

Research Paper

The Response to Brain Tumor-Derived Growth Factors is Altered in Radioresistant Human Brain Endothelial Cells

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ABSTRACT

Introduction: Radioresistant brain tumor vasculature is thought to hamper the efficiency of adjuvant cancer therapies. However, little is known regarding the signalling pathways involved in the angiogenic response to brain tumor-derived growth factors in irradiated human brain microvascular endothelial cells (HBMEC). The goal of this study is to assess the effect of ionizing radiation (IR) on HBMEC survival, migration and tubulogenesis.

Methods: HBMEC were cultured and irradiated at sublethal single doses. Cell survival was assessed by nuclear cell counting and flow cytometry. HBMEC migration in response to brain tumor-derived growth factors (U-87 GF) and tubulogenesis were assayed using modified Boyden chambers and Matrigel, respectively.

Results: We observed that single administration of 3-10 Gy IR doses only reduced cell survival by 30%. Radioresistant HBMEC overexpressed RhoA, a small GTPase protein regulating cellular adhesion and migration, and Rho-kinase (ROK), a serine-threonine protein kinase and one of RhoA's major targets. HBMEC migration was induced by vascular endothelial growth factor (VEGF), but even more so in response to sphingosine-1-phosphate (S1P) and to U-87 GF. Following IR exposure, HBMEC basal migration increased more than two-fold, whereas the response to S1P and to U-87 GF was significantly diminished. Similarly, the inhibitor of ROK Y-27632 decreased HBMEC migration in response to S1P and U-87 GF. Overexpression of RhoA decreased tubulogenesis, an effect also observed in irradiated HBMEC.

Conclusion: Our results suggest that radioresistant HBMEC migration response to tumor-secreted growth factors and tubulogenesis are altered following IR. The RhoA/ROK signalling pathway is involved in the IR-altered angiogenic functions and may represent a potential molecular target for enhancing the impact of radiotherapy on tumor-associated endothelial cells.

INTRODUCTION

Glioblastoma multiform (GBM) represents the most common but also the most lethal primary cerebral neoplasm in the adult population.¹ In fact, median length of survival rarely exceeds one year after diagnosis.^{1,2} From the highly cellular rim of viable tumor which surrounds the necrotic core, GBM cells infiltrate the adjacent cerebral tissue.³ Although single invasive glioma cells do not solely depend on tumor-associated angiogenesis, the growth of primary GBM and the development of a recurrent mass critically depends on neovascularisation. This explains why malignant gliomas are amongst the most intensively vascularized solid tumors.⁴

The process of tumor angiogenesis begins with increased vessel permeability via degradation of basement membrane by proteases secreted by activated endothelial cells (EC), which migrate and proliferate, leading to the formation of solid EC sprouts into the stroma.^{5,6} This process is regulated by a host of growth factors such as the vascular endothelial growth factor (VEGF) or the platelet-derived growth factor (PDGF). These are produced either by the tumor itself or by infiltrating inflammatory cells.^{5,7,8} It is noteworthy that several studies have recently defined platelet phospholipids as potential angiogenic factors, most notably sphingosine-1-phosphate (S1P), which is released by activated platelets as well as by glioblastoma cells⁹ and which is a very potent EC chemoattractant.^{10,11}

Radiotherapy is generally believed to exert its anti-cancerous effects by targeting tumor cells.¹² To this end, we have shown that ionizing radiation (IR) decreases in vitro cell proliferation of malignant glioma cells.¹³ Paradoxically, the glioma cells that resist the acute cytotoxic effects of radiation may acquire an increased migratory potential and enhanced invasiveness.^{14,15} It was recently suggested that IR prevents tumor growth by

directly targeting tumor vasculature and inducing pro-apoptotic and necrotic processes.¹⁶⁻²⁰ It is recognized that both single-dose and fractionated radiotherapy induce EC damage.²¹ Interestingly, endothelial apoptosis and microvascular dysfunction contribute more significantly to tumor cell lethality by the single-dose approach (8–10 Gy) than the low-dose exposures to fractionated therapy (1–3 Gy) since adaptive responses are simultaneously induced in the latter regiment.²¹ Surprisingly, EC can also survive single radiation exposure similarly to cancer cells, in part due to of the tumor-derived microenvironment in which the phenotypic and functional properties of the EC differ from those of normal brain EC.²² The signaling pathways and altered angiogenic functions in radioresistant human brain EC have not yet been investigated.

