Modulation of TRAIL-Induced Tumor Cell Apoptosis in a Hypoxic Environment

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ABSTRACT

Hypoxia induces Hif-1α and selects for loss of wild-type p53 function, both of which can promote tumor cell survival. We evaluated the ability of TRAIL to induce apoptosis of human tumor cell lines exposed to hypoxia. H460 lung cancer cells express low levels of Hif-1α, stabilize wild-type p53 during hypoxia, and undergo TRAIL-induced apoptosis. In U2OS osteosarcoma or PA1 ovarian teratocarcinoma cells, high levels of Hif-1α and low levels of stable p53 are detected during hypoxia, and cells undergo low levels of TRAIL-induced apoptosis as compared to H460 cells. H460 cells are sensitized to TRAIL-induced apoptosis, whereas U2OS are protected, and little apoptosis is observed in relatively TRAIL-resistant PA1 during hypoxia. Forced expression of Hif-1α is also surprisingly a potent inducer of apoptosis in wild-type p53 expressing H460 cells and further promotes TRAIL-induced apoptosis. TRAIL-sensitive wild-type p53-expressing HCT116 colon carcinoma cells modestly elevate Hif-1α levels and are equally or slightly more sensitive to TRAIL during hypoxia. In contrast, p53-null HCT116 have higher levels of Hif-1α during normoxia and are extremely sensitive to TRAIL, but are protected from TRAIL-induced apoptosis during hypoxia. We hypothesize that a hypoxic tumor microenvironment may alter sensitivity to TRAIL, which may be impacted by Hif-1α levels and p53 status. These findings suggest that particular attention to hypoxic regions of tumors and sensitizers to hypoxia-induced cell death may be required to optimize therapeutic combinations using TRAIL.

INTRODUCTION

As tumors grow they acquire regions of hypoxia, which provides a selection pressure to promote tumor progression and therapeutic resistance. In addition, hypoxia is a potent stimulus for angiogenesis and other forms of metabolic adaptation that provide tumors with a survival advantage.

A great deal of study has been focused on the regulation of Hif-1α expression and the identification of Hif-1α targets. Hif-1α expression is negatively regulated by the E3 ligase function of the VHL tumor suppressor gene. Hif-1α is hydroxylated at prolyl residues in normoxia and thereby targeted for proteolysis. During hypoxia, Hif-1α acts as the primary transcription factor functioning to activate multiple target genes. Hif-1α targets include cytokines and growth factors, genes that promote angiogenesis, cell cycle progression, glucose uptake and metabolism, and cell survival. As a result, Hif-1α has been implicated as an oncogene that is overexpressed in human cancer and recent efforts have been directed at blockade of Hif-1α as a therapeutic target alone or in combination with ionizing radiation. Hif-1α has also been implicated as a coregulator of transcription with p53 to promote apoptosis. This has been recently illustrated by the identification of Bnip3L as a p53 and Hif-1α regulated gene that promotes apoptosis of hypoxic tumor cells. Loss of Bnip3L conferred resistance to hypoxia and promoted tumor growth. Thus Hif-1α has been implicated in prosurvival as well as proapoptotic functions during hypoxia.

TRAIL is a promising tumor-specific cytotoxic agent that is currently being tested in Phase I clinical trials. A number of studies have investigated molecular determinants of response to TRAIL in normal and tumor cells. Factors that promote TRAIL-induced apoptosis include expression levels of its pro-apoptotic receptors DR4 and DR5, expression of caspase 8, expression of bax (in some cells), and the expression of the c-Myc oncogene. One early study found that a number of tumor cell lines expressing wild-type p53 were significantly more sensitive to TRAIL as compared to when p53 was targeted for degradation by HPV E6 protein. Negative regulators of TRAIL-induced apoptosis include expression of its decoy receptors TRID and TRUNDD as well as FLIP, Bcl-XL, and XIAP.

