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Stabilisation and Knockdown of HIF - Two Distinct Ways Comparably Important in Radiotherapy

Mareike Ströfer[1,2], Wolfgang Jelkmann[1], Eric Metzen[3], Ulf Brockmeier[3], Jürgen Dunst[2] and Reinhard Depping[1]

[1]Department of Physiology, Center for Structural and Cell Biology in Medicine, University of Lübeck, Lübeck, [2]Department of Radiotherapy, University of Lübeck, Lübeck, [3]Institute of Physiology, University of Duisburg-Essen, Essen

Key Words
Radiotherapy • HIF • Hypoxia • Cancer • Stabiliser

Abstract
Background: Radiotherapy is one of the most widely used treatments for cancer. The benefit of radiation is known to be negatively affected by tumor hypoxia and the expression of hypoxia-inducible factors (HIF), respectively. HIF-1α/β and HIF-2α/β are transcriptional activators of oxygen-regulated genes. The aim of the study was to examine cell type-specific effects of HIF-1α and -2α knockdown or oxygen-independent HIF-stabilisation on radiosensitivity. Methods: Herein, we treated four different wildtype and HIF-1α- or HIF-2α-deficient human cancer cell lines, cultured under normoxic or hypoxic conditions, with ionising radiation in doses from 2 to 6 Gy and examined clonogenic survival. Furthermore, the cells were partly preincubated with a HIF-stabiliser (di-tert-butyroyl-oxymethyl-2,4-pyridine-dicarboxylate, ^{t}Bu-2,4-PDC). Results: The results show that both hypoxia exposure and treatment with ^{t}Bu-2,4-PDC increased the radioresistance of human cancer cells. The HIF-mediated decrease of radioresponsiveness induced by the chemical stabiliser emerged to be as strong as the one caused by hypoxia. Clonogenic survival assays furthermore revealed that HIF-1 expression enhanced resistance to radiation, whereas knocking-down HIF-1 increased the sensitivity to radiation under normoxic as well as under hypoxic conditions. Conclusion: These data extend previous observations of HIF-1α and broaden the view by showing HIF-2α inverse correlation between HIF expression and prognosis for the outcome of radiotherapy.

Introduction

In vivo oxygen levels have been found to naturally cycle up and down in individual blood vessels - a phenomenon called cycling hypoxia [1]. In rapidly growing tumors blood supply often becomes limited, leaving portions of the tumor in a situation where the oxygen concentration is significantly lower than in healthy tissues [2, 3]. In response, adaptative proteins including glycolytic

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Reinhard Depping
Center for Structural and Cell Biology in Medicine, University of Lübeck
Ratzeburger Allee 160, 23562 Lübeck (Germany)
Tel. +49451500-4712, Fax +49451500-4151
E-Mail Depping@physio.uni-luebeck.de

enzymes, glucose transporters and survival factors such as the hypoxia-inducible factors (HIFs) are induced [4]. HIFs play a central role in the adaptation and response to low oxygen levels in metazoan cells. HIF-1 and HIF-2 are transcriptional activators of oxygen-regulated genes via binding to specific regulatory DNA sequences termed hypoxia responsive elements. HIFs are composed of one of three O_2-labile α-subunits (HIF-1α, -2α or -3α) and a constitutive β-subunit (HIF-1β or ARNT) [5]. HIF-α degradation in the presence of oxygen is mediated through a set of non-heme, Fe^{2+} and α-ketoglutarate-dependent prolyl hydroxylases (PHD1-3), which strictly require oxygen as a co-substrate. In the presence of O_2, HIF-α is degraded by the proteasome after being marked by hydroxylation by the PHDs at the oxygen dependent degradation domain.

