The Akt/mTOR Pathway Assures the Synthesis of HIF-1α Protein in a Glucose- and Reoxygenation-dependent Manner in Irradiated Tumors*

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Transcriptional activity of HIF-1 (hypoxia-inducible factor-1) has been reported to be up-regulated in solid tumors after ionizing radiation; however, the molecular mechanism underlying the response remains to be elucidated. In the present study, we performed a series of molecular imaging experiments using a HIF-1-dependent reporter gene, SHREp-ODD-luc, and found an essential role of the Akt/mTOR pathway. Hypoxic tumor cells distant from blood vessels were dramatically reoxygenated at 24 h postirradiation, and HIF-1 activity increased as HIF-1α accumulated in the reoxygenated regions. The accumulation was inhibited with a nonmetabolizable glucose analog, 2-deoxy-D-glucose, through the suppression of radiation-induced phosphorylation of Akt in the reoxygenated regions. Akt knockdown at 24 h postirradiation, and HIF-1 activity increased as HIF-1 is stabilized and activated under hypoxic conditions and consequently up-regulated HIF-1α translation. Moreover, both the accelerated translation and the previously reported reactive oxygen species-mediated stabilization of HIF-1α protein were essential to the activation of HIF-1. All of these results indicate that Akt/mTOR-dependent translation of HIF-1α plays a critical role in the postirradiation up-regulation of intratumoral HIF-1 activity in response to radiation-induced alterations of glucose and oxygen availability in a solid tumor.

Aberrantly accelerated proliferation and metabolism are typical features of cancer cells and lead to an imbalance of oxygen supply and oxygen consumption in solid tumors. Such disequilibrium is a major causative factor of hypoxia (1, 2). Most chronic hypoxia occurs far from tumor blood vessels at the edges of well oxygenated viable regions and is associated with resistance to radiation therapy and recurrence after treatment (1, 3). On the other hand, well oxygenated tumor cells located near tumor blood vessels are relatively sensitive to radiation therapy. Such a difference in radiosensitivity has been associated with postirradiation alterations of the tumor microenvironment. Namely, it has been reported that the distribution of oxygen from tumor blood vessels to hypoxic tumor cells is dramatically improved after radiotherapy as a result of the death of well oxygenated tumor cells and a subsequent decrease in oxygen consumption in these cells (4, 5). This phenomenon is known as tumor reoxygenation and has been well analyzed because it influences the efficacy of radiation therapy. HIF-1 (hypoxia-inducible factor-1), a transcription factor responsible for cellular adaptive responses to hypoxia, is also known to affect the radiosensitivity of tumors (6, 7). HIF-1 is a heterodimer composed of an α-subunit (HIF-1α) and a β-subunit (HIF-1β), and its activity is mainly dependent on the stability and modification of the former (8). Under normoxic conditions, the oxygen-dependent degradation (ODD) domain of HIF-1α is hydroxylated by prolyl hydroxylases and ubiquitinated by a pVHL-containing E3 ubiquitin ligase, resulting in rapid degradation of the HIF-1α protein (9, 10). On the other hand, HIF-1α is stabilized and activated under hypoxic conditions and interacts with HIF-1β (8). The resultant heterodimer, HIF-1, binds to its cognate DNA sequence, the hypoxia-responsive element (HRE), and induces the expression of various factors, such as vascular endothelial cell growth factor (11–13). This factor was reported to not only induce angiogenesis but also protect endothelial cells from the cytotoxic effects of irradiation and consequently increase tumor radioresistance (14, 15). Therefore, it is important to understand changes in intratumoral HIF-1 activity after radiation therapy and the underlying molecular mechanisms.

Recently, it was found that intratumoral HIF-1 activity began to increase steadily over time ~12–24 h after radiation (16). Reactive oxygen species (ROS)-dependent stabilization of
HIF-1α protein was identified as an important mechanism in the up-regulation (16). However, the molecular mechanism, especially genes responsible for the postirradiation up-regulation of intratumoral HIF-1 activity has not been fully elucidated.

In the present study, we monitored HIF-1 activity in a subcutaneous HeLa tumor xenograft with a novel SHREp-ODD-luc reporter gene (17), in which an ODD-luciferase (ODD-Luc) fusion protein is expressed under the regulation of a HIF-1-dependent 5SHRE promoter (18). Because the 5HRE promoter and ODD domain are responsible for HIF-1-dependent transcription of the down stream gene and oxygen-dependent degradation of the ODD-Luc protein, respectively (10, 13), the reporter gene enables us to accurately image absolute HIF-1 activity as luciferase bioluminescence in real time (17). Performing imaging experiments and immunohistochemical analyses, we revealed here that the up-regulation of intratumoral HIF-1 activity 24 h postirradiation was mainly caused by the accumulation of HIF-1α protein in regions of radiation-induced reoxygenation of the solid tumor. Moreover, we found that the Akt/mTOR pathway plays an essential role in the accumulation of HIF-1α in both a glucose- and reoxygenation-dependent manner. Furthermore, we obtained important results indicating that the Akt/mTOR pathway and the previously reported ROS-related mechanism (16) are responsible for the synthesis of HIF-1α protein and the stabilization of the product, respectively, and both were critical in the accumulation of HIF-1α.

