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Modulation of the tumor vasculature functionality by ionizing radiation accounts for tumor radiosensitization and promotes gene delivery

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ABSTRACT

The ultimate goal of radiotherapy is to induce irreversible damages in genetically unstable, fast-growing cancer cells while minimizing the cytotoxic effects on host tissues. The satus of the tumor vasculature is particular because it is located within the tumor but mostly arises from host cells. The aim of this study was to characterize the effects of low-dose irradiation on the function of endothelial cells lining tumor vessels. Using isolated arterioles mounted on a pressure myograph, we first documented that the nitric oxide (NO)-mediated vasorelaxation that was defective in tumor vessels was completely restored following local tumor irradiation. Immunoblot analyses revealed that this was attributable to an increase in the abundance of the endothelial NO synthase while the expression of its physiological inhibitor, caveolin-1, was reduced. We further showed that the potentiation of the NO-dependent pathway induced a marked increase in tumor blood flow and oxygenation that determined the higher sensitivity of the tumor to further irradiation. Finally, we documented that the NO-mediated effects of irradiation on the tumor vasculature increased the delivery and expression of a reporter gene into the tumor. Thus, low-dose irradiation of endothelial cells within a tumor is a key determinant of the effectiveness of radiotherapy and may offer a new strategy to increase gene and/or drug delivery to the tumor.

Key words: nitric oxide • tumor endothelium • oxygenation • irradiation

Since the identification of angiogenesis as a key process in tumor development, endothelial cells have acquired the status of major targets to tackle cancer progression (1, 2). However, although starving a tumor from oxygen and nutrients can suppress cancer in animal models (3), slow-growing human tumors are more likely to be affected by anti-angiogenic approaches in combination with treatments directly targeting cancer cells (4–6). Yet, this may appear counterintuitive since destruction of vascular structures in the tumor will impede drug delivery and reduce tumor oxygenation (required for an efficient radiotherapy). Common

sense therefore suggests that chemotherapy and radiotherapy should precede anti-vascular strategies, that is, that the already poorly efficient tumor vasculature should initially be spared. R.K. Jain (7) recently extended this paradigm by proposing that before starting chemo- or radiotherapy, tumor vasculature could even be normalized by the early effects of antiangiogenic treatments. More classical pharmacological interventions aiming to improve tumor blood vessel dilation or to induce decompression have also been tested with some success to optimize radiotherapy or chemotherapy (8–12). Though promising, these strategies require fine tuning in order to gain in selectivity for the tumor vascular bed and to limit systemic toxicity. Interestingly, radiotherapy itself could provide a solution for tumor targeting, but whether vasomotion in tumors can be actively influenced by irradiation has never been specifically addressed.

A direct link between irradiation and endothelial function is however suggested by the nature of one of the most powerful vasorelaxant identified so far, nitric oxide (NO). Irradiation, indeed, induces the production of reactive oxygen-derived species (ROS) which are known to interfere with the biology of NO at different levels. Accordingly, opposite effects of ROS, including a decrease in the bioavailability of NO by radical scavenging (13) and an increase in the activation of the endothelial NO synthase (eNOS) (14), have been reported. To determine which of these effects, if any, will predominate in the context of tumor radiotherapy should help to address the seminal question of the role of endothelial cells in this therapeutic process. Indeed, although radiotherapy in its fractionated mode (as administered nowadays) has empirically established itself as a gold standard, the *a posteriori* explanations for both its higher efficacy (over a single total dose) and its limitations (due to development of radio-resistance) have largely neglected the implication of the endothelial cells lining the tumor vessels. Legitimate questions are still unanswered. For instance, how important are the deleterious effects of irradiation on the endothelial cells of tumor vessels in the process of tumor eradication? Conversely, do tumor vascular structures provide surviving tumor cells with the means (e.g., access to O_2 and nutrients) to more easily re-grow after X-ray exposure? Alternatively (but not exclusively), is the tumor vasculature playing a role in stimulating oxygenation after each fractionated irradiation and therefore taking an active part in the treatment? Could this be exploited to tailor anti-cancer strategies? More generally, how relevant is the concept of (therapeutically) targeting tumor vascularization to acutely increase blood flow considering that chronic tumor hypoxia led to the selection of cells with defects in apoptosis (15)?

The aims of the present study were to characterize the effects of irradiation on the tumor endothelium-derived NO pathway, to examine the influence of these effects on tumor sterilization and to evaluate their possible exploitation to improve cancer treatment.

MATERIALS AND METHODS

Mouse and cells

Male RJ NMRI and C3H/He N Rj IOPS mice (7–8 wk old) (Elevage Janvier, Le Genest-St-Isle, France) were used in experiments with TLT cells (mouse hepatocarcinoma) (16) and FSA-II (fibrosarcoma) (17), respectively. In the experiments with eNOS null and backcrossed control mice (The Jackson Laboratory, Bar Harbor, ME), Lewis lung carcinoma (LLC) cells were used. Each procedure was approved by the local authorities according to national animal care regulations. Tumor cells (10^5–10^6) were injected intramuscularly in the posterior right leg of

mice, in the vicinity of the saphenous artery. Transversal and antero-posterior measurements of the tumor were daily determined with an electronic caliper. When the tumor-bearing leg reached 8.0 ± 1 mm in diameter, the mice were randomly assigned to a treatment group and irradiated. When required, the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 500 mg/L) was added in the drinking water, daily renewed, and maintained for the periods of time mentioned. Endothelial cells (BAEC, Clonetics, Walkersville, MD) were routinely cultured in 100-mm dishes in medium containing 10% serum.

