Irradiation-induced Angiogenesis through the Up-Regulation of the Nitric Oxide Pathway: Implications for Tumor Radiotherapy

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ABSTRACT

The combination of radiotherapy and antiangiogenic strategies has been shown to increase the tumor response in various experimental models. The rationale for this cotherapy was initially related to the expected gain in efficacy by acting on two different targets, e.g., tumor cells and endothelial cells (ECs). However, recent studies have documented more than additive effects due to apparent mutual potentiation of these approaches. In this study, we tested the hypothesis that these synergistic effects could stem from the stimulatory effects of ionizing radiations on angiogenesis, which would then need to be restrained to avoid tumor regrowth after irradiation. We found that irradiation dose-dependently induced the activation of the proangiogenic NO pathway in ECs through increases in endothelial nitric oxide synthase abundance and phosphorylation. Using 2- and 3-dimensional cultures of ECs and isolated mouse tumor arterioles, we documented that the irradiation-induced enhanced production of NO accounted for EC migration and sprouting. Irradiation was also shown to stimulate the colonization of Matrigel plugs implanted in mouse by ECs, where they formed capillary-like structures in a NO-dependent manner. These findings were confirmed by documenting the NO-mediated infiltration of CD31-positive ECs after local irradiation of Lewis lung carcinoma tumor-bearing mice. Finally, we measured a consistent increase in endothelial nitric oxide synthase mRNA by real-time PCR experiments in human biopsies of head and neck squamous cell carcinoma after low-dose irradiation. In conclusion, we have demonstrated that the potentiation of the NO signaling pathway after irradiation induces profound alterations in the EC phenotype leading to tumor angiogenesis. Moreover, our demonstration that the inhibition of NO production suppresses these provascular effects of irradiation highlights new potentials for the coordinated use of antiangiogenic strategies and radiotherapy in clinical practice.

INTRODUCTION

The rationale for combining anticancer therapies is predominantly based on the enhanced antitumor efficacy and the nonoverlapping toxicity of the associated strategies. Accordingly, the combination of antiangiogenic approaches that target the supportive vessel network of tumors with more conventional antitumor therapies appears particularly well suited (1). Several independent investigators have recently established that treatments with growth factor antibodies or tyrosine kinase inhibitors can indeed increase the antitumor effects of ionizing radiations (2–9). Interestingly, such a combination could surpass the mere additive effects, and several mechanisms were proposed to account for these synergistic effects on tumor growth. For instance, irradiation was shown to lead to the production by the tumor of survival factors, including VEGF (2, 10), that may induce antiapoptotic pathways and, if unopposed, promote tumor regrowth after irradiation. Taken together, these studies suggest that anti-VEGF approaches may act as radiosensitizing compounds, increasing the deleterious effects of X-rays on tumor cells. Another (although not exclusive) reading of these observations is that irradiation could actively exert proangiogenic effects that, if not repressed, could limit its efficacy. Indeed, in most of the studies mentioned above, the final read-out of the cotherapy was the effects on tumor growth, but the strict impact of irradiation on the tumor vasculature was not addressed. In this study, we specifically examined the effects of irradiation on ECs to identify signaling cascades induced by ionizing radiations that could lead to alterations in EC phenotype and/or angiogenesis. Mechanistic insights into the effects of irradiation on ECs may indeed bring some additional rationale for the combination of radiotherapy with antiangiogenic strategies and could also lead to the identification of new therapeutic targets.

Among the potential targets of irradiation on ECs, we chose to focus on the isoform of NOS (eNOS) expressed in these cells for two major reasons. First, NO is a key mediator of the proangiogenic effects of different growth factors, including VEGF (11). Second, in the context of cardiovascular diseases, transcriptional and posttranslational regulations of eNOS have been reported on exposure to ROS (12–14). ROS are produced in large amounts at the time of irradiation through chain reactions initiated by water radiolysis. Moreover, beside this regulation of the enzyme activity, NO and ROS are known to scavenge each other and thereby mutually modulate their biological effects (15).

Therefore, we set up a series of expression and functional experiments, with emphasis on the regulation of NO, to evaluate the effects of irradiation in various models including EC cultures, isolated tumor microvessels, mouse-implanted Matrigel (basement membrane matrix) plugs and tumors as well as human tumor biopsies. Our data reveal that low-dose irradiation can induce NO-mediated pathways leading to EC migration, colonization of host tissues, and organization in vascular network. These findings underline a hitherto downplayed but distinctive angiogenic response of tumors to irradiation and emphasize the need for associating radiotherapy with antiangiogenic approaches to enhance its overall therapeutic efficacy.

