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BIOLOGY CONTRIBUTION

IRRADIATION PROMOTES AKT-TARGETING THERAPEUTIC GENE DELIVERY TO THE TUMOR VASCULATURE

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Purpose: To determine whether radiation-induced increases in nitric oxide (NO) production can influence tumor blood flow and improve delivery of Akt-targeting therapeutic DNA lipocomplexes to the tumor.

Methods and Materials: The contribution of NO to the endothelial response to radiation was identified using NO synthase (NOS) inhibitors and endothelial NOS (eNOS)-deficient mice. Reporter-encoding plasmids complexed with cationic lipids were used to document the tumor vascular specificity and the efficacy of *in vivo* lipofection after irradiation. A dominant-negative Akt gene construct was used to evaluate the facilitating effects of radiotherapy on the therapeutic transgene delivery.

Results: The abundance of eNOS protein was increased in both irradiated tumor microvessels and endothelial cells, leading to a stimulation of NO release and an associated increase in tumor blood flow. Transgene expression was subsequently improved in the irradiated vs. nonirradiated tumor vasculature. This effect was not apparent in eNOS-deficient mice and could not be reproduced in irradiated cultured endothelial cells. Finally, we combined low-dose radiotherapy with a dominant-negative Akt gene construct and documented synergistic antitumor effects.

Conclusions: This study offers a new rationale to combine radiotherapy with gene therapy, by directly exploiting the stimulatory effects of radiation on NO production by tumor endothelial cells. The preferential expression of the transgene in the tumor microvasculature underscores the potential of such an adjuvant strategy to limit the angiogenic response of irradiated tumors. © 2007 Elsevier Inc.

Nitric oxide, Akt, Angiogenesis, Vasodilation, Gene therapy.

INTRODUCTION

Although it takes advantage of a spatially controlled delivery, radiotherapy is, like chemotherapy, subject to resistance through a variety of mechanisms. Besides hypoxia, which prevents the fixation of radiation-induced damage to DNA and thereby reduces the efficacy of radiotherapy (1), reactive signaling cascades may participate in the recovery of tumor growth after irradiation (2–5). The production of vascular endothelial growth factor (VEGF) by tumor cells in response to ionizing radiation is a good example of the induction of prosurvival pathways by radiotherapy itself (2, 4, 6). The consecutive re-stepping of the threshold level of apoptosis initiation is particularly critical in the context of fractionated radiotherapy. Indeed, by inducing VEGF-dependent pro-survival pathways, the administration of radiotherapy necessarily preconditions the tumor for next-day radiation delivery, and so on (4). Another consequence of the higher VEGF expression in irradiated tumors is the activation of pro-angiogenic signaling cascades, including the activation of Akt and endothelial nitric oxide synthase (eNOS) in the endothelial cells of the tumor vascular bed (3, 5). Furthermore, Akt phosphorylation was also documented to occur in endothelial cells in response to radiation without any requirement in tumor-derived growth factors (7). Targeting eNOS and Akt

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therefore seem to be potential adjuvant strategies to prevent postradiation angiogenesis.

Limitations are, however, present in developing therapeutic strategies to block the activity of eNOS and Akt in tumors. Indeed, although NO is involved in very specific signals governing tumor angiogenesis, it is also a powerful vasodilating agent that maintains cardiovascular homeostasis (8). Thus, the involvement of NO in the physiologic regulation of vascular tone impedes the use of pharmacologic NOS inhibitors to selectively target tumors. Similarly, Akt is a broad-spectrum kinase that exerts cytoprotective effects against the many pro-apoptotic insults that face living organisms (9). Therefore, inhibiting the phosphorylation-dependent activation of Akt would require a selective targeting of the tumor endothelium to exert antiangiogenic effects without altering healthy tissues.

In this study, we reasoned that the vasodilating effects of NO, if occurring in the tumor in response to radiation, could be exploited to enhance the delivery of drugs targeting postradiation, Akt-driven tumor angiogenesis. To add another potential layer of specificity, we chose to use, as an antiangiogenic drug, a plasmid encoding a dominant-negative form of Akt complexed with cationic lipids, which *per se* present a high tropism for the tumor vascular bed.

METHODS AND MATERIALS

Mice and cells

Male Rj:NMRI mice (Elevage Janvier, Le Genest Saint-Isle, France) were used in experiments with transplantable liver tumors (TLT) hepatocarcinoma cells (10), and mice deficient for the eNOS gene ($eNOS^{-/-}$) and the littermate control mice (The Jackson Laboratory, Bar Harbor, ME) with Lewis lung carcinoma (LLc) cells. Anesthetized mice (ketamine/xylazine) received intramuscular (i.m.) injections of 10^6 syngeneic tumor cells in the posterior right leg. The tumor diameters were tracked with an electronic caliper. When the tumor diameter reached 4.0 ± 0.5 mm (Day 0), mice were randomly assigned to a treatment group. Where indicated, they also received the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) (500 mg/L) in the drinking water from Day-1. Each procedure was approved by local authorities according to national animal care regulations. Human umbilical vein and bovine aortic endothelial cells were routinely cultured in 100-mm dishes in fetal calf serum–containing endothelial growth medium (Cambrex, Verviers, Belgium).

Irradiation

Anesthetized tumor-bearing mice were locally irradiated using the RT-250 device (Philips Medical Systems, Brussels, Belgium) with a dose delivery of 0.76 Gy/min. The tumor was centered in a circular irradiation field, and healthy tissues were protected by a lead mask. Confluent endothelial cells were irradiated using the same device.

