The Redox Protein Thioredoxin-1 (Trx-1) Increases Hypoxia-inducible Factor 1α Protein Expression: Trx-1 Overexpression Results in Increased Vascular Endothelial Growth Factor Production and Enhanced Tumor Angiogenesis

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ABSTRACT

Hypoxia-inducible factor 1 (HIF-1), a heterodimer of HIF-1α and HIF-1β subunits, is a transcriptional activator central to the cellular response to low oxygen that includes metabolic adaptation, angiogenesis, metastasis, and inhibited apoptosis. Thioredoxin-1 (Trx-1) is a small redox protein overexpressed in a number of human primary tumors. We have examined the effects of Trx-1 on HIF activity and the activation of downstream genes. Stable transfection of human breast carcinoma MCF-7 cells with human Trx-1 caused a significant increase in HIF-1α protein levels under both normoxic (20% oxygen) and hypoxic (1% oxygen) conditions. Trx-1 increased hypoxia-induced HIF-1α transactivation activity measured using a luciferase reporter under the control of the hypoxia response element. Changes in HIF-1α mRNA levels did not account for the changes observed at the protein level, and HIF-1β protein levels did not change. Trx-1 transfection also caused a significant increase in the protein products of hypoxia-responsive genes, including vascular endothelial growth factor (VEGF) and nitric oxide synthase 2 in a number of different cell lines (MCF-7 human breast and HT29 human colon carcinoma and WEHI7.2 mouse lymphoma cells) under both normoxic and hypoxic conditions. The pattern of expression of the different isoforms of VEGF was not changed by Trx-1. Transfection of a redox-inactive Trx-1 (C32S/C35S) markedly decreased levels of HIF-1α protein, HIF-1α transactivating activity, and VEGF protein in MCF-7 cells compared with empty vector controls. In vivo studies using WEHI7.2 cells transfected with Trx-1 showed significantly increased tumor VEGF and angiogenesis. The results suggest that Trx-1 increases HIF-1α protein levels in cancer cells and increases VEGF production and tumor angiogenesis.

INTRODUCTION

Disruption of oxygen homeostasis is a major aspect of disease pathophysiology, particularly in heart disease, stroke, cerebrovascular disease, tumor angiogenesis, and metastases (reviewed in Ref. 1). The transcription factor HIF-1α is one of the key regulators of oxygen homeostasis (2). HIF-1α is a heterodimer of an α and β subunit, both of which belong to the basic-helix-loop-helix PER-ARNT-SIM family of transcription factors (3). HIF-1β, also known as ARNT-1, is a constitutive nuclear protein involved in a range of transcription systems, whereas the major pathway regulating gene induction in response to hypoxia is under the control of the HIF-1α and HIF-2α subunits (reviewed in Refs. 4, 5). HIF-1α expression is increased in a number of human cancers (6–13).

In cells replete with oxygen, HIF-1α and HIF-2α subunits are rapidly degraded by the ubiquitin-proteasome pathway (3, 14). Destruction is mediated by a ubiquitin E3 ligase complex in which the pVHL, in association with elongin B and elongin C, binds oxygen-dependent destruction domain(s) in the HIF-1α subunits (15). The interaction between human pVHL and HIF-1α is regulated through hydroxylation of HIF-α oxygen-dependent destruction domain proline residues (P402 and P564) by one or more members of the prolyl-4-hydroxylase family (reviewed in Ref. 16). Because such enzymes require molecular oxygen as a cosubstrate, it has been suggested that these enzymes provide a link between HIF regulation and the availability of molecular oxygen. However, pVHL is unlikely to be the only ubiquitin protein ligase that regulates the half-life of HIF-1α. The MDM2 ubiquitin protein ligase is recruited to HIF-1α by the binding of the tumor suppressor p53, which may also result in a decrease in HIF-1α levels (17, 18). In addition, activation of the phosphatidylinositol 3′-kinase pathway has been shown to increase HIF-1α protein levels under nonhypoxic conditions (19, 20).

During hypoxia, HIF degradation is suppressed and HIF-1α and HIF-2α protein levels increase and dimerize with HIF-1β subunits whose expression is not changed by hypoxia (21). The HIF complex interacts with coactivators such as p300/CBP (mediated by hydroxylation of asparagine 851 of HIF-1α; Ref. 22), SRC-1, and TIF2 (23–27) and binds to specific enhancer elements called HREs to activate transcription of a range of genes (28). The number of known HIF-1 target genes continues to increase and includes genes such as the VEGFs, iNOS, endothelin-1, and others that attract new vasculature and increase oxygenation (29). This may be the reason that hypoxic tumors are the most proangiogenic (30) and exhibit an aggressive tumor phenotype (31, 32).