MATERIALS AND METHODS

ECs and Mouse Tumor Models. Bovine aortic endothelial cells and human umbilical vein endothelial cells were routinely cultured in 100-mm dishes in DMEM containing 10% serum and EGM (Clonetics, Walkersville, MD), respectively. Male 7–8-week-old RJ NMRI and C57Bl/6J mice (Elevage Janvier, Le Genest-St-Isle, France) were used in experiments with TLT (16)

Received 9/18/02; accepted 1/6/03.

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1 Supported by grants from the Fonds de la Recherche Scientifique Médicale, the Belgian Federation Against Cancer, the Fortis Cancerology Research Fund, the J. Maisin Foundation, the Association Sportive Contre le Cancer, the Pôle d’Attraction Interuniversitaire P5/02, C. D. and O. F. are Fonds National de la Recherche Scientifique (FNRS) Research Associates. P. S. is a FNRS Research Assistant, and A. B. is the recipient of a FNRS-Télévie grant.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; EC, endothelial cell; ROS, reactive oxygen species; TLT, transplantable liver tumor; Llc; Lewis lung carcinoma; GFR-Matrigel, growth factor-reduced Matrigel; IP, immunoprecipitation; SN, supernatant; L-NAME, Nω-nitro-L-arginine methyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PFK, phosphofructokinase.
and LLC cells (17), respectively. Each procedure was approved by the local authorities according to national animal care regulations. Tumor cells were injected i.m. in the posterior right leg of mice, in the vicinity of the saphenous artery. When the tumor transversal diameter reached 4.0 ± 0.5 mm, the mice were randomly assigned to a treatment group. When required, the NOS inhibitor L-NAMe (500 mg/liter) was added in the drinking water, renewed daily, and maintained for the periods of time mentioned.

Irradiation. Cells and anesthetized mice were irradiated using the RT-250 device (Philips) for a dose delivery of 0.86 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, IP, and NO Determination. Immunoblotting and IPs were carried out, as described previously (18, 19), with antibodies directed against caveolin-1, eNOS, and Akt (Becton Dickinson, Lexington, KY) and with phospho-specific antibodies against phospho-Ser1177-eNOS and phospho-Ser473-Akt (NEB Cell Signaling Technologies, Beverly, MA). The determination of NO level, e.g., the 24-h accumulation of NO derivatives in the cell-bathing medium (separated from total DNA using random hexamers, and PCR amplification was performed using SYBR Green reagent (Applied Biosystems, Warrington, United Kingdom). The specific primers were as follows: eNOS sense, 5'-ctctggctagggaggta-3'; eNOS antisense, 5'-accagctcactaatgaatcc-3'; GPHD sense, 5'-ccctgtagccagtccg-3'; GPHD antisense, 5'-cgagcagacagctggtctgc-3'; CD31 sense, 5'-gtacctgatcagcatgtacc-3'; and CD31 antisense, 5'-caagatgagtctggaagcagc-3'. Fluorescence data were analyzed, after PCR completion, with the ABI PRISM 7500 Sequence Detection System instrument (Applied Biosystems). Results were expressed as Ct (number of cycles needed to generate a fluorescent signal above a predefined threshold). Relative quantitation for a given gene, expressed as fold variation over control, was calculated using the 2^(-DeltaCt) formula after normalization to GAPDH (ΔΔCt) and determination of the difference in ΔCt (ΔΔCt) between control and irradiated tumors.

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

RESULTS

Irradiation Stimulates the NO Pathway in ECs. We first evaluated whether a single-dose irradiation was able to regulate eNOS expression in cultured ECs. Accordingly, we documented that ionizing radiations induce a time-dependent (Fig. 1A) and dose-dependent (Fig. 1B, top row) increase in eNOS expression (n = 3-4). Of note, under these experimental conditions (as well as in all other models used in this study), we did not detect any induction of inducible NOS expression after irradiation.

The eNOS activation is known to be regulated by posttranslational modifications, including the inhibitory interaction by the structural protein caveolin-1 and the stimulatory phosphorylation on serine 1177 (18). Co-IP experiments revealed that, independently of the X-ray dose, the same absolute amounts of eNOS were associated with caveolin-1 (Fig. 1B, middle row). In fact, the dose-dependent increase in the abundance of eNOS protein corresponded to an increased caveolin-free fraction of the enzyme, as shown by the amounts of eNOS left behind in the caveolin IP SN (Fig. 1B, bottom row and bar graph). Of note, the bar graph accompanying the blots in Fig. 1B gives the exact proportion of caveolin-free and -bound eNOS because it integrates the dilution factor (~16x) of SN samples and the differences in film exposures.

