Exposure to hypoxia following irradiation increases radioresistance in prostate cancer cells

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Abstract

**Background:** Tumor hypoxia is a common feature of prostate tumors associated with the stabilization of hypoxia-inducible-factor 1 alpha (HIF-1\textalpha) and poor prognosis following radiation therapy. Lack of oxygen at the time of irradiation is associated with radioresistance, but recent reports suggest radioreponse is also modulated by the dynamic nature of tumor hypoxia.

**Objective:** We proposed to evaluate the effect of post-irradiation hypoxic exposure on the radioreponse of 2 prostate cancer (CaP) cell lines (22Rv1, DU145) and to examine whether it correlates with modified cellular responses induced by hypoxia.

**Methods and results:** Aerobic and hypoxic CaP cells exposed to hypoxia (24 h) after irradiation (4 Gy) gained a survival advantage compared with cells fully oxygenated after irradiation. This survival advantage was associated with induction of a G2/M cell cycle arrest, reduced induction of apoptosis and decreased amount of senescent cells. These modified cellular responses appeared mediated by HIF-1\textalpha.

**Conclusion:** Our data suggest that targeting hypoxia after irradiation may benefit patients with aggressive hypoxic prostate tumors. © 2011 Elsevier Inc. All rights reserved.

**Keywords:** Radiation; Hypoxia; Prostate cancer; HIF-1; Resistance

1. Introduction

The presence of hypoxic regions in prostate cancer (CaP) is widely documented in the literature, both through the use of physical measurements (pO\textsubscript{2}) probes [1], imaging modalities (positron emission tomography, magnetic resonance imaging) [2], and the identification of specific biomarkers [3]. For 50 years, tumor hypoxia has been associated with radioresistance and poor clinical outcome. Hypoxic tumor cells can be up to 3 times more resistant to radiation damage than those in a normal oxygen environment. It is proposed that hypoxic cells are resistant to radiation therapy because of a lack of oxygen as a source of radiation induced radicals and indirect DNA damage [4]. It was initially demonstrated that hypoxia induces radioresistance at the time of irradiation, but recent reports suggest radioreponse is related to the duration and degree of hypoxia prior to irradiation [5].

The major transcriptional regulator of the hypoxic response and regulator of homeostasis and cellular metabolism to the stress of low oxygen tension is hypoxia inducible factor 1 alpha (HIF-1\textalpha). This is a critical transcription factor that is responsible for activating a number of genes in hypoxia ultimately resulting in cellular adaptation to low oxygen conditions [6]. To date, over 60 genes have been discovered to be induced by HIF-1\textalpha. These genes encode for products such as erythropoietin, vascular endothelial growth factor (VEGF), a variety of glycolytic enzymes, transferrin, and other proteins essential for cellular homeostasis [7]. Overexpression of HIF-1\textalpha has been associated with increased patient mortality in several cancer types including breast, stomach, cervical, endometrial, and ovarian cancers [8]. Furthermore, the overexpression of HIF-1\textalpha in patients with oropharyngeal cancer is associated with increased risk of failure to achieve complete remission after
radiation therapy [9]. However, the identification of hypoxic regions within tumors has not, to date, altered the clinical management of patients. HIF-1α has been proposed as a marker for radiosensitivity [9,10] but to date, the role of ionizing radiation on the expression patterns of HIF-1α and its downstream genes remains to be elucidated in CaP.

Hypoxia appears to contribute to reduced recognition of DNA DSB, with ataxia-telangiectasia-mutated (ATM) and (ataxia telangectasia and Rad3-related (ATR) protein kinases both playing a part in the cellular DNA damage response to hypoxia/re-oxygenation [11]. Hypoxia may also downregulate DSB repair by preferentially preventing homologous recombination (HR) in vitro [12,13], and base excision repair [14]. In addition, hypoxia is associated with an increased α/β ratio (8.5 Gy) [15].

We proposed to determine whether post-irradiation hypoxic exposure affects the radioresistance of CaP cells and to determine the role of HIF-1 in promoting cell survival in hypoxia in these cells, by investigating the effects of HIF-1 on cell cycle, apoptosis, senescence, and cell death.

2. Materials and methods

2.1. Cell culture and growth conditions

The 22Rv1 human CaP cell line was obtained from the ATCC (ATCC-CRL-2505, Teddington, UK). The DU145 and PC3 human CaP cell lines were obtained from DSMZ, Braunschweig, Germany (ACC261, ACC465, respectively). The lines were maintained in RPMI 1640 medium (Lonza, BE12-702F, Bray, Ireland) supplemented with 10% fetal calf serum (Lonza, DE14-801F), and 1% streptomycin-penicillin (Lonza, DE17-603E).

