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AKT-mediated enhanced aerobic glycolysis causes acquired radioresistance by human tumor cells

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ABSTRACT

Background and purpose: Cellular radioresistance is a major impediment to effective radiotherapy. Here, we demonstrated that long-term exposure to fractionated radiation conferred acquired radioresistance to tumor cells due to AKT-mediated enhanced aerobic glycolysis.

Material and methods: Two human tumor cell lines with acquired radioresistance were established by long-term exposure to fractionated radiation with 0.5 Gy of X-rays. Glucose uptake was inhibited using 2-deoxy-D-glucose, a non-metabolizable glucose analog. Aerobic glycolysis was assessed by measuring lactate concentrations. Cells were then used for assays of ROS generation, survival, and cell death as assessed by annexin V staining.

Results: Enhanced aerobic glycolysis was shown by increased glucose transporter Glut1 expression and a high lactate production rate in acquired radioresistant cells compared with parental cells. Inhibiting the AKT pathway using the AKT inhibitor API-2 abrogated these phenomena. Moreover, we found that inhibiting glycolysis with 2-deoxy-D-glucose suppressed acquired tumor cell radioresistance.

Conclusions: Long-term fractionated radiation confers acquired radioresistance to tumor cells by AKT-mediated alterations in their glucose metabolic pathway. Thus, tumor cell metabolic pathway is an attractive target to eliminate radioresistant cells and improve radiotherapy efficacy.

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Radiotherapy (RT) is one of the main treatment modalities for tumors, although tumor cells that survive after fractionated RT limit its efficacy [1]. Repopulating tumors is the major cause of RT failure, and these tumors often acquire radioresistance [1]. We recently demonstrated that human tumor cells acquired radioresistance when they were exposed to fractionated radiation (FR) of X-rays every 12 h for 1 month [2]. This acquired radioresistance was associated with a series of molecular changes; constitutive activation of DNA-PK and AKT with concomitant downregulation of glycogen synthase kinase-3β (GSK3β), which resulted in suppression of cyclin D1 proteolysis [2].

Constitutive activation of the AKT pathway was responsible for the radioresistance phenotype of long-term FR cells because this phenotype was completely abrogated by treating FR cells with API-2, an AKT inhibitor [2,3]. AKT regulates various biological processes, such as cell proliferation, cell survival, cellular metabolism, and vascularization [4,5]. Any alterations in these activities may affect radiosensitivity of tumor cells.

Most cancer cells generate large amounts of lactate as the result of their energy supply, although this is less efficient for ATP production compared to mitochondrial respiration. This phenomenon is known as aerobic glycolysis or the Warburg effect [6]. High glucose uptake is observed during a clinical diagnosis of cancer using positron emission tomography (PET) with fluorodeoxyglucose (^{18}F-FDG) [7].

The PI3K/AKT signaling pathway enhances aerobic glycolysis for cell survival [8]. AKT regulates multiple steps in glycolysis through post-transcriptional mechanisms that included inducing glucose transporter Glut1 gene expression and enhancing hexokinase activity [9,10]. AKT induces hexokinase activity, which has a key role in regulating glucose uptake by converting glucose to glucose-6-phosphate.

In this study, we investigated the role of the glucose metabolic pathway on acquired radioresistance by tumor cells that were established by long-term FR exposure. Increased lactate production indicated that enhanced aerobic glycolysis occurred in acquired radioresistant cells compared with parental cells. Suppressing glucose uptake using 2-deoxy-D-glucose (2-DG) resulted

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in eradicating these acquired radioresistant cells. Thus, enhanced aerobic glycolysis contributes to acquire radioresistance by tumor cells after long-term FR exposure.

Methods

Cell culture and drugs

HepG2 (human liver cancer) and HeLa (cervical cancer) cell lines were from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. Cells were grown in RPMI1640 medium (NacalaiTesque, Kyoto, Japan) supplemented with 5% heat-inactivated fetal calf serum. API-2 (AKT inhibitor) was from Calbiochem (San Diego, CA, USA) and dissolved in DMSO. Cells were incubated with 20 μM API-2 for 24 h [2,3]. 2-DG was from Sigma (Sigma, St. Louis, MO, USA) and used at 5 mM [11].

Irradiation experiments

A general protocol for fractionated radiotherapy consists of daily exposures to approximately 2 Gy for 5–7 weeks. HepG2 and HeLa cell growth was completely stopped by FR of 2-Gy within 30 days [3]. Therefore, 0.5 Gy-X-ray fraction doses were administrated twice daily for 6 days/week for 31 days in order to establish acquired radioresistant cells. [2]. Cells that were treated with these radiation exposure protocols for 31 days were referred to as 31FR cells. We also established 31FR-31NR cells, for which 31-day FR exposure was followed by a 31-day non-radiation (NR) period. 31FR-31NR cells are radioresistant to 2 Gy of FR [3]. 31FR-31NR cells were further irradiated in this study using a 150-KVp X-ray generator (Model MBR-1520R or Model MBR-1505R2, Hitachi, Tokyo, Japan) with 0.5 mm Cu and 0.1 mm Al filters at 1.0 Gy/min or 0.7 Gy/min.