The Rho signalling pathway has been proposed to be involved in the radioresistance and IR-induced invasiveness of primary GBM.^{13,23,24} Rho GTPase proteins function as molecular switches that modulate the activation of enzymes involved in different biological processes related to tumor progression, such as cell proliferation, apoptosis, cytoarchitecture, adhesion, migration, cell polarity, and transcriptional regulation.^{25,26} The roles of Rho proteins in the cell motility and cell morphology of EC have only recently begun to be uncovered.^{26,27} The role(s) of this pathway in the functional properties of radioresistant EC has not yet been assessed. To our knowledge the human brain microvascular endothelial cell (HBMEC) model used in this study is the closest in vitro model for approximating the human brain tumor-derived EC phenotype in long term studies.

The goal of our study is to investigate the effects of IR on HBMEC angiogenic responses, i.e., cell survival, 3D tubulogenesis, and migration in response to VEGF, S1P and to human glioblastoma cell-derived growth factors. The RhoA/ROK signalling pathway was also investigated in relation to the adaptive properties of radioresistant HBMEC.

MATERIALS AND METHODS

Materials. Sodium dodecyl sulfate (SDS), bovine serum albumin (BSA) and S1P were purchased from Sigma (Oakville, ON). Lipofectamine-2000 transfection reagent and trypsin were from Invitrogen (Burlington, ON). Mouse anti-RhoA and anti-ROK monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-Caveolin-1 monoclonal antibody was from BD Pharmingen (Mississauga, ON) and mouse anti-GAPDH monoclonal antibody was from Advanced Immunochemical (Long Beach, CA). Horseradish peroxidase-conjugated anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). BCA protein assay kit was purchased from Pierce (Rockford, IL) and enhanced chemiluminescence (ECL)-Western blot kit from Chemicon International (Temecula, CA). Products for electrophoresis were obtained from Bio-Rad (Mississauga, ON) and polyvinylidene difluoride (PVDF) membranes were from Boehringer Mannheim (Laval, QC). Human recombinant VEGF (isoform 165) was produced and purified as previously described.²⁸ Y-27632 was purchased from Calbiochem (San Diego, CA).

Cell culture and cDNA transfection method. Human brain microvascular endothelial cells (HBMEC) were obtained from Dr Kwang Sik Kim (John Hopkins University School of Medicine, Baltimore, MD). These cells were positive for factor VIII-Rag, carbonic anhydrase IV, Ulex Europaeus Agglutinin I, took up fluorescently labelled, acetylated low-density lipoprotein and expressed

gamma glutamyl transpeptidase, demonstrating their brain EC specific phenotype.²⁹ HBMEC were immortalized by transfection with simian virus 40 large T antigen and maintained their morphological and functional characteristics for at least 30 passages.³⁰ HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 10% (v/v) NuSerum (BD Bioscience, Mountain View, CA), modified Eagle's medium nonessential amino acids (1%) and vitamins (1%), heparin (5 U/ml), sodium pyruvate (1 mM), L-glutamine (2 mM), EC growth supplement (30 µg/ml), 100 units/ml penicillin and 100 µg/ml streptomycin. Culture flasks were coated with 0.2% type-I collagen to support the growth of HBMEC monolayers. Cells were cultured at 37°C under a humidified atmosphere containing 5% CO₂. All experiments were performed between cell passages 16 and 28. HBMEC were transiently transfected with the Myc-tagged WT-RhoA cDNA construct generously provided by Dr. Allan Hall (University College London, London, UK). All experiments involving these cells were performed 36 hours following transfection. Mock transfection of HBMEC cultures with empty pcDNA (3.1+) expression vector was used as a control.

Irradiation treatment. Cells were irradiated with a 6 MV photon beam from an Elekta SL75 linear accelerator. The delivered radiation doses were measured using a thermoluminescence dosimetry (TLD) system with an accuracy of 7%. During irradiation, cells were in RPMI containing 10% FBS and 10% NuSerum. Radioresistant cells were allowed to recuperate for 48 hours. Nonirradiated control cells were handled similarly to those which were subjected to IR treatment.

Cell survival assay. Cells were collected by gentle scraping and were resuspended in the overlaying medium. From each experimental sample, 150 µl of cell suspension were saved for nuclear cell counting using an automatic cell counter (New Brunswick Scientific Co., Edison, NJ). Viable cell number determination was also assessed using Trypan blue dye exclusion. Cells stained dark blue were not considered viable. Cell survival data are expressed as a mean value for at least four independent experiments.