EXPERIMENTAL PROCEDURES

Cell Culture—The human cervical epithelial adenocarcinoma cell line (HeLa) was purchased from the American Type Culture Collection and maintained in 10% fetal bovine serum-Dulbecco’s modified Eagle’s medium with (for glucose-available conditions) or without (for glucose-deprived conditions) 4.5 mg/ml d-glucose. For normoxic cultures, cells were incubated in a well humidified incubator with 5% CO₂ and 95% air at 37 °C. For hypoxic cultures, cells were incubated in a Bactron anaerobic chamber, BACLITE-2 (O₂ < 0.02%: Sheldon Manufacturing Inc., Cornelius, OR).

Reagents—Stock solutions of 2-deoxy-d-glucose (2-DG; 4.5 g/ml), catalase (3 × 10⁶ units/ml; Wako Pure Chemical Industries Ltd., Osaka, Japan), 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; 2 μM; Invitrogen), cycloheximide (CHX; 10 μg/ml), and rapamycin (20 μM) were prepared in MilliQ water (for 2-DG and catalase) or DMSO (for the others). The working concentrations of 2-DG, catalase, H₂DCFDA, CHX, and rapamycin for in vitro experiments were 4.5 mg/ml, 3,000 units/ml, 2 μM, 10 μg/ml, and 20 nm, respectively.

Stable Transfectants—HeLa/EFP-Luc and HeLa/5SHREp-ODD-Luc cells were established as described previously (17, 19). The HeLa/5SHREp-ODD-Luc cells were further transfected with the plasmid, SureSilencing shRNA plasmid for human Akt1 (SABiosciences Corp., Frederick, MD), by the calcium phosphate method (20), and cultured for 10 days in medium containing puromycin (Nacalai Tesque, Kyoto, Japan) to establish HeLa/AktKD/5SHREp-ODD-Luc cells. A negative control short hairpin RNA plasmid (SABiosciences Corp.) was used in the same way to establish the HeLa/NC/5SHREp-ODD-Luc cells. Almost 90% of Akt expression was suppressed only in the HeLa/AktKD/5SHREp-ODD-Luc cells.

siRNA Treatment in Vitro—Cells were seeded in 24-well plates (2 × 10⁴ cells/well) and transfected with the Validated Stealth RNAi for the Akt gene (Invitrogen Corp.) using Lipofectamine RNAiMAX (Invitrogen Corp.) according to the manufacturer’s instructions. The cells were cultured for an additional 24 h to assure the effect of the RNAi and subjected to Western blot analyses and luciferase assays (see the legend to Fig. 5 for details). As a negative control, a Stealth RNAi Negative Control Kit (Invitrogen Corp.) was used.

Luciferase Assay and Western Blotting—Cells were seeded in 24-well plates (2 × 10⁴ cells/well) and subjected to each experiment (see each figure legend for details). The cells were washed with phosphate-buffered saline twice and lysed with 100 μl of passive lysis buffer (Promega, Madison, WI) for luciferase assays or with 100 μl of 1 × SDS-PAGE loading buffer for Western blot analyses. The luciferase assay was performed by using luciferase assay reagent (Promega) according to the manufacturer’s instructions. The Western blot analyses for HIF-1α, phospho-Akt, Akt, phospho-S6, S6, ODD-β-galactosidase, and β-actin were performed using anti-HIF-1α mouse monoclonal antibody (BD Biosciences), anti-phospho Akt (Ser⁴⁷³) rabbit polyclonal antibody (Cell Signaling Technology, Inc., Tokyo, Japan), anti-Akt rabbit polyclonal antibody (Cell Signaling Technology, Inc.), anti-phospho-S6 ribosomal protein (Ser²⁴⁰/²⁴⁴) rabbit polyclonal antibody (Cell Signaling Technology, Inc.), anti-S6 ribosomal protein rabbit monoclonal antibody (Cell Signaling Technology, Inc.), anti-β-galactosidase mouse monoclonal antibody (Cell Signaling Technology, Inc.), and anti-β-actin mouse monoclonal antibody (BioVision Research Products, Mountain View, CA), respectively. Proteins were detected using anti-mouse or anti-rabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare) and an ECL-PLUS system (GE Healthcare) according to the manufacturer’s instructions.

