling time was linearly related to scVEGF/^{177}Lu dose (Supplemental Fig. 2). The differences between treated and control mice were statistically significant at each dose ($P < 0.05$ at 7.4 MBq and $P < 0.01$ at 14.8 and 18.9 MBq), and there was a statistically significant difference between treatments with 7.4 and 18.9 MBq/mouse ($P < 0.05$, Fig. 3B). For further comparison, tumors were binned into groups with rapid ($4 < Td < 7$ d), intermediate ($7 < Td < 10$ d), and slow ($Td > 10$ d) growth rates. All control tumors were in the rapid growth group, whereas 60%–80% of tumors treated with 7.4, 14.8, and 18.9 MBq/mouse grew slower than any control tumor (Fig. 3C). Interestingly, even at the highest dose of scVEGF/^{177}Lu, there were individual mice with tumor growth not affected by the treatment. In mice treated with 2 lower doses, 1.35 and 3.7 MBq/mouse, the doubling times for all tumors were in the 4- to 7-d range (data not shown). On the other hand, in a more aggressive syngeneic 4T1luc tumor model, a higher dose of 34.6 MBq/mouse did not increase tumor growth inhibition beyond approximately 3-fold (Supplemental Fig. 3).

Considering that fractionated scVEGF/^{177}Lu might be better tolerated than bolus dosing, we compared the effects of a total dose of 18.9 MBq per mouse given either as a single injection or as three 6.3-MBq doses injected at intervals of 1 wk between the first and the second injections and 2 wk between the second and the third ones. Indeed, as shown in Figure 3D, fractionated dosing led to a significantly smaller body weight loss, relative to bolus treatment. On the other hand, a mean tumor doubling time for fractionated treatment was not statistically different from that for bolus treatment (mean Td, 8.0 vs. 8.7 d; $P = 0.34$), suggesting that fractionated dosing provided for better tolerability without a decrease in efficacy.

Effects of scVEGF/^{177}Lu Treatment on EC and Tumor Cell Survival

To assess the effects of scVEGF/^{177}Lu on survival of EC and tumor cells, tumors harvested at 4–5 wk after the beginning of treatment were processed for immunohistochemical staining for the endothelial markers CD31 and VEGFR-2 and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining for apoptosis. In agreement with previous reports (*9,11*), in highly vascularized MDA231-luc tumors there was an abundant immunostaining for the panendothelial marker CD31, with a significant fraction of ECs expressing VEGFR-2, whereas tumor apoptosis was barely detected (Fig. 4A, top). In contrast, in tumors harvested 33 d after a single injection (18.9 MBq/mouse), the prevalence of CD31 and VEGFR-2 was dramatically decreased, leading to the appearance of large avascular areas,

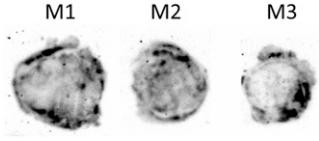

FIGURE 1. scVEGF/^{177}Lu preferentially accumulates in tumor angiogenic rim. MDA231luc tumors were excised and processed along with contralateral pectoralis muscles at 3 h after injection of scVEGF/^{177}Lu (3.2 MBq/mouse). Representative 60-μm sections of tumor for 3 individual mice (M1, M2, and M3) are shown. Images of contralateral pectoralis muscles are not visible at this contrast.