For Fig. 1C (left panels) shows that the extent of eNOS phosphorylation dose-dependently increases with irradiation. Importantly, when the data were normalized for the amounts of eNOS protein, the extents of eNOS phosphorylation at 2 and 6 Gy reached 125 ± 9% and 168 ± 21% of control levels (n = 4-10), respectively. We also found that the PI3K/Akt signaling pathway was involved in the process of irradiation-induced eNOS phosphorylation. Indeed, irradiation led to a significant increase in the amounts of the Ser-473-phosphorylated (activated) form of Akt (n = 4-10; Fig. 1C, right panels), and LY294002, the inhibitor of PI3K (an upstream activator of Akt), completely prevented eNOS phosphorylation in 6-Gy-irradiated ECs (94 ± 3% of control levels; n = 3; Fig. 1D).

Finally, to examine whether changes in eNOS expression and posttranslational regulations had a real impact on the activity of the enzyme, we measured the production of nitrates and nitrates (the decomposition products of NO) in ECs exposed to ionizing radiations; these measurements were repeated in the presence of the NOS inhibitor L-NAMe (5 mm). Accordingly, low-dose irradiations (2 and 6 Gy) led to a significant increase (+65% and +72%, respectively) in L-NAMe-sensitive NO production (P < 0.05; n = 6).
Irradiation Promotes NO-dependent EC Migration and Tube Formation. We then examined whether the up-regulation of the NO pathway was associated with functional changes in the ability of ECs to migrate and form capillary-like networks.

To evaluate migration, we used the scratch injury test based on the ability of ECs to sprout into the wounded area. Preliminary studies revealed that under the experimental conditions used in this assay, proliferation was almost absent [as also validated by others (20)], and only migration could account for the colonization of the scratched area. Of note, the cells found in the scratched area consistently presented the typical migratory spindle shape (see Fig. 2A).

Fig. 2 reveals that a 6-Gy dose dramatically stimulated the ability
of ECs to migrate toward the center of the wound. At 72 h, the scratched area was almost entirely repopulated. However, the evaluation of the promigratory effects of irradiation needed to take into account the fact that a proportion of ECs did not survive to the X-ray exposure. Accordingly, the migration index, which is corrected for cell death (see “Materials and Methods”), revealed a maximal 5.2-fold higher capacity of ECs to sprout 48 h after irradiation (Fig. 2B). Conversely, this migration index (n = 3–11) was not significantly different from controls when cells were exposed to the NOS inhibitor L-NAME after irradiation. Importantly, this promigratory impact of ionizing radiations was dose dependent because the effects were detected with doses as low as 2 Gy and increased progressively up to a dose of 20 Gy (data not shown).

We then examined whether irradiation could also lead to the reorganization of ECs in tubes or precapillary structures when cultured on extracellular matrices. Accordingly, ECs were irradiated at 6 and 20 Gy and plated 24 h later on Matrigel. Fig. 3A shows that contrary to nonirradiated cells, cells preexposed to X-rays rapidly formed tubes or precapillary structures. Because of the use of GFR-Matrigel, the irradiation-induced network then progressively faded. In fact, a bell-shaped effect was observed in the time course of network formation and disassembly (see Fig. 3B). Importantly, when the cells were treated with the NOS inhibitor L-NAME (n = 4), tube formation was completely prevented (Fig. 3B).

Irradiation Promotes NO-dependent Angiogenesis. We next documented the physiological effects of irradiation on angiogenesis using isolated mouse tumor microvessels and implanted Matrigel plugs.

When tumor microvessels were collected from TLT-bearing mice and cultured in GFR-Matrigel, the tumor vessels isolated from locally irradiated mice showed the sprouting of a vascular network (Fig. 4); von Willebrand labeling (data not shown) confirmed the endothelial nature of >90% of the outgrowing cells. When microvessels were grown in the presence of L-NAME, they did not lead to tube formation when incubated in Matrigel, confirming the key role of NO in this proangiogenic process (Fig. 4).