Tumor blood flow monitoring

Tumor blood flow was measured with a laser Doppler imager (Moor Instruments, Axminster, United Kingdom), which maps cortical tumor perfusion (with a tissue penetration of approximately 2 mm), and with laser Doppler microprobes (OxyFlo; Oxford Optronics, Oxford, United Kingdom). For laser Doppler imaging, mice were anesthetized and fur was removed with a depilatory cream. Perfusion was evaluated on the basis of colored histogram pixels and normalized per unit of surface. The persistence of changes in perfusion when the tumor was surgically exposed to laser Doppler imaging ruled out any significant participation of skin vessels to our observations (not shown). For OxyFlo measurements, fiberoptic microprobes were inserted into the tumor and into the muscle of the opposite (healthy) leg. Backscattering measurements were used to validate the absence of movement artifacts. A 5-min stable recording line was acquired at a sampling frequency of 20 Hz. For both assays, the animals were placed on a heating pad (37°C) to minimize variations in temperature. Tumor blood flow was normalized against the perfusion level of the healthy opposite leg of the same animal.

Myograph assay

In our experimental tumor model (i.m. injection of tumor cells in the rear leg), saphenous arterioles and their branches are progressively co-opted as the tumor grows. After 2 weeks (of influence by the tumor microenvironment), they can then be isolated for evaluation of vasoreactivity (11). For this assay, tumor-bearing and healthy mice were locally irradiated (6 Gy or sham); 24 h later, tumor-co-opted vessels and size-matched healthy arterioles (diameters between 250 and 300 μm) were microdissected. After the vessels were mounted on a 110P pressure myograph (DMT, Aarhus, Denmark), changes in vessel diameters were tracked by videomicroscopy using Myoview software (DMT), as previously reported (11). Acetylcholine (100 μM) and the NOS inhibitor N^{ω}-nitro-L-arginine (100 μM) were used to determine the changes in eNOS activation.

In vivo and in vitro lipofection

Twenty-four hours after irradiation, tumor-bearing mice were injected (through the tail vein) with Effectene (Qiagen, Venlo, The Netherlands) cationic lipids complexed with a plasmid (2 μg per mouse) encoding either a hemagglutinin (HA)-tagged reporter protein, a green fluorescent protein (GFP), or a dominant-negative Akt form (DN-Akt) (12), all under the control of a cytomegalovirus (CMV) promoter. Sham-irradiated animals were used as controls. The GFP- and DN-Akt-encoding plasmids complexed with the Effectene cationic lipids were also used to transfect cultured endothelial cells; in these experiments, cells were first exposed to tumor necrosis factor α (40 U/mL) for 2 h (before transfection) to mimic *in vivo* tumor endothelial cell activation.

Immunoblotting and immunoprecipitation

At Day 0, TLT tumor-bearing mice were exposed (or not) to a single 6-Gy radiation dose. At Day +1, tumor microvessels were microdissected and cleared from connective tissues. Pools of five different vessels were used to reach sufficient amounts of proteins, as done previously (11). Immunoblotting was performed with antibodies directed against eNOS (BD Pharmingen, Erembodegem, Belgium), Akt and phospho-Akt (Cell Signaling Technology, Danvers, MA), or β-actin (Sigma Life Science, St. Louis, MO). In the experiments in which the HA-tagged reporter transgene was used, the whole tumors were collected at Day +4 and corresponding lysates were immunoprecipitated using a polyclonal anti-HA antibody before immunoblotting with a monoclonal HA-tagged antibody; all antibodies were from BD Pharmigen.

NOx and cyclic guanosine monophosphate measurements

Quantitative analysis of NOx species (NO_2^- and NO_3^-) production in cultured endothelial cells was used as an index of NO production (5). The determination of NO level (*e.g.*, the 24-h accumulation of NO derivatives in the cell-bathing [serum-deprived] medium) was carried out using the Nitric Oxide Colorimetric Assay (Roche Diagnostics, Mannheim, Germany).

Immunostaining

At Day +4, tumors were cryosliced, analyzed for GFP expression, and probed for the tumor vasculature detection with monoclonal antibodies against CD31 (BD Pharmingen) and tetramethylrhodamine isothiocyanate (TRITC)–labeled secondary antibodies. Sections were finally counterstained with Mayer's hematoxylin. Cryoslices of collected tissues were then examined with a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) equipped for fluorescence.

Statistical analyses

Data were normalized for the total amounts of proteins and are presented for convenience as mean ± SEM. Statistical analyses were performed using Student's *t* test or one-way analysis of variance where appropriate. Synergism was tested by comparing expected and observed tumor doubling times (after fitting individual lognormal tumor growth curves) using Welsch's test.