Few studies have investigated the effect of hypoxia on TRAIL-induced apoptosis. An early study found that hypoxia significantly protected A549 human lung carcinoma cells
from apoptosis induced by TRAIL. This study found that levels of Bcl2 and Bcl-X, as well as cIAP1, cIAP2 and XIAP were increased in A549 cells exposed to hypoxia. A later study by the same group demonstrated that hypoxia inhibited TRAIL-induced apoptosis of HCT116 human colon cancer cells, whereas Bax-null HCT116 were resistant to TRAIL regardless of whether the cells were under normoxia or hypoxia. On the other hand, a recent study using A549 found those cells to be relatively resistant to TRAIL under normoxia but more sensitive to TRAIL during hypoxic exposure. A number of other cell lines were tested including HCT15, H460 and Jurkat and all appeared to both stabilize Hif-1α and to become more sensitive to TRAIL during hypoxia while no increase in Bcl-2 expression was observed. Thus the effect of hypoxia on TRAIL-induced apoptosis remains unclear.

In the present studies we investigated the expression of Hif-1α and p53 in hypoxia-exposed cells that were also treated with TRAIL. We found that H460 cells were sensitized to TRAIL-induced apoptosis during hypoxia possibly related to their induction of p53. While H460 cells express relatively low levels of Hif-1α during prolonged exposure to hypoxia, unexpectedly we observed that Hif-1α overexpression markedly sensitized them to TRAIL-induced apoptosis. We also found that hypoxia had little effect on the sensitivity of HCT116 cells carrying both wild-type p53 and wild-type bax alleles. In contrast p53-null HCT116 cells that were sensitive to TRAIL during normoxia became significantly more resistant to TRAIL during hypoxia. U2OS cells were also significantly protected from TRAIL-induced apoptosis during hypoxia, which was associated with highly induced levels of Hif-1α. These results suggest that some human tumor cells may be resistant to TRAIL-induced apoptosis during hypoxia and the possibility that p53 status and Hif-1α expression may impact on TRAIL sensitivity. The results have implications for studies of TRAIL efficacy as a hypoxic tumor microenvironment may adversely affect response to TRAIL therapy. The observed inhibitory effects of hypoxia on TRAIL-induced apoptosis suggest that additional strategies may need to be developed and combined with TRAIL to improve tumor cell killing, especially in the hypoxic tumor microenvironment.

**MATERIALS AND METHODS**

**Cell Lines.** H460 lung carcinoma, U2OS osteosarcoma and PA1 ovarian teratocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC). HCT116 p53+/− and HCT116 p53−/− colon carcinoma cells were previously described.

**Hypoxia.** All experiments in which cells were exposed to hypoxic conditions; the INVIVO2 hypoxia workstation (TOUCAN Technologies) was used. The chamber was set at 0.2% O2, 5.0% CO2 and 37°C for all experiments. Cells for western blotting analysis were treated with Laemmli sample buffer (Tris-HCl (pH 6.8), 2-mercaptoethanol, 10% SDS, Glycerol). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Labs) and adjusted accordingly, prior to SDS-PAGE. Proteins were transferred to a PVDF membrane (Immobilon-P Millipore Corporation) by a semi-dry transfer apparatus from Bio-Rad Laboratories. The membrane was blocked in 5% non-fat dry milk diluted in TBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl) and 0.1% Tween-20. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. The p53 antibody (DO-1; Santa Cruz) was used at 1:1000 dilution, Hif-1α antibody (BD Biosciences) was used at 1:250 dilution, and the HA-tag antibody (Cell Signaling) was used at 1:250 dilution, and the HA-tag antibody (Cell Signaling) was used at 1:1000 dilution. The RAN antibody (BD Biosciences) at a dilution of 1:2500 was used to provide an immunoblot for a protein loading control. The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG (Amersham) used at a 1:10,000 dilution.

**Flow cytometry.** Cells were treated with Trypsin (Invitrogen) and collected along with detached cells in the media for flow cytometric analysis. The collected cells were ethanol-fixed and stained with propidium iodide (Sigma) as described. The DNA content of the stained cells was then measured using an Epics Elite flow cytometer (Beckman-Coulter).