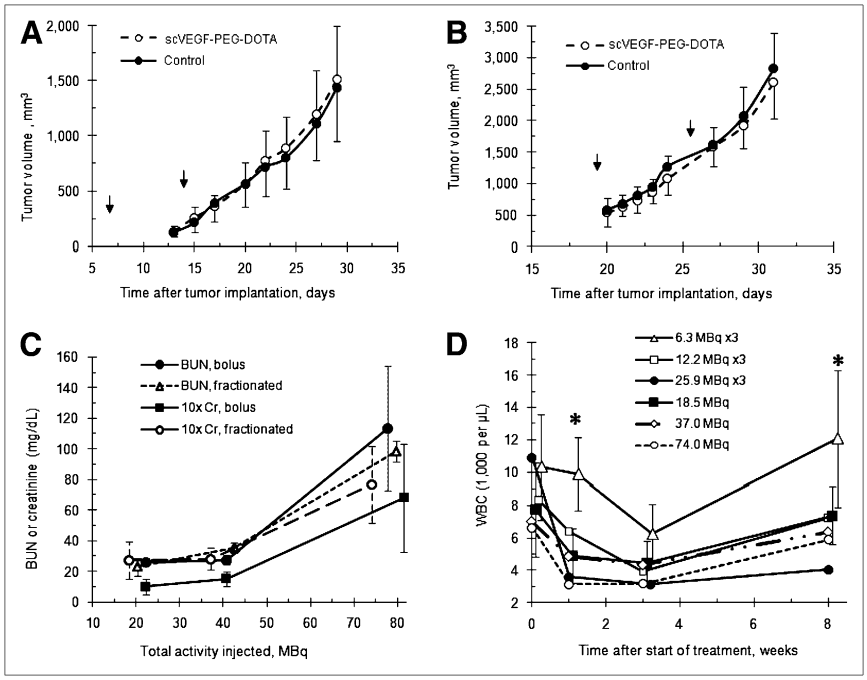

FIGURE 2. Safety of scVEGF/^{177}Lu. BALB/c mice (n = 11) received scVEGF-PEG-DOTA (43 μg/mouse, intravenously), as indicated by arrows, on days 8 and 15 (A) or 15 and 26 (B) after 4T1 cell implantation (10,000 cells/mouse). Control mice (n = 11) received saline injections on same days. (C) Levels of BUN and creatinine after scVEGF/^{177}Lu injection. Values for creatinine were multiplied by 10, to be displayed with the BUN values. (D) Total white blood cell count after scVEGF/^{177}Lu injection. Average values ± SD are shown. t test results comparing 6.3 MBq × 3 weekly doses to bolus of 18.5 MBq are shown. *$P < 0.05$. Cr = creatinine.

whereas apoptosis became widespread (Fig. 4A, bottom). To take into account the significant heterogeneity in CD31 and VEGFR-2 immunostaining, low-magnification images obtained for a large number of immunohistochemical sections from several mice in each group were overlaid with a lattice of 100 × 100 μm squares, and the percentage of avascular squares without immunostaining was determined for each image. Treatment with scVEGF/^{177}Lu induced a statistically significant increase in total avascular cross-sectional areas (Fig. 4B). In agreement with observations of tumor growth (Fig. 3D), bolus (18.9 MBq/mouse) and fractionated (3 × 6.3 MBq/mouse) scVEGF/^{177}Lu dosing led to a similar prevalence of avascular cross-sectional areas (Supplemental Fig. 4).

The sustainable effects of scVEGF/^{177}Lu on tumor vasculature and apoptosis were in a sharp contrast to the results

