Ionizing Radiation Antagonizes Tumor Hypoxia Induced by Antiangiogenic Treatment

Oliver Riesterer,1 Michael Honer,3 Wolfram Jochum,2 Christoph Oehler,1 Simon Ametamey,3 and Martin Pruschy1

Abstract Purpose: The combined treatment modality of ionizing radiation with inhibitors of angiogenesis is effective despite the supposition that inhibition of angiogenesis might increase tumor hypoxia and thereby negatively affect radiation sensitivity. To directly assess this still controversial issue, we analyzed treatment-dependent alterations of tumor oxygenation in response to inhibition of angiogenesis alone, irradiation, and combined treatment.

Experimental Design: Serial measurements with high-resolution [18F]fluoromisonidazole positron emission tomography and immunohistochemical detection of the endogenous hypoxia marker glucose transporter-1 were done to determine tumor hypoxia in a murine mammary carcinoma allograft model.

Results: Inhibition of angiogenesis with the clinically relevant vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584 reduced microvessel density but had only minimal effects on tumor growth, tumor cell apoptosis, and proliferation. However, PTK787/ZK222584 treatment increased overall and local tumor hypoxia as revealed by extended expression of the hypoxia marker glucose transporter-1 and increased uptake of [18F]fluoromisonidazole. Fractionated irradiation induced a strong growth delay, which was associated with enhanced apoptosis and reduced proliferation of tumor cells but only minor effects on microvessel density and allograft oxygenation. Combined treatment with fractionated irradiation resulted in extended tumor growth delay and tumor cell apoptosis but no increase in tumor hypoxia.

Conclusions: These results show that irradiation antagonizes the increase of hypoxia by vascular endothelial growth factor receptor tyrosine kinase inhibition and abrogates the potential negative effect on tumor hypoxia. Thus, the risk of treatment-induced hypoxia by inhibitors of angiogenesis exists but is kept minimal when combined with a cytotoxic treatment modality.

Hypoxia reduces the radiosensitivity of tumor cells. Cells irradiated in normoxic conditions are twice to thrice more radiosensitive than cells irradiated under severe hypoxia. Thus, a putative reduction of oxygen delivery in result to inhibition of angiogenesis and destruction of the tumor vasculature could render the tumor more radioresistant and as such would be of major clinical concern (ref. 1 and references therein). Chronic and acute hypoxia result from diffusion-limited processes and intermittent blood flow due to an imbalance of rapid tumor growth, insufficient tumor angiogenesis, and supply of oxygen. This can not only directly reduce the efficacy of chemotherapy and radiotherapy but also cause the selection of more aggressive and even highly angiogenic tumor cell populations, which are also potentially resistant to inhibitors of angiogenesis (2).

Various classes of angiogenesis inhibitors exist. They either directly target the microvascular endothelial cell and in particular its endothelial growth factors and corresponding receptors or indirectly block the tumor stress response to a more hypoxic environment by preventing the expression of proangiogenic factors in tumor cells. Inhibitors of angiogenesis suppress microvessel outgrowth and reduce the tumor vascular density, which might lead to a further increase of hypoxia in the remaining tumor (2, 3). On the other hand, antiangiogenic agents were reported to also increase tumor blood flow and oxygen delivery, and a recent model, termed normalization of tumor vasculature, describes the destruction of immature vessels, concomitant recruitment of pericytes, and stabilization of intact vessels in response to antiangiogenic therapy (4, 5).

This transient normalization window with increased blood flow and tumor oxygenation provides a rationale for concomitantly applied radiotherapy and chemotherapy (6). Although numerous preclinical studies have shown that inhibitors of angiogenesis enhance the efficacy of a combined
treatment modality with concomitant irradiation, the mechanisms of this synergistic interaction have not been identified yet (5, 7–10). Therefore, reservations exist to fully promote this treatment combination into clinical trials (11, 12).