Real Time PCR—After the treatment of the cells as explained in the legend to Fig. 4C, total RNA was extracted using a FastPure RNA Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer’s instructions. Reverse transcription was performed using an RNA LA PCR Kit (AMV) version 1.1 (Takara Bio). The HIF-1α mRNA level was quantified by the real time PCR technique using a Thermal Cycler Dice Real Time System (TP-800; Takara Bio) with forward (5’-TCATCCAAGAAGC-CCTAAGGTG-3’) and reverse (5’-TTTCGCTTTCTCTGGACTTCTCGT-3’) primers (Takara Bio) and a SYBR Premix Ex Taq kit (Takara Bio).

Intracellular ROS Detection—After reglucose and/or reoxygenation treatment in vitro (see the legend to Fig. 8A for details), cells were treated with 2 μM H₂DCFDA for 30 min, washed twice with phosphate-buffered saline, and fixed with a 10% formalin neutral buffer solution (pH 7.4; Wako Pure Chemical Industries).

Tumor-bearing Mice and Radiation Conditions—The suspensions of HeLa-derived cells (2 × 10⁶) were subcutaneously inoculated into the right hind leg of 6-week-old nude mice (BALB/c nu/nu mice, SHIMIZU Laboratory Supplies Co. Ltd., Kyoto, Japan). When the tumor volume reached about 150...
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**A**

**B**

**C**

**D**

**E**

**F**

FIGURE 1. Intratumoral HIF-1α expression and HIF-1 activity after ionizing radiation. A, HeLa/EFP-Luc or HeLa/SHRP-ODD-Luc xenografts were irradiated at a dose of 0 or 5 Gy. Intratumoral HIF-1α activity was monitored as luciferase bioluminescence at the indicated time after irradiation, and representative images are shown. B, the bioluminescent intensity at 0 and 24 h after the irradiation in A was quantified, and the intensity at 24 h was compared with that at 0 h to calculate the relative luciferase activity. Results are means ± S.D., n = 6, ***, p < 0.01. C, the HeLa/SHRP-ODD-Luc tumor xenografts were surgically excised 24 h after irradiation at a dose of 0 or 5 Gy and subjected to immunohistochemical analyses for HIF-1α (red) or pimonidazole (green). Counterstaining was conducted with DAPI (blue). A thinner frozen section (3 μm) was observed under high magnification to analyze the intracellular localization of HIF-1α protein (e). Bar, 50 μm. D and E, fractions of HIF-1α-positive cells (D) and pimonidazole-positive cells (E) in C were quantified. Results are means ± S.D., n = 5, *, p < 0.05; **, p < 0.01. F, immunohistochemical analyses of the HeLa/SHRP-ODD-Luc xenografts with anti-HIF-1α antibody (red) and a perfusion marker, Hoechst 33342 (blue), at 24 h after irradiation at a dose of 0 or 5 Gy. Bar, 100 μm.

mm³, the tumor xenografts were locally irradiated with 5 Gy of ¹³⁷Cs γ-rays using a Gammacell 40 Exactor (MDS Nordion International, Inc., Ontario, Canada). Local irradiation of tumor xenografts was achieved with a specific collimator (MDS Nordion International, Inc.). For in vitro experiments, cells were irradiated at a dose of 5 Gy (1.468 Gy/min) with an x-ray irradiation machine (Shimadzu Corp., Kyoto, Japan).

**Immunohistochemical Analyses—**Tumor xenografts were surgically excised after each treatment (see each figure legend for details), embedded in OCT compound, and frozen at −80 °C. For pimonidazole staining, pimonidazole hydrochloride (Natural Pharmacia International, Inc., Belmont, MA) was intraperitoneally injected into the tumor-bearing mice (60 mg/kg) 20 min before the tumor excision. The frozen sections (8 μm) were treated with fluorescein isothiocyanate-conjugated anti-pimonidazole monoclonal antibody (Natural Pharmacia International) according to the manufacturer’s instructions. Counterstaining was conducted with DAPI (Wako Pure Chemical Industries). For HIF-1α staining, frozen sections were fixed in 2% paraformaldehyde and ice-cold methanol, blocked with blocking solution (serum-free protein block solution (Dako, Glostrup, Denmark) containing 0.1% cold water fish skin gelatin (Sigma)) and treated with anti-HIF-1α monoclonal antibody (BD Biosciences) in the blocking solution. For HIF-1α and phospho-Akt double staining, the sections were further treated with anti-phospho-Akt (Ser473) rabbit monoclonal antibody (Cell Signaling Technology, Inc.) in the blocking solution. After extensive washing, the specific signals of HIF-1α and phospho-Akt were detected with Alexa Fluor 546 goat anti-mouse IgG (Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen), respectively. For the analysis of perfusion (Hoechst 33342 distribution), tumor-bearing mice were intravenously injected with 100 μl of Hoechst 33342 trihydrochloride tris(hydroxymethyl)aminomethane solution (10 mg/ml; Invitrogen) 1 min before excision of each xenograft. To calculate the percentages of pimonidazole-positive and HIF-1α-positive cells, random 15 fields in five tumor xenografts were prepared for each treatment group, and the positive areas were quantified using NIH Image 1.63 software (National Institutes of Health, Bethesda, MD) and compared with the entire tumor. The quantitative analyses were conducted in a double-blind fashion, and the reproducibility was sufficiently confirmed three times.