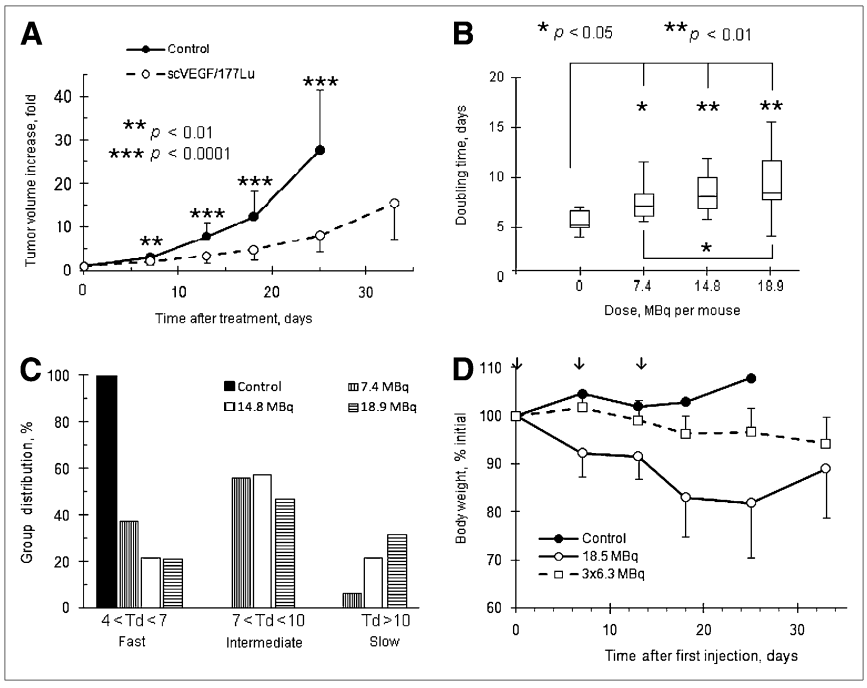

FIGURE 3. Effects of scVEGF/^{177}Lu on tumor growth. (A) MDA231luc tumor volume after single dose of scVEGF/^{177}Lu (18.9 MBq/mouse, n = 13). Control mice (n = 10) received single saline injection. For each mouse at each time point, measured volume was normalized on volume at day of treatment (day 0). (B) Tumor doubling time (Td) for scVEGF/^{177}Lu–treated mice is increased in dose-dependent manner. Td for control mice (n = 18) and mice treated with 7.4 MBq/mouse (n = 16), 14.8 MBq/mouse (n = 14), and 18.9 MBq/mouse (n = 19) are shown by box plots for which rectangles represent middle 50% of data points, whiskers represent minimum and maximum values, and lines represent median for each group. One-way ANOVA and post hoc multiple-comparison analysis revealed that Tds were significantly prolonged, compared with control group at 7.4-MBq dose ($P < 0.05$) and at 14.8- and 18.9-MBq doses ($P < 0.01$). Among scVEGF/^{177}Lu treatment groups, significant differences were observed only between 7.4- and 18.9-MBq doses ($P < 0.05$). (C) Distribution of tumors between rapid, intermediate, and slow growth at different scVEGF/^{177}Lu doses. (D) Fractionated 3 × 6.3 MBq/mouse dosing of scVEGF/^{177}Lu given at days 0, 7, and 14 is better tolerated than bolus dose of 18.9 MBq/mouse given at day 0.

obtained with antiangiogenic drugs currently used in the clinic, the VEGF neutralizing antibody bevacizumab and the VEGFR-2 tyrosine kinase inhibitor sunitinib. Although these drugs inhibited MDA231luc tumor growth, at 4 wk after the beginning of treatment (bevacizumab was given intravenously, on days 1, 4, 7, and 11, whereas the sunitinib group received the drug orally, daily, on days 1–7), there were no statistically significant changes in tumor vascularization in treated versus control mice (Supplemental Fig. 5).

Combination Therapy with scVEGF/^{177}Lu and Antiangiogenic Drugs Bevacizumab and Sunitinib

Because scVEGF/^{177}Lu appeared to inhibit tumor revascularization, we tested whether pretreatment of MDA231-luc tumor–bearing mice with the lowest effective dose of scVEGF/^{177}Lu (7.4 MBq/mouse) could enhance the efficacy of bevacizumab or sunitinib. Bevacizumab alone resulted in a significant tumor growth inhibition, with 80% of tumors growing with intermediate ($7 < Td < 10$ d) or slow ($Td > 10$ d) rates (Fig. 5A). Nevertheless, adding scVEGF/^{177}Lu pretreatment to bevacizumab therapy notably increased the fraction of slow-growing tumors from 10% to 44% (1/10 vs. 4/9 mice). Similarly, adding scVEGF/^{177}Lu pretreatment to the sunitinib regimen increased the fraction of slow-growing tumors from 10% to 33% (1/10 vs. 3/9 mice, Fig. 5B).

DISCUSSION

Several groups are exploring targeted delivery of radiotherapeutic isotopes to the tumor vasculature, relying on antibodies against biomarkers selectively expressed or overexpressed on tumor ECs, such as cadherins (*16*) or prostate-specific membrane antigen (*17*). However, 2 interlinked problems typically hinder clinical development of antibodies, namely, the relatively slow, natural internalization of antibody or antigen complexes, which necessitates high doses of antibodies to achieve continuous target occupancy, and the potential of an immune response against long-circulating antibodies, which is not always circumvented by humanized antibodies. In contrast, scVEGF-based tracers are rapidly internalized by ECs via VEGFR-2–mediated endocytosis and are rapidly cleared from the circulation (*6,7*), decreasing potential problems associated with the in vivo stability and off-target toxicity. As VEGFR-2 overexpression is a general feature of ECs in the tumor vasculature in a wide variety of solid and hematologic malignancies, the potential of scVEGF loaded with ^{177}Lu (or other therapeutic isotope) in treating many types of cancer is particularly attractive.