Besides its adaptive function, the HIF system has a prominent role in cancer growth and malignant progression because HIF promotes angiogenesis and, thus, affects the efficiency of tumor therapy via tumor oxygenation. Radiation therapy is an important tool in cancer treatment. However, hypoxic tumor cells are often found to be resistant to radiotherapy and chemotherapy [6-8]. Therefore, HIF-inhibitors are potentially clinically relevant as enhancers of cancer radiosensitivity. Cell type-specific effects of siRNAs downregulating HIF-1α and -2α protein levels were analysed in this study to demonstrate their properties as radiosensitisers. On the other hand, an upregulation of HIF and its downstream target gene erythropoietin (EPO) by PHD inhibitors as ^{t}Bu-2,4-PDC can be helpful in patients with kidney diseases or persons receiving chemotherapy to treat anaemia but can negatively influence radiotherapy which can be inferred from the data demonstrated here.

Materials and Methods

Antibodies and Chemicals

Antibodies used were anti-HIF-1α (1:1000, BD Biosciences) and anti-HIF-2α (1:1000, Novus Biochemicals, Inc.). Puromycin was optained from Calbiochem. Small interfering RNA (siRNA) against HIF-1α, Lipofectamine 2000 and Lipofectamine RNAiMAX were purchased from Invitrogen, MISSION shRNA vectors targeting HIF-1α were obtained from Sigma-Aldrich. The cell membrane-permeable HIF-stabiliser di-tert-butyroyl-oxymethyl-2,4-pyridine-dicarboxylate (^{t}BU-2,4-PDC) was synthesised by the addition of butyric acid chloromethyl ester to a solution of 2.4 pyridine dicarboxylic acid in dimethyl formamide in a one step reaction (incubation at 60°C for 6 h followed by room temperature for 18 h; performed by Sohena GmbH, Tübingen).

The human cell lines U2OS (osteosarcoma), MCF-7 (breast adenocarcinoma) and HEK-293 (embryonic kidney) were grown in DMEM and Hep3B (hepatoma) in RPMI-1640 culture medium (Gibco) containing 10% fetal calf serum (FCS; Gibco) and 100 IU/ml penicillin/ 100 µg/ml streptomycin (PAA Laboratories) at 37°C in a 5% CO_2 incubator. To study hypoxic conditions, cells were placed in a humidified atmosphere containing 1%, O_2, 5% CO_2 and balanced N_2. When noted, specific plates with a gas-permeable fluorocarbon bottom were used (Imaging Plate 24 FC; Zell-Kontakt GmbH, Nörten-Hardenberg).

Transient Transfection

The cell lines Hep3B and MCF-7 were transiently transfected with siRNA using Lipofectamine RNAiMAX transfection reagent. Cells were grown 80% confluent in 24-well culture plates before transfection which was done following the instructions of the manufacturer´s protocol.

Stable Transfection

The cell lines HEK-293 and U2OS were stably transfected with short hairpin RNA (shRNA) using Lipofectamine 2000. Cells were grown 80 % confluent in 24-well culture plates before transfection following the instructions of the manufacturer´s protocol. Successfully transfected cells were selected using puromycin (2 µg/ml). Cells were replated in 96-well culture plates, one cell per well. Grown cell clones were tested for HIF-1α deficiency by immunoblot analysis. The most sufficient clones were designated HEK19A and U2OS91.

Immunoblot Analyses

Following transfection or pretreatment with the HIF-stabiliser ^{t}BU-2,4-PDC, cells were kept under normoxic or hypoxic (1% O_2) conditions for 4 h. Cells were rinsed with ice-cold PBS and lysed in 7 M urea lysis buffer. Protein concentrations were determined using Bio-Rad DC Protein Assay (Bio-Rad). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes by semi-dry electroblotting. After blocking (5% non-fat milk in phosphate buffered saline), blots were incubated with primary antibodies overnight and HRP-conjugated corresponding secondary antibodies (DAKO) were added in a 1:2000 dilution for 1 h, and finally detected by ECL (GE Healthcare).