To directly assess this still controversial issue, serial measurements of tumor hypoxia prior and in response to treatment is of high demand (13, 14). To investigate treatment-dependent alterations of tumor oxygenation in response to inhibition of angiogenesis alone, ionizing radiation, and combined treatment, we studied tumor hypoxia in a murine mammary carcinoma allograft model using immunostaining for the hypoxia marker glucose transporter-1 (GLUT-1) and quantitative, serial high-resolution positron emission tomography (PET) with the hypoxia-sensitive radiotracer \[^{18}F\]FMISO. Detection of primary antibody was done with a Histofine/Plus; Menzel, Braunschweig, Germany), deparaffinized, rehydrated, and fixed in 4% PBS-buffered formalin and embedded in paraffin. Three-tissue immersion intact deoxyribonucleic acid (DNA) fragmentation assay, or terminal deoxynucleotidyl transferase–mediated nick-end labeling assay.

Histology, immunohistochemistry, and terminal deoxynucleotidyl transferase–mediated nick-end labeling assay. Tissues were immersion fixed in 4% PBS-buffered formalin and embedded in paraffin. Three-micrometer-thick sections were mounted on glass slides (SuperFrost Plus; Menzel, Braunschweig, Germany), deparaffinized, rehydrated, and stained with H&E using standard histologic techniques.

For CD31 staining, a goat polyclonal anti-PECAM1 antibody (M20, Santa Cruz Biotechnology, Santa Cruz, CA) at a final dilution of 1:50 was used. Detection of primary antibody was done with a Histostain/ diamidobenzidine staining kit (Nichirei Corp., Tokyo, Japan). Microvessel density was determined in 5 to 10 randomly chosen visual fields in each of three similarly treated vital tumor tissues at ×100 magnification (0.3 mm² visual field size).

For Ki67 staining, CCI-pretreated sections were incubated with monoclonal rabbit Ki67 antibody (clone SP6; NeoMarkers, Fremont, CA). Detection of primary antibody was done with a biotinylated anti-rabbit IgG antibody (Jackson Immunoresearch, West Grove, PA). For Glut-1 immunostaining, CCI-pretreated sections were incubated with polyclonal rabbit antihuman Glut-1 antibody (MIM AB 1351; Chemicon International, Temecula, CA) at a final dilution of 1:1,000 at room temperature. Ki67 and Glut-1 staining procedures were done on a Benchmark immunohistochemistry staining system (Ventana Medical Systems, Tucson, AZ). Apoptotic cells were identified by the terminal deoxynucleotidyl transferase–mediated nick-end labeling method according to the protocol of the In situ Cell Death Detection kit (Roche, Penzberg, Germany). Terminal deoxynucleotidyl transferase–mediated nick-end labeling– or Ki67-positive cell count from these fields was determined.

**PET scanning.** The radioisynthesis of \[^{18}F\]FMISO was carried out at the Paul Scherer Institute according to the two-step procedure reported by Lim and Berridge (15). PET experiments were undertaken using the 16-module variant of the NanoPET tomograph (Oxford Positron Systems; Weston-on-the-Green, United Kingdom) with performance characteristics as reported elsewhere (16). The animals were lightly restrained and injected with the radiotracer via a lateral tail vein (5.9–38.6 MBq; max, 200 μL). At 80 minutes after injection, the animals were anesthetized with isoflurane (Abbott, North Chicago, IL) in an air/oxygen mixture and scanned in the PET camera as described previously (17). PET data were acquired from 90 to 120 minutes after injection in list mode and reconstructed using the FAIR algorithm (18) with a bin size of 0.3 mm and a matrix size of 120 × 120 × 200. Reconstruction did not include scatter, random, and attenuation correction.