Although it is technically simple to randomly conjugate radionuclide chelators to amino groups in natural dimeric VEGF (*18–20*), the robustness and simplicity of scVEGF expression and facile, site-specific conjugation of the PEGylated chelator DOTA could greatly facilitate clinical development of scVEGF/^{177}Lu. Furthermore, the high specific radioactivity of scVEGF/^{177}Lu (~2.5 MBq/μg or ~78 MBq/nmol of scVEGF) achieved through a simple and scalable radiolabeling procedure allows for effective radiotherapy using as little as 3–5 μg of tracer per mouse (450–750 μg/m^2). These doses would be equivalent to human doses of 12–20 μg/kg or 0.72–1.2 mg for a 60-kg adult using body surface–based scaling (*21*). Although the safety of such doses for humans remains to be established, we report here that they are well tolerated by mice.

For therapeutic studies, we selected a scVEGF-PEG-DOTA conjugate with a 3.4-kDa PEG linker, because it provides for a better combination of specific and nonspecific scVEGF/^{177}Lu uptake than tracers with 2.0- or 5.0-kDa PEG linkers. As expected for a 32-kDa protein conjugate (and observed previously for scVEGF-based imaging tracers (*6,7*), the major off-target uptake of scVEGF/^{177}Lu occurs in the kidney. However, therapeutically effective doses of 18.9 MBq/mouse (2,835 MBq/m^2 or 76.6 mCi/m^2) or less are well tolerated, and systemic toxicity might be further decreased by fractionated dosing. Considering that similar doses are being used in current clinical trials with ^{177}Lu-radiolabeled antibodies (www.clinicaltrials.gov), we expect that radiotoxicity would not be a barrier for translational development of scVEGF/^{177}Lu. Nevertheless, several general strategies, such as coinjection with lysine or the use of plasma expanders, are available for decreasing kidney uptake of radiopharmaceuticals (*22*). Another strategy might be introducing certain point mutations that could selectively

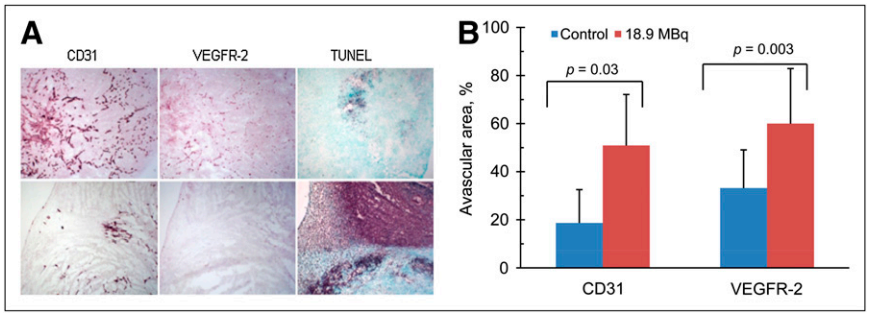

FIGURE 4. scVEGF/^{177}Lu causes sustainable regression of tumor vasculature and widespread apoptosis. (A) Representative immunohistochemical staining for CD31, VEGFR-2, and apoptosis in control mice (top) and mice treated with scVEGF/^{177}Lu (18.9 MBq/mouse), 33 d after treatment (bottom). (B) scVEGF/^{177}Lu–induced increase in avascular areas at 33 d after injection of 18.9 MBq/mouse: analysis of low-magnification (×4) fields (23 fields on CD31 immunostained cryosections from 6 control mice, and 17 fields on CD31 immunostained cryosections from 5 treated mice). Avascular areas are defined as fraction of immunostaining-free 100 × 100 μm squares in lattice covering tissue cross-section in low-magnification field of view. TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

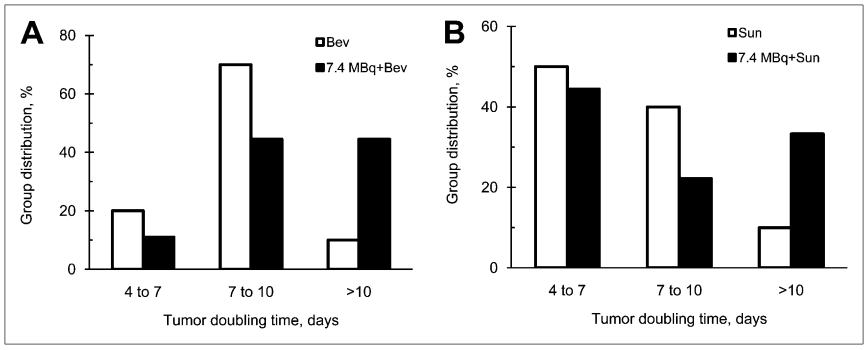

FIGURE 5. Effects of scVEGF/^{177}Lu pretreatment on therapy with bevacizumab or sunitinib. (A) Mice (n = 10) treated with bevacizumab alone (10 mg/kg, intravenously at days 1, 4, 7, and 11) or with scVEGF/^{177}Lu (7.4 MBq/mouse) at day 0, followed by similar bevacizumab treatment (n = 9). (B) Mice (n = 10) treated with sunitinib alone (80 mg/kg, via gavage feeding, daily for days 1–7) or with scVEGF/^{177}Lu (7.4 MBq/mouse) at day 0, followed by similar sunitinib treatment (n = 10).