**Real Time Imaging of Luciferase Activity in Tumor Xenografts—**When the average tumor volume reached 150 mm³, an osmotic pump (Alzet Osmotic Pumps, Cupertino, CA) loaded with 200 μl of α-luciferin (20 mg/ml in phosphate-buffered saline; Promega) was subcutaneously transplanted into the left flank of tumor-bearing mice. The mice were treated with γ-ray irradiation in combination with a drug, such as rapamycin (1 mg/kg, intraperitoneal injection) or 2-DG (20 mg/kg, with an osmotic pump), in each experiment. Optical imaging to detect the luciferase bioluminescence was carried out with an IVIS-200 in vivo imaging device (Xenogen, Alameda, CA). During the imaging, the mice were anesthetized with 2.5% isoflurane gas in the oxygen flow (1.5 liters/min). Images were analyzed using Living Image 2.50-Igor Pro 4.09 software (Xenogen). In all imaging experiments, reproducibility was confirmed with 5–6 independent tumor-bearing mice, and representative images are shown.

**Ethics of Animal Experiments—**All of our animal experiments were approved by the Animal Research Committee of Kyoto University and carried out in accordance with its guidelines.
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RESULTS

HIF-1α Expression Is Induced in Radiation-induced Reoxygenated Cells 24 h after Ionizing Radiation—To analyze the effect of ionizing radiation on intratumoral HIF-1 activity, we used a novel HIF-1-dependent reporter gene, SHREP-ODD-luc, in which the SHRE promoter enhances expression of the ODD-Luc fusion under hypoxic conditions (17). We previously reported that this reporter gene is suitable for the real time imaging of absolute HIF-1 activity in tumor xenografts (17). We subcutaneously transplanted HeLa cells stably transfected with the SHREP-ODD-luc gene (HeLa/SHREP-ODD-Luc cells) and monitored the postirradiation dynamics of intratumoral HIF-1 activity using an optical in vivo imaging system as we described previously (19, 21). The HIF-1 activity dramatically increased, peaked at 24 h postirradiation (p < 0.01), and decreased thereafter (Fig. 1, A and B). On the other hand, the HeLa/EF-Luc tumor xenograft, which constitutively expresses the luciferase gene, showed steady bioluminescence after the irradiation (Fig. 1, A and B), indicating that the postirradiation changes in bioluminescence from HeLa/SHREP-ODD-Luc xenografts depended on the SHREP and ODD. Immunohistochemical analyses using the HeLa/SHREP-ODD-Luc tumor xenografts revealed that the level of HIF-1α protein at the edges of DAPI-positive viable regions significantly increased 24 h after the radiation treatment (Figs. 1, C (c) and D, p < 0.05) and that the HIF-1α protein predominantly localized in nuclei of the cells (Fig 1C (e)). These results indicate that the changes in the HIF-1α expression level seemed to be mainly responsible for the change in postirradiation HIF-1 activity in tumor xenografts.

Because HIF-1 activity is mainly dependent on the deprivation of oxygen, we assumed that the accumulation of HIF-1α at 24 h postirradiation resulted from the increase in tumor hypoxia caused by the radiation treatment. To examine such a possibility, we conducted an immunohistochemical analysis using a marker of hypoxia, pimonidazole (22) (Fig. 1C). Predictably, pimonidazole-positive cells were detected only at the edges of viable regions (Fig. 1C (b)). Surprisingly, their numbers significantly decreased 24 h after irradiation (Figs. 1, C (d) and E; p < 0.01), although HIF-1α expression and HIF-1 activity increased at this time point (Figs. 1, A–C). The distribution of a perfusion marker, Hoechst 33342, among the tumor xenograft was not decreased at 24 h postirradiation, supporting the interpretation that the decrease in pimonidazole-positive cells was caused from the radiation-induced reoxygenation but not from the decrease in permeability of pimonidazole (Fig. 1F). Subsequently, we analyzed the molecular mechanism responsible for the up-regulation of HIF-1α expression at the edges of viable regions in such a reoxygenated environment after radiation treatment.