RESULTS

We first examined the influence of local tumor irradiation on the NO-dependent response of tumor blood vessels. Microvessels from irradiated TLT tumors were microdissected 24 h after irradiation and compared with size-matched vessels collected from nonirradiated tumors. Figure 1a reveals that a 6-Gy dose delivery induced a strong increase in vascular eNOS expression. To determine whether this approximately twofold increase in eNOS abundance (see Fig. 1b) was attributable to direct effects of irradiation on the tumor vessel endothelium, we repeated this experiment on cultured endothelial cells. Figure 1c shows that a 6-Gy irradiation similarly increased eNOS abundance in endothelial cells. Also, by measuring the amounts of NO end products (NOx) found in the culture supernatants, we confirmed that irradiation did stimulate NO release from endothelial cells and that eNOS was involved because the NOS inhibitor L-NAME completely

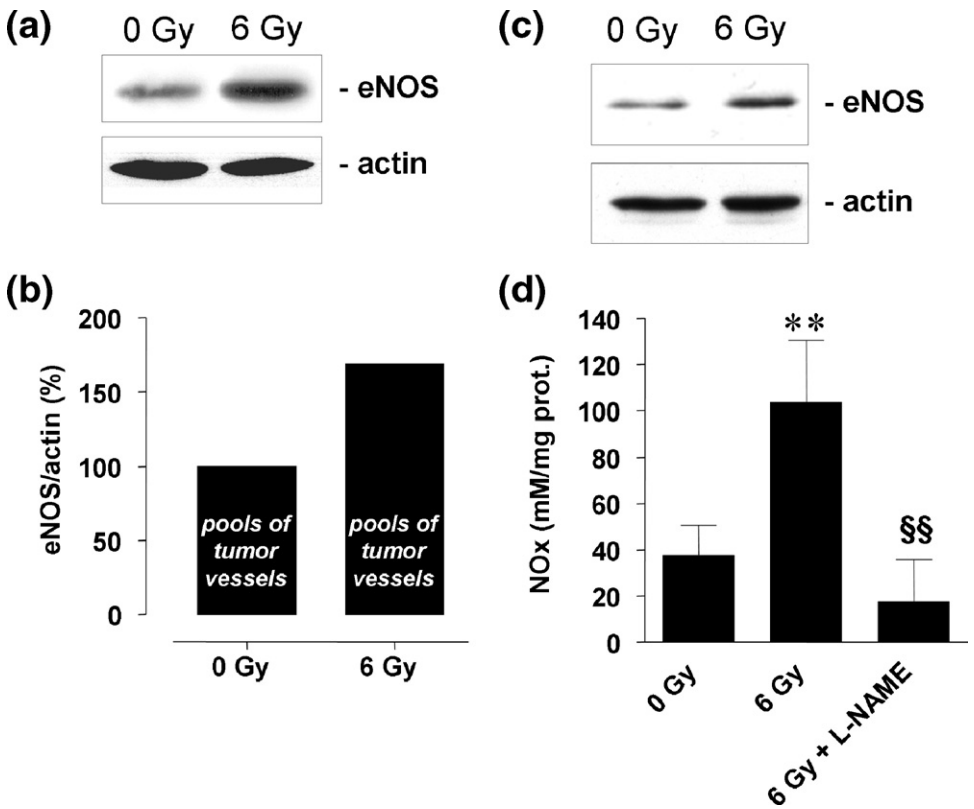

Fig. 1. Irradiation increases endothelial nitric oxide synthase (eNOS) expression and activity in isolated tumor blood vessels and cultured endothelial cells. (a) Microvessels were isolated from irradiated (6 Gy) or nonirradiated transplantable liver tumors (TLT) and pooled ($n = 5$) to reach sufficient amounts of material for eNOS immunoblotting. (b) The quantification of two independent experiments is presented; eNOS abundance is normalized vs. β-actin expression levels. (c) Cultured endothelial cells were irradiated (6 Gy) or not, and the effects on eNOS expression were determined 24 h later on corresponding lysates. (d) Bar graph represents the effects of irradiation on NO production determined in the same conditions; N^{ω}-nitro-L-arginine methyl ester (L-NAME) was also used to block NOS activity. $^{**}p < 0.01$ vs. nonirradiated endothelial cells; $^{\S\S}p < 0.01$ vs. 6 Gy; $n = 3$.