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**Materials and Methods**

**Tumor allografts, treatment modalities, administration of PTK787/ZK222584, and irradiation.** Murine c-neu-overexpressing mammary carcinoma cells (NF9006) were injected s.c. (2 × 10⁶) on the back of 4- to 8-week-old athymic nude mice. Tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula \( L \times W^2 / 2 \). Tumors were allowed to expand to a volume of 200 mm³ ± 10% before start of treatment. Three different treatment modalities were applied: mice were given a strictly locoregional radiotherapy of \( 4 \times 3 \) Gy at four consecutive days using a PANTAK Therapax 300-kV X-ray unit at 0.7 Gy/min, or PTK787/ZK222584 (dissolved in 5% DMSO, 1% Tween 80, and 94% H₂O) applied orally for four consecutive days. The combined treatment group received locoregional radiotherapy and PTK787/ZK222584 orally 0.5 hour before irradiation for 4 days.

**Histology, immunohistochemistry, and terminal deoxynucleotidyl transferase–mediated nick-end labeling assay.** Tissues were immersion fixed in 4% PBS-buffered formalin and embedded in paraffin. Three-micrometer-thick sections were mounted on glass slides (SuperFrost Plus; Menzel, Braunschweig, Germany), deparaffinized, rehydrated, and stained with H&E using standard histologic techniques.

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**Regions of interest (ROI)** were defined for each tumor on all subsequent coronal planes containing tumor tissue using the dedicated software PMOD (ref. 19; PMOD Technologies Ltd., Adliswil, Switzerland). Tumor volumes obtained by ROI analysis of PET data were compared with caliper measurements done after the PET scans. Brain and muscle ROIs were defined for each animal accordingly to tumor ROIs. Normalization of tumor hypoxia was done against tumor volume and against the activity in the reference tissue brain. Because brain ROIs showed a more uniform radioactivity uptake than muscle ROIs, brain was considered as a superior reference tissue compared with muscle.

**Statistical analysis.** Statistical analysis of the in vivo tumor growth data was done with the Mann-Whitney U test. The absolute tumor growth delay was defined as the time for tumor volume in the treated groups to triplicate the initial treatment size minus the time in the untreated control group to reach the same size. T test analysis for mean hypoxia was done with the SPSS program using inverse uptake ratios. The percentual distribution of voxel intensities in the hypoxic tumor areas was analyzed using the PMOD software. Results of this voxel-wise analysis were illustrated as a so-called “percentile plot” showing the numbers of voxels (given in % total voxel number) in relation to the normalized activity in the voxel. The number of columns in the percentile plot was determined by the hottest voxel in the reconstructed field of view and thus varies within our experimental series. Descriptive statistical analysis using percentile blots was done with the Statview program.

**Results**

**Growth delay of mammary carcinoma on treatment with ionizing radiation and PTK787/ZK222584.** We first analyzed the effects of ionizing radiation or treatment with the clinically relevant vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor PTK787/ZK222584 (PTK787) on the growth of murine tumor allografts derived from c-neu-overexpressing mammary carcinoma cells (NF9006; ref. 20). When treated with a biologically effective dose of PTK787 (4 × 100 mg/kg) alone, allograft tumor growth was minimally delayed compared with untreated control tumors but was significantly inhibited in response to 4 × 3 Gy irradiation. Tumor growth delay was most enhanced by combined treatment with PTK787 (4 × 100 mg/kg) and 4 × 3 Gy (Fig. 1A). Resumed tumor growth was least aggressive in the combined treatment group. The absolute growth delay to triple the initial tumor volume at start of treatment (200 mm³ ± 10%) was at least additive when compared with tumor growth in response to PTK787 or ionizing radiation alone [15 days (PTK787/ionizing radiation) versus 3 days (PTK787) versus 11 days (ionizing radiation), respectively].

**Tumor cell apoptosis and proliferation** were investigated to analyze the effects of the three treatment modalities on the cellular level. At day 4, ionizing radiation and combined PTK787/ionizing radiation treatment resulted in a 3- and 4-fold
increase of terminal deoxynucleotidyl transferase–mediated nick-end labeling–positive cells per visual field, respectively, whereas PTK787 treatment alone had no major effect on the apoptotic index (Fig. 1B). Ten days after treatment end, the apoptotic indexes were comparable among the three treatment groups, most probably due to further tumor growth and the dissimilation of the apoptotic cells (data not shown).