Reoxygenation Treatment Up-regulates HIF-1α Expression in a Glucose-dependent Manner in Vitro—To examine whether cultured cells induce HIF-1α expression and HIF-1 activity in response to reoxygenation in vitro, we treated the HeLa/SHREP-ODD-Luc cells under hypoxic and subsequent normoxic conditions. The reoxygenation treatment had little impact on the HIF-1α expression and HIF-1 activity in vitro (Fig. 2, A and B, lane 2). We then assumed that ionizing radiation has the propensity to induce HIF-1α expression; however, it also had little effect on HIF-1α expression and HIF-1 activity under normoxic or reoxygenated conditions (Fig. 2, A and B, lanes 3 and 4).

To better mimic the microenvironment in the region of radiation-induced reoxygenation, we focused on the change in glucose availability as well as reoxygenation, because hypoxic tumor cells far from tumor blood vessels are not supplied with a sufficient volume of nutrients and oxygen and because irradiation is assumed to improve their supply (23, 24). We cultured HeLa/SHREP-ODD-Luc cells in glucose-deprived and hypoxic...
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changes in intratumoral HIF-1 activity by imaging (Fig. 3). To continuously administer 2-DG during the imaging experiment, we subcutaneously implanted a 2-DG-loaded osmotic pump into the left flank of the tumor-bearing mice. The up-regulation of intratumoral HIF-1 activity 24 h postirradiation was significantly suppressed by the 2-DG treatment (Fig. 3, A and B; p < 0.05). Immunohistochemical analysis revealed that the suppression was accompanied by a decrease in HIF-1α expression with the 2-DG treatment at the edges of viable regions (Fig. 3, C and D; p < 0.01). These results clearly show that metabolizable glucose plays an essential role in the increase of both HIF-1α expression and HIF-1 activity at 24 h postirradiation in vivo.

Improved Glucose Availability Along with Reoxygenation Treatment Accelerates HIF-1α Translation in Vitro—To identify the molecular mechanism responsible for the reglucose- and reoxygenation-dependent induction of HIF-1α expression, we first focused on the stability of HIF-1α, because HIF-1 activity is reported to mainly depend on it. After hypoxic treatment in glucose-deprived conditions, the HeLa/5HREp-ODD-Luc cells were cultured with a translation inhibitor, CHX, under normoxic conditions in the presence or absence of glucose for 0, 10, 30, and 60 min. The influence of glucose availability on the decrease in HIF-1α protein and HIF-1 activity was analyzed by Western blotting and luciferase assay, respectively (Fig. 4, A and B). Glucose availability did not influence the rate of decrease in HIF-1α protein or HIF-1 activity. We then examined another possibility, that the increase in glucose availability accelerates the synthesis of HIF-1α protein (Fig. 4, C and D). Treatment of the HeLa/5HREp-ODD-Luc cells with CHX significantly suppressed the increase in HIF-1α expression and HIF-1 activity at 6 h after the reoxygenation along with the reglucose treatment (Fig. 4, C and D, lane 3; p < 0.01). Real time PCR experiments clearly showed that the reoxygenation along with reglucose treatment or the CHX treatment had no impact on the HIF-1α mRNA level (Fig. 4, C and E). All of these results clearly showed that the translation of HIF-1α is accelerated in response to improved glucose availability along with reoxygenation treatment in vitro.

The Akt/mTOR Signaling Pathway Functions in Translation of HIF-1α after Reoxygenation Along with Reglucose Treatment—It was reported that the 5′-untranslated region of HIF-1α conditions for 18 h, fed the cells with glucose, and cultured them under normoxic conditions for 6 h. Such in vitro reoxygenation along with reglucose treatment significantly induced HIF-1α expression and HIF-1 activity in the cells (Figs. 2, C and D, lane 3; p < 0.01). This induction was completely suppressed with a nonmetabolizable glucose analog, 2-DG (Fig. 2, C and D, lane 4; p < 0.01) but not influenced by irradiation (Fig. 2, C and D, lane 6). Moreover, reglucose had no impact on the HIF-1α expression and HIF-1 activity under normoxic conditions (Fig. 2, E and F). All of these results clearly show that reoxygenation along with reglucose treatment leads to the accumulation of HIF-1α protein. Such a property raised the possibility that radiation-induced improvement of glucose availability in the reoxygenated regions played an essential role in the accumulation of HIF-1α protein.