Irradiation

Cells and anesthetized mice were irradiated using the RT-250 device (Philips) for a dose delivery of 0.86 Gy/min and 0.76 Gy/min, respectively. For the *in vivo* experiments, mice were anesthetized with ketamine/xylazine and the tumor was centered in a 3-cm-diameter circular irradiation field.

Immunoblotting, immunoprecipitation and cGMP determination

Endothelial cells, isolated microarteries or whole tumors were collected and lysed as previously described (18, 19). Immunoblotting (IB) and immunoprecipitations (IP) were carried out with antibodies directed against eNOS, nNOS, iNOS, Hsp90, caveolin-1 and HA-tag (polyclonal Ab for IP and monoclonal Ab for IB); all antibodies from Beckton Dickinson (Lexington, KY). The cGMP content was determined by enzyme immunoassay (Amersham, Freiburg, Germany).

Myograph assay

Tumor arterioles and size-matched arterioles (saphenous artery branch) from healthy mice were dissected under a stereoscopic microscope and mounted on a 110P pressure myograph (DMT, Aarhus, Denmark). Isolated arterioles were left to recover for 45–60 min in no-flow condition (60 mm Hg, 37°C) in Krebs medium supplemented with indomethacin (to inhibit cyclooxygenases). They were then preconstricted with 50 mM KCl, and the vasodilation in response to increasing acetylcholine (Ach) concentrations was determined in the presence or in the absence of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NA, 100 μM). Changes in the outer diameters were tracked and measured with the Myoview software (DMT, Aarhus, Denmark).

Transfection procedure

Cationic lipid-mediated transfections were carried out by injecting the plasmid-lipid complex in the tail vein of mice irradiated or not. The plasmid was chosen to encode for an HA-tagged reporter protein and was mixed with a DNA-condensing enhancer and coated with cationic lipids (Effectene, Qiagen, CA).

Tumor blood flow monitoring

Tumor perfusion was measured with a Laser Doppler perfusion imager (LDI, Moor Instruments). In brief, mice were anesthetized and fur was removed from the limbs using a depilatory cream. Before initiating scanning, mice were placed on a heating pad (37°C) to minimize variations in temperature. The average perfusions of the tumor-bearing leg and the control leg were evaluated on the basis of colored histogram pixels. To minimize variables including ambient light and

temperature, calculated perfusion was expressed as the ratio of right (tumor-bearing) to left (control) limb perfusion.

EPR oximetry

Electron paramagnetic resonance (EPR) oximetry was used to track the changes in pO_2 induced by irradiation. For *in vivo* pO_2 measurements, this real-time, O_2-nonconsuming technique relies on the oxygen-dependent broadening of the EPR linewidth of a paramagnetic oxygen sensor preimplanted in the tumor. Accordingly, 50 μl of a suspension (100 mg/ml) of the O_2-sensitive probe (charcoal wood powder, CX0670-1, EM Science, Gibbstown, NJ) were injected in the center of the tumor 24 h before X-ray irradiation, as previously described and validated (10, 20, 21). At the indicated day postirradiation, the tumors of anesthetized mice were placed in the center of the surface coil. EPR spectra were recorded using an EPR spectrometer (Magnettech, Germany) with a low frequency microwave bridge operating at 1.1 GHz and extended loop resonator. In these experimental conditions, the measured pO_2 corresponds to the mean value in a tumor volume of ~10 mm^3 (i.e. the volume of charchoal dispersion as determined by histology) (21).

For oxygen consumption rate evaluation (*ex vivo* measurements), the method developed by P. James was used (22). In brief, the tumors isolated from treated mice were pieced in trypsin-containing medium, filtered and pooled as indicated. Cell viability was then evaluated with the trypan blue exclusion method and viable cells (2×10^7/ml) were suspended in complete medium. A neutral nitroxide, ^{15}N PDT (4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-^{15}N-1-oxyl) at 0.2 mM (CDN isotopes, Quebec, Canada), was added to 100-μl aliquots of tumor cells that were then drawn into glass capillary tubes. They were rapidly placed into quartz ESR tubes and maintained at 37°C during the recording on a Bruker EMX EPR spectrometer operating at 9 GHz.

Statistical analyses

Data are normalized for the amount of protein in the dish (or for the number of cells engaged) and are presented for convenience as mean ±SE. Statistical analyses were made using Student's *t* test or one-way ANOVA where appropriate.

RESULTS

Irradiation oppositely regulates the expression of key signaling proteins of the endothelial NO pathway *in vitro* and *in vivo*

As a first approach, we examined the effects of a gradual increase in single irradiation doses on cultured endothelial cells. The expression levels of different proteins regulating NO production were determined from surviving endothelial cells after a period of 24 h. Irradiation induced a progressive up-regulation of eNOS expression, reaching a plateau of twice its basal expression at 6 Gy ($P<0.01$, $n=3$) (Fig. 1a). Further increases in the delivered dose only slightly impacted on eNOS expression. In parallel, irradiation dose-dependently induced the down-regulation of caveolin-1 (a physiological inhibitor of eNOS) (18) up to a dose of 12 Gy where its abundance amounted to 30% of the control value ($P<0.01$; $n=3$) (Fig. 1a). These data suggest that irradiated, surviving endothelial cells have an enhanced potential to produce NO because eNOS is up-regulated and its activity less repressed. Of note, the expression of heat-shock protein 90

(Hsp90), another regulator of eNOS activity (19), was not altered by the X-ray exposure (Fig. 1a) and the other NOS isoforms (iNOS and nNOS) were not detected in any of the experimental conditions (data not shown).

