Effect of Radiation and Ibuprofen on Normoxic Renal Carcinoma Cells Overexpressing Hypoxia-Inducible Factors by Loss of von Hippel–Lindau Tumor Suppressor Gene Function

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ABSTRACT

Purpose: Tumor hypoxia is a major limiting factor for radiation therapy. Hypoxia-inducible factors (HIFs) are overexpressed in several human cancers and are considered prognostic markers and potential targets for cancer therapy. The purpose of the present study was to investigate the impact of HIFs on radiosensitivity.

Experimental Design: Renal clear cell carcinoma (RCC) cell lines overexpressing HIFs under normoxic conditions because of inactivation of von Hippel–Lindau tumor suppressor gene function (VHL-ve) and their matched pairs in which overexpression of HIFs was abolished by expression of functional VHL (VHL+ve) were irradiated. Radiosensitivity was determined by clonogenic assay. HIF and VHL protein levels were evaluated by Western blot analysis. RCC cells were also treated with ibuprofen, a radiosensitizer and HIF inhibitor in prostate cancer cells. The effect of ibuprofen on radiosensitization and HIF and VHL proteins was compared in RCC matched-pair cell lines.

Results: The data showed only small differences in the radiosensitivity between the cells overexpressing HIFs and cells with basal HIF levels. The dose-modifying factors for C2, 786-0, and A498 RCC cells were 1.14, 1.14 and 1.15, respectively. Radiation did not alter HIF or VHL protein levels. Ibuprofen inhibited HIFs in VHL+ve cells expressing basal levels of HIFs. In VHL-ve cells overexpressing HIFs, the inhibition was very modest. Ibuprofen radiosensitized C2 RCC cells to the same extent irrespective of their HIF status.

Conclusions: Overexpression of HIFs in RCC cells harboring VHL mutations has only a modest effect on the radiosensitivity. Radiosensitization by ibuprofen appears to be independent of HIF status.

INTRODUCTION

The majority of human tumors overexpress the hypoxia-inducible transcription factors HIF-1 and -2 compared with the surrounding normal tissues (1–3). Immunohistological studies of clinical tumor samples show a positive correlation between HIFs and the angiogenic factor vascular endothelial growth factor (VEGF; Refs. 4–7), which is associated with an increase in microvessel density (8). In addition to angiogenesis, HIFs regulate the expression of genes involved in metabolic adaptation to hypoxia, genes encoding growth factors, growth factor receptors, cell cycle regulators, and metastasis (9). For these reasons HIFs are considered not only as prognostic markers but also as potential targets for cancer therapy (9–11). HIF proteins are highly unstable under normoxic conditions (12). In the presence of oxygen the prolyl residues in the degradation domain of the α-subunit of HIFs are hydroxylated, facilitating an interaction of the HIFs with the tumor suppressor von Hippel–Lindau protein (pVHL; Refs. 13, 14). pVHL acts as a recognition component of ubiquitin ligase complex and targets HIF-1α and -2α for ubiquitination and degradation (15). HIF proteins are stabilized and up-regulated by hypoxia because HIF/VHL interaction cannot take place at low oxygen levels (1, 13, 14, 16, 17). However, in tumor cells, genetic alterations, activation of autocrine growth factors, or inactivation of tumor suppressor genes results in constitutive activation of HIFs even under normoxic conditions (9, 18–20). For example, overexpression of functional HIF-1α or -2α is commonly observed in hereditary and sporadic renal carcinomas as a consequence of deletion, hypermethylation, or mutations in the VHL tumor suppressor gene (20–25). Reintroduction of functional wild-type VHL gene in these renal carcinoma cells abrogates the overexpression of HIFs and HIF-regulated gene products (20, 22, 26).

Although hypoxic tumors are relatively resistant to cancer therapy (27), the precise role of HIFs in chemoresistance or radioresistance is not clear at present. In our previous studies we observed that the nonspecific nonsteroidal anti-inflammatory drug (NSAID) ibuprofen (Ibu) enhanced radiosensitivity of prostate cancer cells in vitro as well as in vivo (28, 29). Recently we showed that ibuprofen inhibited HIFs under normoxic and hypoxic conditions in prostate cancer cells (30). However, the role of HIFs as targets for radiation was not addressed in those studies. The objective of the present study was to determine the role of HIFs in cellular response to radiation. For this purpose we compared the radiosensitivity of VHL-ve renal cell carcinoma (RCC) cells overexpressing HIF-1α or HIF-2α with the radiosensitivity of their VHL+ve counterparts that did not overexpress HIFs. These studies showed only modest differences in the radiosensitivity between the cells that overexpressed HIF
proteins and the cells that did not. Furthermore, ibuprofen sensitized the VHL-ve (C2) and VHL+ve (C2VHL) RCC cells to the same extent at a dose at which there was a significant difference in the HIF1 protein level in the two cell lines.