To test the in vivo relevance of these observations, we developed a model in which mice were injected with Matrigel at day 0 and irradiated (or not irradiated) at day 10, e.g., when the matrix plug was already partially invaded by host cells. The Matrigel plugs were harvested at day 15 and analyzed by immunohistochemistry for eNOS and von Willebrand factor expressions. Whereas nonirradiated plugs did not reveal a significant EC infiltration, a robust eNOS-positive capillary-like network was detected in the irradiated Matrigel (Fig. 5A). As found in the ex vivo experiments, the irradiation-induced proangiogenic process was completely abolished when mice received L-NAME (500 mg/liter) from day 9 to sacrifice (Fig. 5A, right panel). Of note, hypercellular areas were clearly visible at the host tissue-Matrigel plug interface. In the Matrigel plugs exposed to ionizing radiations, this compact cell rim was much larger and gave rise to infiltrating septa positive for von Willebrand factor staining (Fig. 5B). L-NAME treatment repressed both the the interface thickening and the cellular path formation (Fig. 5B, right panel).

Irradiation Induces EC Recruitment in Murine Tumors. We next determined whether a fractionated protocol of radiotherapy could also induce endothelial sprouting in tumors. Accordingly, we performed local successive irradiations each 24 h for 5 days in LLC tumor-bearing mice. As early as 24 h after the last dose delivery, CD31-positive punctate labeling was visible at the tumor-host tissue interface in irradiated tumors but not in untreated tumors, where CD31 antibodies only labeled mature host vessels (data not shown). The irradiation-induced endothelial sprouting appeared to be a dynamic process because in tumors collected 120 h after the last dose delivery (Fig. 5C), the extent of EC infiltration was 7-fold higher than that in nonirradiated tumors. Interestingly, this irradiation-induced EC recruitment in tumors was repressed when mice were treated with L-NAME (from day −1 to sacrifice; Fig. 5C, right panel). Moreover, the tumor-host interface appeared to be a privileged zone for EC recruitment because irradiated host muscular tissues distant from the tumor were not colonized by ECs (Fig. 5C, middle panel, inset). These findings were confirmed by using other EC markers including eNOS and the von Willebrand factor (data not shown).

Irradiation Induces eNOS Expression in Human Tumors. To determine whether irradiation also increases eNOS expression in human tumors, we used real-time PCR to determine the abundance of eNOS mRNA in squamous cell carcinoma biopsies collected before and after a 2-Gy irradiation. Because of the intrinsic variability of vascular content in tumors, eNOS gene expression was normalized...
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Fig. 6 represents the changes in eNOS expression, e.g., the ratio of eNOS:CD31 gene expression, in repetitive biopsies collected before and after irradiation in five patients (n = 14 biopsies). These data indicate that a 2-Gy irradiation was sufficient to induce a significant increase in eNOS expression in this human tumor type.

DISCUSSION

In this study, we report that low-dose irradiation exerts proangiogenic effects through the up-regulation of the endothelial NO pathway. Indeed, we provide in vitro, ex vivo, and in vivo evidence that NO acts as a key mediator of the irradiation-induced formation of new vascular network and tumor invasion by ECs. Two mechanisms leading to the increased NO production by ECs on exposure to ionizing radiations have been identified. First, eNOS expression was time- and dose-dependently increased on exposure to irradiation (Fig. 1, A and B). Furthermore, the activability of this increased pool of eNOS did not appear to be counterbalanced by a higher level of interaction with its functional, allosteric inhibitor caveolin-1 (see Fig. 1B). Second, the level of eNOS phosphorylation was also enhanced (to a larger extent than the up-regulation of eNOS expression itself), suggesting a higher ability of these cells to exhibit a sustained production of NO (see Fig. 1, C and D). Accordingly, such increases in both abundance and activation of eNOS led to a robust increase in NO release compatible with the angiogenic phenotype shift.

It is important to stress that although irradiation produces large amounts of ROS known to react with NO and neutralize its action, in our study the blocking effects of the NOS inhibitor L-NAME on functional read-outs such as EC migration (Fig. 2) and tube formation (Fig. 3) demonstrate that NO production reached sufficient levels to overcome the scavenging effects of ROS. Nevertheless, ROS production is likely to be involved in the transcriptional and posttranslational regulation of eNOS. Several investigators have indeed reported that ROS can induce AP-1 activation, which in turn promotes eNOS gene transcription (12, 21), whereas others have documented that cell exposure to ROS can lead to Akt activation (14), which, in turn, phosphorylates eNOS on serine 1177 (19). This Akt pathway is also likely to account, at least in part, for the survival of a proportion of ECs exposed to ionizing radiations, as recently documented by Edwards et al. (22).