Analysis of apoptosis/necrosis by flow cytometry. Cell death was assessed 48 hours after irradiation by flow cytometry. Cells floating in the supernatant and adherent cells harvested by trypsin solution were gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with PBS. Then, 2×10^5 cells were pelleted and suspended in 200 µL of buffer solution and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) as described by the manufacturer (BD Bioscience). The cells were diluted by adding 300 µL of buffer solution and processed for data acquisition and analysis on a Becton Dickinson FACS Calibur flow cytometer using CellQuest Pro software. The X- and Y-axes indicated the fluorescence of annexin-V and PI respectively. It was possible to detect and quantitatively compare the percentages of gated populations in all of the four regions delineated. In the early stages of apoptosis, phosphatidylserine is translocated to the outer surface of the plasma membrane, which still remains physically intact. As annexin-V binds to phosphatidylserine but not to PI, and the dye is incapable of passing the plasma membrane, it is excluded in early apoptosis (annexin-V⁺/PI⁻). Cells in late apoptosis are stained with annexin-V and PI (annexin-V⁺/PI⁺). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin-V⁻/PI⁺).

Analysis of HBMEC migration. HBMEC migration was assessed using modified Boyden chambers. The lower surfaces of

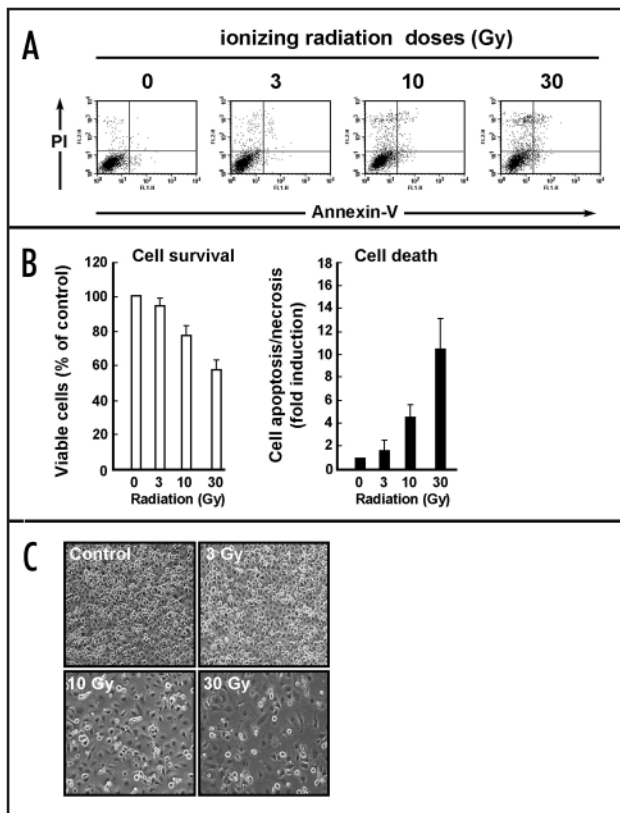


Figure 1. Ionizing radiation decreases HBMEC survival. Subconfluent HBMEC were exposed to single doses of ionizing radiation of different strengths (IR). (A) Cell survival was assessed 48 hours after IR exposure by using Annexin-V/PI staining and trypan blue exclusion (B) as described in the Methods section. Cell survival data are expressed as a mean value of at least three independent experiments. Error bars represent standard deviation of values. C) Pictures of adherent HBMEC were taken 48 hours after IR.

Transwells (8- μ m pore size; Costar, Acton, MA) were precoated with 0.2% type-I collagen for 2 hours at 37°C. The Transwells were then assembled in a 24-well plate (Fisher Scientific Ltd, Nepean, ON). The lower chamber was filled with serum-free HBMEC medium or growth factor-enriched conditioned medium isolated from 48 hours serum starved-U-87 human glioblastoma cells (U-87 GF). Control HBMEC or cells exposed to IR were collected by trypsinization, washed and resuspended in serum-free medium at a concentration of 10^6 cells/ml and 10^5 cells were then inoculated onto the upper side of each modified Boyden chamber. The plates were placed at 37°C in 5% CO₂/95% air for 30 minutes after which VEGF (50 ng/ml), S1P (10 μ M), or Y-27632 (10 μ M) (an inhibitor of Rho-kinase (ROK)) were added to the lower chambers of the Transwells. Migration then proceeded for 20 hours at 37°C in 5% CO₂/95% air. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% crystal violet-20% methanol (v/v). Images of at least five random fields per filter were digitized (100X magnification). The average number of migrating cells per field was quantified using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON). Migration data are expressed as a mean value of at least four independent experiments.