2.2. Irradiation and hypoxic conditions

Hypoxia was defined as 0.5% oxygen. This environment was achieved by exposing cells in a 1000 in vivo hypoxic chamber (BioTrace, Bracknell, UK) to a mixture of nitrogen, CO₂ (5%) and compressed air to achieve a 0.5% oxygen concentration. Radiation was delivered under hypoxic conditions using a RS225 cell irradiator (Xstrahl, Camberley, UK), at a dose rate of 3.25 Gy/min.

2.3. Clonogenic assays

Between 1000 and 10,000 cells/well were plated onto 6-well plates and exposed to hypoxia (4, 24, 48 h) prior to and/or following to irradiation (4, Gy). Immediately post-irradiation the cells were either returned to the hypoxic chamber for 24 h or returned directly to an aerobic incubator. Following post-irradiation hypoxic exposure for 24 h the cells were transferred to an aerobic incubator for 1 to 2 weeks to allow colony formation. After incubation at 37°C for 14 days the resultant colonies were stained with crystal violet in 95% ethanol, and those consisting of greater than 50 cells were scored as representing surviving cells using CoulCount automatic cell counter (Oxford Optronix Ltd., Oxford, UK). The response of aerobic cells was used as a control. The plating efficiency was calculated as the ratio of the number of colonies counted over the number cells plated. The surviving fraction in the treated wells was subsequently calculated as the ratio of the number of clones counted over the number of cell plated corrected with the appropriate plating efficiency.

2.4. Cobalt chloride treatment

Cobalt Chloride (CoCl₂) induces HIF-1α expression by binding to the PAS domain resulting in blockage of HIF-1α pVHL binding and thereby HIF-1α stability [16]. To simulate hypoxic exposure and HIF-1α expression, cells were exposed to a 100 μM solution of CoCl₂ for 4 hours and irradiated (Sigma Aldrich, 15862, Poole, UK). Cells were washed and fresh media added immediately or 24 hours later to simulate post-irradiation hypoxic exposure. Cells were incubated for 1 to 2 weeks to allow colony formation and were analyzed by clonogenic assays.

2.5. Western blotting

CaP cell lines, 22Rv1 and DU1245, were grown to 80% confluence in T75 cell culture flask and were exposed to cobalt chloride, hypoxic conditions, and irradiation as required. The cells were scraped and stored on ice. The pellet was re-suspended in cold radio-immunoprecipitation assay (RIPA) lysis buffer supplemented with a protease inhibitor cocktail (Santa Cruz Biotechnology, sc-24948, Santa Cruz, CA). Following 10 minutes incubation on ice, the lysates were centrifuged at 4°C for 20 minutes (15,000 rpm), and the supernatants were stored at −70°C until required. The amount of protein present in both CaP cell lines lysates was determined by a Bradford assay. Whole cell lysates were separated on 12% SDS-polyacrylamide gels and transferred to nylon/nitrocellulose membranes. The membranes (GE Healthcare, RPN303F, Buckinghamshire, UK) were then probed with rabbit anti-HIF-1α (1:500, Cell Signaling Technology, NEB.13716, Wicklow, Ireland), mouse anti-VEGF (1:1000, Abcam, ab1319, Dublin, Ireland), rabbit anti-CHK1 (1:1000, Cell signaling technology, 9931), rabbit anti-CHK2 (1:1000, Cell Signaling Technology, NEB1.9931), rabbit anti-p16INK4a (1:1000, Cell Signaling Technology, NEB.1.4824). The Pierce Luminal kit (Pierce, 34080, Rockford, IL) was used for protein detection. Membranes were stripped prior to reprobing with either a mouse monoclonal anti-actin antibody (1:10,000, Sigma-Aldrich, A3853) or anti-rabbit HRP-labeled antibody (1:5000, DakoCytomation, K0675, Glostrup, Denmark).
2.6. Flow cytometric analysis of cell cycle distribution

Cells grown in 25 cm² cell culture flasks to 70% confluence were harvested, counted, washed with PBS (Lonza, BE17-516F), fixed with 90% ethanol and stored at 4°C. The cells were then washed once with PBS, incubated in PBS containing RNase A (100 μg/ml, Sigma, R6513) for 30 minutes at 37°C, stained with propidium iodide (50 μg/ml, Sigma, P4864) and incubated on ice in the dark for 30 min. Cell cycle distribution was analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), using the CellQuest software (Becton Dickinson).