To establish the *in vivo* relevance of these observations, we then locally irradiated TLT tumor-bearing mice. Control and irradiated tumor microvessels (100–250 μm diameter) were dissected and pooled to be processed in immunoblotting experiments. Twenty-four hours after a local 6-Gy irradiation, the tumor microvasculature (eight to ten microvessels per condition) exhibited a fourfold increase in eNOS abundance and a simultaneous decrease in caveolin-1 expression (Fig. 1b), thereby confirming the *in vitro* data.

Irradiation restores NO-dependent vasomotion in tumor afferent arterioles

Having documented the acute effects of X-ray exposure on the expression of tumor vasculature proteins (i.e., eNOS and caveolin), we next evaluated whether irradiation could modulate NO-mediated arteriole reactivity. Accordingly, a pressure myograph system was used to study the agonist response of arterioles isolated from healthy mice and from TLT tumor-bearing mice that were locally irradiated or not. After a precontraction induced by 50 mM KCl, the mounted vessels were challenged by various agonists, including bradykinin, substance P, and acetylcholine (Ach), known to elicit NO-mediated responses in other vascular beds. Only Ach induced a dose-dependent dilation of control saphenous arterioles, reaching 61.0 ± 9.1% of the maximal contraction at the dose of 10^{-4} M Ach ($P<0.01$, $n=4$) (Fig. 2a, left panel). This pharmacological response appeared to be entirely NO-dependent since it was totally abolished in the presence of the NOS inhibitor L-NA (100 μM) (Fig. 2a). By contrast, Ach (up to 10^{-4} M) failed to induce any relaxation of diameter-matched TLT tumor arterioles (Fig. 2b); no difference was seen in presence of L-NA. We then tested the reactivity of tumor arterioles collected 24 h after a local 6-Gy irradiation. The Ach-induced NO-mediated vasorelaxation appeared completely restored in these irradiated vessels (Fig. 2c). Indeed, we observed a significant dose-dependent vasodilation starting as early as 5.10^{-7} M Ach ($P<0.01$, $n=4$) and reaching 67.0% ± 1.0% of the maximal contraction. In addition, as in control arterioles, the Ach-induced relaxation in irradiated arterioles was completely abolished in the presence of L-NA (100 μM) (Fig. 2c).

Further validation of the increased NO-dependent endothelial functionality arose from the determination of the cGMP content in microvessels isolated from tumor-bearing mice irradiated (or not) and treated (or not) with the NOS inhibitor L-NAME (e.g. from day –1 to day +1 after irradiation). Accordingly, the NOS-dependent cGMP content of tumor vasculature (pools of 5 saphenous arterioles per condition) was consistently higher (+22–30%) after irradiation than in control conditions.

Nitric oxide conditions the vascular-dependent increases in tumor blood flow and oxygenation after irradiation

We then examined whether the observed changes in NO-dependent vasomotion induced by irradiation accounted for *in situ* changes in tumor blood flow and oxygenation. We used Laser Doppler imaging and EPR oximetry to evaluate blood flow and pO_2, respectively, in irradiated TLT tumors from mice treated or not with the NOS inhibitor L-NAME (500 mg/L). A local 6-Gy irradiation significantly increased tumor blood flow (+36 ± 3%; $P<0.01$, $n=5$) and tumor oxygenation (+ 75 ± 29%; $P<0.05$, $n=8$) as measured 24 h after treatment (Fig. 3a and 3b). The

effect was maximal at 48 h and then progressively faded to reach undetectable levels 3–4 days after irradiation (see Fig. 3b). Conversely, no significant difference in tumor blood flow and oxygenation was induced by irradiation of animals receiving L-NAME ($P>0.05$; $n=5$). In nonirradiated tumors, pO_2 amounted to 4.5 ± 1.0 mmHg ($n=6$) and was not significantly altered in the 3-day interval of the experiments. We also verified that only eNOS was immunodetectable from whole tumor lysates and that isolated tumor cells, irradiated or not, did not express eNOS, iNOS or nNOS (data not shown), indicating that the only source of NO in the tumor was the vascular eNOS.

The tumor oxygenation level results from the net balance between O_2 delivery from the tumor vasculature and the local consumption by tumor cells. To rule out the eventuality of NO-mediated effects on the O_2 consumption in our experimental conditions, we collected viable tumor cells after a local 6-Gy irradiation and analyzed the effect of L-NAME treatment on the O_2 consumption rate by EPR measurements. The average O_2 levels in sealed tubes decreased linearly from 21% to nearly 0% (Fig. 3c). No difference was seen in the O_2 consumption rates between the viable tumor cells isolated from irradiated mice pretreated with L-NAME or not (mean slopes of –0.76 ± 0.03 and –0.75 ± 0.01, respectively; $P>0.05$, two pools of five tumors). This set of data illustrates that in our experimental conditions, the NO-mediated increase in pO_2 observed in irradiated tumors can not be explained by a reduced O_2 consumption but, instead, strongly supports the paradigm of a higher, X-ray-induced vascular-dependent O_2 delivery.