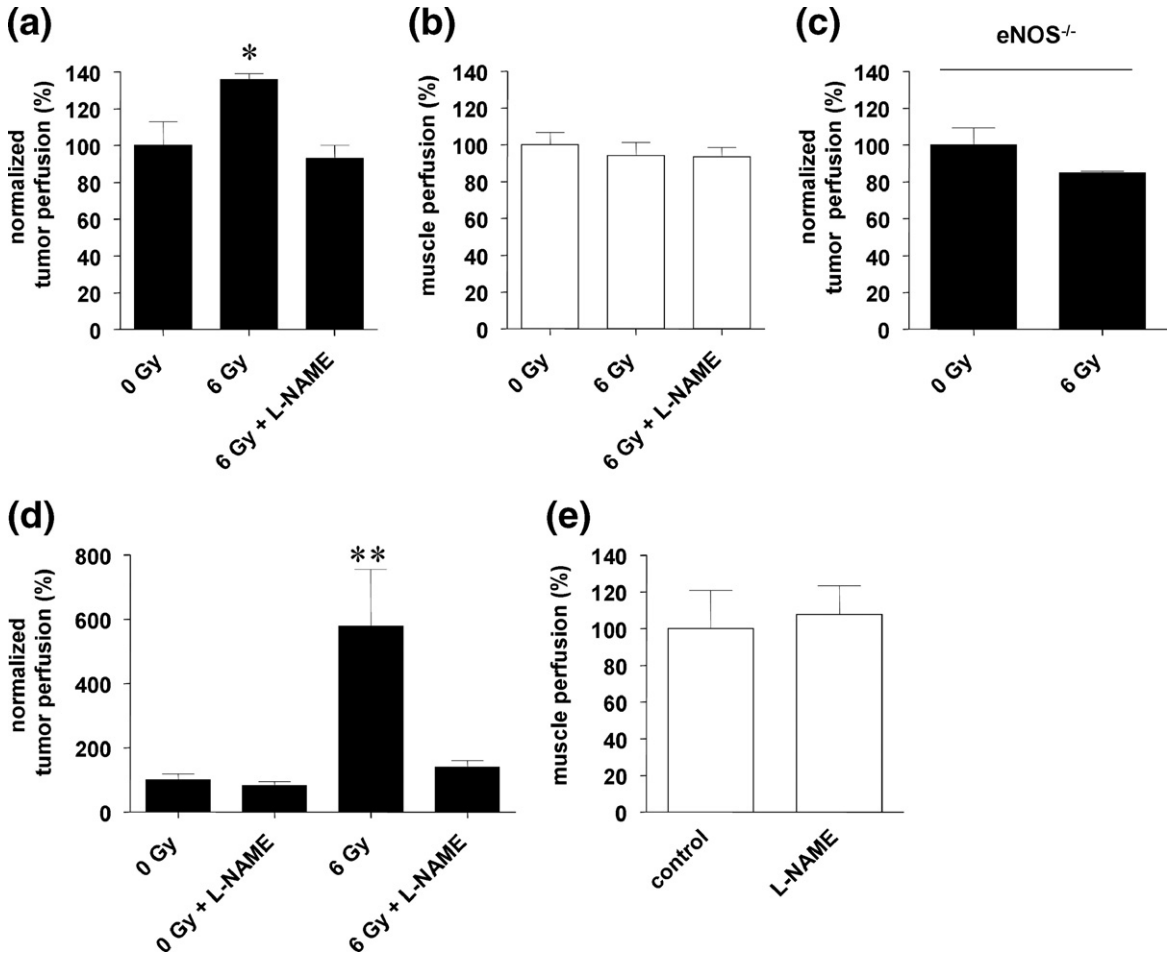

Fig. 2. Local tumor irradiation increases tumor blood flow through a vascular nitric oxide (NO)-dependent pathway. Laser Doppler imaging (a–c) and microprobes (d, e) were used to measure perfusion. (a) Bar graph presents the mean (± SEM) of tumor perfusion measured by laser Doppler imaging; data are normalized against the perfusion (shown in b) in a matched area of the contralateral healthy leg of the same animal. (c) Blood flow in sham-irradiated or 6 Gy-irradiated Lewis lung carcinoma tumors established in endothelial $NOS^{-/-}$ mice; data are normalized against the opposite leg perfusion. (d) Bar graph presents the mean (± SEM) of tumor perfusion measured by the OxyFlo microprobes; data are normalized against the perfusion (shown in e) of the contralateral healthy leg of the same animal. Data are expressed as percentage of perfusion in the untreated tumors. *$p < 0.05$; **$p < 0.01$ vs. 0 Gy; $n = 4–10$. L-NAME = N^{ω}-nitro-L-arginine methyl ester.

prevented the radiation effects (Fig. 1d). We also verified by immunoblotting that the expression of inducible NOS was not induced in irradiated endothelial cells (not shown).

We then used laser Doppler–based techniques to examine the potential impact of the NO increase on tumor perfusion in response to tumor radiotherapy. When compared with nonirradiated tumors, local 6-Gy irradiation induced a robust increase in the surface tumor blood flow, which peaked 24 h after treatment (Fig. 2a). Interestingly, the irradiation-induced increase in blood flow seemed to be exquisitely NO-dependent because it was completely prevented by the use of the NOS inhibitor L-NAME (Fig. 2a) and could not be reproduced in genetically eNOS-deficient mice (Fig. 2c). Also, the effects of radiation exposure on blood blow were restricted to the irradiated tumors, as witnessed by the unaltered perfusion in the contralateral leg (Fig. 2b) and the persistence of an increased perfusion when the tumor was surgically exposed to laser scanning (not shown). To further validate the above surface laser Doppler imaging data, we also used OxyFlo probes to measure perfusion deeper into the tumor mass. A similar L-NAME-sensitive increase in tumor blood flow was observed after irradiation (Fig. 2d). Of note, the 2-day administration of L-NAME in the drinking water (although sufficient to prevent eNOS activation in tumors) did not exert systemic hypertensive effects (see Fig. 2b and 2e), consistent with previous studies using this mode of administration (13).

To address the vascular specificity of this response, we measured the changes in eNOS activation in isolated microarterioles. Tumor and size-matched healthy vessels were irradiated (6 Gy or sham) *in vivo*, microdissected 24 h later, and mounted on a myograph. Endothelial NOS-mediated vasodilation was identified by challenging the arterioles with acetylcholine in the presence of an NOS inhibitor. As