2.7. AnnexinV-PI apoptosis assays

22Rv1 and DU145 cells exposed to hypoxia and/or radiation as required were trypsinized, and pelleted (1300 rpm, 3 minutes). The pellets obtained were labeled with AnnexinV-FITC (IQ Products, Groningen, The Netherlands) according to manufacturer’s instructions. Propidium iodide (125 ng/ml) was added immediately before analysis on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Analysis of data was performed using CellQuest software (Becton Dickinson). According to the manufacturer’s instructions, cells staining negative for Annexin V and PI were defined as viable, those staining Annexin V positive and PI negative as undergoing early apoptosis and those staining positive for both Annexin V and PI as undergoing late apoptosis.

2.8. Detection of reactive oxygen species

Intracellular ROS levels were measured by flow cytometry with 5-((6)-chloromethyl-2%oo,7’-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) (Invitrogen, C2938, Dublin, Ireland). Cells in the exponential growth phase were harvested by trypsinization and pelleted by centrifugation at 1300 RPM for 3 minutes. Cells were resuspended in PBS at a concentration of 1 × 10⁷ cells (per sample) in 5 ml falcon tubes (BD Biosciences, 352052). Lyophilized CM-H₂DCFDA probe was re-suspended in dimethyl sulfoxide (DMSO, Sigma-Aldrich D8418) to a 1 M stock solution. Cells were loaded with 10 μM CM-H₂DCFDA (diluted in PBS) except for appropriate controls, and incubated for 30 minutes at 37°C in 5% CO₂/95% humidified air, in the dark. Control samples were incubated in PBS. Cells were pelleted by centrifugation at 1300 RPM × g for 3 min, and re-suspended in 0.5 ml PBS. A volume of 5μL PI solution (1 mg/ml, Sigma-Aldrich, H3410) was then added to all samples, except appropriate controls. Appropriate samples were then irradiated with 2Gy of radiation, and all samples were placed on ice in the dark. Positive controls were treated with 100 μM H₂O₂ for 30 minutes. Fluorescence was analyzed immediately using a CyAnADP flow cytometer. A minimum of 10,000 events, in a live gate were collected. Mean fluorescence intensity was used as a measure of ROS, using Summit ver. 4.3 software (Dako, Glostrup, Denmark).

2.9. Estimation of reactive oxygen species by glutathione detection

22Rv1 and DU145 cells were plated onto a 96-well plate and exposed to hypoxia/radiation as required. Immediately after the treatment, the media was removed from each well of the 96-well plates and replaced with GSH-Glo reaction buffer (Promega V6911, Dublin, Ireland). The 96-cell plate was gently mixed on a plate shaker and left to incubate at room temperature for 30 minutes. Reconstituted luciferin detection reagent was then added to each well of the 96-well plate and again allowed to incubate at room temperature for 15 minutes. Luminescence was read by transferring the 96-well plate to a SpectraFlour Plus fluorometer (Tecan, Männedorf, Switzerland) and quantifying sample luminescence. The net GSH-dependent luminescence of each sample was calculated by subtracting the average luminescence of the negative control reactions (reactions without GSH) from that of GSH-containing reactions.

2.10. Detection of cellular senescence

Cellular senescence was determined using the senescence detection kit (Cambridge Bioscience, K320-250, Cambridge, UK), according to manufacturer’s instructions. Briefly, 22Rv1 and DU145 cells were plated onto a 12-well plate and exposed to hypoxia/radiation as required. The next day, the media was removed and the cells were washed once with 1× PBS and a fixative solution was added to each well for 10 minutes at room temperature. Cells were washed 2 further times with 1× PBS, and staining solution mix was added. This was comprised of staining solution, staining supplement, and 20 mg/ml X-gal in DMSO. The cells were covered and incubated overnight at 37°C. The cells were observed under a microscope for the development of a blue color (marker of SA-β-Gal activity) and cell morphology at 200× total magnification. A minimum of 200 cells was manually scored at random according to SA-βgal staining (positive or negative). For each experiment a mean of 600 cells was counted. Digital images were taken with an Olympus CKX41 microscope, Southernd-on-Sea, UK) and were processed with DP Controller version 1.2.1.108 software (Olympus).