X-ray-induced NO production participates in the effectiveness of radiotherapy

As tumor oxygenation is a key factor conditioning the effectiveness of irradiation, we then examined whether the NO-dependent increase in pO_2 induced by a first irradiation was required to potentiate the effects of a second irradiation. Accordingly, we daily measured the tumor diameters of mice submitted (or not) to one or two X-ray exposure(s) and evaluated the effects of L-NAME treatment. While the nonirradiated TLT tumors exhibited a fast linear growth rate, a single (day 0) and a double (days 0 and 2) local 6-Gy irradiations considerably delayed tumor development, increasing the mean regrowth delay from 5.0 to 9.4 days, respectively (Fig. 4a and Table 1). Importantly, when tumor-bearing mice were treated with L-NAME from day –1 to day 2, the benefit of the second irradiation was completely abolished. The retardation in tumor growth did not differ from mice exposed to a single 6-Gy dose (Fig. 4a and Table 1). As controls, we verified that L-NAME administered after and before the first irradiation had *per se* no effect on tumor development.

We also examined whether the paradigm of NO-mediated increase in radiosensitivity was applicable in a model of fibrosarcoma (FSA-II) implanted in another mice strain; of note, baseline pO_2 amounted to 3.6 mmHg in this second tumor type. Data are presented in Table 1 and also reveal the suppression of the tumor growth delay induced by the second irradiation when mice were treated with L-NAME in the time interval between the two X-ray exposures.

Irradiation promotes liposomal gene delivery into the tumor

We finally examined whether the functional up-regulation of the NO pathway induced by ionizing radiation (i.e., the tumor vessel dilation and the consecutive increase in blood flow) could be exploited to increase gene delivery to the tumor. Accordingly, we evaluated the efficacy of cationic lipid-based transfection (23) of a reporter protein-encoding plasmid DNA in tumors.

TLT tumor-bearing mice where locally irradiated and, after 24 h, were injected i.v. with the plasmid-lipid complex. Tumors were collected 60 h later, and lysates were immunoprecipitated using polyclonal anti-HA antibody and immunoblotted with a mouse anti-HA antibody. Strikingly, although the HA-tagged reporter protein was barely detectable in nonirradiated tumors, the transgene was robustly expressed in the tumors preexposed to a single 6-Gy dose (Fig. 4b).

Importantly, this process of irradiation-induced DNA-liposome addressing to the tumor was quasi-exclusively dependent on NO since the administration of L-NAME almost completely prevented the reporter protein expression in TLT tumors (Fig. 4b). Furthermore, when these experiments were repeated using eNOS null mice bearing LLC tumor, irradiation failed to induce gene delivery into the tumor whereas a net increase in reporter gene expression was observed in backcrossed control mice bearing LLC tumor (data not shown).

DISCUSSION

In this study, we report that the functional NO-mediated changes induced in the tumor vasculature by irradiation directly influence tumor response. Unexpectedly, this tumoricidal effect does not arise from induced vascular cytotoxicity/apoptosis as previously reported with large doses of ionizing radiation (24) but, instead, is observed with clinically more relevant lower doses (see comments in ref 25). Indeed, we showed that in cultured endothelial cells as well as in the tumor microvasculature, limited doses of ionizing irradiation (2–6 Gy) can spare a large fraction of vascular cells and lead to a significant increase in eNOS abundance. This was associated with a reduction in the endogenous eNOS inhibitor caveolin-1 (18), thereby reinforcing the stimulatory effect of irradiation on eNOS activity. Importantly, the functional relevance of the expressional up-regulation of the NO pathway was verified by administering L-NAME to tumor-bearing mice: the NOS inhibitor completely abrogated the irradiation-induced increases in tumor blood flow, pO_2, and radiosensitivity.

Although preliminary experiments using mice invalidated for the eNOS gene led to similar results (data not shown), the use of the NOS inhibitor L-NAME was preferred after verification that eNOS was the only isoform expressed in the tumor (i.e., in the tumor vasculature). Indeed, this drug treatment offered the advantage to be easily administered for short periods of time and at very specific time points (see Fig. 4). Accordingly, while L-NAME had *per se* no effect on the tumor growth following a single irradiation (see Table 1), its use during the time interval between two X-ray doses completely prevented the sterilization effect of the second exposure to ionizing radiations (see Fig. 4a and Table 1). These data (obtained with two different tumor models) indicate that the tumor growth retardation was strictly dependent on the eNOS-mediated increase in NO production induced by the first irradiation. Mechanistically, our data document that the local exposure of the tumor to limited doses of ionizing radiations can effectively rescue the function of the arterioles perfusing the tumor. Indeed, in our experimental model, the NO-dependent vasorelaxation observed in arterioles of healthy animals was lost when the arterioles were located into the tumor but completely recovered after local irradiation of the tumor. The major impact of irradiation on the tumor vasculature was further confirmed by functional (i.e., the cGMP elevation) and expressional changes (i.e., the up-regulation of eNOS and the down-regulation of caveolin). ROS are likely to mediate the latter transcriptional effects of irradiation on endothelial cells. Indeed, AP-1 activation by ROS has been shown to induce eNOS gene expression (26), whereas caveolin gene transcription is under the (negative) control of

extracellular signal-regulated kinase (ERK) (27), itself known as one of the major ROS-responsive serine/threonine kinases (28).