MATERIALS AND METHODS

Materials. The effect of overexpression of HIFs on the radiosensitivity was analyzed in several different VHL+ve and VHL-ve cell line pairs. C2 and C2VHL renal carcinoma cells were generously provided by Dr L. M. Neckers (National Cancer Institute, NIH, Rockville, MD) and were grown as monolayer cultures in DMEM-F12 medium (Life Technologies) supplemented with 10% fetal bovine and penicillin/streptomycin. C2 cells overexpressed HIF-1α and HIF-2α (20, 31). Stably transfected 786-0-PRC (empty vector; clone B2) and 786-0-WT (wild-type VHL; clone G37) renal carcinoma cells (32) were generously provided by Dr. W. M. Linehan (Urological Oncology Branch, NIH, Bethesda, MD) and were grown as monolayer cultures in DMEM high-glucose medium (33) supplemented with 10% fetal bovine serum, 1× MEM nonessential amino acids, and penicillinstreptomycin. C2 cells overexpressed HIF-1α and HIF-2α (20, 31). Stably transfected 786-0-PRC (empty vector; clone B2) and 786-0-WT (wild-type VHL; clone G37) renal carcinoma cells (32) were generously provided by Dr. W. M. Linehan (Urological Oncology Branch, NIH, Bethesda, MD) and were grown as monolayer cultures in DMEM high-glucose medium (33) supplemented with 10% fetal bovine serum, 1× MEM nonessential amino acids, and penicillin/streptomycin. 786-0-PRC cells overexpressed HIF-2α (20). VHL-ve (infected with empty vector) and VHL+ve (infected with HA-VHL) A498 renal carcinoma cells and CHO cells with loss of pVHL (10.8) and their counterpart normal cells (E48.4.51) were generously provided by Dr. W. Kaelin (Dana-Farber Cancer Institute, Boston, MA). A498 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 1.0 μg/ml puromycin. CHO cells were grown in F12 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

The antibodies used for immunoblotting were as follows: HIF-1α monoclonal (Transduction Labs), HIF-2α (Novus Biologicals), VHL (PharMingen), actin (Chemicon), and topoisomerase-1 (Santa Cruz Biotechnology). Ibuprofen (11892; Sigma Chemicals) was prepared fresh as a 100 mM stock in distilled water and filter-sterilized before being added to culture media.

Clonogenic Cell Survival Assay. To determine the effect of Ibuprofen on clonogenic survival, cells were treated with different concentrations of ibuprofen, trypsinized after 24 h, and plated in drug-free medium. To determine the effect of radiation on clonogenic survival, cells were irradiated and after 1 h trypsinized and plated. For the combination treatment, cells were treated with 1.5 mM ibuprofen for 24 h, irradiated, and plated after 1 h. Colonies were stained with crystal violet after 12 days, and colonies containing ≥50 cells were counted.

Statistical Analysis. Data on clonogenic survival of ibuprofen-treated cells were analyzed by two-tailed paired t test. The survival curves of VHL+ve and VHL-ve RCC matched pair cell lines were analyzed by a modified single-hit multitarget model (34).

Western Blot Analysis. After treatment with ibuprofen and radiation, cell extracts were prepared as described previously (30). Sixty μg of proteins were separated on a 6% gel for HIF-1α and -2α and a 14% gel for VHL analyses. Topoisomerase-1 and actin were used as loading controls. Membranes were processed by the enhanced chemiluminescence method (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by autoradiography and scanned with a Hewlett-Packard (Palo Alto, CA) ScanJet 5470c scanner. Signal intensities were quantitated by ImageQuant (version 5.2) software (Molecular Dynamics, Sunnyvale, CA). HIF and VHL values were normalized to their loading controls and expressed as fold change compared with the untreated control sample.

VEGF Levels and Irradiation. Cells were plated in 6-well plates, and media from cells were collected 24 h after plating and analyzed by ELISA as described previously (30). Cells were irradiated at a dose rate of 1.6 Gy/min in a PANTAK high-frequency X-ray generator (East Haven, CT), operated at 300 V and 10 mA with a 2-mm Al filtration.