Trx-1 is a small (104 amino acids) redox protein that undergoes reversible NADPH-dependent reduction by selenocysteine containing flavoprotein Trx-1 reductases (for reviews see Refs. 33, 34). Trx-1, through its redox activity, regulates the activity of enzymes such as apoptosis signal-regulating kinase 1 (35) and protein kinases C α, δ, ε, and ζ (36) and increases the DNA binding and transactivating activity of transcription factors, including nuclear factor κB (37), the glucocorticoid receptor (38), and p53 (39). Trx-1 is a potent cell growth and survival factor. Mouse WEHI7.2 lymphoma cells transfected with human Trx-1 form tumors in immunodeficient scid mice that grow more rapidly and show less spontaneous and drug-induced apoptosis than vector-alone-transfected cells. A redox inactive mutant Trx-1 acts as a dominant negative to inhibit MCF-7 human breast cancer xenograft tumor growth in scid mice (40, 41). Trx-1 expression is increased in several human primary cancers, including lung, colon, cervix, liver, pancreatic, colorectal, and squamous cell cancer (42–46), and has been linked to aggressive tumor growth and inhibited apoptosis (45, 47). More recently, increased Trx-1 levels have been correlated with decreased patient survival in non-small cell lung cancer (48).

To further understand the role of Trx-1 in promoting tumor growth, we have studied the effects of increased Trx-1 expression and of a
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reduced inactive mutant Trx-1 on HIF-1α levels and the activation of HIF downstream genes. We have shown that increased Trx-1 expression is associated with increased HIF-1α levels and HIF transactivation in cancer cells together with an increase in VEGF production and enhanced tumor angiogenesis.

MATERIALS AND METHODS

Cell Culture and Hypoxia Treatment. MCF-7 human breast cancer, HT-29 colon cancer, and WEHI7.2 mouse lymphoma cells were obtained from the American Tissue Type Collection (Manassas, VA). The stably transfected cell lines were used as MCF-7 human breast cancer cells transfected with Trx-1 (MCF-7/Trx; clone 9), with the redox inactive Cys32→Ser/Trx35→Ser mutant Trx-1 (MCF-7/C325/C355; clone 4) or with empty vector (MCF-7neo; Ref. 40). The corresponding HT-29 human colon cancer cells were HT-29/Trx (clone 7), HT-29/C325/C355 (clone 7), and HT-29/Neo cells and WEHI7.2 mouse lymphoma cells WEHI7.2/Trx (clone 5), WEHI7.2/C325/C355 (clone 5), and WEHI7.2/Neo cells (47). Cells were grown under humidified 95% air, 5% CO2 in an incubator at 37°C in DMEM supplemented with 10% fetal bovine serum and 200 μg/ml G418 where appropriate. For exposure to hypoxia, the culture flasks were incubated at 37°C for various times with a humidified gas mixture containing 5% CO2 /95% N2 and air. A Pro-Ox 110 oxygen sensor (Reming Bioinstruments Co., Redfield, NY) was used to regulate the oxygen level in the gas phase to 1% oxygen. After treatment, cells were washed twice with PBS (pH 7.5) at 4°C and stored as cell pellets at −80°C for further analysis. One ml of media from each flask was removed after treatment and stored at −80°C for measurement of VEGF levels. Recombinant human Trx-1 (40) was stored at −80°C before the protein content was determined using the Bio-Rad assay (Bio-Rad, Hercules, CA). The amount of human VEGF in cell lysates and VEGF secreted into the medium was determined using an ELISA kit that measures VEGF165 and VEGF121 isoforms (Human VEGF-ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Mouse VEGFs in the cell lysate and medium of mouse WEHI7.2 cells were determined using a mouse VEGF-ELISA kit (R&D Systems) that also measures VEGF165 and VEGF121 isoforms. Cell lysate VEGF was expressed as pg VEGF protein/mg of total cell protein and VEGF in the medium corrected for protein content of total cell protein from the same flask.