2.11. Statistical analysis

The data is presented as mean ± standard error of the mean of at least 3 independent experiments. Student’s t-tests were used to compare means between 2 groups, an ANOVA was used to compare means between 2 or more groups, as appropriate. Statistical analysis was performed using Prism,
3. Results

3.1. Post-irradiation hypoxia increases the surviving fraction of CaP cells

The impact of post irradiation hypoxic exposure (24 hours) on the radioresistance of aerobic or hypoxic (4 hours) 22Rv1 and DU145 cells exposed to a single dose of radiation (4 Gy) was determined using clonogenic assays (Fig. 1). The response of aerobic cells and cells reoxygenated following irradiation were used as a control. DU145 were intrinsically more radioresistant than 22Rv1 cells ($P = 0.0041$). Hypoxia increased the radioresistance of both cell lines significantly ($22Rv1, P = 0.04, DU145, P = 0.0067$). Post irradiation hypoxic exposure significantly increased the surviving fraction of aerobic cells ($22Rv1 P = 0.0017; DU145 P = 0.0002$) but not that of hypoxic cells ($22Rv1 P = 0.2462; DU145 P = 0.0727$). Aerobic cells exposed to hypoxia after irradiation were as radioresistant as hypoxic cells ($22Rv1 P = 0.1020; DU145 P = 0.7338$) and hypoxic cells exposed to hypoxia following irradiation for $22Rv1$ cells only ($22Rv1 P = 0.2874; DU145 P = 0.0359$). The effect of post-irradiation hypoxia on the radioresistance of chronically hypoxic CaP cells (24, 48, 72 hours) was also determined and did not further increase the surviving fraction of cells following post-irradiation hypoxic exposure, compared with cells that were in hypoxia for 4 hours prior to irradiation ($22Rv1$ ANOVA, $P = 0.2865$, DU145 ANOVA, $P = 0.1675$) (data not shown).

3.2. Hypoxia decreases reactive oxygen species and glutathione levels following irradiation

The effect of hypoxia on intracellular levels of reactive oxygen species (ROS) and Glutathione were determined. Aerobic or hypoxic (4 hours) $22Rv1$ and DU145 cells were exposed to the delivery of a single dose of radiation (4 Gy). Median intracellular ROS levels were detected by flow cytometry (Fig. 2A, C) and glutathione levels by luminescence (Fig. 2B, D). The response of aerobic cells and hypoxic cells were used as controls. The mean baseline ROS levels in aerobic cells were significantly higher in $22Rv1$ cells (687.6 ± 125.3) than in DU145 cells (294.0 ± 52.65) ($P = 0.0007$). Irradiation with 4 Gy increased the median...
ROS levels in both aerobic cell lines significantly (22Rv1 \( P = 0.0179 \), DU145 \( P < 0.0001 \)). The amount of intracellular ROS was significantly higher in hypoxic cells, when compared with aerobic cells (22Rv1 \( P = 0.0021 \) and DU145 \( P < 0.0034 \)). In hypoxic cells, irradiation did not lead to increased intracellular ROS levels in both DU145 cells (\( P = 0.6384 \)) and 22Rv1 cells (\( P = 0.1136 \)). Following irradiation, the intracellular ROS levels were significantly reduced in hypoxic cells compared with aerobic cells in 22Rv1 (\( P = 0.0154 \)) but not in DU145 cells (\( P = 0.0534 \)). Baseline GSH levels were significantly higher in DU145 cells compared with 22Rv1 cells (\( P < 0.0001 \)). Irradiation with 4 Gy decreased the intracellular GSH levels in aerobic DU145 cells (\( P = 0.0020 \)) but not in aerobic 22Rv1 cells (\( P = 0.1592 \)). Hypoxia significantly decreased the intracellular levels of GSH in both cell lines (22Rv1 \( P = 0.0026 \), DU145 \( P < 0.0001 \)). Following irradiation, the intracellular levels of GSH were further decreased in 22Rv1 cells (\( P = 0.0148 \)) but not DU145 cells (\( P = 0.3590 \)).

### 3.3. Post-irradiation hypoxia increases HIF-1α and VEGF expression

The effect of hypoxia on HIF-1α protein expression was determined and correlated with VEGF protein expression. Aerobic and hypoxic (4 hours) 22Rv1 and DU145 cells were exposed to the delivery of a single dose of radiation (4 Gy) and allowed to recover under aerobic or hypoxic conditions for 24 hours. HIF-1α and VEGF expression was determined using Western blots (Fig. 3). The response of aerobic cells and hypoxic cells were used as controls. HIF-1α expression was not demonstrated in aerobic samples of 22Rv1 and DU145 cells. Hypoxia (4 hours) increased HIF-1α protein expression in both cell lines. HIF-1α was expressed in irradiated hypoxic 22Rv1 and DU145. Cells placed in hypoxia post-irradiation had the greatest levels of HIF-1α expression. The expression of HIF-1α was comparable between both cell lines. To confirm the effect of hypoxia VEGF protein expression was also evaluated. VEGF protein expression was evident in both cell lines under all conditions. VEGF expression appeared greatest in hypoxic cells exposed to hypoxia post-irradiation.