Postirradiation Up-regulation of Intratumoral HIF-1 Activity Is Dependent on Metabolizable Glucose—To examine whether the availability of glucose is an important factor in the postirradiation up-regulation of HIF-1α expression and HIF-1 activity in solid tumors, we treated HeLa/5HREp-ODD-Luc tumor-bearing mice with 2-DG and observed the postirradiation
The Akt/mTOR signaling pathway has been reported to stimulate HIF-1α expression after reoxygenation along with reglucose treatment in vitro. Moreover, we confirmed that the pathway functions under irradiated conditions too (Figs. 5, A–C (lane 5) and D–F (lane 4)).

The Akt/mTOR Signaling Pathway Plays an Important Role in Postirradiation Up-regulation of Intratumoral HIF-1 Activity—To examine whether Akt/mTOR signaling functions in the up-regulation of HIF-1α expression and HIF-1 activity 24 h after radiation therapy in vivo, we treated HeLa/5HREp-ODD-Luc tumor-bearing mice with rapamycin prior to the radiation treatment and observed the changes in intratumoral HIF-1 activity. The radiation-induced up-regulation of HIF-1 activity was significantly suppressed by the treatment (Figs. 6, A and B). These results were caused by the decrease in HIF-1α expression at the edges of viable regions (Fig. 6, C and D; p < 0.01). To examine the participation of the Akt/mTOR pathway more directly, we performed the same kind of imaging experiment by using mice bearing a HeLa/AktKD/5HREp-ODD-Luc tumor xenograft, in which Akt expression was dramatically suppressed by short hairpin RNA. The HeLa/AktKD/5HREp-ODD-Luc tumor xenografts showed no induction of HIF-1 activity 24 h after radiation treatment, whereas their counterparts, in which Akt expression was normal, showed the same radioreponse as HeLa/5HREp-ODD-Luc tumor xenografts (Figs. 7, A and B). Moreover, immunohistochemical analyses in the HeLa/AktKD/5HREp-ODD-Luc tumors showed that knockdown of Akt led to a significant reduction in the radiation-induced accumulation of HIF-1α in the reoxygenated regions (Fig. 7, C and D; p < 0.01). All of these results clearly showed the involvement of the Akt/mTOR pathway in the postirradiation up-regulation of HIF-1α expression and HIF-1 activity in solid tumors.

Phosphorylation of Akt Depends on Metabolizable Glucose under Reoxygenated Conditions and Assures the Synthesis of HIF-1α Protein—In the present study, we revealed the importance of both glucose and the Akt/mTOR pathway in the postirradiation accumulation of HIF-1α protein in reoxygenated regions of solid tumors. We next analyzed the interplay between them in vitro (Fig. 8). Western blotting revealed that phosphorylation of Akt induced by reoxygenation along with reglucose treatment was dramatically suppressed by 2-DG treatment, resulting in the suppression of HIF-1α expression...
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Fig. 5. Akt/mTOR signaling pathway plays an important role in the accelerated translation of HIF-1α after reglucose and reoxygenation treatment in vitro. A, HeLa/5HREp-ODD-Luc cells were cultured in the glucose-deprived medium under hypoxic conditions for 18 h. The cells were then subjected to 5 Gy (lane 5) or 0 Gy (other lanes) of IR and cultured under normoxic or hypoxic conditions, with or without glucose, in the presence or absence of vehicle or rapamycin for 6 h. B and C, after being treated as shown in A, cell extracts were subjected both to Western blot analyses for the indicated proteins (B) and to luciferase assays (C). D, HeLa/5HREp-ODD-Luc cells were transfected with negative control siRNA or Akt siRNA and cultured for 18 h. The cells were cultured in the glucose-deprived medium under hypoxic conditions for an additional 18 h, exposed to 5 Gy (lanes 3 and 4) or 0 Gy (other lanes) of IR, and subjected to reglucose along with reoxygenation treatment for 6 h. E and F, after being treated as shown in D, cell extracts were subjected to the same experiments as in B and C, G, HeLa/5HREp-ODD-Luc cells were transfected with control siRNA or two concentrations of Akt siRNA (+ or ++), which targets different regions of Akt gene from the one used in D–F, and cultured for 18 h. The cells were cultured in the glucose-deprived medium under hypoxic conditions for an additional 18 h and subjected to reglucose along with reoxygenation treatment for 6 h. H and I, cell extracts were subjected both to Western blot analyses for the indicated proteins (H) and to luciferase assays (I). Closed arrow, open arrow, Glu(+), and Glu(-), hypoxic, normoxic, glucose-deprived, and glucose-available cultures, respectively. Vehicle, Rap, Scr siRNA, and Akt siRNA, vehicle, rapamycin, scramble siRNA, and Akt siRNA treatment, respectively. All results are means ± S.D., n = 3. **, p < 0.01.

(Fig. 8, A and B, compare lane 4 with lane 5). This result indicates that glucose affects the phosphorylation of Akt in the induction of HIF-1α expression under reoxygenated conditions.