Clonogenic survival Assay

For clonogenic survival assays cells were transfected or preincubated with ^{t}Bu-2,4-PDC (4 h, 75 µM). Cells transfected with siRNA were kept under normoxic or hypoxic conditions for another 4 h. Following irradiation with intensities of up to 6 Gy (Siemens Mevatron-74 linear accelerator), cells were harvested, counted and known numbers of living cells, estimated by trypan-blue staining, were reseeded in 6-well culture dishes. After being incubated for 12 d, colonies were fixed with 3.7% formaldehyde and 70% ethanol, stained with Coomassie G250 and subsequently counted. Clonogenic survival was calculated on a percentage of basis by dividing the number of colonies of the irradiated cells by the number of colonies of

the appropriate nonirradiated control cells for every treatment design.

Statistics

Statistical analysis was performed using GraphPad InStat Software. To compare two treatment groups (normoxia vs. hypoxia or wildtype cells vs. HIF-α deficient cells) the unpaired t test with Welch correction was used. Graphs are shown as means ± standard deviation (SD). All experiments of clonogenic survival assays were performed independently at least three times (n = 3) in duplicates (m = 6 measured values).

Results

HIF-α deficiency increases radioresponsiveness of tumor cells

To investigate the role of the HIF-α subunits in radiotherapy different cell lines were transiently transfected with siRNA targeting HIF-1α or -2α for 48 h followed by 4 h of hypoxic incubation and irradiation. Stable HIF-1α deficient cell clones of U2OS and HEK-293 cells were generated by means of shRNA transfection. Using this method, a DNA vector is introduced into the genome of the cells to ensure that shRNA against the targeted protein is continuously expressed. As shown in Fig. 1, HIF-1α was completely knocked down in all cell lines. HIF-2α levels were markedly decreased (Fig. 1).

Clonogenic survival assays of wildtype cells as compared to HIF-α deficient cells irradiated with 2, 4, or 6 Gy displayed a higher susceptibility of HIF-1α knockout cells to irradiation when compared to wild-type cells irrespective of oxygenation status before irradiation. Knockdown of HIF-2α radiosensitised cells in the majority of cases in normoxia and hypoxia as well, indicating significant importance of expression of either HIF-α subunit, HIF-1α and HIF-2α, in radioresistance. Data are shown as percentage of survival fractions of irradiated HIF-1α or HIF-2α deficient or wildtype cells grown under normoxic (Fig. 2 left) or hypoxic (Fig. 2 right) conditions versus unirradiated control cells (normalised data). Comparing normoxic versus hypoxic HIF-1α (Fig. 3 right) or HIF-2α (Fig. 3 left) knockdown cells, a weaker influence of remaining HIF-2α as compared to HIF-1α on radioresistance was demonstrated, as for example shown in Hep3B cells.

^{t}Bu-2,4-PDC stabilises HIF-1α and HIF-2α under normoxic conditions and induces radioresistance

Different cell lines were incubated with ^{t}Bu-2,4-PDC for 4 h. As shown in Fig. 4, HIF-1α and HIF-2α are stabilised by ^{t}Bu-2,4-PDC to the same degree as under hypoxic conditions.

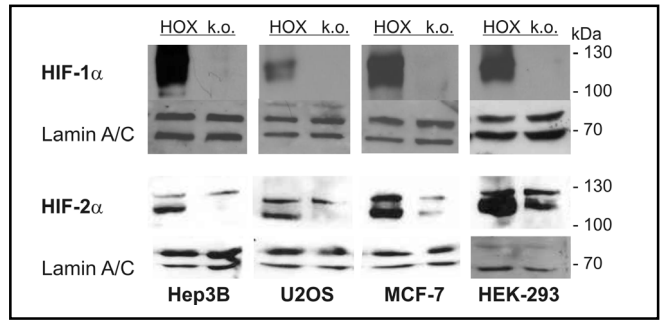


Fig. 1. Western Blots showing HIF-1α and HIF-2α levels in hypoxic (Hox) and transfected hypoxic (k.o.) cells. Cells were transiently transfected with siRNA for 48 h or stably transfected using shRNA (HEK-293, U2OS). Hypoxic and HIF-1α or -2α deficient cells were incubated under hypoxic conditions (1% O_2) for 4 h before immunoblotting.