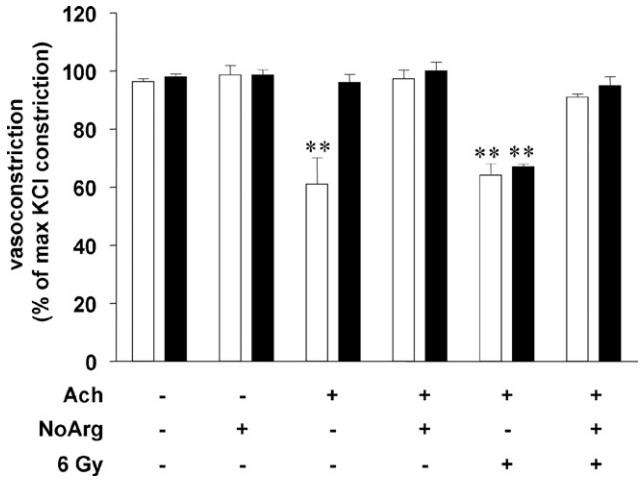

Fig. 3. Irradiation selectively promotes a functional nitric oxide (NO) pathway in tumor vs. healthy microvessels. Tumor-bearing and healthy legs of mice were locally irradiated (6 Gy) or not at Day 0. At Day +1, tumor (black bars) and size-matched healthy (white bars) microarterioles were dissected, mounted on a pressure myograph, and preconstricted with a KCl depolarizing medium. Changes in vessel diameters were tracked after exposure to 100 μM acetylcholine (Ach) in the presence (or not) of the NOS inhibitor N$^{\omega}$-nitro-L-arginine (NoArg, 100 μM). Results are expressed as percentage of maximal contraction. $^{**}p < 0.01$ vs. corresponding untreated microvessels; $n = 4$.

reported in Fig. 3, eNOS-mediated vasodilation, which was absent from tumor vessels, was fully restored after irradiation to the level reached in healthy vessels. Importantly, irradiation did not modify the eNOS-mediated dilation of healthy vessels, underlying the tumor specificity of the radiation effects on the vascular reactivity (Fig. 3).

Radiation-induced NO-mediated dilation of tumor blood vessels should theoretically lead to a better delivery of drugs to tumors through an increase in the exchange surface (14, 15). In this study, we chose to use cationic lipid vectors to carry therapeutic DNA to the tumor vasculature (16). In a first set of experiments, cationic lipids were complexed with plasmids encoding a reporter HA-tagged protein or the GFP and directly infused through the tail vein of tumor-bearing mice.

First, the use of the HA-tagged reporter allowed us to evaluate the expression of the transgene in the whole tumor by performing quantitative immunoprecipitation before detection of the reporter protein by immunoblotting. Figure 4a shows that the HA-tagged reporter protein was robustly expressed in the irradiated tumor-bearing wild-type mice, whereas it was barely detectable in the tumors of nonirradiated mice. To further examine the role of NO in the radiation-induced enhanced gene delivery, we also implanted LLc tumor cells in eNOS$^{-/-}$ mice. We found that the benefit of irradiation was totally lost when tumors were implanted in these mice (Fig. 4a), confirming the exquisite NO-dependency of the phenomenon. Of note, immunoblot analyses allowed us to exclude the presence of other enzymatic sources of NO (*i.e.*, inducible NOS or neuronal NOS) in tumor vessels as well as in tumor cells (not shown).

After the quantitative analysis of transgene expression, we focused on the topology of the irradiation-driven transgene expression using a GFP-encoding plasmid. Figure 4b shows that GFP expression was restricted to the vascular compartment of irradiated TLT tumors, as authenticated by CD31 co-immunostaining. Of note, although the lungs and the liver of the transfected mice were collected, no positive GFP signal (over background fluorescence) could be detected. To gain further insight as to whether the observed NO-dependent increase in tumor blood flow (see Fig. 2) or other effects of NO were involved, we also transfected cultured (activated) endothelial cells using the same cationic lipid complexes. Figure 4c shows that, *in vitro*, irradiation did not increase the efficacy of GFP-encoding plasmid transfection. Moreover, NOS inhibition did not significantly alter the extent of irradiated endothelial cells transduced with the plasmid.

We then explored the therapeutic potential of the combination of radiotherapy with the lipofection of a relevant gene targeting tumor angiogenesis. Accordingly, we used a plasmid encoding a dominant-negative form of Akt (DN-Akt), which we first validated in irradiated endothelial cells (considering the technical difficulties in detecting phosphoproteins in whole tumor). Figure 5a shows that the phospho-Akt signal was significantly decreased ($p < 0.01$) when endothelial cells were transfected with the DN-Akt construct (see Fig. 5b for quantitative analyses). Of note, no phospho-Akt signal was detectable from the lysates of nonirradiated endothelial cells (not shown).

We then examined the antitumor effects of the combination of a single 6-Gy radiation dose and the consecutive administration of the DN-Akt construct lipocomplex. Figure 5c shows that, whereas nonirradiated tumors with or without DN-Akt transfection grew very rapidly, the combination of radiation with the Akt-inactivating transgene therapy remarkably delayed tumor growth. Although irradiation alone also partly inhibited tumor growth, statistical analysis of the tumor growth curves revealed that transgene administration and ionizing radiation had synergistic effects ($p < 0.05$). Finally, we used radiotherapy in its fractionated mode and daily delivered a local 2-Gy dose to tumor-bearing mice over 5 days. The bar graph presented in Fig. 5d shows that the combination therapy was again more potent in delaying tumor growth than fractionated radiotherapy alone.