decrease kidney uptake, as was reported for a VEGF-DOTA/^{64}Cu tracer based on human VEGF (*19*).

Tumor uptake of scVEGF/^{177}Lu (78 MBq/nmol) at 4 h after injection was approximately 2–3 %ID/g (4.7–7 pmol or ~2.9–4.35 × 10^{12} molecules of tracer per gram of tumor tissue), with the overall dose of radiation being 60–70 cGy/MBq per gram of tumor tissue. Assuming that tumor ECs constitute 3% of approximately 10^9 cells in 1 g of tissue, the calculated average uptake would be approximately 10^5 scVEGF/^{177}Lu molecules per EC; this value might be significantly higher for tumor angiogenic rim. Recent dosimetry modeling for several β-emitting radionuclides suggested that approximately 10^4–10^5 radionuclides per cell would be cytotoxic for ECs (*23*). Thus, it appears that a cumulative dose of 60–70 cGy/MBq distributed in ECs per gram of tumor tissue would be sufficiently cytotoxic for ECs and might provide for significant bystander killing. Notably, we did not observe any stimulation of tumor growth by scVEGF-PEG-DOTA, even at cumulative doses approximately 25-fold higher than the therapeutic doses of scVEGF/^{177}Lu, most likely because scVEGF, by design, lacks a mitogenic C-terminal domain (*24*).

Tumor growth inhibition induced by scVEGF/^{177}Lu is dose-dependent and appears to be sustainable for 4–5 wk after a single intravenous scVEGF/^{177}Lu injection. However, it is necessary to emphasize that there are significant variations in responses of individual MDA231luc tumor–bearing mice to treatment with scVEGF/^{177}Lu. Further studies will be required to characterize host factors that could affect responses of angiogenic vasculature to scVEGF/^{177}Lu treatment (*25,26*).

Immunohistochemical examination of MDA231luc tumors at 4–5 wk after treatment with scVEGF/^{177}Lu revealed the presence of large avascular areas, reflecting a sustained and dose-dependent vascular regression. Widespread apoptosis observed in tumor tissue at the same time points could be caused by starvation of tumor cells deprived of a functioning vascular network and by bystander radiotoxicity to tumor cells. These long-term effects of scVEGF/^{177}Lu treatment are in contrast with the transient effects of current VEGF/VEGFR inhibitors and vascular disrupting agents. Both drug types effectively induce vascular regression, which is followed by a rebound of drug-resistant tumor vasculature, which apparently reutilizes the emptied vascular channels (*3–5,27–31*). From this perspective, more sustainable scVEGF/^{177}Lu–induced vascular regression might be due to the bystander effects of ^{177}Lu that specifically radioablate vascular channels, damaging supporting cells, such as pericytes, or supporting proteins, such as EGFL7. Furthermore, damage to vascular channels might explain why combining pretreatment with the lowest effective dose of scVEGF/^{177}Lu (7.4 MBq/mouse) with the antiangiogenic drugs bevacizumab or sunitinib increased the proportion of slow-growing tumors and the appearance of large avascular areas.

CONCLUSION

We have demonstrated that targeting VEGRs in tumor vasculature with well-tolerated doses of a novel radiopharmaceutical, scVEGF/^{177}Lu, has a marked and persistent effect on the growth of orthotopic breast tumors in mice that is directly associated with dose-dependent destruction of the tumor vasculature and widespread intratumoral apoptosis. The effects of scVEGF/^{177}Lu on tumor vasculature appear to be more sustainable than those seen with sunitinib or bevacizumab monotherapy, and scVEGF/^{177}Lu pretreatment potentiates the effects of those drugs. Further studies of scVEGF/^{177}Lu in antiangiogenic therapy on other tumor models are warranted to expand on these promising results.

DISCLOSURE STATEMENT

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