Immunofluorescence (IF) staining

Cells (3×10^5) were seeded onto 18 mm × 18 mm cover slips, placed in 35-mm tissue culture dishes and cultured overnight. Cells on cover slips were fixed with 4% paraformaldehyde for 5 min and washed twice with 0.1% Triton X-100 in PBS. Cells were then blocked for 15 min at room temperature with 5% bovine serum albumin (BSA) in PBS. An anti-Glut1 antibody (Santacruz, CA, USA) was diluted 1:100 with 0.5% BSA in PBS and added to cells on cover slips. After incubating for 1 h, cover slips were washed thrice with 0.1% Triton X-100 in PBS. Cells were then incubated for 1 h with a secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA: diluted 1:100 with 0.5% BSA in PBS), washed thrice with 0.1% Triton X-100 in PBS, and counterstained for DNA with Hoechst 33258 (4 μg/mL in 50% glycerol). Images were acquired using a CCD camera attached to a fluorescence microscope (Keyence, Osaka, Japan).

Western blot analysis

Cytoplasmic and membrane proteins were isolated using a Subcellular Protein Fractionation Kit (Thermo Scientific). Western blotting was performed as described previously [2]. For γ-H2AX analysis, cells were incubated with 5 mM 2-DG for 30 min before irradiation experiments. Histone extracts were prepared as described by Tung and Winn [12]. Proteins were separated by sodium lauryl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to PVDF membranes (Bio-Rad). Membranes were blocked with 5% (w/v) phospho-blocker (Cell Biolabs, Inc.) for 1 h and incubated with each primary antibody, including anti-Glut1 antibody (Santacruz, CA, USA), anti-β-actin (Sigma, A2066) and anti-γ-H2AX (Upstate), either at room temperature for 1 h or at 4 °C overnight. The membranes were then incubated at room temperature for 1 h with either HRP-conjugated goat anti-rabbit IgG (Nichirei Bioscience) or HRP-conjugated goat anti-mouse IgG (R&D Systems). Protein bands were visualized with Chemi-Lumi One L Western blotting substrate (NacalaiTesque).

Measurements of lactate concentrations

Lactate concentrations were determined using a lactate assay kit (Bio Vision, Mountain View, CA, USA) according to the manufacturer's instructions. Samples and lactate standard with lactate assay buffer (50 μl) was prepared to a 96-well plate. Add 50 μl of the reaction mix containing the lactate enzyme mix to each well. Incubate the reaction for 30 min. at room temperature. OD values at 570 nm were measured with a microplate reader (SpectraMax M2e, Molecular Devices).

ROS detection

Cells (5×10^5) were seeded in a T-25 flask and cultured overnight. At 6 h after irradiation, cells were stained with 20 μM DCFDA (Sigma) in RPMI medium without serum for 30 min. Cells were washed twice with PBS(−) and then trypsinized. Cells were placed on glass slides and embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a CCD camera attached to a fluorescence microscope (Keyence, Osaka, Japan). Cells were incubated with 5 mM 2-DG for 3 h. DCFDA-stained cells were quantified with a FACScan (Becton Dickinson, USA).

Clonogenic assay

Cells were seeded in 60-mm dishes at a density of 1×10^3 cells/dish, and then incubated with 5 mM 2-DG for 3 h before irradiation. After irradiation, cells were incubated in RPMI1640 medium without 2-DG for 10 days (until colonies were visible), fixed with ethanol for 30 min, and stained with Giemsa solution (Merck & Co., Inc., Rahway, NJ, USA). Using a light microscope, colonies with >50 cells were counted as survivors.

Annexin V staining

Apoptotic cells were identified and quantified using an annexin V-FITC apoptosis detection kit (Bio Vision, Mountain View, CA, USA) according to the manufacturer's instructions. Cells were incubated with 5 mM 2-DG for 3 h before irradiation. Following staining with annexin V-FITC 48 h after irradiation, cells were analyzed with a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Oxford, UK).

Statistical analysis

Results are presented as means with error bars in figures indicating standard deviations. All experiments were repeated at least thrice with independent samples. Student's *t*-tests were used to compare the results of lactate concentrations between control 0FR and control 31FR-31NR cells. One-way ANOVA test followed by the Dunnett's test was used to compare the results of annexin V staining after different treatments. Single and double asterisks indicate significant differences of $p < 0.05$ and $p < 0.01$, respectively.

Results

Glut1 expression by 31FR-31NR cells

Constitutive AKT activation induced by long-term FR exposure may increase glucose uptake in acquired radioresistant 31FR-31NR

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