Importantly, our results do not refute the existence of cytotoxic effects of irradiation on ECs, as reported previously to participate in the antitumor treatment (23). In fact, our results support the nonexclusive proposition that a proportion of ECs did survive the radiation stress and that this selected cell population underwent the phenotypic changes observed. Doses and schemes of radiation administration are likely to influence the outcomes of ECs exposed to X-rays, e.g., the proapoptotic or prosurvival signaling pathways (24). Interestingly, in the clinical context of fractionated radiotherapy as administered nowadays, we consistently found an increase in eNOS mRNA transcript abundance in tumor biopsies collected after local exposure to a 2-Gy irradiation (Fig. 6). These data support the paradigm of the rapid induction of the NO pathway as elicited in our experimental models. Together with recent reports documenting the X-ray-induced increase in VEGF (2, 10), an upstream activator of eNOS signaling (11), our findings strongly suggest that an angiogenic phenotype shift is also likely to occur in human tumors. Furthermore, our data obtained with mouse-implanted Matrigel plug and tumors suggest that irradiation induced an increase in total density of ECs. The origin of ECs lining tumor vessels is still under debate. Recent findings suggest that hematopoietic and/or circulating endothelial progenitor cells participate in tumor neovascularization (25). In our mouse models, the hypercellularity and the presence of vascular structures (observed after irradiation) at the interface between host tissues and tumor or Matrigel (Fig. 5, A and B) suggest the participation of an inflammatory process in the initiation of the angiogenic pathway as observed in...
other models (26). Interestingly, the inhibitory effect of L-NAME on EC colonization of the implanted Matrigel plugs and tumors strongly suggests that NO is not only involved in the network reorganization of ECs but also participates in their recruitment. Additional experiments on slow-growing tumors (e.g., which are more angiogenesis dependent for their growth than the tumor models used in this study) are required to evaluate whether this NO-mediated process originates from endothelial progenitor cells recruitment and/or EC pruning from existing or inflammation-induced local vascularization.

Beside the mechanistic dissection of the irradiation-induced up-regulation of the NO pathway in the tumor vessel ECs, our data provide an additional rationale for associating antiangiogenic strate-

Fig. 4. Irradiation induces NO-dependent angiogenic sprouting ex vivo. TLT-bearing mice, treated or not treated with L-NAME (500 mg/liter drinking water), were locally irradiated and sacrificed 24 h later. Tumor arterioles were harvested, cultured in GFR-Matrigel, and studied for their ability to give rise to endothelial sprouting (bar = 100 μm).

Fig. 5. Irradiation induces NO-dependent EC recruitment in Matrigel plugs and tumors. A and B. Matrigel plugs were s.c. implanted in mice at day 0 and allowed to be colonized by host cells until day 10. They were then locally irradiated (6 Gy) and harvested 5 days later. When indicated, mice were treated with L-NAME from day 9 to day 15. Slices of the host tissue-Matrigel plug transitions were immunostained with antibodies against eNOS (A) and the von Willebrand factor (B). Insets show eNOS staining of a newly formed endothelial tube at higher magnification and CD31 staining of rich cellular paths through the Matrigel plug, respectively. Arrows delineate the hypercellular regions at the host-plug interface, and arrowheads point to vascular structures in the cell rim and septum. C. LLc-bearing mice (treated or not treated with L-NAME) were locally irradiated according to a fractionated scheme (each 24 h from day 0 to day 4) and sacrificed 120 h after the last dose delivery. Slices of the host tissue-tumor transitions were immunostained with antibodies directed against the EC marker, CD31. Inset shows the absence of CD31 staining in the irradiated host tissue (muscle) at a more distal location from the muscle-tumor interface. These experiments were repeated two to three times with similar results (bar = 100 μm).
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Fig. 6. Irradiation induces an increase in ENOS expression in human tumors. Biopsies of head and neck squamous cell carcinoma were collected before and 24 h after local irradiation (2 Gy). RNA was extracted, cDNA was synthesized, and real-time PCR amplifications were performed to evaluate both CD31 and eNOS gene expressions in each sample. Shown is the logarithmic representation of the changes in abundance of eNOS transcripts normalized per amounts of vascular structure (as determined by the abundance of CD31 transcripts in a given sample) in paired biopsies. Lines connect the expression levels in biopsies taken from the same patient before and after irradiation. Note that for one patient ( ), three biopsies were obtained before and after irradiation: only the mean values (±SE) are presented.

ACKNOWLEDGMENTS

We thank Delphine De Mulder for excellent technical assistance.

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