Because reoxygenation treatment is reported to generate ROS and stabilize HIF-1α protein (16), it was important to analyze the relationship between the Akt/mTOR-mediated synthesis and the ROS-related stabilization of HIF-1α. To simply analyze the stability of HIF-1α without the influence of translational regulation via the 5′-terminal oligopyrimidine tract of HIF-1α mRNA (25), we utilized the ODD-β-galactosidase fusion protein (30) as an indicator of HIF-1α stability (Fig. 8B). Additionally, we utilized a specific fluorescent dye for H2O2, H2DCFDA, to monitor the intracellular ROS level (Fig. 8C). Reoxygenation treatment in glucose-deprived conditions generated ROS in the cultured cells and stabilized the ODD-β-galactosidase fusion protein but did not lead to the phosphorylation of Akt and expression of HIF-1α (Figs. 8, A–C, lane 4). On the other hand, reoxygenation along with reglucose treatment led to both the phosphorylation of Akt and the expression of HIF-1α in addition to the generation of ROS and stabilization of ODD-β-galactosidase protein (Fig. 8, A–C, lane 4). The Akt phosphorylation and HIF-1α expression observed in lane 4 were suppressed by 2-DG treatment (Fig. 8B, lane 5), whereas levels of both ROS and ODD-β-galactosidase were maintained even in the presence of 2-DG (Fig. 8C, panel 5). A decrease in the ROS level caused by catalase treatment resulted in a loss of both HIF-1α induction and ODD-β-galactosidase stabilization (Fig. 8, B and C (lane 6)). All of these results indicate that phospho-Akt-dependent translation and ROS-related stabilization of HIF-1α function in the synthesis and stabilization of HIF-1α protein, respectively, and both reactions are essential for HIF-1α expression under reoxygenation along with reglucose treatment.

Radiation Therapy Activates Akt at the Edges of Viable Regions in a Glucose-dependent Manner—To analyze the importance of the interplay between glucose and the Akt/mTOR pathway in the postirradiation activation of HIF-1α in vivo, we conducted immunohistochemical analyses using anti-HIF-1α antibody and anti-phospho-Akt antibody (Fig. 9). In nonirradiated tumors, phospho-Akt-positive cells were detected only in viable areas, whereas HIF-1α-positive cells were located only at the edges of viable regions; they complemented each other (Figs. 9, A and B). On the other hand, in irradiated tumors, Akt was activated not only in viable areas but also in HIF-1α-positive areas (Fig. 9, C and D). The radiation-
induced activation of Akt and accumulation of HIF-1α at the edges of viable regions were suppressed by 2-DG treatment (Fig. 9, A and B). These results clearly show that irradiation leads to the activation of Akt at the edges of viable regions in a glucose-dependent manner and up-regulates HIF-1α expression in these regions.

**DISCUSSION**

In the present study, we performed a series of optical imaging experiments and analyzed the molecular mechanisms responsible for the postirradiation activation of HIF-1 in solid tumors. The in vivo experiments revealed that the ionizing radiation induces accumulation of HIF-1α protein in the reoxygenated regions, and the phenomenon depended on metabolizable glucose and the Akt/mTOR pathway. In addition, our in vitro experiments revealed that reoxygenation treatment accelerates the translation of HIF-1α in a glucose- and Akt/mTOR-dependent manner. Taken together, all of our results indicate that the radiation-induced improvement of glucose and oxygen availability leads to the accumulation of HIF-1α at the edges of viable regions of solid tumors.

It has been assumed that cancer cells far from tumor blood vessels are supplied with little glucose as well as little oxygen, but reoxygenation improves their microenvironment (23, 24). Therefore, we examined the impact of changes in glucose and/or oxygen availability on the up-regulation of HIF-1 activity in vitro. Although reoxygenation treatment alone did not induce HIF-1 activity in our in vitro experiment, reoxygenation along with reglucose treatment significantly induced HIF-1α expression and HIF-1 activity. 2-DG treatment suppressed this regulation in vitro. The administration of 2-DG significantly suppressed the induction of intratumoral HIF-1α expression and HIF-1 activation at 24 h postirradiation in vivo. These results revealed the involvement of metabolizable glucose in the response and, moreover, are consistent with reports that irradiation improves the distribution of glucose in solid tumors (23, 24). To actually confirm the involvement of glucose in the regulation of HIF-1 activity, it is necessary to directly chase the distribution of glucose in solid tumors after irradiation. Although a microautoradiographic technique using 2-deoxy-2-[18F]fluoro-D-glucose has already been established, the resolution was not good enough for our purposes (31). Instead, using an excellent technique for the geographic mapping of glucose with quantitative bioluminescence and single photon imaging, we will be able to elucidate the reoxygenation-induced alteration of the distribution of glucose and its influence on intratumoral HIF-1 activity (32–34).