### 3.4. HIF-1α expression is associated with a survival advantage following irradiation

The effect of HIF-1α in the induction of radioresistance by hypoxia was next evaluated in CaP cells treated with the HIF-1α inducer cobalt chloride (CoCl2). Aerobic and CoCl2 treated (4 hours) 22Rv1 and DU145 cells were exposed to a single dose of radiation (4 Gy) and allowed to recover under aerobic or CoCl2 treated media for 24 hours. HIF-1α and VEGF expression were determined using Western Blotting (Fig. 4A, B) and survival was measured using clonogenic
assays (Fig. 4C, D). The response of aerobic cells and cells reoxygenated following irradiation were used as a control. CoCl2 treatment (4 h) increased HIF-1α protein expression in both irradiated and unirradiated cell lines. Cells treated with CoCl2 post irradiation had the greatest levels of HIF-1α expression. The expression of VEGF appeared enhanced in cells incubated with CoCl2, specifically 22Rv1 and DU145 cells treated with CoCl2 post-irradiation. Treatment with CoCl2 resulted in increased radioresistance, compared with aerobic controls (22Rv1 \( P = 0.0287 \); DU145 \( P = 0.0002 \)). The surviving fraction of CoCl2 treated cells was not significantly different to that of hypoxic cells, in cells exposed to both post-irradiation aerobic conditions (22Rv1 \( P = 0.7192 \); DU145 \( P = 0.0002 \)) and post-irradiation hypoxic/CoCl2 treated conditions (22Rv1 \( P = 0.2180 \), DU145 \( P = 0.3754 \)).

3.5. Post-irradiation hypoxia induces G2/M cells cycle arrest

The impact of post irradiation hypoxic exposure on the cell cycle was determined. Aerobic and hypoxic (4 hours) 22Rv1, DU145 cells were exposed to a single dose of radiation (4 Gy) and allowed to recover under aerobic or hypoxic conditions for 24 hours. Cell cycle phase distributions were determined by flow cytometry (Fig. 5A, B). The response of aerobic cells and cells reoxygenated following irradiation were used as a control. The proportion of cells in the G1 phase was not modified by hypoxia in irradiated cell lines. (22Rv1, ANOVA, \( P = 0.7961 \); DU145, ANOVA, \( P = 0.9975 \)). Exposure to hypoxia post irradiation significantly reduced the proportion of cells in the S phase in aerobic cells (22Rv1 cells \( P = 0.0034 \); DU145 \( p = 0.006 \)) but not in hypoxic cells (22Rv1 cells \( P = 0.27 \); DU145 \( p = 0.28 \)). The proportion of aerobic cells exposed to hypoxia post-irradiation in the S phase was not statistically different from that of hypoxic cells and hypoxic cells exposed to hypoxia post-irradiation (22Rv1, ANOVA, \( P = 0.439 \); DU145, ANOVA, \( P = 0.2854 \)). Similarly, exposure to hypoxia post-irradiation significantly increased the proportion of cells in the G2/M phase in aerobic cells (22Rv1 cells \( P = 0.0164 \); DU145 \( P < 0.001 \)) but not in hypoxic cells (22Rv1 cells \( P = 0.7673 \); DU145 \( P = 0.9689 \)). The proportion of aerobic cells exposed to hypoxia post-irradiation in the G2/M phase was not statistically different from that of hypoxic cells and hypoxic cells exposed to hypoxia post-irradiation (22Rv1, ANOVA, \( P = 0.3228 \); DU145, ANOVA, \( P = 0.5162 \)).