Detection of VEGF Isoforms by RT-PCR. Total RNA was isolated from cells using Trizol (Invitrogen Life Technologies, Inc., Carlsbad, CA) following standard protocols, and murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals, Indianapolis, IN) was used to generate cDNA. The cDNA was then amplified by the PCR using Platinum Taq DNA polymerase (Invitrogen Life Technologies, Inc.). The PCR primers for the VEGF isoforms also measures VEGF165 and VEGF 121 isoforms. Cell lysate VEGF was expressed as pg VEGF protein/mg of total cell protein and VEGF in the medium corrected for protein content of total cell protein from the same flask.

VEGF ELISA. Approximately 105 cells were lysed at 4°C for 1 h in 200 μl of lysis buffer [150 mM NaCl, 50 mM Tris buffer (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1 mM potassium orthovanadate, 1% NP40, and 0.2% SDS). The lysate was centrifuged (15 min, 4°C, 12,000 × g) and the supernatant was collected. A 20-μl aliquot was removed and stored at −80°C before the protein content was determined using the Bio-Rad assay (Bio-Rad, Hercules, CA). The amount of human VEGF in cell lysates and VEGF secreted into the medium was determined using an ELISA kit that measures VEGF165 and VEGF121 isoforms (Human VEGF-ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Mouse VEGFs in the cell lysate and medium of mouse WEHI7.2 cells were determined using a mouse VEGF-ELISA kit (R&D Systems) that also measures VEGF165 and VEGF121 isoforms. Cell lysate VEGF was expressed as pg VEGF protein/mg of total cell protein and VEGF in the medium corrected for protein content of total cell protein from the same flask.

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Western Blotting. Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to the manufacturer’s instructions. Total protein extracts were prepared as described for the VEGF ELISA. The protein concentration of each sample was measured using the Bio-Rad Protein assay (Bio-Rad), and Western blotting was performed as described previously (43). Blots were probed overnight at 4°C with a 1:250 dilution of mouse antihuman HIF-1α (Transduction Labs, Lexington, KY), a 1:200 dilution of mouse antihuman HIF-1β (Santa Cruz Biotechnology, Santa Cruz, CA), a 1:100 dilution of mouse antihuman iNOS (Transduction Labs), a 1:1000 dilution of goat antihuman actin (Santa Cruz Biotechnology), or a 1:1000 dilution of goat antihuman lamin A (Santa Cruz Biotechnology). Antimouse or antiglot horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia, Uppsala, Sweden) were used at a dilution of 1:5000 for detection by chemiluminescence, and blots were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

HIF-1α mRNA Measurement. Northern blotting was carried out as described previously (42) using 15 μg of total RNA. Full-length cDNA probes for HIF-1α and 18S rRNA were used. Blots were imaged using the MD Storm 860 phosphorimager and quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

HRE Reporter Assay. The pGL3 firefly luciferase reporter plasmid containing the HRE from phosphoglycerate kinase (50) was supplied by Professor Ian Stratford (University of Manchester, United Kingdom). Plasmid DNA was prepared using a commercial kit (Qiagen, Valencia, CA). The empty pGL3 control plasmid and the pRL-CMV Renilla luciferase containing plasmid used as a control for transfection efficiency were obtained from Promega (Madison, WI). Cells were transfected with 5 μg of HIF-1α reporter plasmid or pGL3 control plasmid and 0.025 μg of pRL-CMV Renilla luciferase plasmid using LipOfXAX mammalian transfection reagent (Stratagene, TX). Forty-eight h later, cells were exposed to hypoxia as described previously. Firefly and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions.

Immunohistochemistry. WEHI7.2 cells (105) or WEHI7.2/Trx cells were injected s.c. into the flanks of scid mice, and tumors were allowed to reach ~0.5 g before being removed, formalin fixed, and embedded in paraffin. Sections were stained with antibodies to factor VIII-related antigen (Dako-Patts, Santa Barbara, CA) or VEGF (Santa Cruz Biotechnology) using an automated immunostainer (GenII; Ventana Medical Systems, Tucson, AZ). Detection of bound antibody was assessed through the use of indirect avidin-biotin-peroxidase methodology with 3,3'-diaminobenzidine as the color substrate (Dako-Patts). A Ventana Medical Systems antibody diluent was used as a negative control and human placental tissue as a positive control. Nuclei were counterstained with hematoxylin. Microvessel density was assessed by light microscopy using the criteria of Weidner et al. (51). Vessels were identified...
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Fig. 2. Effect of Trx-1 and redox-inactive Trx-1 transfection on HIF activity in MCF-7 cells. Human MCF-7 cells stably transfected with empty vector (MCF-7/neo), Trx-1 (MCF-7/Trx), or redox-inactive Trx-1 (MCF-7/C32S/C35S) were transiently transfected with a vector encoding firefly luciferase with or without the HRE from phosphoglycerate kinase and were exposed to air (normoxia) or hypoxia (1% oxygen) for 16 h. Luciferase activity was normalized using transfection with Renilla luciferase, and the activation of the HRE was determined by calculating the increase in firefly:Renilla luciferase activity compared with the empty vector control activity under normoxia (relative fluorescence). Results show one experiment that was representative of three separate experiments carried out in triplicate; bars, SD; * denotes a value significantly different (P < 0.05) to the appropriate empty vector (normoxia) control.