Radioresistance of cells incubated with ^{t}Bu-2,4-PDC emerged to be at least as strong as the effect of hypoxia in 3 of 4 cell lines (Fig. 5). ^{t}Bu-2,4-PDC-treatment of HIF-1α (Fig. 6 left) or HIF-2α (Fig. 6 right) knockdown cells provides information regarding the influence of the remaining upregulated HIF-α subunit on radioresponsiveness. Results from the clonogenic survival assays of these cells suggest that HIF-2α in this case leads to a stronger influence on radioresponsiveness induced through HIF-stabilisation by ^{t}Bu-2,4PDC as compared to HIF-1α.

Discussion

The benefit of HIF-knockdown in radiotherapy

Solid tumors in many cases develop hypoxic regions resulting from a low rate of blood vessel formation compared to the rate of tumor cell proliferation. In addition, tumor oxygenation is limited by the formation of a chaotic dysfunctional vascular network [9, 10]. That sort of hypoxic tumor cells were prevalently found to be resistant to radiotherapy and oxygen-dependent chemotherapies [7, 8, 11, 12]. This effect is in part due to an increase in HIF-1 production. Moreover, HIF-1 is constitutively upregulated in several tumor types and may thus be implicated in therapy resistance of the tumor cells. But on the other hand, renal clear cell carcinoma cells, HT 1080 human fibrosarcoma cells and SQ5 human lung carcinoma cells show only modest or no effect of the oxy-