Endothelial cell morphogenesis assay. Tubulogenesis was assessed using Matrigel aliquots of 50 μ L, plated into individual wells of 96-well tissue culture plates (Costar, Amherst, MA) and

allowed to polymerize at 37°C for 30 minutes. After brief trypsination, control or irradiated HBMEC were washed and resuspended at a concentration of 10^6 cells/ml in serum-free medium. Next, 25 μ L of cell suspension (25,000 cells/well) and 75 μ L of serum with medium were added into each culture well. Cells were allowed to form capillary-like tubes at 37°C in 5% CO₂/95% air for 20 hours. The structures formed within the Matrigel were digitized and quantified (100X magnification). For each experiment, four randomly chosen areas were quantified by counting the number of tubes formed. Tubulogenesis data are expressed as a mean value of at least three independent experiments.

Immunoblotting procedures. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred onto polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were then washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% BSA, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-mouse IgG (1/2,500 dilution) in TBST containing 5% non-fat dry milk. The secondary antibodies were visualized by ECL and quantified by densitometry.

Statistical data analysis. Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test and was used to compare migration and extent of capillary-like structure formation to mock nonirradiated HBMEC migration and tube formation. Probability values of less than 0.05 were considered significant and are indicated by an asterisk (*).

RESULTS

Ionizing radiation reduces HBMEC survival. HBMEC survival to increasing doses of IR was first examined. Subconfluent cells were exposed to single doses of IR and the cell survival was assessed 48 hours after exposure to IR using flow cytometry (Fig. 1) and trypan blue dye exclusion (Fig. 1). We observed a dose-dependent decrease in cell survival (lower left quadrants in Fig. 1) that was quantified by up to 40% at 30 Gy (Fig. 1, left panel). Induction of significant cell death was also observed with increasing IR doses, as 10–30 Gy induced a combined necrosis/apoptosis of 4–10-fold over control (Fig. 1, right panel). The appearance of a heterogeneous cellular morphology (100X magnification) was also apparent at 10–30 Gy confirming some cell death (Fig. 1). These observations suggest that approximately 60–70% cells escaped the IR-induced cytotoxic effect and potentially exhibited some radioresistant phenotype. This sub-population was further analyzed for the rest of our study.

HBMEC migration in response to pro-angiogenic factors is diminished in radioresistant cells. EC migration in response to pro-angiogenic stimuli is a feature of angiogenesis. Several reports have previously shown that both tumor-derived VEGF and the angiogenic, platelet-derived S1P stimulated brain EC migration.^{9,31,32} Therefore we assessed cell migration of control and irradiated HBMEC. A single 10 Gy radiation dose was found to trigger a significant 2.15-fold increase in HBMEC migration (Fig. 2). As expected, basal migration of control HBMEC was increased by 1.75-fold in response to VEGF (50 ng/ml), 2.5-fold with S1P (10 μ M) and 4.4-fold with U-87 GF. Interestingly, the pro-migratory effect of

S1P tended to decrease in irradiated HBMEC, while it was significantly abrogated in response to U-87 GF (Fig. 2).

Single dose IR induces the expression of RhoA, ROK and caveolin-1. We previously found that a single IR dose induced the expression of RhoA, a small GTPase protein regulating cellular adhesion, migration and invasion,²⁵ in radioresistant U-87 malignant glioma cells.¹³ Therefore we examined, in irradiated HBMEC, expression of the RhoA protein, expression of ROK (a serine-threonine protein kinase known to be one of RhoA's major targets) and the expression of Caveolin-1, a protein associating RhoA to endothelial caveolin-enriched membrane domains,³³ which are proposed to participate in cell survival and angiogenesis.³⁴ After exposure to 10 Gy IR, a 2-fold increase was observed in RhoA expression, while that of ROK increased by 1.5-fold (Figs. 3A and B). Interestingly, the expression of caveolin-1 also significantly increased by 1.6-fold after IR, while that of the house-keeping protein GAPDH remained unaffected.