To examine the effect of the different treatment modalities on the tumor vasculature, microvessel density was determined by CD31 immunohistochemistry at the end of treatment (day 4). Although ionizing radiation alone minimally reduced the microvessel density, treatment with PTK787 alone in combination with irradiation resulted in a 20% and 48% reduction, respectively (Fig. 1C). Determination of microvessel densities 10 days after treatment end (ionizing radiation, PTK787, or combined) revealed a normalization of the tumor vasculature independent of the treatment modality (data not shown). Hence, reduction of microvessel density inversely correlated with tumor cell apoptosis induced by the combined treatment modality. These results support the mechanistic concept that ionizing radiation and inhibitors of angiogenesis cooperatively induce vascular damage, which subsequently results in enhanced tumor cell apoptosis.

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Figure 1. Differential tumor response to PTK787, ionizing radiation (IR), and in combination. Tumor growth delay, microvessel density, and tumor cell apoptosis of NF9006-derived allografts in nude mice. A, treatment was started after tumors reached a minimal volume of 200 mm³ ± 10% on four consecutive days with ionizing radiation (4 × 3 Gy), or PTK787 alone (100 mg/kg), or in combination. Tumor cell apoptosis (B) and microvessel density (C) were assessed by terminal deoxynucleotidyl transferase–mediated nick-end labeling and CD31 staining, respectively.

Tumor cell proliferation was determined using immunohistochemistry for the Ki-67 protein, which is expressed during all phases of the cell cycle except G0. At the end of treatment, PTK787 treatment was not associated with reduced proliferative activity, whereas irradiation alone or in combination with PTK787 reduced the proliferative activity to 50% and 46% of control tumors, respectively (data not shown). Combined treatment of NF9006 tumor cells in vitro did not result in an enhanced antiproliferative and cell death–inducing response (data not shown). Thus, the extended tumor growth delay in response to irradiation alone and the combined treatment modality associated with high apoptotic indices and reduced proliferation of tumor cells.

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Treatment-dependent modulation of tumor hypoxia analyzed by immunohistochemistry. Treatment-induced changes of the tumor vasculature may induce tumor hypoxia, which is a major limitation for effective radiotherapy. To investigate treatment effects on allograft hypoxia, tumor sections were immunohistochemically analyzed for the expression of the GLUT-1 protein, an established marker for hypoxia. At day 4 of treatment, an increase of GLUT-1-positive regions was detected in tumor sections from mice treated with PTK787 alone compared with untreated control tumors (Fig. 2A and B), indicating that inhibition of angiogenesis renders the tumor more hypoxic. In contrast, tumor sections derived from animals treated with ionizing radiation alone or in combination with PTK787 displayed no increase and a comparable speckled pattern of GLUT-1 positivity (Fig. 2C and D). H&E staining of adjacent sections (Fig. 2E-H) did not reveal significant treatment-induced necrosis. This result indicates that PTK787 induces allograft hypoxia, which can be antagonized by ionizing radiation.

Treatment-dependent modulation of tumor hypoxia analyzed by high-resolution FMISO-PET. Because immunohistochemical analysis of tumor hypoxia by GLUT-1 staining is restricted to the microregional level, we quantitatively assessed global (mean) tumor hypoxia using FMISO-PET with a unique submillimeter resolution, HIDAC-based small animal tomograph (for experimental setup, see Materials and Methods and Fig. 3A). FMISO is a nitroimidazole derivative that forms bioreductive metabolites and accumulates in hypoxic tissue at pO2 levels below 5 to 10 mm Hg, which are relevant for hypoxia-dependent changes of radiosensitivity. Global (mean) FMISO uptake was quantified with the software PMOD.

To corroborate FMISO accumulation with tumor hypoxia, FMISO distribution in digitalized transaxial tumor slices was first compared with the GLUT-1 staining pattern of corresponding paraffin-embedded transaxial tumor sections
(Fig. 3B and C). For this, tumors were excised and fixed immediately after PET measurement. Extensive staining for Glut-1 and misonidazole accumulation were detected in corresponding tumor sections with a spared central necrotic area and normoxic zones at the rim of the tumor.