In our in vitro experiments, the level of S6 protein was increased after reoxygenation along with reglucose treatment (Fig. 5, A and B, lane 2). Because ischemic conditions are known to induce cell cycle arrest, the improvement of both oxygen and glucose availability might have triggered cell cycle progression through the increase in S6 protein. The precise mechanism underlying the phenomenon remains to be elucidated; however, our results may provide useful information. Knockdown of Akt suppressed the increase in S6 protein partially (35). Instead, using an excellent technique for the geographic mapping of glucose with quantitative bioluminescence and single photon imaging, we will be able to elucidate the reoxygenation-induced alteration of the distribution of glucose and its influence on intratumoral HIF-1 activity (32–34).
Based on the present study, the glucose dependence of the postirradiation activation of HIF-1α can be explained by the glucose dependence of Akt phosphorylation under reoxygenated conditions. In nonirradiated tumors, the phosphorylated form of Akt was detected in well oxygenated normoxic tumor cells but not at the edges of viable regions. Irradiation dramatically altered the distribution of phospho-Akt in tumors; phospho-Akt was detected at the edges of viable regions in addition to in the well oxygenated regions 24 h after irradiation. The radiation-induced phosphorylation of Akt at the edges of viable regions was dramatically suppressed by the administration of 2-DG, leading to a decrease in HIF-1α expression in the cells. These results indicate that metabolizable glucose is essential for the phosphorylation of Akt and resultant induction of HIF-1α expression at the edges of viable regions after radiotherapy. This model is further supported by our in vitro experiment, in which reglucose treatment induced the phosphorylation of Akt and accumulation of HIF-1α under reoxygenated conditions and consequently led to the accumulation of HIF-1α.

It was reported that radiation-induced reoxygenation leads to the generation of ROS and inhibits prolyl hydroxylase activity, resulting in the stabilization and accumulation of HIF-1α protein (16). On the other hand, we demonstrated here that glucose- and Akt/mTOR-dependent translation of HIF-1α protein also plays an important role in the up-regulation. The relationship between the ROS-related stabilization and the Akt/mTOR-related translation of HIF-1α has not yet been elucidated; however, we raised the important possibility that both mechanisms function coordinately in the induction of HIF-1α after irradiation in vivo. Our in vitro experiment showed that, in the absence of reglucose treatment, reoxygenation treatment could not induce the phosphorylation of Akt and accumulation of HIF-1α, although the level of reoxygenation-induced ROS increased the stability of the ODD-β-galactosidase fusion protein. These results indicate that the ROS-mediated stabilization of HIF-1α protein alone could not be fully responsible for the activation of HIF-1 without newly translated HIF-1α. In this context, it is very reasonable that rapamycin and knockdown of Akt almost completely suppressed the induction of HIF-1 activity in both in vitro and in vivo experiments.

The present immunohistochemical analyses suggest the existence of an unreported mechanism for
repopulation occurs as the tumor bulk diminishes and the surviving tumor cells find themselves in a more favorable growth environment with less competition for oxygen and nutrients. Accelerated repopulation as well as tumor hypoxia has been recognized as a negative factor for radiation therapy; however, the molecular mechanism responsible for the phenomenon is not yet fully understood. The present immunohistochemical analysis showed that Akt was dramatically activated at the edges of viable regions after radiotherapy. Moreover, we have obtained direct evidence that the perinecrotic tumor cells survive radiation treatment and become sources of tumor recurrence after treatment. Therefore, we assume that activation of the Akt/mTOR pathway in the cells is responsible for the accelerated tumor cell repopulation by inducing the proliferation of cells after radiation therapy. The radiosensitizing effect of various Akt/mTOR pathway inhibitors might be caused by the inhibition of such a molecular mechanism.

To our knowledge, this is the first report to show the importance of Akt/mTOR-dependent mechanisms in the radiation-induced activation of HIF-1 in solid tumors. Moreover, the present study strengthens the importance of the tumor microenvironment concerning glucose and oxygen in the postirradiation dynamics of intratumoral HIF-1 activity. The present information will help to optimize protocols for radiation therapy. However, all of these findings were based on research using no more than a subcutaneous tumor xenograft model. Various factors in a tumor xenograft model, such as growth speed, structure of blood vessels, time course of radiation-induced reoxygenation, distribution of nutrients among a tumor, etc., are known to be different from those of real human tumors. Therefore, it is necessary to examine whether our findings hold true for clinical tumors.

3 Harada, S. Itasaka, M. Inoue, S. Kizaka-Kondoh, K. Shibuya, A. Morinibu, K. Shinomiya, and M. Hiraoka, manuscript in preparation.
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