Fig. 3. Effect of Trx-1 on HIF-1α mRNA. MCF-7 cells transfected with empty vector (MCF-7/neo), Trx-1 (MCF-7/Trx), or redox-inactive Trx-1 (MCF-7/C32S/C35S) were exposed to air (−) or hypoxia (+) for 16 h. mRNA was prepared, and Northern blotting was performed to examine levels of HIF-1α. 18S was used as a loading control. The blot is representative of three separate experiments. Lane 1 = MCF-7/neo (−), Lane 2 = MCF-7/neo (+), Lane 3 = MCF-7/C32S/C35S (−), Lane 4 = MCF-7/C32S/C35S (+), Lane 5 = MCF-7/Trx (−), and Lane 6 = MCF-7/Trx (+).

Effect of Trx-1 on Activation of HRE. MCF-7/Trx cells showed a significantly increased activation of the HRE compared with MCF-7/neo cells with a 24-fold hypoxia-induced increase seen in Trx-1 transfects compared with a 7.5-fold hypoxia-induced increase in MCF-7/neo cells (P = <0.001). MCF-7/C32S/C35S cells showed a 0.7-fold decrease in the hypoxia-dependent response compared with MCF-7/neo cells, although it was still increased compared with normoxic controls (Fig. 2). Similar effects of Trx-1 and C32S/C35S on HRE activation were observed in cells grown in air. The ratios of luciferase activity under hypoxia:normoxia were not significantly different between MCF-7/neo, MCF-7/C32S/C35S, and MCF-7/Trx cells with values of 9.4 ± 3.7, 12 ± 5 and 10 ± 4.5, respectively (P = <0.05 in all cases).

Effect of Trx-1 on HIF-1α mRNA. Transfection of MCF-7 cells by Trx-1 or redox inactive Trx-1 did not significantly affect levels of HIF-1α mRNA measured by Northern blotting (Fig. 3).

Exogenous Trx-1 Increases VEGF Production during Hypoxia. Recombinant human Trx-1 added to the medium of MCF-7/neo cells gave small but significant increases in cell lysate and medium VEGF protein levels, achieving a plateau value after 16 h of exposure (Fig. 4). Approximately half of the VEGF was in the cell lysate and half in the culture medium. Similar effects of hypoxia were observed on VEGF formation by HT-29 and WEHI7.2 cells (results not shown).

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in response to hypoxia. The effect was maximal at 0.5 μM Trx-1 (Fig. 6). No effect on VEGF formation was seen in the absence of hypoxia.

**VEGF Isoform Expression.** VEGF isoform mRNA expression was studied in MCF-7 breast cancer cells by semiquantitative RT-PCR (Table 1). In all MCF-7 cell lines, VEGF 121 and VEGF 165 were the predominant isoforms under both normoxic and hypoxic conditions with VEGF 189 only weakly expressed. There was no change in the pattern of VEGF isoform expression in MCF-7/neo, MCF-7/Trx, or MCF-7/C32S/C35S cells under normoxic or hypoxic conditions.

**Effect of Trx-1 on iNOS Expression.** Trx-1 transfection increased levels of iNOS protein after 16 h of hypoxia compared with MCF-7/neo cells (Fig. 7). Levels of iNOS were barely detectable under normoxia.