Inhibition of RhoA signalling pathway diminishes HBMECs' response to pro-angiogenic factors. In order to partially mimic the RhoA/ROK-activated signalling pathway of irradiated cells, HBMEC were transfected with the cDNA construct encoding for RhoA protein. Transfection efficiency was confirmed by immunodetection for ectopic RhoA protein in RhoA-transfected cells (Fig. 4A). Mock, irradiated and RhoA-transfected HBMEC were inoculated onto the upper side of each Transwell and migration proceeded as described in the Methods section. Both IR and overexpression of RhoA significantly increased basal HBMEC migration by 1.75-fold and 2.6-fold, respectively (Fig. 4B). We further studied the migration of RhoA-transfected HBMEC in the presence of the two most potent pro-angiogenic conditions used, namely, S1P and U-87 GF. The migration of HBMEC which overexpressed recombinant RhoA increased respectively 3.2-fold and 6.4-fold in response to S1P and U-87 GF (Fig. 4B, black bars), relatively to mock cells in the absence of added stimulatory agent. Whereas RhoA-transfected HBMEC exhibit increased migration, as reflected with a RhoA-transfected/Mock ratio of 2.6, their migration response was lower showing a ratio of 1.26 for S1P and 1.46 for U-87 GF. Indeed, RhoA-transfected HBMEC present a weaker migration response to pro-angiogenic agents but irradiated HBMECs' migration response is abrogated (Figs. 2 and 4). This suggests that other signalling

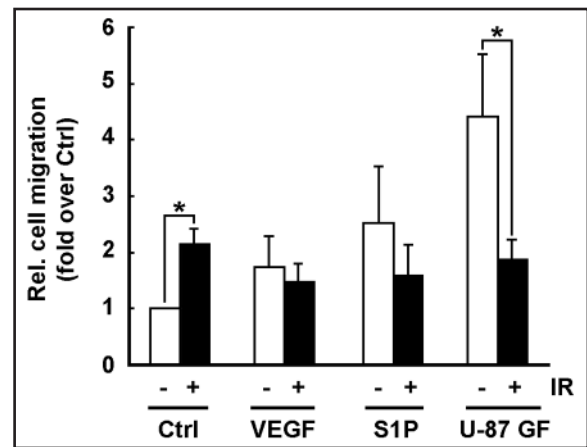


Figure 2. Ionizing radiation inhibits HBMECs' migratory response to brain tumor-derived growth factors. Control and irradiated (10 Gy) HBMEC were seeded on type-1 collagen-coated filters, allowed to adhere for 30 min and migration performed as described in the Methods section in the presence or absence of VEGF (50 ng/ml), S1P (10 μ M), or U-87 GF. Migration data are expressed as a mean value for the relative migration rate from at least four independent experiments. Error bars represent standard deviation of values.

pathways, in addition to the RhoA pathway, may be implicated in mediating the altered angiogenic functions of radioresistant HBMEC. We also assessed the effect of inhibiting the RhoA/ROK signalling pathway on HBMEC migration. The addition of Y-27632, an inhibitor of ROK, into the lower chamber of the Transwells did not affect the migration of control HBMEC but significantly decreased that of RhoA-transfected cells as well as decrease the migration of irradiated HBMEC as compared to cells untreated with the ROK inhibitor. The addition of Y-27632 to S1P and CM U-87 significantly inhibited mock, irradiated and RhoA-transfected HBMECs' migration response to these angiogenic agents.

IR and RhoA overexpression decrease HBMEC in vitro tubulogenesis. We next investigated HBMEC's capacity to form tube-like structures on Matrigel. Matrigel is a reconstituted basement membrane containing various growth factors including basic fibroblast growth factor, platelet-derived growth factor, transforming growth factor beta, epidermal growth factor and insulin-like growth factor 1.³⁵