Pharmacological interventions aiming to increase tumor blood flow and O_2 are the subject of many experimental studies, using either vasoconstrictors to increase perfusion pressure (29) or vasodilators to decrease vascular resistance to flow (11, 20, 21, 30). Positive and negative results have been observed with both approaches (9, 30). The lack of specificity for the tumor vs. the host vessels led, indeed, to vasomotor effects mostly dependent on the overall arrangement of these vessels (i.e., opposite if both tumor and host tissue vessels are located in parallel and similar if placed in series). Also, the proportion of functional vs. neo-formed immature vessels into the tumor is known to influence the net effects of these drugs (29). Here, we show that local delivery of ionizing radiation may bypass the specificity problem and can selectively modulate flow resistance. Although changes in tumor blood flow have already been reported to occur after irradiation (31, 32), our data provide the first evidence, to our knowledge, that such changes are induced by the direct effects of ionizing radiation on the reactivity of mature tumor vessels (i.e., those having the ability to vasorespond). An obvious correlate is the increase in tumor oxygenation and the radiosensitization that we have documented in this study. Interestingly, it suggests that the frequency of exposure to X-rays could be fine-tuned in order to clinically exploit the best window of reoxygenation and/or increase in perfusion. Moreover, the provascular effects of radiotherapy should be carefully evaluated because they could vary with the radiotherapy fractionation schedule, the degree of tumor vascularization and the baseline pO_2 values (33, 34). Furthermore, it should be emphasized that although there are obvious limitations in the methodology used in this study for measuring tumor oxygenation and perfusion, predictive assays aiming to evaluate tumor pO_2/blood flow are under constant development (35). This should permit, in the future, to easily adapt the fractionated scheme of irradiation to a given tumor in a given individual.

Finally, our data also indicate that beside the strict context of tumor radiosterilization, low-dose irradiation could also act as an adjuvant for a more selective delivery to the tumor (Fig. 4b). Indeed, we have documented that cationic lipid DNA complex can be more efficiently targeted to locally irradiated tumors. Cationic liposomes are known to possess a particularly high tropism for tumors and are thought to be taken up by virtue of angiogenic endothelial cell endocytosis (23) or extravasation from the leaky tumor vasculature (36, 37). In our study, the process leading to plasmid delivery into the tumor appeared exquisitely dependent on the induction of NO production because the transgene expression was barely detectable when mice were treated with L-NAME at the time of the irradiation or when eNOS null mice were used. Therefore, beside the well-known intrinsic limitations in the size of both the cargo-liposome complexes and the tumor pores (36, 37), our observations emphasize the importance of NO-regulated parameters, such as blood flow and vessel permeability (see ref 38), for efficient uptake and expression of the transgene by the tumor. More importantly, we showed that this NO-dependent delivery of DNA to the tumor is radioinducible. This could allow the limitation of potential systemic undesired effects and/or the reduction of the amount of injected DNA construct and the unspecific uptake by the liver. Further studies are now required to evaluate whether such provascular effects of low-dose radiotherapy are potentially adaptable to a large variety of tumors and pharmaceutical formulations.

In conclusion, we report here that low doses of ionizing radiations profoundly alter the function of endothelial cells lining tumor arterioles. These data unravel part of the rationale behind the

effectiveness of fractionated radiotherapy and offer new perspectives for the individual profiling of anticancer treatments. More generally, the current study gives the bases of future investigations aiming to exploit the vascular effects of ionizing radiation as an adjuvant to promote selective delivery of genes and/or drugs to tumors.

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Table 1

Mean regrowth delays for TLT and FSA-II tumors as a function of ionizing radiation exposure(s) and L-NAME treatment (n=6 per condition)

Tumor Type	Dose (Gy) at day 0	Dose (Gy) at day 2	L-NAME (500 mg/L)	Mean regrowth delay (days)
TLT Liver carcinoma	6	0	—	5.0
	6	6	—	9.4
	6	6	Day –1 to day 2	5.3
	6	0	Day –1 to end	5.1
	0	6	Day –1 to day 2	4.9
FSA-II Fibrosarcoma	6	0	—	4.6
	6	6	—	7.2
	6	6	Day –1 to day 2	4.7