**Hif-1α transfection.** The HA-tagged Hif-1α pCMV expression vector was provided by Dr. Mikhail Blagosklonny (Ordway Institute, Albany, NY). Transfection was performed using the manufacturers recommended protocol for Lipfectamine 2000 (Invitrogen). H460 cells were seeded at approximately 90% confluency prior to transfection. To each well 4µL of HA-Hif-1α or pCMV-βgal diluted in Opti-MEM® was added to serum and antibiotic-free media. At six hours after transfection media was removed and complete RPMI media was added. Cells were incubated for 24 hours prior to harvesting for flow cytometry or Western blotting.

**RESULTS**

Variable expression of Hif-1α and p53 in tumor cell lines exposed to the hypoxia-mimetic agent desferoxamine. Western blot analysis probing for Hif-1α and p53 in H460, U2OS and PA1 human tumor cells treated with the hypoxia-mimetic agent desferoxamine or adriamycin. All three cell lines show p53 stabilization following treatment with adriamycin and to a lesser extent with desferoxamine. Hif-1α expression however, is markedly lower in H460 cells after desferoxamine treatment than in U2OS or PA1 cells.

**Western blot analysis.** Cells were lysed directly in culture plates by scraping in Laemmli sample buffer (Tris-HCl (pH 6.8), 2-mercaptoethanol, 10% SDS, Glycerol). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Labs) and adjusted accordingly, prior to SDS-PAGE. Proteins were transferred to a PVDF membrane (Immobilon-P Millipore Corporation) by a semi-dry transfer apparatus from Bio-Rad Laboratories. The membrane was blocked in 5% non-fat dry milk diluted in TBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl) and 0.1% Tween-20. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. The p53 antibody (DO-1; Santa Cruz) was used at 1:1000 dilution, Hif-1α antibody (BD Biosciences) was used at 1:250 dilution, and the HA-tag antibody (Cell Signaling) was used at 1:1000 dilution. The RAN antibody (BD Biosciences) at a dilution of 1:2500 was used to provide an immunoblot for a protein loading control. The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG (Amersham) used at a 1:10,000 dilution.

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there may be variability in Hif-1α expression in human tumor cell lines exposed to hypoxia-mimetic agents.

Hypoxia sensitizes H460 human lung cancer cells to TRAIL-induced apoptosis. Because H460 cells express wild-type p53 and low levels of Hif-1α upon exposure to the hypoxia-mimetic agent desferrioxamine (Fig. 1), we hypothesized that hypoxia may sensitize H460 cells to TRAIL-induced apoptosis. To perform these studies we treated H460 cells in the presence or absence of exposure to severe hypoxia (0.2% O2) for four hours followed by

Figure 2. Hypoxia sensitizes H460 and SW480 cells to TRAIL-induced apoptosis. Western blot showing moderate levels of Hif-1α and increased stabilization of p53 in H460 cells over prolonged hypoxic exposure (A, left panels). Flow cytometry showing increased sub-G1 percentage of total DNA following TRAIL treatment of H460 cells under hypoxic conditions compared with normoxic conditions (A, right panels). A second % sub-G1 DNA content experiment showing sensitization of H460 cells to TRAIL-induced apoptosis during hypoxia after a six instead of four hour pre-incubation with 0.2% O2 (B). Increased sensitivity of SW480 cells to TRAIL-induced apoptosis under hypoxia shown by propidium iodide % sub-G1 DNA analysis (C, top panel) or active caspase 3 (C, bottom panel).
the addition of increasing doses of TRAIL for an additional six hours (Fig. 2). As expected, Hif-1α protein expression was elevated in cells exposed to hypoxia (Fig. 2A, left panels), and this was accompanied by increased p53 expression in the hypoxia-exposed cells. Flow cytometric analysis of the hypoxia-exposed TRAIL-treated cells revealed increased sub-G1 DNA content consistent with increased apoptosis in hypoxia versus normoxia. This was particularly evident at 50 ng/ml TRAIL where 44% of the hypoxic H460 cells had a sub-G1 content as compared to 30% of the normoxic H460 cells (Fig. 2A, right panels). In a second experiment where H460 cells were exposed to severe hypoxia for a total of 12 hours (six hours prior to TRAIL and an additional six hours of exposure to TRAIL), we observed a clear sensitization of the H460 cells during hypoxia to killing by TRAIL (Fig. 2B). These effects were observed at all doses of TRAIL used. Thus, H460 lung cancer cells that harbor a wild-type p53 gene and express modest