3.6. Post-irradiation hypoxia decreases expression of cell cycle checkpoint proteins

The effect of hypoxia and HIF-1α on CHK1 and CHK2 protein expression was determined. Aerobic, hypoxic, and CoCl2 treated (4 hours) 22Rv1 and DU145 cells were ex-
posed to the delivery of a single dose of radiation (4 Gy) and allowed to recover under aerobic, hypoxic or CoCl2 treated media for 24 hours. CHK1 and CHK2 protein expression was then determined using Western blots (Fig. 5C, D). The response of aerobic cells and hypoxic cells were used as controls. Expression of CHK1 and CHK2 proteins was comparable between 22Rv1 and DU145 cells. Hypoxia pre-irradiation and hypoxic exposure post-irradiation increased the expression of CHK1 and CHK2 proteins in both cell lines. CoCl2 treatment pre and post irradiation increased expression of CHK2 and decreased CHK1 expression in both 22Rv1 and DU145 cells.

3.7. Post-irradiation hypoxic exposure reduces apoptosis levels

The effect of hypoxia on apoptosis was determined. Aerobic or hypoxic (4 hours) 22Rv1 and DU145 cells were exposed to the delivery of a single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hours. Percentage early apoptosis was determined by flow cytometry (Fig. 6A, B) and correlated with p53 protein expression using Western blotting (Fig. 6C). The response of aerobic cells and hypoxic cells were used as controls. Exposure to hypoxia following irradiation significantly reduced apoptosis levels in aerobic cells (22Rv1 $P = 0.0228$; DU145 $P = 0.0032$), hypoxic 22Rv1 cells ($P = 0.029$) but not in hypoxic DU145 cells ($P = 0.9708$). Hypoxic exposure following irradiation but not radiation alone increased p53 expression in aerobic 22Rv1 and DU145 cells. In both hypoxic cell lines, p53 expression was increased by radiation alone, but reduced when cells were exposed to hypoxia following irradiation.

3.8. Hypoxia can reduce cellular senescence following irradiation

The effect of hypoxia on cellular senescence was determined. Aerobic or hypoxic (4 hours) 22Rv1 and DU145 cells were exposed to the delivery of a single dose of radiation (4 Gy) and allowed to recover in aerobic or hypoxic conditions for 24 hours. The presence of senescent cells was determined by histochemical detection of β-galactosidase (SA-β-Gal) activity (Fig. 7). The response of positive controls, aerobic cells, and hypoxic cells were used as controls. The mean baseline cellular senescence of 22Rv1 cells was 15.4% ± 0.9% and 8.7% ± 2.4% in DU145 cells. Oxygenated 22Rv1 and DU145 that were irradiated and allowed to recover under aerobic conditions, demonstrated increased cellular senescence compared with controls. The percentage of irradiated senescent cells was 24.9% ± 2.1% in 22Rv1 ($P = 0.002$) and 11.73% ± 1.3% in DU145 cells. Post-irradiation hypoxia significantly reduced the percentage of cells in the senescent state in both cell lines compared...
with irradiated aerobic controls. The percentage of senescent cells was reduced to 9.314% /H11006 1.0% in 22Rv1 cells (P/H11005 0.0003), and to 5.536 /H11006 0.74 in DU145 cells (P/H11005 0.002).

3.9. Hypoxia and HIF-1α can reduce p16ink4a expression

The effect of HIF-1α on p16ink4a protein expression was finally determined. Aerobic, hypoxic and CoCl2 treated (4 hours) 22Rv1 and DU145 cells were exposed to the delivery of a single dose of radiation (4 Gy) and allowed to recover in aerobic, hypoxic or CoCl2 treated media for 24 hours and p16ink4a protein expression was determined using Western blots (Fig. 8). The response of aerobic cells was used as control. Expression of p16ink4a was greater in DU145 aerobic control cells compared with 22Rv1 aerobic control cells. In both cell lines hypoxic irradiation followed by hypoxic exposure for 24 hours increased the expression of p16ink4a in 22Rv1 cells only. CoCl2 treatment reduced the expression of p16ink4a in both cell lines.

4. Discussion

This study proposed to evaluate the effect of post-irradiation hypoxic exposure on the radioresponse of CaP cells and to examine whether it correlates with modified cellular responses induced by hypoxia and mediated by HIF-1α. We report that CaP cells hypoxic after irradiation gain a survival advantage compared with cells fully oxygenated after irradiation. This survival advantage was associated with induction of a G2/M cell cycle arrest, reduced induction of apoptosis, and a reduced population of senescent cells. Furthermore, we demonstrate that these modified cellular responses could be mediated by HIF-1α. Our data indicate that post-irradiation hypoxic exposure is important in the development of radioresistance and suggests that targeting hypoxia after irradiation may benefit patients with aggressive hypoxic prostate tumors.