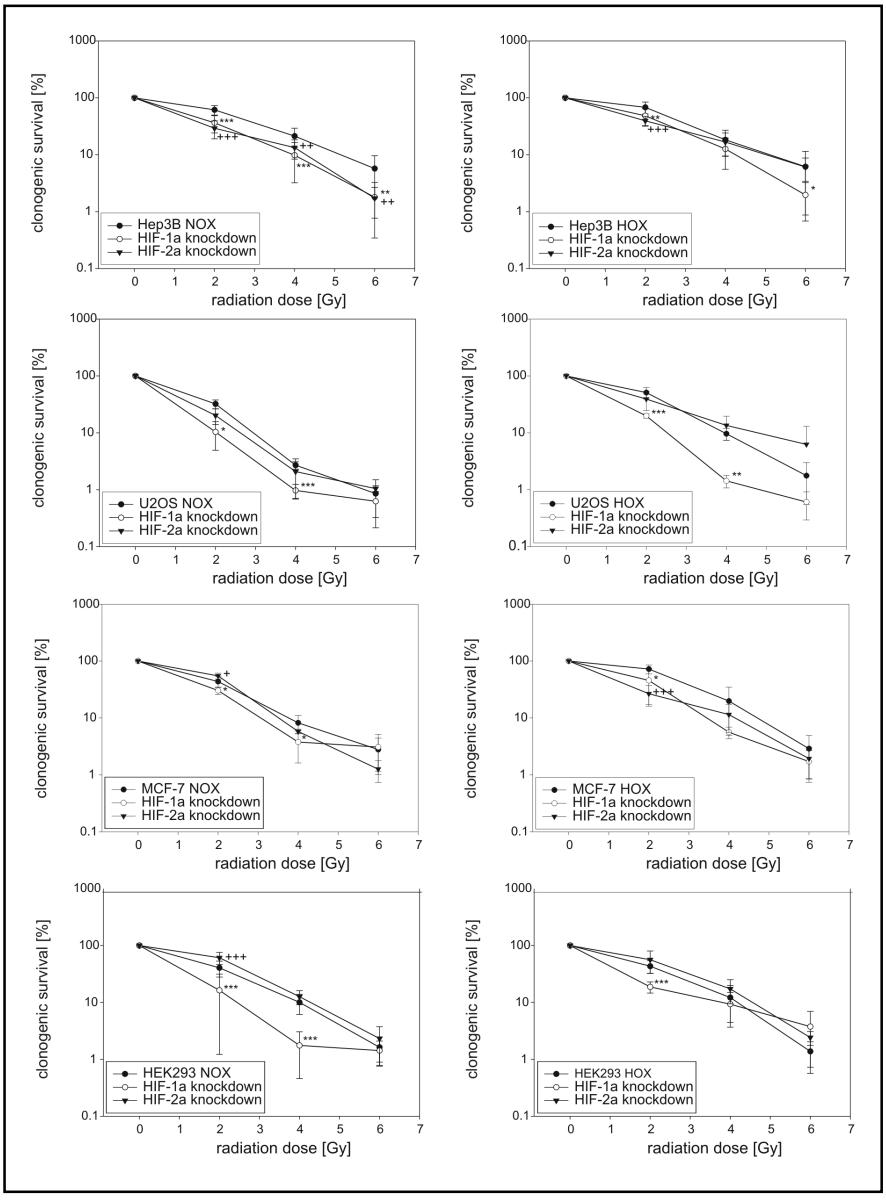


Fig. 2. Clonogenic survival assay of various wildtype and HIF-α knockdown cancer cells. Normoxic (left) or hypoxic (right) wildtype and HIF-1α or HIF-2α deficient cells were irradiated, replated, incubated for 12 d and stained. The percentage of clonogenic survival was calculated by dividing the number of irradiated colonies by the number of the appropriate nonirradiated control colonies. Mean ± SD; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$wildtype vs. HIF-1α knockdown; + $P < 0.05$; ++ $P < 0.01$; +++ $P < 0.001$ wildtype vs. HIF-2α knockdown (unpaired t test with Welch correction; n = 3, m = 6).

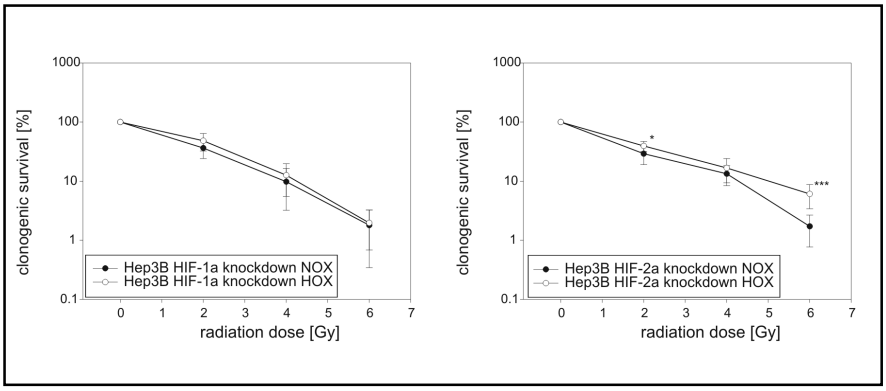


Fig. 3. Clonogenic survival assay of Hep3B HIF-α knockdown cells. Normoxic or hypoxic HIF-1α (left) or HIF-2α (right) deficient cells were irradiated, replated, incubated for 12 d and stained. The percentage of clonogenic survival was calculated by dividing the number of irradiated colonies by the number of the appropriate nonirradiated control colonies. Mean ± SD; * $P < 0.05$; *** $P < 0.001$ wildtype vs. HIF-1α knockdown; (unpaired t test with Welch correction; n = 3, m = 12).

genation status or the HIF-expression level on radiation sensitivity [13-15].