**Effect of Trx-1 on Tumor Angiogenesis.** WEHI7.2/neo and WEHI7.2/Trx cells were grown as tumors in scid mice. VEGF levels

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Table 1  Effect of Trx-1 and redox-inactive Trx-1 transfection on VEGF isoform expression by MCF-7 human breast cancer cells

<table>
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* Cells were exposed to hypoxia for 0 or 16 h, and RNA was prepared. The expression of VEGF isoforms was determined using RT-PCR. Data show the relative amount of each isoform (+++ high level of expression, ++ lower level of expression, + weak expression).

* Indicates the time of hypoxia exposure (h).

* Indicates the size (number of amino acids) in each isoform studied. No expression of the 205 isoform was observed in these cell lines.
were almost undetectable in the WEHI7.2/neo tumors but were markedly increased in the WEHI7.2/Trx tumors (Fig. 8). There was a 2.5-fold increase in tumor angiogenesis measured by microvessel vascular density in the WEHI7.2/Trx compared with the WEHI7.2/neo tumors.

**DISCUSSION**

The transcriptional activator complex HIF-1 is a key regulator of oxygen homeostasis and mediates many cellular and physiological responses necessary to adapt to changes in oxygen tension (reviewed in Ref. 2). HIF-1 has been implicated in many important aspects of tumor behavior, including increased angiogenesis, metastasis, and inhibited apoptosis (reviewed in Refs. 1, 3).

When cultured in air, cells have low levels of HIF-1α protein that markedly increase under hypoxic conditions. We observed that human MCF-7 breast cancer, human HT-29 colon cancer, and mouse WEHI7.2 lymphoma cell lines stably transfected with Trx-1 have increased levels of HIF-1α protein compared with wild-type or empty vector-transfected cells under both normoxic and hypoxic conditions. Stable transfection of the cells with a redox inactive mutant C32S/C35S Trx-1 markedly decreased HIF-1α protein levels under both normoxic and hypoxic conditions. C32S/C35S Trx-1 has previously been shown to inhibit the effects of wild-type Trx-1 by acting in a dominant negative manner (40) through competitive inhibition of Trx reductase (52). There was no change in HIF-1β/ARNT protein by hypoxia, as has been previously reported (21), or by Trx-1 or C32S/C35S transfection. The changes in HIF-1α protein were mirrored by changes in HIF transactivating activity measured using luciferase under the control of a HRE. Thus, Trx-1 enhances HIF-1α protein levels and HIF-1 activity in several cancer cell lines. Interestingly, we have previously shown that Trx-1 mRNA levels are increased 14-fold by exposure to hypoxia for 16 h (43). The mechanism by which Trx-1 increases HIF-1α protein levels remains to be established.

The increase in HIF-1 transactivating caused by Trx-1 (3-fold) exceeds the increase in HIF-1α protein (2-fold), suggesting that mechanisms in addition to an increase in HIF-1α protein contribute to the effects of Trx-1. HIF-1 transactivating activity has been reported to be increased by Ref-1, a dual function DNA repair endonuclease and redox regulatory protein (53). This occurs through a redox-dependent increase by Ref-1 of the interaction between the transcription factor

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**Fig. 7.** Effect of Trx-1 on iNOS production in MCF-7 human breast cancer cells. MCF-7 cells transfected with empty vector (MCF-7/neo), Trx-1 (MCF-7/Trx), or redox-inactive Trx-1 (MCF-7/C32S/C35S) were exposed to air (−) or hypoxia (1% oxygen) (+) for 16 h. Total protein extracts were prepared, and Western blotting was performed to examine levels of iNOS. Actin was used as a loading control. The blot is representative of two separate experiments. Lane 1 = MCF-7/neo (−), Lane 2 = MCF-7/neo (+), Lane 3 = MCF-7/Trx (−), Lane 4 = MCF-7/Trx (+), Lane 5 = MCF-7/7/C32S/C35S (−), and Lane 6 = MCF-7/C32S/C35S (+).

**Fig. 8.** Effect of Trx-1 on angiogenesis and VEGF production in tumors WEHI7.2/neo (vector control) and WEHI7.2/Trx cells were grown as tumors in scid mice. Top panel: typical sections. A and D, H&E-stained tumor sections; B and E, low power (×40) of factor VIII-related antigen-stained sections; C and F, high power (×200) of factor VIII-related antigen-stained sections; placenta, factor VIII-related antigen-stained placenta as a positive control. Lower panel: microvessel vascular density and VEGF expression measured for four tumor samples for each group with WEHI7.2/neo (□) and WEHI7.2/Trx (■) cells. Bars are SE of the mean.
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