To follow a treatment-dependent effect on tumor hypoxia, serial FMISO-PET measurements were done on day 0 (24 hours before first treatment), day 4 (last day of treatment), and day 10 following treatment end. Such serial noninvasive measurements offer the advantage to quantitatively assess and directly compare the treatment responses in limited heterogeneous treatment groups.

Radioactivity uptake in the tumor was normalized against tumor volume and radioactivity uptake in the mouse brain to accommodate for fluctuations due to varying specific radio-ligand activities and injected quantities. Brain instead of whole body was selected as reference tissue to avoid inclusion of radioactivity in the biliary tract, kidney, or urinary bladder. At day 4 of treatment, normalized tumor FMISO uptake in control, irradiated and in animals treated with PTK787/ionizing radiation was comparable, but a significant increase in radioactivity uptake was observed in response to treatment with PTK787 alone (155%, $P_{day\ 4/day\ 0} = 0.048$). Importantly, PTK787-treated animals displayed significantly higher tumor radioactivity uptake on day 4 when compared with animals treated with PTK787/ionizing radiation (ratio = 1.63; $P = 0.041$; Fig. 4A). Ten days following treatment with PTK787, the increased tumor hypoxia status was reversed to the level determined before treatment start. Only minor fluctuations were observed in response to the other treatment modalities. Interestingly, the apoptotic index and microvessel density level in all tumors and independent of treatment and tumor size at this time point returned to the level determined before treatment (data not shown). This is most probably due to the adaptation of the tumor vasculature to resumed tumor growth.

A quantitatively increased hypoxic tumor fraction in response to treatment with PTK787 could be due to overall expanded tumor areas with insufficient oxygen supply or due to elevated levels of hypoxia in the preexisting low oxygen tumor areas. Therefore, we analyzed the percentual distribution of voxel intensities in the hypoxic tumor areas before and after treatment with PTK787 as a result of different FMISO uptake at this resolution (0.73 mm$^3$; Fig. 4). Percentile plots of individual tumors revealed a strong rightshift towards higher voxel intensities and up to 1.5- to 2.3-fold higher mean voxel intensities in response to treatment with PTK787 (Fig. 4B-D). In contrast, combined treatment did only minimally change the overall distribution of voxel intensities and mean values (Fig. 4E-G). This differential response occurred independently of initial mean levels of hypoxia or different tumor sizes.

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**Fig. 2.** Enhanced GLUT-1 expression in response to PTK787. Representative GLUT-1 and corresponding H&E staining of tumors from untreated mice (control) and mice treated with PTK787, ionizing radiation (IR) alone, or in combination on four consecutive days. Tumors were excised 6 hours after the last treatment, and tumor sections were stained with polyclonal rabbit antihuman Glut-1 antibody. Bar, 500 μm.
of a NF9006-derived tumoral lograft. Of note, we observed that treatment with the antiangiogenic substance PTK787 induces tumor hypoxia in a range that is associated with enhanced radioresistance.

This observation is consistent with reduced oxygenation in histochemically analyzed tumor sections after treatment with the anti-vascular endothelial growth factor receptor-2 antibody DC101 alone but not in response to combined treatment with irradiation (21). However, our results and the results from Fenton et al. are in contradiction to the “tumor vascular normalization” concept, which claims that treatment with low doses of antiangiogenesis inhibitors preferentially targets immature vessels and by that creates a normalization window with improved functionality of the remaining tumor vasculature. This could avoid tumor hypoxia by improved delivery of oxygen (4). The differences between our study and the results by Winkler et al. may also relate to the tumor models, the angiogenesis inhibitors, and the dosing regimens used (6).

In contrast to PTK787, irradiation as a single treatment modality induced a strong growth delay that was associated with enhanced apoptosis and reduced proliferation of tumor cells but only minor effects on microvessel density and allograft oxygenation based on GLUT-1 immunostaining and FMISO uptake. This observation indicates that the tumor cells are the primary target relevant for the observed ionizing radiation-induced growth delay.