Oxidative stress, as occurring in radiation therapy, furthermore results in nuclear accumulation of HIF-1 in response to reactive oxygen and enhances translation of HIF-1-regulated transcripts secondary to stress granule depolymerisation [16]. The subsequent increase in HIF-1-regulated cytokines enhances cellular radioresistance.

Fig. 4. Western Blot showing HIF-1α and HIF-2α levels in normoxic (Nox), hypoxic (Hox) and ^{t}Bu-2,4-PDC -treated (PDC) cells. Cells were subjected to hypoxia (1% O_2) or preincubated with ^{t}Bu-2,4-PDC (75μM) for 4 h prior to immunoblotting. ^{t}Bu-2,4-PDC containing medium was renewed every 2 h

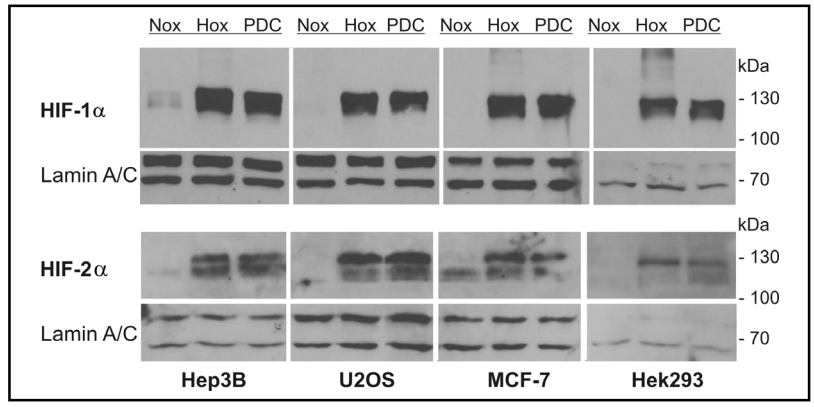


Fig. 5. Increased clonogenic survival of several ^{t}Bu-2,4-PDC pretreated versus hypoxic cells. Cells were grown under hypoxic conditions (4 h, 1% O_2) or pretreated with ^{t}Bu-2,4-PDC (75 μM, 4 h). Cells were irradiated, replated, incubated for 12 d and stained. The percentage of clonogenic survival was calculated by dividing the number of irradiated colonies by the number of the appropriate nonirradiated control colonies. Mean ± SD; * P < 0.05; ** P < 0.01; *** P < 0.001 (unpaired t test with Welch correction; n = 3, m = 6)