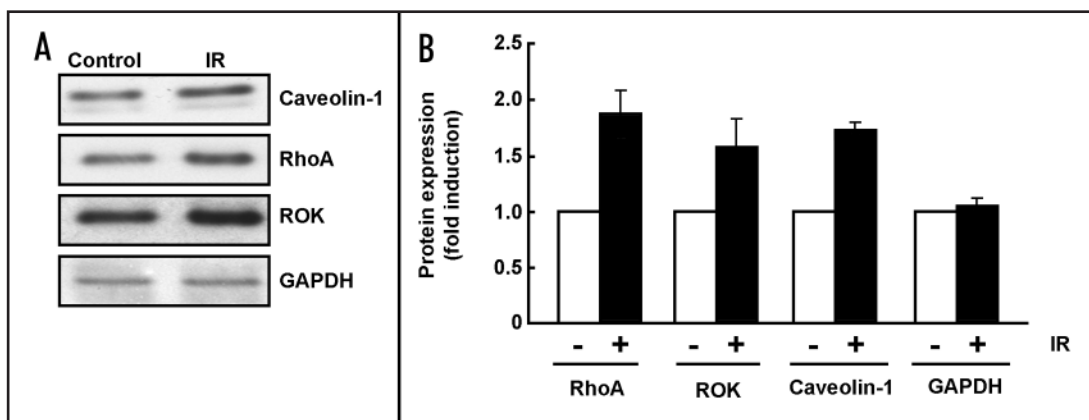


Figure 3. Ionizing radiation induces RhoA, ROK and Caveolin-1 expression in radioresistant HBMEC. Subconfluent HBMEC were irradiated at 10 Gy, left to recuperate at 37°C for 48 hours and then radioresistant cells were harvested. Cell lysates from each condition were electrophoresed on SDS gels and (A) immunodetection was carried out as described in the Methods section. (B) Quantification of RhoA, ROK and Caveolin-1 protein expression was performed by scanning densitometry of samples from control and from irradiated HBMEC. Protein expressions were normalized to GAPDH expression.

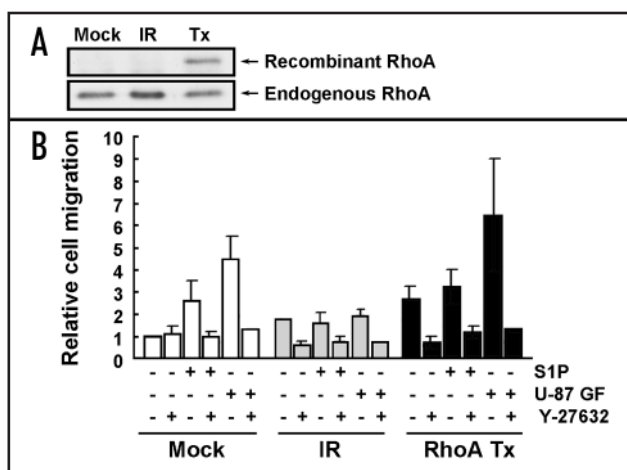


Figure 4. Inhibition of RhoA/ROK signalling pathway produces similar diminution in migration responses of RhoA-transfected and of irradiated HBMEC. (A) HBMEC cultured at 70% confluence were transfected with the Myc-tagged WT-RhoA cDNA construct. The transfection efficiency was confirmed by Western blotting and immunodetection for anti-RhoA as described in the Methods. Endogenous RhoA was detected after a 30 second exposition and recombinant RhoA after a two minute exposition. (B) RhoA-transfected (RhoA Tx) and irradiated (IR, 10 Gy) HBMEC were seeded on type-1 collagen-coated filters and allowed to adhere for 30 min. Migration was performed for 20 hours in the presence or absence of S1P (10 μ M), U-87 GF or Y-27632 (10 μ M). Migration data are expressed as a mean value of at least four independent experiments. Error bars represent standard deviation of values.

In a first series of experiments, irradiated and RhoA-transfected HBMEC were seeded on Matrigel and allowed to form capillary-like tubes. While nonirradiated HBMEC were capable of forming an extensive network of thin tubules (Fig. 5A), these capillary-like structures were decreased in irradiated and RhoA-transfected HBMEC by 50% and 42%, respectively (Fig. 5B). Tubules formed under these conditions presented aggregated cells with heterogeneous tubule thickness and diameter (Fig. 5A). Therefore HBMEC that overexpress RhoA or that survive IR present a decreased tubulogenesis potential. Moreover, we assessed the inhibitor of ROK, Y-27632, on tubulogenesis. Mock, irradiated and RhoA-transfected HBMEC were resuspended in medium containing Y-27632 and were seeded on Matrigel. In the presence of Y-27632, mock HBMEC's capacity to form tubes was completely abolished (Fig. 5A and B). Furthermore, although the inhibitor of RhoA/ROK pathway completely disrupted the abnormal tubes formed by radioresistant HBMEC, it partially reversed the abnormal appearance of tubes formed by RhoA-transfected HBMEC (Fig. 5A and B). Indeed, small diameter tube-like structures were observed in HBMEC overexpressing recombinant RhoA and treated with the RhoA/ROK inhibitor (Fig. 5A and B).