RESULTS

C2 RCC cells overexpressed HIF-1α protein and a small amount of HIF-2α protein under normoxic conditions as a result of nonfunctional VHL (VHL-ve), whereas C2VHL cells stably transfected with wild-type VHL (VHL+ve) showed minimal HIF-1α and HIF-2α expression (Fig. 1A). The difference in the HIF levels was reflected in the amounts of the HIF-regulated protein VEGF secreted into the medium. VEGF concentrations in the media were 4.38 ± 0.26 and 2.11 ± 0.24 ng/10^6 cells (mean ± SD; n = 4) in C2 and C2VHL cells, respectively. 786-0-PRC cells overexpressed HIF-2α because of the presence of a nonfunctional VHL gene (VHL-ve), whereas 786-0-WT cells transfected to express wild-type VHL (VHL+ve) showed minimal endogenous HIF2 expression under normoxic conditions (Fig. 1B). The amount of VEGF secreted by 786-0-PRC
and 786-0-WT cells was 12.03 ± 2.86 and 4.26 ± 0.21 ng/10^6 cells, respectively (mean ± SD; n = 3).

**Effect of Ibuprofen on HIF-1α and HIF-2α Proteins.** Our previous study on prostate cancer cells demonstrated that at a concentration of 2 mM ibuprofen completely inhibited HIF1 protein expression under normoxic conditions, whereas hypoxia-up-regulated HIF1 was only partially inhibited (30). To determine the effect of ibuprofen on cells overexpressing HIF1, we treated C2 RCC cells with ibuprofen and analyzed HIF1 protein levels at 24 h. In C2 cells, which overexpressed HIF1, the inhibition of HIF1 was incomplete and was observed at only higher ibuprofen concentrations (≥2 mM; Fig. 1A). In contrast, in C2VHL cells, which expressed only basal levels of HIF1, HIF1 was completely inhibited at 1 mM ibuprofen (Fig. 1A). In addition to HIF1, C2 cells also expressed low levels of HIF2 under normoxic conditions, which was inhibited by ibuprofen in a dose-dependent manner (data not shown).

The effect of ibuprofen on HIF2 protein was analyzed in 786-0 renal carcinoma cells. 786-0-PRC cells overexpressed HIF2, whereas 786-0-WT cells showed basal levels of HIF2. Ibuprofen up to 2 mM had no effect on HIF2 overexpressed in PRC cells (Fig. 1B). In contrast, ibuprofen inhibited endogenous HIF2 in WT cells in a dose-dependent manner (Fig. 1B). Thus, ibuprofen inhibited the basal HIF proteins in VHL+ve cells but was not effective in VHL-ve cells that overexpressed HIFs.

**Effect of Radiation on HIF-1α and HIF-2α Protein Levels.** The effect of radiation on HIF1 protein was examined in the absence and presence of 2 mM ibuprofen. For these experiments cells were irradiated 3 h after the addition of ibuprofen, and cell extracts were prepared after 24 h. There was no significant change in HIF1 levels in C2 and C2VHL cells after exposure to 8 Gy (Fig. 2A). At 2 mM, ibuprofen showed only marginal inhibition of HIF1 in the irradiated and unirradiated C2 cells that overexpressed HIF1, whereas at this concentration, ibuprofen markedly inhibited HIF1 in irradiated and unirradiated C2VHL cells (Fig. 2A). The effect of radiation on HIF2 protein levels was examined in 786-0 RCC cells. HIF2 levels were not affected by radiation in VHL+ve or VHL-ve 786-0 cells (Fig. 2B). In combination treatment, 2 mM ibuprofen showed no inhibition of HIF2 in irradiated and unirradiated 786-0-PRC cells, which overexpressed HIF2 whereas at this concentration ibuprofen inhibited HIF2 in both unirradiated and irradiated 786-0-WT cells. Thus, HIF protein levels were not significantly affected by radiation, and ibuprofen inhibited HIFs to the same extent in unirradiated or irradiated cells.

**Effect of Ibuprofen and Radiation on VHL Protein.** Because the difference in HIF1 levels in C2 and C2VHL cells and HIF2 levels in 786-0-PRC and 786-0-WT cells is a result of expression of wild-type functional VHL protein, the effect of ibuprofen and radiation on VHL protein was examined in RCC cells (Fig. 3). VHL was not detected in C2 and 786-0-PRC cells. Ibuprofen treatment had no marked effect on VHL protein level.
in C2VHL cells (Fig. 3A), but in 786-0-WT cells VHL levels increased with ibuprofen concentration (Fig. 3B). The increase in VHL protein was $1.26 \pm 0.17$-fold (mean $\pm$ SE; $n = 5$) and $3.75 \pm 1.52$-fold (mean $\pm$ SE; $n = 5$) in C2VHL and 786-0-WT cells, respectively. The level of VHL protein was not significantly changed by radiation in C2VHL or 786-0-WT cells (Fig. 3C).