DISCUSSION

In contrast to tumor cells, which are genetically unstable, host cells retain full capacity of repairing postradiation damage. Endothelial cells in particular, which survive radiation exposure, are known to participate in tumor regrowth through processes involving angiogenesis (2–7). Here, we document that irradiated tumors exhibit an increase in tumor blood flow in response to radiotherapy and that the supporting mechanism, at least in part, involves eNOS. Hence, although irradiation has been reported to promote angiogenesis by increasing long-term NO production (5), our study demonstrates that more acutely, increased NO production

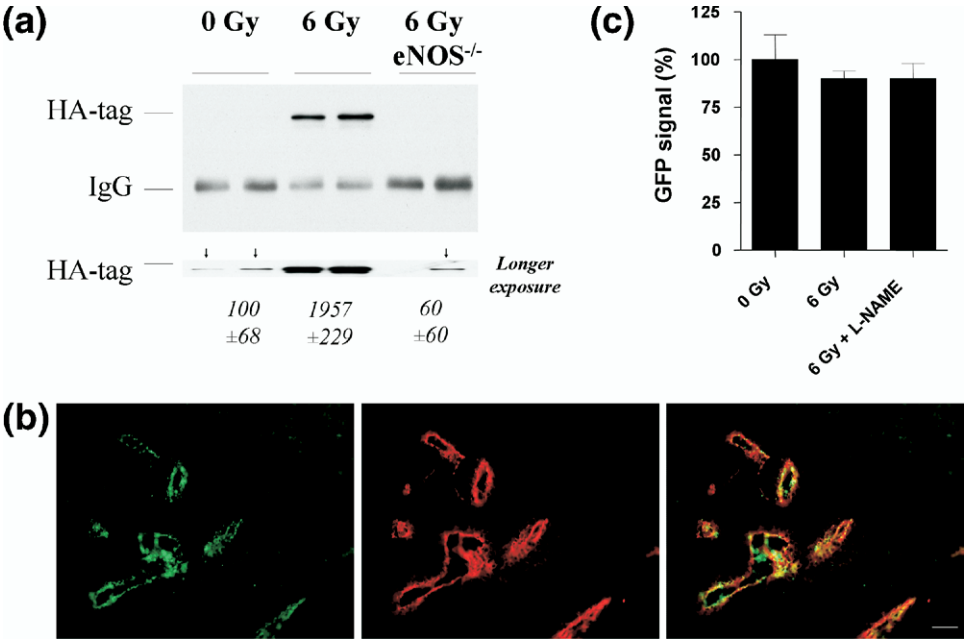

Fig. 4. Irradiation-induced nitric oxide (NO) production promotes the endothelial selective expression of transgenes carried by cationic lipids *in vivo* but not *in vitro*. At Day 0, tumor-bearing mice received local 6-Gy irradiation or not. At Day +1, lipocomplexes containing plasmids encoding either a hemagglutinin (HA)-tagged or green fluorescent protein (GFP) reporter protein were injected intravenously (i.v.). Tumors from control and irradiated animals were collected at Day +4. (a) Lewis lung carcinoma tumors were implanted in the leg of wild-type and endothelial NO synthase $(eNOS)^{-/-}$ mice. Equal amounts of proteins from corresponding lysates were immunoprecipitated with rabbit anti-HA antibodies (IgG) and immunoblotted with mouse anti-HA antibodies (HA-tag). The transgene expression was consistently detected in irradiated tumors of wild-type mice, whereas longer film exposure was required to detect the reporter protein in the other conditions (arrows). This experiment was repeated twice with similar results; densitometry-derived expression values are provided in italics. (b) Transplantable liver tumor (TLT) cryosections reveal the expression of the GFP transgene (green) and the CD31 co-staining of the tumor vasculature (red); merged images are also presented in a third panel. This experiment was repeated twice with similar results. Bar = 25 μm. (c) Bar graph represents the effects of pre-irradiation (6 Gy) on the expression of the GFP transgene in endothelial cells (in the presence or absence of N^{ω}-nitro-L-arginine methyl ester [L-NAME]). Data are expressed as percentage of the extent of endothelial cells expressing GFP in nonirradiated conditions. $n = 3$.

accounts for a stimulation of the tumor perfusion in response to radiation exposure. Interestingly, we also provide evidence that the NO-mediated effects of irradiation may be exploited to increase the specific delivery of DNA vectors to the tumor vascular endothelium. Using reporter-encoding plasmids, we showed that (*1*) quantitatively, the extent of transgene expression in the whole tumor was almost 20-fold higher after irradiation (Fig. 4a), and (*2*) qualitatively, only the vascular cells were reached by the GFP-encoding plasmid lipocomplex (Fig. 4b). Interestingly, we have also found that the delivery of a therapeutic DNA vector may benefit from the co-administration of ionizing radiation. Accordingly, additional tumor growth delay was observed by combining DN-Akt-encoding plasmid with low-dose irradiation. In fact, we showed that the DN-Akt-encoding plasmid had no effect on tumor growth without irradiation preconditioning (Fig. 5c).

That radiotherapy induces other eNOS-dependent alterations in tumors, which accounts for the higher recruitment of the DNA lipocomplex independently of the increase in blood flow, is very likely. Nevertheless, in this study, the effects of radiation appeared to be highly specific of the tumor vasculature and dependent on the tumor microenvironment. Hence, the irradiation of cultured endothelial cells did not stimulate the extent of transduced endothelial cells (Fig. 4c), despite a threefold increase in NO produced (Fig. 1d), and L-NAME did not reduce the proportion of *in vitro* transfected endothelial cells (Fig. 4c). Also, vasoreactivity experiments revealed that increased eNOS activation and consecutive vasorelaxation occurred in irradiated tumor vessels but not in irradiated healthy vessels. On the basis of these data, a model can be proposed according to which in response to irradiation, endothelial cells express more eNOS and the tumor cells (and more largely, the cells in the tumor microenvironment) provide the stimulus to activate the enzyme that consecutively drives the NO-dependent dilation of tumor blood vessels. VEGF is very likely to be one of these eNOS-triggering stimuli. VEGF is indeed known to be produced by tumor cells in response to radiotherapy (2, 4, 6) and has been documented, in other contexts, to induce NO-dependent vasodilation (17, 18). In tumor cells, other factors acting either as inducers of eNOS expression or eNOS agonists (or both) could also derive from the induction of hypoxia inducible factor 1α, which