Figure 3. Hypoxia protects U2OS cells from TRAIL-induced apoptosis. Western blot showing high levels of Hif-1α expression and little p53 expression over basal levels during prolonged hypoxic incubation after TRAIL treatment (A, left panels). Flow cytometric analysis showing decreased %sub-G1 DNA content following TRAIL treatment under hypoxic conditions as compared with normoxic conditions in U2OS cells (A, right panels). Western blot analysis showing substantial Hif-1α expression and no apparent change in p53 expression over the basal level in PA1 cells (B, left panels). Flow cytometric analysis showing insignificant sensitization to TRAIL-induced apoptosis by hypoxia in PA1 cells (B, right panels).
levels of Hif-1α during hypoxia are significantly more sensitive to the apoptotic effects of TRAIL.

We made similar observations in SW480 human colon cancer cells exposed to hypoxia and cytotoxic doses of TRAIL. SW480 cells are normally sensitive to TRAIL and express mutated p53 genes and low levels of Hif-1α upon exposure to severe hypoxia. We found that SW480 cells were significantly more sensitive to TRAIL-induced apoptosis during hypoxia and these effects were noted using both the sub-G1 content assay as well as the flow cytometric active caspase 3 assay (Fig. 2C).
Figure 5. Exogenous Hif-1α induces apoptosis in H460 cells in normoxia and potentiates TRAIL-induced apoptosis during hypoxia. Western blots showing relative Hif-1α expression levels in HA-Hif-1α transfected cells as compared to pCMVβgal transfected cells (Hif-1α antibody [A, top panel], HA-tag antibody [A, bottom panel]). Graph showing significant percent sub-G1 DNA content increase in Hif-1α transfected cells as compared to controls (B).

U2OS osteosarcoma cells are protected from TRAIL-induced apoptosis during hypoxia. Because U2OS cells were found to express high levels of Hif-1α in response to the hypoxia-mimetic agent desferrioxamine (Fig. 1), we wondered whether they might be more resistant to TRAIL under hypoxia even though they also express wild-type p53, as do the H460 cells. We therefore investigated the effect of hypoxia on TRAIL sensitivity in the U2OS cells by exposing them for four hours of severe hypoxia (0.2% O2) followed by incubation with increasing doses of TRAIL for an additional six hours (Fig. 3A). Similar to H460 cells, exposure of U2OS to hypoxia led to increased expression of Hif-1α over the duration of the exposure but less obvious stabilization of p53 over basal levels (Fig. 3A, left panels). The results reveal that unlike the H460 cells that were sensitized to TRAIL-induced apoptosis by hypoxia (Fig. 2A and B), U2OS were significantly protected from TRAIL-induced apoptosis during severe hypoxia. Whereas 24.9% of the normoxic U2OS cells exposed to 50 ng/ml TRAIL had a sub-G1 DNA content indicative of apoptosis, only 9.6% of the hypoxic U2OS cells had a sub-G1 fraction (Fig. 3A, left panel). Thus even though U2OS cells express wild-type p53 and Hif-1α, their sensitivity to TRAIL is reduced substantially by exposure to severe hypoxia.

In PA1 teratocarcinoma cells we observed substantial Hif-1α stabilization for the duration of the exposure to severe hypoxia with no apparent change in p53 expression after four hours of hypoxia followed by incubation with TRAIL for an additional six hours (Fig. 3B, left panels). Similar to U2OS cells, PA1 were not significantly sensitized to TRAIL-induced apoptosis by hypoxia under these experimental conditions (Fig. 3B, right panels).