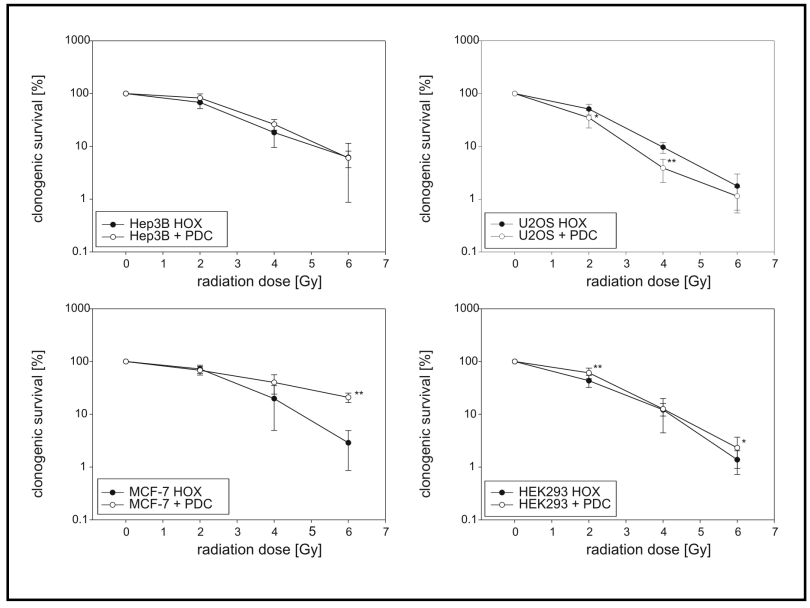
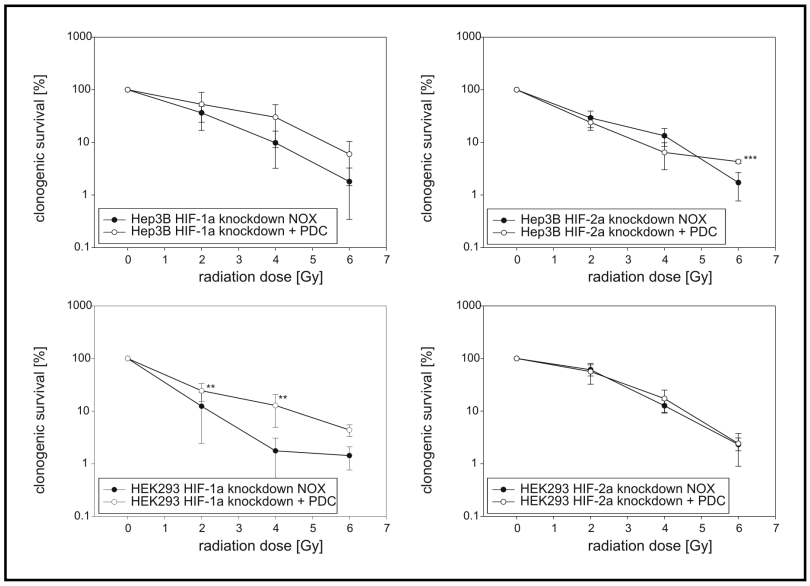


Fig. 6. Differences in clonogenic survival of normoxic HIF-α deficient cells versus ^{t}Bu-2,4-PDC pretreated counterparts. Normoxic and ^{t}Bu-2,4-PDC (75 μM, 4 h) treated HIF-1α or HIF-2α deficient cells were irradiated, replated, incubated for 12 d and stained. The percentage of clonogenic survival was calculated by dividing the number of irradiated colonies by the number of the appropriate nonirradiated control colonies. Mean ± SD; * P < 0.05; ** P < 0.01 (unpaired t test with Welch correction; n = 3, m = 6).



The role of HIF-2α in regard to these effects is not known. Therefore, HIF-inhibitors are potentially clinically relevant enhancers of cancer radiosensitivity through inhibiting pre- and postradiation HIF-1 activation, tumor HIF-1 signalling and reducing the innate radiation resistance of hypoxic tumor cells. Blocking HIF-1 activity reduces the

tumor's ability to stimulate glycolysis (ATP production) in low-oxygen conditions which is supposed to reduce tumor growth.