Fig. 1

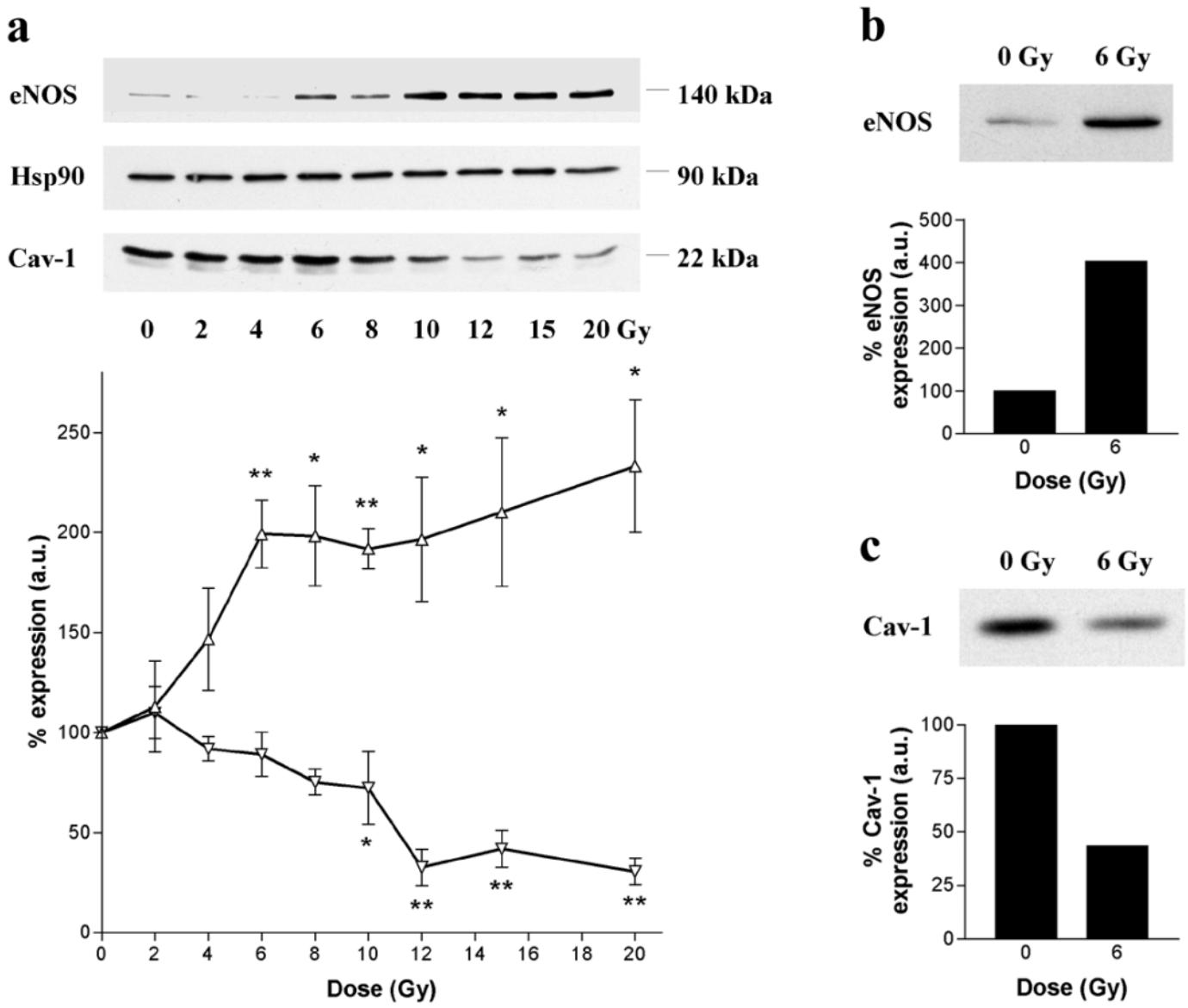

Figure 1. Irradiation potentiates the NO-dependent pathway. a) Cultured endothelial cells (EC) were irradiated at increasing doses and harvested 24 h later. Corresponding lysates were immunoblotted with monoclonal antibodies directed against eNOS, Hsp90, and caveolin-1. The upper panels show representative immunoblots; densitometric analyses for eNOS (Δ) and caveolin-1 (∇) are presented below (**P*<0.05, ***P*<0.01, *n*=3–11). **b)** TLT-bearing mice were locally irradiated (or not) at 6 Gy, and microvessels were isolated from resected tumors 24 h later. Lysates from pools of 8–10 microvessels were immunoblotted for eNOS and **c)** caveolin-1; this experiment was repeated twice with similar results.