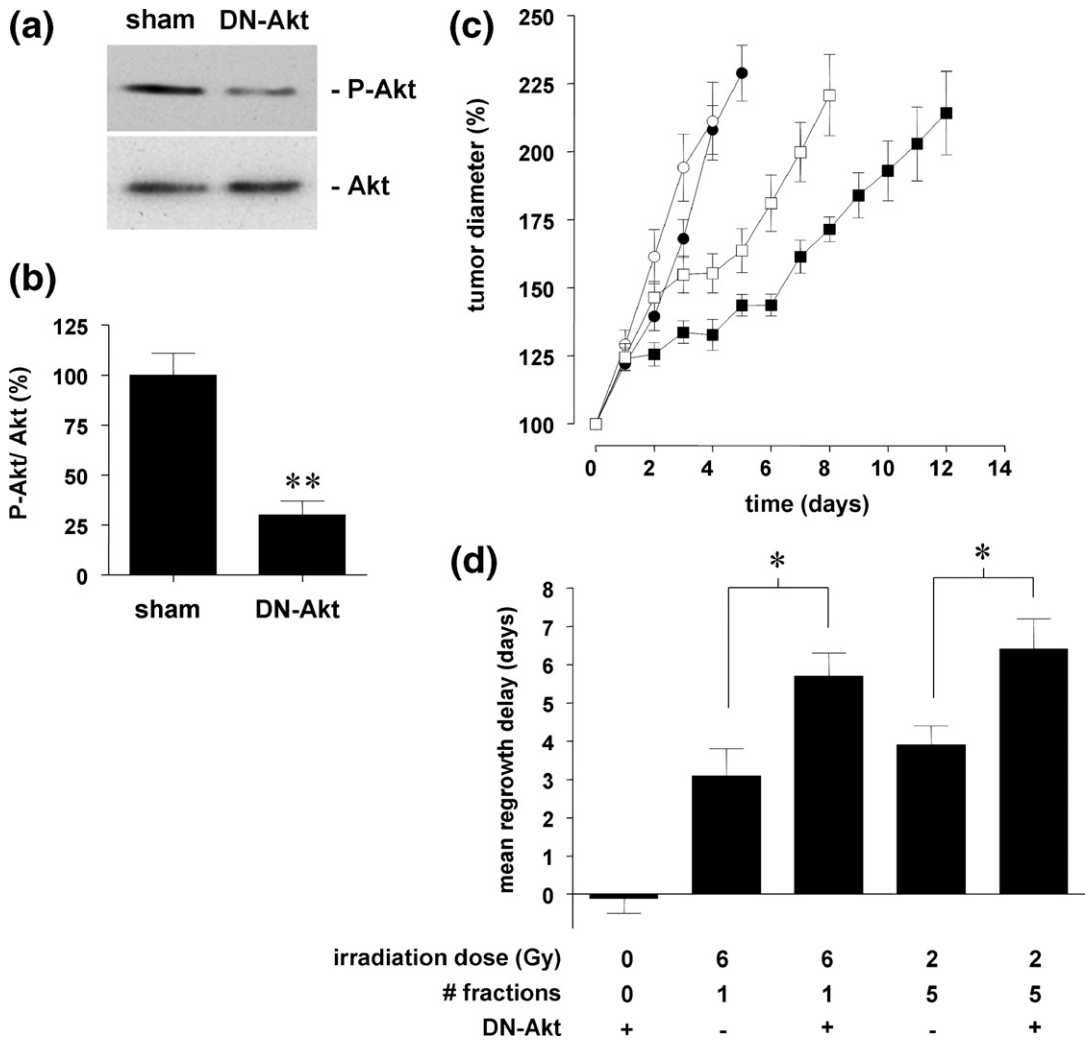

Fig. 5. Irradiation and administration of a dominant-negative Akt-encoding plasmid-lipocomplex exert synergistic antitumor effects. The extent of Akt phosphorylation was determined in irradiated endothelial cells expressing or not DN-Akt. Representative immunoblots for the expression of phospho-Akt and total Akt (a) and quantitative analyses (**$p < 0.01$; $n = 3$) (b) are presented. (c) Transplantable liver tumor (TLT) diameters were determined in untreated mice (open circles) and in X-ray-treated mice (6 Gy at Day 0) that received intravenously (i.v.) (at Day +1) the DN-Akt transgene (filled squares) or an empty vector (open squares). We also checked tumor growth in nonirradiated mice treated with the DN-Akt plasmid (filled circles). Data are expressed as percentage of the tumor diameters at Day 0. (d) Mean growth delays to reach twice the initial tumor diameter were determined for the indicated combinations of X-rays and DN-Akt lipocomplex delivery. The therapeutic transgene (+) or empty vector (−) was administered i.v. 24 h after each daily irradiation. *$p < 0.05$; $n = 6$–11.

has recently been documented to occur in response to radiotherapy (6). In endothelial cells, reactive oxygen species may also act as triggers of the stimulation of eNOS expression/activity. Reactive oxygen species–dependent inductions of activator protein 1 (19, 20) and nuclear factor κB (21, 22) have both been reported to promote eNOS gene transcription and activity. Further investigation is needed to confirm/identify which signaling intermediaries within both tumor cells and endothelial cells drive the stimulation of the eNOS pathway in response to radiotherapy.