**Effect of Ibuprofen on Clonogenic Survival of C2 and C2VHL Cells.** Our previous studies showed that ibuprofen was cytotoxic and enhanced the radiosensitivity of PC3 and DU-145 cells. Because ibuprofen inhibited HIF1 protein in C2VHL cells but not in the C2 cells, we examined the clonogenic survival of VHL-ve and VHL+ve C2 RCC cells treated with different concentrations of ibuprofen (Fig. 4). The plating efficiencies (mean $\pm$ SE; $n = 5$) of C2 and C2VHL cells were $0.43 \pm 0.04$ and $0.33 \pm 0.01$, respectively. Ibuprofen reduced the clonogenic survival of both cell lines. C2VHL cells appeared to be more sensitive to ibuprofen at concentrations $>1.5$ mM; however, the difference was not statistically significant.

**Radiosensitivity of Renal Carcinoma Cells as a Function of HIF Expression under Normoxia.** To determine the significance of HIF1 in radiation response, we irradiated C2 and C2VHL cells and then plated 1 h after irradiation for clonogenic assay (Fig. 5). The plating efficiencies (mean $\pm$ SE; $n = 5$) of the untreated C2 and C2VHL cells were $0.44 \pm 0.02$ and $0.38 \pm 0.1$, respectively. HIF1-overexpressing C2 cells appeared to be slightly more radiosensitive than the C2VHL cells (dose modifying factor: 1.14), but the difference was not statistically significant. When cells were treated with 1.5 mM ibuprofen and exposed to radiation, ibuprofen enhanced the radiosensitivity of both cell lines. At a 0.1 survival fraction, ibuprofen enhanced the radiosensitivity of C2 and C2VHL cells by a factor of 1.5 and 1.42, respectively (Fig. 5). At this concentration of ibuprofen, HIF1 protein was completely inhibited in C2VHL cells, but substantial amounts of HIF1 protein were still present in the C2 cells (Fig. 1A).

To determine the role of HIF2 in radiation response, we compared the radiation survival curves of 786-0-PRC and 786-0-WT cells (Fig. 6). The plating efficiencies (mean $\pm$ SE; $n = 3$) of 786-0-PRC and 786-0-WT cells were $0.31 \pm 0.04$ and $0.26 \pm 0.04$, respectively. PRC cells were slightly more radioresistant than the WT cells (dose modifying factor, 1.14). Similarly, in A498 cells HIF2 overexpression had minimal impact on radiosensitivity (dose modifying factor, 1.15; data not shown). There was no difference in the radiosensitivity of VHL-ve and normal CHO cell lines (data not shown). Thus, overexpression of HIFs had only a small effect on radiosensitivity.
DISCUSSION

Tumor hypoxia is one of the major limiting factors of cancer therapy because hypoxic tumors are relatively resistant to radiation as well as to certain chemotherapeutic agents (27, 35). Pretreatment oxygenation measurements in diverse human carcinomas have indicated that tumor hypoxia adversely affects the outcome of radiotherapy and patient prognosis (36–39). The majority of human tumors overexpress HIF1 and HIF2 (2, 24, 40), and several studies have shown a correlation between an adverse response to radiotherapy and elevated levels of HIFs (4, 5, 41–43). However, the precise role of HIF1 and HIF2 in the observed resistance to radiation therapy is poorly understood. The data presented here show that radiation does not affect HIF protein levels. Although HIF-overexpressing RCC cells appeared to be more resistant to radiation, the difference was only modest. Ibuprofen sensitized HIF1-overexpressing cells to radiation to an extent similar to that of cells in which HIF1 overexpression was abolished by transfection of functional VHL.

In our previous study we showed that ibuprofen was cytotoxic to PC3 and DU-145 prostate cancer cells (28). In the present study ibuprofen reduced the plating efficiency of RCC cells. The C2 RCC cells, which lacked functional VHL and overexpressed HIF1, appeared to be slightly more resistant to ibuprofen than were C2VHL cells, but the difference was not statistically significant. This is in agreement with an earlier report comparing the cytotoxicity in VHL-ve and VHL+ve RCC cells, in which exposure to various cytotoxic treatments produced similar cytotoxicity in both cell types (44). The cytotoxic treatments included H2O2, arsenite, UVC irradiation, mimosine, hypoxia, heat shock, glucose deprivation, cycloheximide, phenylacetate, 12-O-tetradecanoylphorbol-13-acetate, tumor necrosis factor-α, and serum withdrawal. Cells lacking VHL function were, however, more affected by glucose deprivation, suggesting that VHL-deficient cells are unable to handle abnormally processed proteins (44). In another study, transformed mouse embryo fibroblasts deficient in HIF1 were more susceptible to the chemotherapeutic agents carboplatin and etoposide as well as to ionizing radiation than were wild-type cells in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (45). Several factors associated either directly or indirectly with tumor hypoxia contribute to an overall decrease in the efficacy of cytotoxic agents in vivo (35). Further studies are required to assess the precise role of HIFs in the response of tumor cells to various therapeutic treatments.