One possible difference between H460 and either U2OS or PA1 under the experimental conditions used was that p53 stabilization was evident only in the H460 after four hours of severe hypoxia. It is therefore possible that sensitization of H460 was in part related to p53 activity. It is also clear that under the experimental conditions described here, some human tumor cell lines are not sensitized and some are protected from the cytotoxic effects of TRAIL under hypoxia.

Loss of p53 in HCT116 human colon cancer cells leads to significant protection from TRAIL-induced apoptosis following exposure to severe hypoxia. Our earlier results implicated p53 stabilization in determining whether severe hypoxia sensitized or protected cells from TRAIL-induced apoptosis. To investigate this possibility more directly, we exposed wild-type p53-expressing and p53-null HCT116 human colon cancer cells to severe hypoxia followed by TRAIL treatment (Fig. 4). As expected, Western blot analysis revealed that the expression of both Hif-1α and p53 was increased in wild-type p53-expressing HCT116 cells exposed to severe hypoxia for four hours followed by incubation with increasing doses of TRAIL for six additional hours under hypoxia (Fig. 4A, left panels). Even though there was p53 stabilization in the HCT116 cells exposed to severe hypoxia, there was a modest additional sensitization to TRAIL-induced apoptosis under hypoxia only at 5 ng/ml TRAIL and no additional sensitization, when compared with normoxia, at 50 ng/ml TRAIL plus severe hypoxia for the total of ten hours (Fig. 4A, right panels).

In contrast to the wild-type p53 expressing HCT116 cells we observed a much higher basal level of Hif-1α in the p53-null HCT116 cells accompanied by additional Hif-1α stabilization during hypoxia (Fig. 4B, left panels). This is consistent with previous observations implicating Hif-1α regulation by p53-dependent MDM2 association.10 Despite the high basal levels of Hif-1α in p53-null HCT116 cells, the cells were found to be extremely sensitive to TRAIL-induced apoptosis under normoxia (Fig. 4B, right panel). However, when p53-null HCT116 cells were exposed to severe hypoxia they were significantly protected from TRAIL-induced apoptosis (Fig. 4B, right panel). Thus loss of p53 in HCT116 cells was associated with protection from TRAIL-induced apoptosis during hypoxia, even though these cells were highly sensitive to TRAIL during normoxia.

Exogenous Hif-1α induces apoptosis in H460 lung cancer cells. Because H460 cells appeared to express relatively low levels of Hif-1α in response to hypoxia mimetic agent desferrioxamine (Fig. 1), we predicted that Hif-1α would be associated with sensitization to TRAIL-induced apoptosis under severe hypoxia (Fig. 2A), we predicted that Hif-1α may protect H460 cells from TRAIL-induced cell death. We therefore overexpressed Hif-1α in H460 cells and determined its effects on TRAIL-induced apoptosis during normoxia or hypoxia (Fig. 5). We verified with Western blot analysis that the exogenous HA-tagged Hif-1α was expressed in the transiently transfected H460 cells (Fig. 5, left panels). Immunoblotting for either Hif-1α or HA-tagged-Hif-1α is shown for cells transfected by HA-Hif-1α (Fig. 5, left panels as indicated). It is clear that there was a higher level of Hif-1α expression due to transfection of HA-tagged Hif-1α under both normoxia or hypoxia (Fig. 5, left panels all blots were immunoblotted and exposed for the same duration of time). It is likely that cell death (see below) was a contributing factor to the lower Hif-1α expression detected in cells exposed to hypoxia and treated with 50 ng/ml TRAIL.