Fig. 2

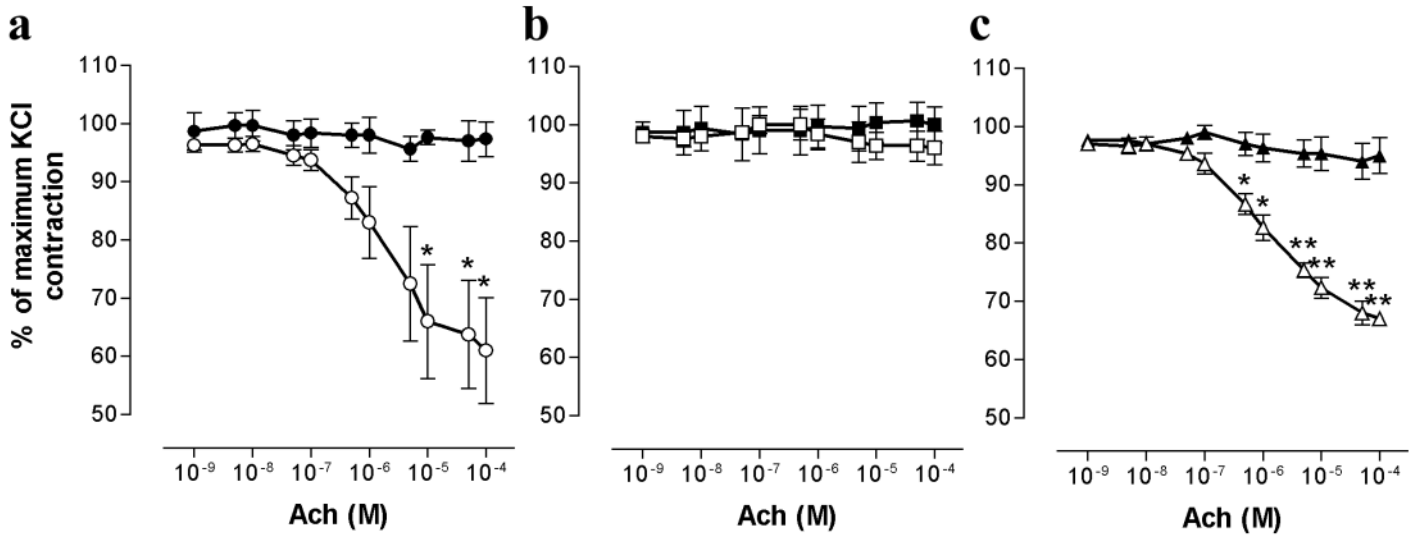

Figure 2. Irradiation rescues the NO-dependent tumor vessel reactivity. Arterioles were isolated from healthy mice **(a)** and from nonirradiated **(b)** or 6 Gy-irradiated **(c)** TLT tumors. Size-matched vessels were mounted in a pressure myograph and allowed to develop a basal myogenic tone in no-flow conditions. After precontraction with 50 mM KCl, the response to cumulative additions of Ach was evaluated (open symbols). This protocol was repeated on the same arterioles in the presence of 100 μM L-NA to block NOS activity (closed symbols). The outer diameters were followed by video microscopy and the mean results from three to four independent experiments are shown as the percentage of the maximal precontraction. *P<0.05, **P<0.01.

Fig. 3

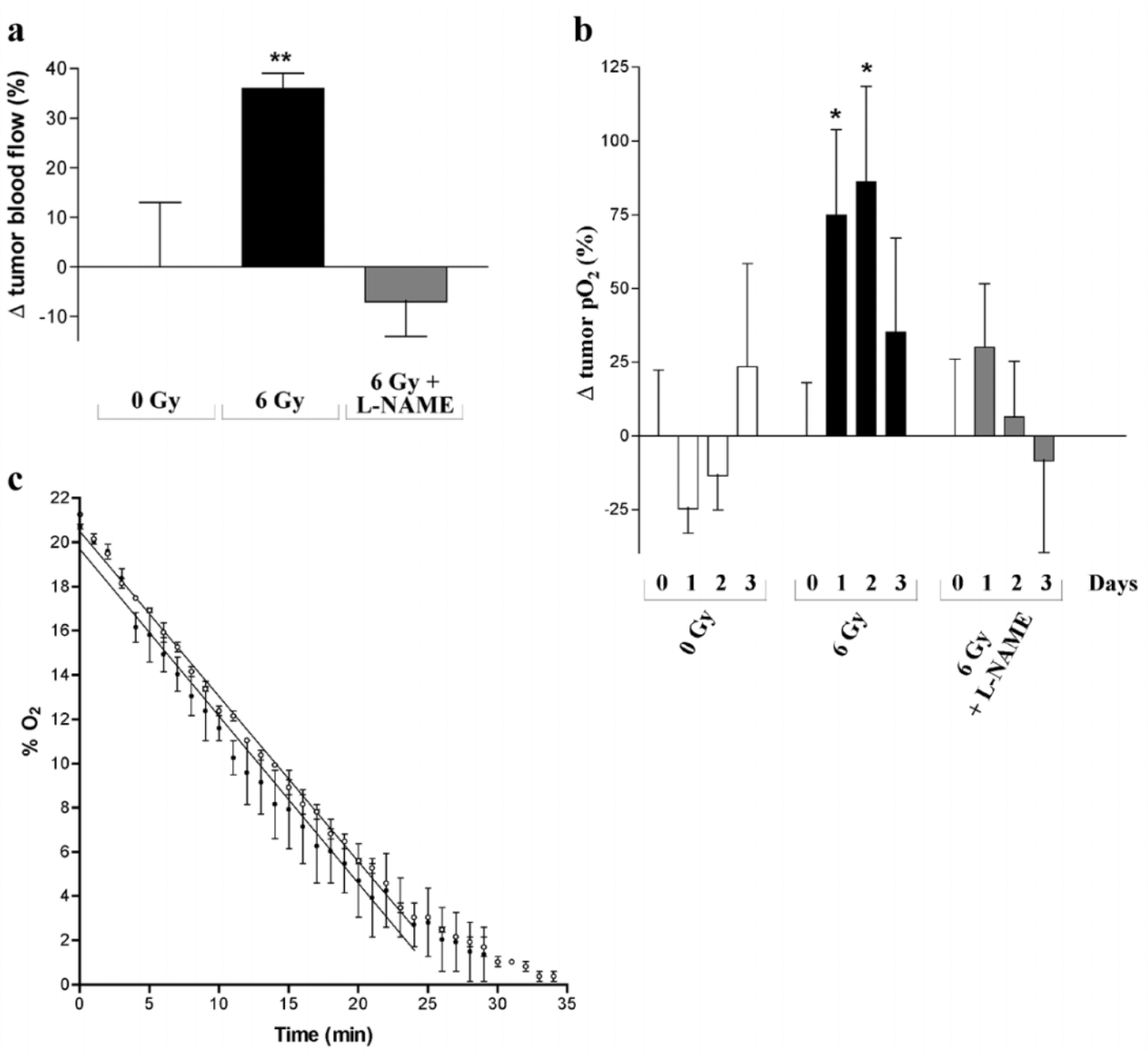

Figure 3. Irradiation increases tumor blood flow and oxygenation through a vascular NO-dependent pathway. **a)** Changes (%) in tumor blood flow measured by Laser Doppler 24 h after irradiation. **b)** Changes (%) in tumor pO_2 measured by EPR oximetry for three consecutive days after the day of irradiation (day 0). Tumor-bearing mice receiving (*n*=5) or not (*n*=8) L-NAME in drinking water were irradiated at a 6-Gy dose and compared with nonirradiated mice (*n*=6). (**P*<0.05; ***P*<0.01 vs. corresponding controls). **c)** The O_2 consumption rate was determined in sealed tubes from equal amounts of viable TLT tumor cells recovered from 6-Gy-irradiated mice receiving (●, *n*=10) or not (○, *n*=10) L-NAME in the drinking water (from day –1 to day 2).