The treatment of 786-0-WT cells with ibuprofen resulted in an increase in VHL protein. The cellular VHL content is regulated by cell density, and the VHL content in dense cultures can be several fold higher than in sparse cultures (46). The increase in VHL protein in ibuprofen-treated 786-0-WT cells cannot be attributed to an increase in cell density because the cell density of ibuprofen-treated cells was not higher than the density of untreated control cells. In addition, the effect of ibuprofen on VHL appears to be cell type specific because no significant change in VHL was observed in ibuprofen-treated C2VHL cells although the treatment inhibited HIF1. In an earlier study, Jones et al. (47) reported that treatment of rat gastric microvascular endothelial cells with the NSAIDs NS398 and indomethacin resulted in an inhibition of HIF1 protein. Interestingly, hypoxia reduced VHL protein, and NSAIDs up-regulated VHL under hypoxic conditions. The authors speculated that NSAIDs up-regulate VHL and thereby enhance HIF degradation, even under hypoxic conditions (47). According to the presently postulated mechanism of HIF regulation, oxygen-dependent hydroxylation of proline residues in the α subunits of HIFs facilitates the binding of HIFs to VHL protein (13–15). VHL then targets HIFs for ubiquitination and proteasomal degradation under normoxic conditions. VHL cannot bind to HIF under hypoxic conditions because HIF is not prolyl-hydroxylated. The modulation of VHL protein by hypoxia and by drugs such as NSAIDs, accompanied by the changes in HIF levels, warrants further evaluation of the role of VHL in HIF regulation.

Hypoxic cells are 2–3-fold more resistant to radiation than are well-oxygenated cells. Radiobiological studies have demonstrated that the biological effect of radiation is greatly influenced by the presence or absence of molecular oxygen at the time of irradiation (48). Oxygen molecules react rapidly with the free radical damage produced by ionizing radiation in DNA, and the DNA damage is “fixed” or made permanent, which ultimately results in cell death (27). The oxygen enhancement ratio for X-rays is ~3 at high doses and possibly lower (2–2.5) at doses <2 Gy (49, 50). In addition to low oxygen tension, HIF is also implicated as an important factor for radioresistance due to hypoxia (4, 5, 41–43). In our previous studies we observed that ibuprofen inhibited constitutively expressed HIFs in PC3 and DU-145 cells at concentrations of 1–2 mm (30) and radiosensitized the cells at 1.5 mm (28), raising a possibility that HIFs may be involved in radiosensitization by ibuprofen. To evaluate the specific role of HIFs in radioresistance, in the present study we used matched VHL+ve and VHL-ve RCC paired cell lines. VHL-ve cells showed only basal levels of HIFs, whereas VHL-ve cells overexpressed HIFs under normoxic conditions. Matched pairs were irradiated under identical normoxic conditions. Despite the large difference in the HIF1 levels, there was only a modest difference in the radiosensitivity of the matched pairs, in contrast to the established 2–3-fold difference in the radioresensitivity of cells irradiated under normoxic versus hypoxic conditions (49). Moreover, ibuprofen had only a marginal effect on HIF1 protein level in C2 cells, whereas, in C2VHL cells, ibuprofen completely inhibited HIF1. However, ibuprofen radiosensitized both cell lines to the same extent, suggesting that the radiosensitivity of normoxic C2 RCC cells is not primarily influenced by HIF expression.

Although HIF proteins are up-regulated in hypoxic cells and hypoxic cells are radioresistant, reports on the effects of radiation on HIF proteins are scarce. The present study showed that HIF protein levels were unaffected by radiation in renal carcinoma cells and is in agreement with a recent report on U251 human glioma cells (51). In that study, DNA-damaging agents, such as ionizing radiation and doxorubicin, did not affect HIF1 protein accumulation.

Taken together, our study on normoxic HIF-overexpressing RCC cells with VHL mutations suggests that in the setting of VHL mutation, HIF may not be a primary target of radiation and radiosensitization in vitro.
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REFERENCES


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