DISCUSSION

Until recently, research on the effects of IR on malignant gliomas was primarily focused on the cancer cells themselves. Although IR suppressed neoplastic glial cell proliferation,^{13,36,37} inhibited cell cycle progression³⁶ and triggered cell death,³⁸ radiotherapy not only still fails to eradicate all tumour cells, but paradoxically enhances glioblastoma cells invasive potential.^{14,15} On the other hand, the effects of IR on the cerebral vascular compartment have been far less

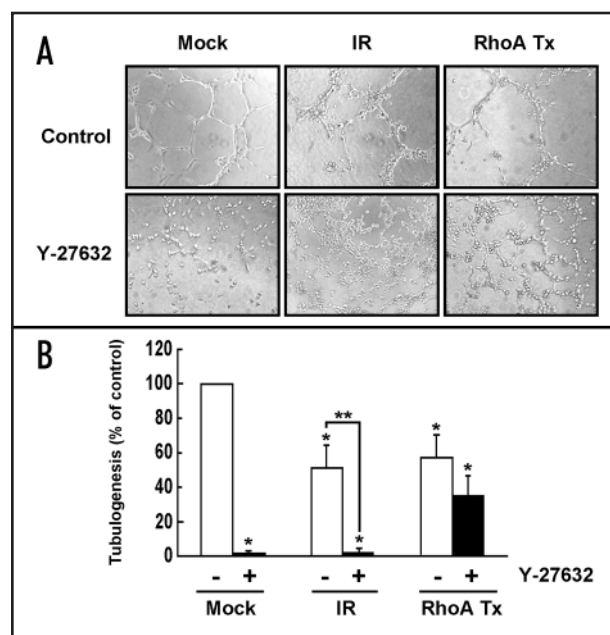


Figure 5. Ionizing radiation and RhoA overexpression decrease HBMEC capacity to form tubes in vitro. HBMEC were seeded on Matrigel and allowed to form capillary-like tubes at 37°C for 20 hours in the presence or absence of Y-27632 (10 μ M). (A) The formation of capillary-like structures was examined microscopically and pictures (100 X magnification) were taken. A representative experiment is presented. (B) Capillary-like structures were quantified as described in the Methods section. For each experiment, four randomly chosen areas were quantified by counting the total number of well formed tubes. Tubulogenesis data are expressed as a mean value of at least three independent experiments. Error bars represent standard deviation of values. (*) represents statistical significance with respect to untreated mock cells, while (**) statistical significance between untreated and Y-27632-treated cells in mock, IR-treated cells, and in RhoA-transfected cells.

documented. It has been suggested that the microvascular damage induced by IR might regulate the tumor response to further radiation and therefore mediate the sensitivity of tissues to radiotherapy, especially in single-dose regimens.^{21,39} In fact, IR is thought to exert antiangiogenic effects on EC of various origins such as human umbilical vein EC (HUVEC).^{19,40,41} It should be noted that HUVEC immortalized with SV40 antigens and the catalytic subunit of human telomerase overexpress the tumor endothelial marker-1/endothelialin, which is regarded as the most differentially expressed molecule in tumor-derived endothelium versus normal endothelium.⁴² Therefore, the fact that HBMEC were immortalized by transfection with SV40 allows us to further approximate the molecular impact of our study on cells that would have acquired some transformed phenotype in the GBM tumor microenvironment. The HBMEC model used in this study is a surrogate model that approximates tumor-derived EC. To our knowledge, it is the closest in vitro model that can approximate the human brain tumor-derived EC phenotype in long term studies.²⁰

We have recently shown that IR decreases in vitro HBMEC cell survival through altered cell cycle progression and induction of apoptosis and necrosis.²⁰ In fact, that study was the first to report a dose response to IR in HBMEC.²⁰ Although EC dysfunction has been observed following fractionated 1–3 Gy therapy, the endothelial damage induced by single 8–10 Gy dose radiotherapy has been reported to induce greater tumor cell lethality.²¹ Given the variability

of radiation regimens applied in clinical practice, one can envision EC to be exposed to different total radiation doses administered in a single or multiple fractions.⁴³ Noteworthy, EC response to IR in mouse and human tumor specimens displayed an apparent threshold at 8–10 Gy²¹ supporting the doses range of our current study. Therefore, the ability of IR to damage HBMEC at doses relevant to clinical radiotherapy suggests that targeting EC may contribute to the overall anti-cancerous effect of radiotherapy.¹⁶⁻²⁰

Since each tumor capillary vascularizes hundreds of tumor cells, targeting tumor vasculature should potentiate the anti-tumorigenic effect of radiation. However, similar to tumour cells, EC can escape IR effects. The signalling pathways through which EC are protected against IR have only begun to be investigated and the activation of the phosphoinositide-3-kinase (PI3-K)-Akt-Bcl-2 survival pathway has been recently suggested.^{44,45} The impact of IR exposure on the angiogenic functions of surviving human brain tumor-derived EC has not previously been investigated.