Fig. 4

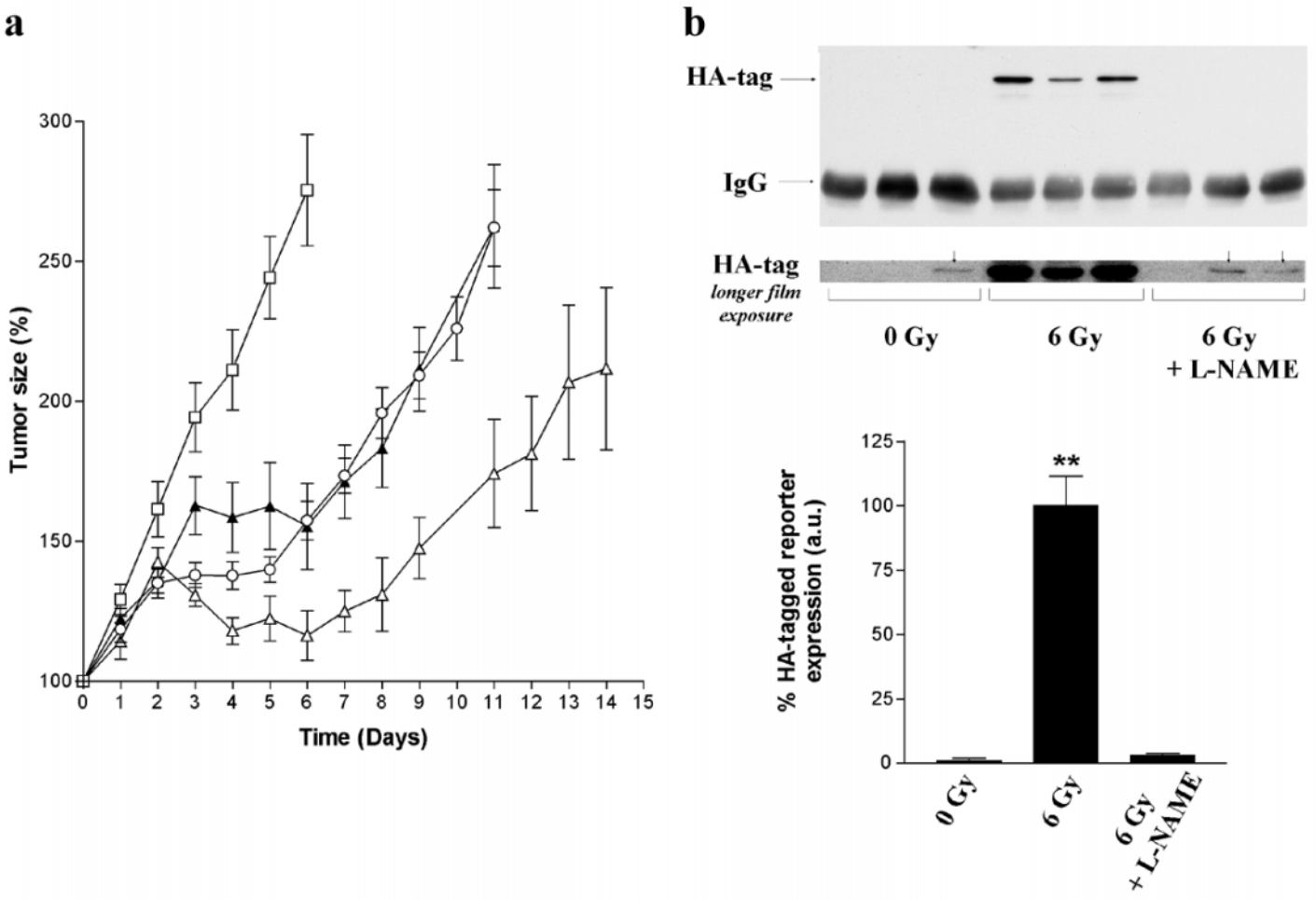

Figure 4. Irradiation-induced NO production accounts for tumor radiosensitization and promotes transgene delivery. a) Evolution of TLT tumor volumes in control mice (□) and in mice irradiated at a single 6-Gy dose at day 0 (○) or irradiated twice (6 Gy at day 0 and 6 Gy at day 2) with (▲) or without (Δ) L-NAME treatment from day –1 to day 2 (n=6–10). **b)** TLT tumor-bearing mice receiving or not L-NAME in drinking water were irradiated at a 6-Gy dose at day 0. The cationic lipid complex containing the HA-tagged reporter gene was injected in the tail vein at day 1. Tumors from control and irradiated mice were collected at day 4. Equal amounts of proteins from corresponding lysates were immunoprecipitated with rabbit anti-HA antibodies (IgG) and immunoblotted with mouse anti-HA antibody (HA-tag). The transgene expression is consistently detected in nontreated, irradiated tumors, whereas longer film exposure is required to detect the reporter protein in the other conditions (see arrowheads). Densitometric analyses are presented below (**P<0.01, n=3).