A specific approach to suppress the synthesis of HIF-α subunits is the use of RNA inhibition methodology. Our data derived from clonogenic survival assays of HIF-expressing hepatoma, osteosarcoma, breast adenocarcinoma and embryonal kidney cells versus their HIF-deficient counterparts are consistent with the observations of other groups on glioma, fibrosarcoma and pancreatic cancer cells [17-20]. The present results furthermore agree with the observed inverse correlation between HIF-1α expression and the prognosis for the outcome of radiotherapy [21, 22]. Determination of tumor cells of different origins underlined a general mechanism of HIF-1α on radioresistance in these cell lines. Although HIF-1α is the best known and widely described isoform we could demonstrate that not only HIF-1α but also HIF-2α induces radioresistance in several tumor cell lines of different origin. In line with these data, HIF-2α was found to be the primary HIF-subunit accounting for renal cell carcinoma aggressiveness, whereas HIF-1α promotes the growth of this tumor [23]. Thus, despite high similarities in protein sequence, our data confirm that differences in tissue distribution, physiology, and mechanism of regulation between HIF-1α and HIF-2α exist [23, 24]. The expression of genes encoding glycolytic enzymes is stimulated by HIF-1α [25]. In contrast, the erythropoietin gene is selectively activated by HIF-2α, but not HIF-1α, under hypoxic conditions [26]. Other selective HIF-2α genes could be found as ADRP (adipose differentiation-related protein), ADM (adrenomedullin), GRO-2 (chemokine growth-regulated oncogene 2) and Oct-4 (Octamer binding transcription factor 4), indicating the importance of this isoform in gene regulation [27, 28]. Furthermore, cell lines differ in induction kinetics of HIF-α stabilisation and in responsiveness to graded oxygen levels [29]. In conclusion, many data suggest that HIF-2α is at least as important as HIF-1α in hypoxic gene regulation. Our results demonstrate increased sensitivity of different cell lines following selective HIF-1α or HIF-2α knockdown which would imply an increased benefit in radiotherapy by blocking both HIF-α subunits. The knockdown of HIF-1α resulted in a more pronounced decrease of clonogenic survival as compared to HIF-2α (e.g. HIF-1α vs. HIF-2α 25.6% as compared to 18.3% at 2 Gy and 7.7% as compared to 0.2% at 4 Gy in hypoxia), suggesting a more crucial role of HIF-1α in radioresistance evoked by hypoxia in the four analysed cell lines. Due to the fact that effects of HIF-1α and HIF-2α on radioresponsiveness emerged not only in hypoxic but also in normoxic cells, basal HIF activity and postradiation HIF-1 activation seem to take part in the induction of radioresistance. Although HIF-1α and HIF-2α are regarded as promising therapeutic targets, the timing of the inhibition is required to be optimised to achieve the most beneficial outcome, especially when combined with other treatments like oxygen-dependent chemotherapy or hyperthermia [30]. Furthermore, radiosensitvity is cell type-specific depending on several factors as the degree of O_2-deprivation and expression of tumor suppressor p53 [14, 31].

Implications of HIF-stabilisation in anaemia

Not only HIF-inactivation but also HIF-stabilisation can be adjuvant in therapy of certain diseases. HIF-1α directly activates transcription of the *vegf* gene encoding vascular endothelial growth factor which promotes endothelial cell survival and angiogenesis [32]. An increased expression of VEGF can be beneficial for therapeutic angiogenesis for example in a situation of ischemia. Furthermore, hypoxia and the induction of HIF-2α are of major importance in the control of Erythropoietin (EPO) production [33]. Current research goals are to develop novel forms of therapy, e.g. for activating endogenous EPO production independent of oxygen availability. Substances similar to α-ketoglutarate can function as competitive PHD inhibitors resulting in HIF stabilisation and possibly increased EPO production [34]. It is a possibility that such HIF stabilisers can therefore be applied in the treatment of anemia. Here, we used the selfmade cell membrane-permeable α-ketoglutarate analogue ^{t}Bu-2,4-PDC to investigate its ability to stabilise HIF and to show its impact on radioresponsiveness of cultured tumor cells. The present study demonstrated that ^{t}Bu-2,4-PDC could efficiently stabilise HIF-1α as well as HIF-2α in normoxia. ^{t}Bu-2,4-PDC in addition increased the radioresistance of several human cancer cells significantly with HIF-2α, in contrast to its role in hypoxia, apparently being the main mediator of this effect. The HIF-mediated decrease of radioresponsiveness emerged to be at least as strong or even slightly stronger than the effect caused by hypoxia in three of four cell lines. Considering the fact that HIF-stabilisers could be beneficial in treatment of anemia but could also increase radioresistance in several tumor cells, the outcome of treatment with these substances in anemic patients undergoing radiation therapy is difficult to predict. Furthermore, since the PHDs belong to a large family of α-ketoglutarate dependent dioxygenases which is not

fully characterised as yet, it seems possible that use of these substances leads to unpredicted side effects. Careful evaluation prior to clinical use is certainly mandatory, in particular in patients undergoing tumor therapy.

Acknowledgements

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