It is proposed that hypoxic cells are more resistant to irradiation because of a lack of oxygen, a source of radiation-induced radicals [4]. Our data suggest that hypoxic radioresistance could be in part due to different intrinsic ROS and antioxidant levels between cell lines. 22Rv1 cells had the highest baseline ROS and lowest GSH levels, whereas the more radioresistant DU145 cells had lower ROS levels and higher GSH. Hypoxia reduced the amount of ROS generated by irradiation, and this was associated with a reduction in GSH. This may have lead to a failure of oxygen containing compounds to fix DNA damage, allowing DNA repair to occur.
Cellular adaptation to hypoxia involves the coordinated expression of a large and diverse group of genes, many of which are transcriptionally regulated by HIF-1α. In hypoxia, HIF-1α levels significantly increase as proteasomal degradation is inhibited due to the inability of von Hippel-Lindau tumor suppressor protein (pVHL) to bind to HIF-1α [7]. This affects many processes, including glycolysis, mitosis, apoptosis, and angiogenesis, all of which have been shown to influence radio-responsiveness [17]. We confirmed the expression of HIF-1α in both hypoxic CaP cell lines (22Rv1 and DU145). We previously reported that HIF-1α protein expression in 22Rv1 and DU145 cell lines was no longer detectable 48 hours into hypoxic exposure and concluded that HIF-1α expression appeared between 4 and 8 hours of exposure [18]. Cells placed into hypoxia post-irradiation showed greatest HIF-1α expression. This suggests that radiation facilitates the continued stabilization of HIF-1α. Radiation alone has indeed been proposed to increase HIF-1α activity between 24 and 48 hours after irradiation in vivo [19]. Similarly, 22Rv1 and DU145 cells treated with CoCl2, a chemical inducer of HIF1α, showed increased radioresistance and increased HIF-1α expression compared with untreated controls. These results further suggest that HIF-1α stabilization governs the radioresponse of CaP cells in hypoxia. Further research is required to investigate direct involvement of HIF-1α in this response.

![Fig. 6. Post-irradiation hypoxia decreases apoptosis Percentage apoptosis of aerobic or hypoxic (A) 22Rv1 and (B) DU145 cells exposed to the delivery of a single dose fraction (4 Gy) and allowed to recover under aerobic (clear bar) or hypoxic (black bar) conditions for 24 hours; n = 3; mean ± SEM; *P < 0.05. Representative p53 and β-actin immunoblots of aerobic and hypoxic (4 hours) (C) 22Rv1 and (D) DU145 cells that were exposed to the delivery of a single dose of radiation (4 Gy) and allowed to recover under aerobic or hypoxic conditions for 24 hours.](image)

Cellular adaptation to hypoxia involves the coordinated expression of a large and diverse group of genes, many of which are transcriptionally regulated by HIF-1α. In hypoxia, HIF-1α levels significantly increase as proteasomal degradation is inhibited due to the inability of von Hippel-Lindau tumor suppressor protein (pVHL) to bind to HIF-1α [7]. This affects many processes, including glycolysis, mitosis, apoptosis, and angiogenesis, all of which have been shown to influence radio-responsiveness [17]. We confirmed the expression of HIF-1α in both hypoxic CaP cell lines (22Rv1 and DU145). We previously reported that HIF-1α protein expression in 22Rv1 and DU145 cell lines was no longer detectable 48 hours into hypoxic exposure and concluded that HIF-1α expression appeared between 4 and 8 hours of exposure [18]. Cells placed into hypoxia post-irradiation showed greatest HIF-1α expression. This suggests that radiation facilitates the continued stabilization of HIF-1α. Radiation alone has indeed been proposed to increase HIF-1α activity between 24 and 48 hours after irradiation in vivo [19]. Similarly, 22Rv1 and DU145 cells treated with CoCl2, a chemical inducer of HIF1α, showed increased radioresistance and increased HIF-1α expression compared with untreated controls. These results further suggest that HIF-1α stabilization governs the radioresponse of CaP cells in hypoxia. Further research is required to investigate direct involvement of HIF-1α in this response.

Our data suggest that hypoxic exposure post-irradiation induces a G2/M cell cycle arrest, and decreases the S-phase cell population. Hypoxia increased the expression of CHK1 and CHK2 in 22Rv1 and DU145 cells. CDK2 expression was HIF-1α dependent but CHK1 expression was reduced by HIF-1α. The regulation of CHK1 expression by hypoxia requires further examination and is likely to involve processes other than HIF-1α. Previous studies suggested that re-oxygenation produces ROS, which generate DNA damage that elicits a CHK2-dependent G2 checkpoint. Indeed, CHK2-deficient cells fail to arrest in G2 in anoxia and instead undergo apoptosis [20,21]. By contrast, chronic hypoxia leading to cellular adaptation may not activate G1.