Surprisingly, transfection of Hif-1α into H460 cells was found to induce apoptosis under both normoxia and hypoxia, with or without TRAIL treatment (Fig. 5, right panel). In these experiments we confirmed the sensitization effect of hypoxia on TRAIL-induced apoptosis of H460 cells in either mock or HA-tagged Hif-1α-transfected cells (Fig. 5, right panel). It is possible that the unexpected effect of Hif-1α to induce apoptosis in H460 cells during normoxia may be related to the wild-type p53 expression in these cells. The results demonstrate that Hif-1α overexpression or se does not necessarily confer a survival advantage to human tumor cells either in normoxia or hypoxia.
DISCUSSION

The uncontrolled proliferation of cancer cells causes the formation of hypoxic regions within solid tumors due to lack of adequate blood supply. Hypoxia exists to varying degrees in most solid tumors, and clinically, tumor hypoxia has been associated with poor therapeutic response and patient prognosis.20 Our data suggests that a hypoxic tumor microenvironment will likely affect responsiveness to TRAIL, in some cases reducing its cytotoxic effects.

Altered gene expression in cells of hypoxic tumor regions likely provides protection to these cells from TRAIL-induced apoptosis. Hif-1α and p53 are two important transcription factors stabilized overexpression may be expression throughout as a tumor oncogene may supersede α1074 Cancer Biology & Therapy 2005; Vol. 4 Issue 10 α overexpression in -1 for cancer therapy. Nat Rev Cancer 2003; 3:721-32. α

This data provide evidence that the expression status of these two transcription factors is a potential determinant in cancer cell sensitivity to TRAIL-induced apoptosis during severe hypoxia.

In light of the conflicting previous reports on gene expression patterns of human cancer cells under hypoxia, and the implication of these proteins in determining responsiveness to TRAIL, we further investigated this effect. Here we have shown that H460 human lung carcinoma cells are significantly more sensitive to TRAIL-induced apoptosis under hypoxia, and that wild-type p53 status and increased stabilization under severe hypoxia in these cells may contribute to this effect. The sensitization to TRAIL under hypoxia is similar to those results reported previously using H460 cells.18 However, in stark contrast to the sensitizing effects of hypoxia in H460 cells, an equivalent hypoxic exposure in U2OS human osteosarcoma cells caused an apparent protective effect from TRAIL-induced apoptosis. We propose that the protective effect in this cell line is due to insignificant p53 accumulation under the hypoxic conditions tested, as well as strong Hif-1α expression throughout the duration of the TRAIL treatment and hypoxic exposure. To support this result we compared HCT116 p53+/- and HCT116 p53-/- to further test the role of p53 in fostering this effect. We found that p53-null HCT116 cells were significantly protected from TRAIL-induced apoptosis under hypoxia, as compared to the insignificant protective effect observed in wild-type p53 expressing HCT116 cells. This is in contrast with previous a previous report using HCT1116 cells.17

It is likely that the relative protein expression determines the cytotoxic response in these cells, and that the length of hypoxic exposure prior to TRAIL treatment and following treatment are determinants to this response. Accordingly, if U2OS cells were treated much longer with hypoxia we might observe p53 stabilization, which may impact the cytotoxic response. This suggests additional experiments to determine effects of relative duration of hypoxia on expression of p53 and Hif-1α proteins and timing of TRAIL treatment with regard to induction of tumor cell death.

Of note was the result that exogenous Hif-1α overexpression in H460 cells caused significant induction of apoptosis independent of the effects of hypoxia or TRAIL. This observation points to the results of previous reports which show that Hif-1α overexpression may be a favorable prognostic indicator in certain cancers.22,23 It is also consistent with a role for Hif-1α as an apoptotic mediator in the context of wild-type p53 expression in some cancers or cell types.5 Additionally, a role for Hif-1α as a tumor oncogene may supersede its role as an apoptotic mediator following loss of wild-type p53 function in cancer cells. Further studies will provide a better understanding of the paradoxical roles of Hif-1α in the context of tumor stage and progression.

The effect of a hypoxic tumor microenvironment on therapeutic response to radiotherapy and chemotherapy has long been established.24 Our data indicates that hypoxia may similarly alter the therapeutic effects of the tumor-specific cytotoxic agent TRAIL. As clinical trials continue with TRAIL, the possibility of altered responsiveness in hypoxic regions of tumors and the appropriate need for adjuvant therapies should be considered. In this regard it may be fruitful to attempt to combine hypoxic cell sensitizers such as Tirapazamine,25 already in clinical trials,26 with TRAIL.

References