CONCLUSION

Our study reveals that collateral effects of radiotherapy on the endothelial NO pathway, in part through the NO-mediated modulation of tumor vascular tone, render the concomitant administration of therapeutic transgenes particularly suited. We have also documented that this strategy may further benefit from mutually reinforcing layers of specificity: (*1*) local tumor irradiation allows, besides direct antitumor effects, the spatial control of the transgene delivery in tumors, and (*2*) the preferential expression of the transgene in the vascular compartment of the tumor allows targeting of pro-angiogenic pathways known to be activated in irradiated endothelial cells. More specifically, we showed that the therapeutic effects of a plasmid encoding a dominant-negative form of Akt were observed at low (clinically relevant) doses of fractionated radiotherapy, further extending the potential of combining irradiation and cationic lipid-based transgene delivery.

REFERENCES

1. Gray LH, Conger AD, Ebert M, *et al.* The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol* 1953;26:638–648.
2. Gorski DH, Beckett MA, Jaskowiak NT, *et al.* Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Res* 1999; 59:3374–3378.
3. Koukourakis MI, Giatromanolaki A, Sivridis E, *et al.* Squamous cell head and neck cancer: Evidence of angiogenic regeneration during radiotherapy. *Anticancer Res* 2001;21: 4301–4309.
4. Gupta VK, Jaskowiak NT, Beckett MA, *et al.* Vascular endothelial growth factor enhances endothelial cell survival and tumor radioresistance. *Cancer J* 2002;8:47–54.
5. Sonveaux P, Brouet A, Havaux X, *et al.* Irradiation-induced angiogenesis through the up-regulation of the nitric oxide pathway: Implications for tumor radiotherapy. *Cancer Res* 2003;63:1012–1019.
6. Moeller BJ, Cao Y, Li CY, Dewhirst MW. Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: Role of reoxygenation, free radicals, and stress granules. *Cancer Cell* 2004;5:429–441.
7. Tan J, Hallahan DE. Growth factor-independent activation of protein kinase B contributes to the inherent resistance of vascular endothelium to radiation-induced apoptotic response. *Cancer Res* 2003;63:7663–7667.
8. Massion PB, Feron O, Dessy C, *et al.* Nitric oxide and cardiac function: Ten years after, and continuing. *Circ Res* 2003;93: 388–398.
9. Lu Y, Wang H, Mills GB. Targeting PI3K-AKT pathway for cancer therapy. *Rev Clin Exp Hematol* 2003;7:205–228.
10. Taper HS, Woolley GW, Teller MN, *et al.* A new transplantable mouse liver tumor of spontaneous origin. *Cancer Res* 1966;26:143–148.
11. Sonveaux P, Dessy C, Martinive P, *et al.* Endothelin-1 is a critical mediator of myogenic tone in tumor arterioles: Implications for cancer treatment. *Cancer Res* 2004;64:3209–3214.
12. Dimmeler S, Assmus B, Hermann C, *et al.* Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: Involvement in suppression of apoptosis. *Circ Res* 1998;83: 334–341.
13. Holm P, Korsgaard N, Shalmi M, *et al.* Significant reduction of the antiatherogenic effect of estrogen by long-term inhibition of nitric oxide synthesis in cholesterol-clamped rabbits. *J Clin Invest* 1997;100:821–828.
14. Hirst DG, Hirst VK, Shaffi KM, *et al.* The influence of vasoactive agents on the perfusion of tumours growing in three sites in the mouse. *Int J Radiat Biol* 1991;60:211–218.
15. Chaplin DJ, Hill SA, Bell KM, *et al.* Modification of tumor blood flow: Current status and future directions. *Semin Radiat Oncol* 1998;8:151–163.
16. Thurston G, McLean JW, Rizen M, *et al.* Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J Clin Invest* 1998;101:1401–1413.
17. Ku DD, Zaleski JK, Liu S, *et al.* Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *Am J Physiol* 1993;265:H586–H592.
18. Takeshita S, Isshiki T, Ochiai M, *et al.* Endothelium-dependent relaxation of collateral microvessels after intramuscular gene transfer of vascular endothelial growth factor in a rat model of hindlimb ischemia. *Circulation* 1998;98:1261–1263.
19. Lopez-Ongil S, Hernandez-Perera O, Navarro-Antolin J, *et al.* Role of reactive oxygen species in the signalling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. *Br J Pharmacol* 1998;124:447–454.
20. Navarro-Antolin J, Rey-Campos J, Lamas S. Transcriptional induction of endothelial nitric oxide gene by cyclosporine A. A role for activator protein-1. *J Biol Chem* 2000;275:3075–3080.
21. Kamata H, Manabe T, Oka S, *et al.* Hydrogen peroxide activates IkappaB kinases through phosphorylation of serine residues in the activation loops. *FEBS Lett* 2002;519:231–237.
22. Davis ME, Grumbach IM, Fukai T, *et al.* Shear stress regulates endothelial nitric-oxide synthase promoter activity through nuclear factor kappaB binding. *J Biol Chem* 2004;279:163–168.