Here we investigated the specific impact of IR on HBMEC, which approximate human brain tumor-derived EC, with a focus on migration and tubulogenesis. Initial characterization of HBMEC migration revealed induction by VEGF and S1P. However, the strong migration response induced by S1P, in comparison to VEGF, suggests that this factor induces agonist-specific regulation of EC angiogenic responses.⁴⁶ The HBMEC migration response was even greater in the presence of U-87 GF, which contains numerous pro-angiogenic growth factors and cytokines secreted by malignant brain tumors. Radioresistant HBMEC had an increased basal migration potential in comparison to nonirradiated HBMEC consistent with our previous observations on irradiated HUVEC.¹⁹ The IR-induced migration of HBMEC correlates with their increased expression of RhoA and caveolin-1, both known to regulate EC migration.^{19,47}

Interestingly, although radioresistant HBMEC have an increased migration potential, their response to angiogenic factors, including VEGF, S1P and U-87 GF, was significantly attenuated. Furthermore, tubulogenesis capacity was also decreased in irradiated HBMEC. The reported effects of IR on tubulogenesis vary widely as some studies reported that IR increased tube formation,¹⁹ while others reported decreased tube formation.^{40,48} This discrepancy may reflect cell type specificity and differences in experimental conditions, notably the maximum IR dose and the time elapsed from radiation to the time that tubulogenesis was assessed.

It is established that tumors can regulate the responsiveness of their associated EC by secreting growth factors, cytokines and mitogens.³⁹ The induction of angiogenic factors has also been proposed to be part of the tumors-associated EC response to IR-induced stress.^{49,50} It has been documented that IR of the tumor cell compartment may induce the coexpression of VEGF^{40,51,52} and of its receptors⁵¹ and stimulate the expression of PDGF.⁵³ Irradiated HUVEC and human dermal microvascular EC showed upregulation of VEGF receptor-2.⁴⁰ IR may also result in increased phosphorylation of tyrosine residues in the cytoplasmic domain of receptor tyrosine kinases, which serve as docking sites for signalling entities of downstream pathways.^{54,55} Therefore, radioresistant HBMEC angiogenic functions, namely migration response to chemoattractants and tubulogenesis, may be attenuated due to alterations in downstream signaling pathways. Although some EC escape radiation's cytotoxicity, they respond weakly to growth factors required for tumor expansion and angiogenesis. This probably contributes to IR's capacity to stabilize or reduce tumor cell burden.

The Rho signalling pathway has been reported to be involved in the radioresistance of glioblastomas.^{13,23,24} Rho, Rac and Cdc42 have been shown, *in vitro*, to affect multiple aspects of cell behavior relevant to tumorigenicity.²⁵ RhoGTPases are critical not only for tumor cells but also for EC shape changes and for the adhesion dynamics that drive migration.^{26,56,57} Although the RhoA/ROK signaling pathway may be activated in radioresistant HBMEC, a balance in the RhoA/ROK pathway may be crucial in regulating EC functions.^{27,57} Tubulogenesis in control and irradiated HBMEC may be diminished by ROK inhibitors if the resultant balance of RhoA/ROK expression is negative. However, tube formation of RhoA-transfected cells may be partially reestablished by RhoA/ROK inhibitors if the balance tends toward equilibrium. Other pathways may also be involved in mediating the altered angiogenic functions of radioresistant HBMEC as one can acknowledge the limitations of the well characterized and specific pharmacological inhibitors used in our study. Specific gene silencing using siRNA, combined to dominant negative molecule approaches, will ultimately provide further evidence on the alternate pathways involved. Our data however strongly suggest that the RhoA/ROK signaling pathway plays an important role in the response of tumor-associated EC to IR.

Collectively, our data suggest that IR significantly alters crucial steps of angiogenesis, namely cell proliferation, migration and tubulogenesis in radioresistant HBMEC. Response to growth factors important for tumor expansion and angiogenesis is significantly attenuated in radioresistant HBMEC. These functional alterations probably contribute to IR's capacity to stabilize or reduce tumor cell burden. We propose that the RhoA/ROK signalling pathway may be involved in mediating the IR-induced altered angiogenic functions. This pathway might represent a potential molecular target for enhancing the impact of radiotherapy on tumor-associated EC.

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