The combined treatment modality with PTK787 and ionizing radiation leads to a supraadditive growth delay of tumor allografts and was associated with the highest and lowest values for tumor cell apoptosis and proliferation, respectively. Furthermore, reduction of microvessel density was most prominent upon combined treatment, representative for a strong antiangiogenic and vascular targeting effect especially of the combined treatment modality (22). This observation on the synergistic action of ionizing radiation and PTK787 is consistent with the results of previous studies using fractionated irradiation together with other antiangiogenic approaches or other tumor models (1).

Most importantly, we observed that tumor oxygenation was in the range of untreated control allografts, indicating that ionizing radiation can antagonize the hypoxia-inducing effects of the antiangiogenic substance PTK787. Although the mechanisms underlying this antagonistic effect remain unclear, it is conceivable that enhanced apoptosis together with reduced proliferation of tumor cells may reduce intratumoral oxygen demand to a level that can still be met by the damaged tumor vasculature and thereby avoid a hypoxic state in the allograft (2, 23). This applies to the combination of angiogenesis inhibitors with irradiation but might be relevant also for the combination with other cytotoxic agents.

In our study, FMISO-PET was used to investigate allograft oxygenation in response to three different treatment modalities. This approach allowed us to quantify the level of allograft hypoxia in vivo, to visualize hypoxia distribution with high resolution, and to perform serial analysis on the same allograft. The FMISO-PET results showed strong correlation with GLUT-1 immunostaining, which was used as an independent approach to assess allograft hypoxia. Thus, FMISO-PET represents a potent method to analyze tumor oxygenation in experimental and clinical settings, to follow treatment responses, and to investigate potential hazards especially of combined treatment modalities (2, 21, 24).

Fig. 3. Overlapping FMISO-PET and GLUT-1 staining. A, representative coronal whole body section (slice thickness = 0.3 mm) of a tumor-bearing mouse injected with [18F]fluoromisonidazole. Comparative [18F]fluoromisonidazole tumor distribution (B) and corresponding GLUT-1 staining (C) in a transaxial section of a NF9006-derived tumor allograft.

Discussion

In this study, we investigated the effects of the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787, fractionated irradiation, and the combined treatment modality on tumor oxygenation using serial high-resolution small-animal PET. The results of this functional imaging approach show that PTK787 enhances tumor hypoxia, but ionizing radiation antagonizes tumor hypoxia induced by this antiangiogenic treatment.

PTK787 treatment for 4 days reduced microvessel density but had only minimal effects on tumor growth and tumor cell apoptosis and proliferation. However, PTK787 treatment reduced allograft oxygenation as revealed by extended expression of the hypoxia marker GLUT-1 and increased uptake of FMISO, a nitroimidazole derivative that accumulates in hypoxic tissue at \( \rho O_2 \) levels below 5 to 10 mm Hg. These results indicate that treatment with the antiangiogenic substance PTK787 induces tumor hypoxia in a range that is associated with enhanced radioresistance.

Overall and consistent with the findings for Glut-1 expression, these FMISO-PET results indicate that inhibition of angiogenesis with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787 results in enhanced tumor hypoxia, which is counteracted by concomitant irradiation with clinically relevant doses of ionizing radiation.

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In conclusion, our study has directly investigated in vivo the effects on tumor oxygenation of an antiangiogenic treatment with PTK787, ionizing radiation, or a combination of PTK787 and ionizing radiation. Using FMISO uptake and GLUT-1 expression as end points, we found that PTK787 alone induced tumor hypoxia, which was reverted by concurrent ionizing radiation. These results indicate that the risk of treatment-induced hypoxia by inhibitors of angiogenesis exists but is kept minimal when combined with irradiation. This may, therefore, provide a mechanistic basis for the concurrent application of antiangiogenic drugs and ionizing radiation in the treatment of solid tumors. Further studies are required to evaluate these biology-guided end points as dynamic surrogate markers.

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