![Fig. 7. Hypoxia reduces the amount of cells in the senescent state. Quantitative assessment of SA-β-Gal staining in a minimum of 200 cells randomly scored. Bar chart representing the mean ± SEM percentage of 22Rv1 and DU145 cells expressing SA-βgal at baseline (black bar), in irradiated aerobic (white bar) and hypoxic (lined bar) cells; n = 3, **P < 0.01.](image)
or S checkpoints and could potentially lead to accumulation of DNA replication errors or DNA breaks over time [5]. However, whether it is the level of oxygen, the duration of exposure, or the re-oxygenation events that determine whether cell cycle progression is affected within the complexity of a hypoxic solid tumor has not been adequately addressed in vivo.

The role of apoptosis in determining tumor response to radiation remains poorly understood and controversial. One of the main controversies is the time course of apoptosis following irradiation. For apoptosis to contribute to a loss in clonogenic survival, it must occur prior to the first post-irradiation mitosis or early apoptosis [22]. Apoptosis that occurs hours to days after the first mitosis is secondary to death of the cell following an aberrant mitotic event caused by radiation-induced chromosome abnormalities. Furthermore, any modulation of secondary or late apoptosis observed due to changes in the tumor microenvironment are not relevant to any observed changes in clonogenic survival [23]. Hypoxic exposure decreased apoptosis levels in both cell lines. Levels were lowest in hypoxic cells placed in hypoxia post-irradiation. This correlated with reduced p53 expression in DU145 cells. Irradiation leads to increased p53 expression in both cell lines and this appeared most apparent in cells hypoxic only pre- or post-irradiation. The expression of p53 protein was reduced by CoCl2 treatment, suggesting regulation by HIF-1. The interaction between HIF-1α and p53 is not fully understood and further research is required. HIF-1α interacts with wild-type p53 but not with tumor-derived mutant p53 [24]. HIF-1α was shown to directly bind to the p53 ubiquitin ligase mdm2 both in vivo and in vitro, thereby stabilizing p53 [25]. However, a direct binding of p53 to the oxygen-dependent degradation (ODD) domain of HIF-1α was also reported [26].

Senescence is thought to be a tumor suppressive mechanism and an underlying cause of aging. A state of irreversible growth arrest, it can be triggered by multiple mechanisms, including telomere shortening, the epigenetic depression of the INK4a/ARF locus, and DNA damage. Together these mechanisms limit excessive or aberrant cellular proliferation. Hypoxia has been demonstrated to decrease the proportion of cells in the senescent population of human HCT116 colon carcinoma cells, via a HIF-1α mediated pathway, and to increase chemotherapeutic resistance [27]. Hypoxia decreased the proportion of cells in the senescent population of 22Rv1 and DU145 CaP cells. Cells hypoxic prior to irradiation and placed in hypoxia post-irradiation showed the least amount of SA-β-Gal expression. There was also a difference between the cell lines; 22Rv1 cells seemed to express more SA-β-Gal than DU145 cells. It therefore must be noted that the most radioresistant cells were also the cells that had the least amount of its population in the senescent state. Hypoxia or CoCl2 treatment decreased the expression of p16Ink4a in both cell lines. This suggests that HIF-1α interacts with p16Ink4a and prevents cells entering the senescent state and thereby increases the radioresistance of the CaP cell. Further research is however required to characterize this relationship. Treatment of MDA-MB-231 cells with small interfering RNA targeting the α-subunit of HIF-1α prevented hypoxia-induced chemotherapeutic resistance [27]. HIF-1α small interfering RNA also selectively abolished the hypoxia-induced changes in the senescent population, indicating that the increased survival was due to protection against drug-induced senescence. These results support a requirement for HIF-1α in the adaptations leading to drug resistance and reveal that decreased drug-induced and possibly radiation-induced senescence are also important contributors to the development of hypoxia-induced resistance [27]. In preclinical studies, inhibition of HIF-1α activity has marked effects on tumor growth [28]. An increasing body of evidence also suggests that targeting and inhibition of the transcription factor HIF-1α may be a viable therapeutic target.

5. Conclusion

Our results identify for the first time a role for post-irradiation hypoxia in the increased radioresistance of hypoxic prostate tumor and support the need for continued research into anti-HIF-1α therapies as an adjuvant treatment for CaP patients receiving radiotherapy.
References


