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Silencing thioredoxin induces liver cancer cell senescence under hypoxia

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Abstract

Aim—Although thioredoxin 1 (TXN) has pleiotropic cellular functions as a redox-sensitive protein, very little is known about its role in tumor survival and growth under hypoxia. MHCC97H hepatocellular carcinoma cells have a high metastatic potential and high thioredoxin expression levels compared with their parent cell line, MHCC97. Thus, we used this cell line to explore the functional connections between TXN and hypoxia.

Methods—MHCC97H cells were cultured under normoxia and hypoxia for specific periods after nucleofection with TXN siRNA or control siRNA. We assessed the β -phenylethyl isothiocyanate (PEITC) sensitivity of the cells, cell proliferation, cell cycle and senescence, and DNA damage response by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, colony formation assays, flow cytometry, β -galactosidase staining, western blotting, and immunohistochemistry.

Results—PEITC treatment shifted reduced TXN to oxidized TXN in MHCC97H cells. Although silencing of TXN via siRNA had no impact on the PEITC sensitivity of the cells, it suppressed cell proliferation and colony formation under both normoxia and hypoxia. Under hypoxia, silencing TXN did not induce apoptosis but induced DNA damage response and cellular senescence.

Conclusions—High TXN levels in MHCC97H cells protect them from DNA damage and cellular senescence under hypoxia. Targeting TXN might enhance the chemotherapeutic effects of some DNA-damaging agents against hepatocellular carcinoma.

Keywords

Thioredoxin; Cellular senescence; DNA damage response; Hepatocellular carcinoma; MHCC97H

Introduction

The annual incidence of hepatocellular carcinoma (HCC) in the United States has risen sharply, tripling in the past 20 years, while the overall incidence of cancer has stabilized.¹ This trend in HCC incidence will certainly continue because of the large pool of people chronically infected with hepatitis C virus. HCC is a particularly lethal cancer: >95% of HCC patients die as a result of disease progression.¹ Therefore, effective prevention and treatment modalities for HCC are vitally needed. Moreover, the molecular mechanisms underlying distant metastasis of HCC remain to be elucidated.

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The thioredoxin family is a group of small, ubiquitous, redox-sensitive proteins that, together with glutathione and peroxiredoxins, act as reducing agents to protect cells from oxidative stress. Reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are elevated in tumor cells and are thought to provide a selective advantage for tumor growth because ROS function as signaling molecules to regulate cell proliferation, differentiation, and angiogenesis. However, ROS are highly toxic to tumor cells.^{2, 3} DNA damage, the most prominent detrimental effect of ROS, can lead to cellular senescence and apoptosis via the p53/p21^{Cip1/Waf1}/p16^{Ink4a} tumor suppressor pathways.⁴ Thus, the level of ROS is tightly regulated through multiple mechanisms.

One member of the thioredoxin family, thioredoxin 1 (also known as thioredoxin; TXN) is present in the cytoplasm and is often upregulated in tumor cells. TXN provides reducing equivalents from cysteine thiol (-SH) at its active site (WCGPCK) to target molecules, including ROS, and oscillates between the inactive, oxidized and active, reduced forms.³ In this way, TXN maintains ROS homeostasis in cancer cells to prevent oxidative damage.² TXN also stimulates cell cycle progression and proliferation, induces hypoxia-inducible factor-1 α (HIF-1 α) and angiogenesis, and regulates transcription factors.⁵ In addition, TXN can change the balance between the matrix metalloproteinases and their tissue inhibitors and enhance human neuroblastoma cell invasion and metastasis.⁶ However, very little is known about the role of TXN in tumor survival and proliferation under hypoxia.

We used a HCC cell line, MHCC97H, which intrinsically expresses high levels of TXN.⁷ MHCC97H cells were derived from their parent cell line MHCC97 through a metastatic cell cloning procedure and have high metastatic potential.⁷ In this study, we silenced TXN expression with siRNA in MHCC97H cells. The resulting phenotypes were characterized under normoxia and hypoxia. We found that silencing TXN expression elicited DNA damage response (DDR) and cellular senescence in MHCC97H cells under hypoxia.

Materials and Methods

Cell line and reagents

The human HCC cell line MHCC97H was kindly provided by Zhao-You Tang of the Liver Cancer Institute, Zhongshan Hospital, Shanghai, China. The pancreatic cancer cell lines MIAPaCa2 and Panc-1 were purchased from American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and 1 mM sodium pyruvate in a humidified incubator at 37°C under normoxia or hypoxia (1% O_2) with 5% CO_2 . β -Phenylethyl isothiocyanate (PEITC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS), respectively.

MTT cell proliferation and colony formation assays

Cell proliferation and cytotoxicity were assessed with the MTT colorimetric assay. Cells were plated at $3-5 \times 10^3$ per well in 96-well plates in triplicate or quadruplicate. Cells were incubated or treated with PEITC for indicated periods. MTT was added to each well, and incubation was continued at 37°C for 4 h. Cell density/viability was measured by dissolving the cellular reduced product of MTT in 200 μ l of DMSO, and the absorbance was read at 570 nm with a microplate reader (FLUOstar Omega, BMG Labtech, Chicago, IL). In addition, approximately 5×10^3 cells were plated on Matrigel (BD Biosciences, San Jose, CA) and incubated for 8–10 days. The colonies were photographed on an Olympus IX71 microscope with Image Pro MC 6.1 software.

Western blotting

The redox forms of TXN were separated on a 12.5% native polyacrylamide gel according to the procedures of Holmgren and Fagerstedt.⁸ Other cellular components were separated on denaturing polyacrylamide gels, according to the molecular sizes of the proteins. After separation, gels were electroblotted to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The filters were incubated with the appropriate primary antibodies, and specific immunoreactivity was detected with fluorescently labeled secondary antibodies (Invitrogen, Carlsbad, CA) using the Odyssey imager and software version 3 (LI-COR, Lincoln, NE).

Cell cycle analyses

The cells were harvested as indicated, washed with PBS, and fixed in 70% ethanol overnight at 4°C. Subsequently, the cells were washed with PBS and suspended in 20 µg/ml RNase A and 10 µg/ml propidium iodide (Sigma-Aldrich, Germany). Cell cycle data were acquired with the BD LSRII cytometer (BD Biosciences, Germany) and analyzed with FlowJo software (Tree Star, Ashland, OR).

siRNA and nucleofection

The negative control siRNA (either #1 or #2) and TXN siRNA (S1) were purchased from Ambion (Austin, TX). MHCC97H cells were passaged for 3 days before nucleofection. We collected $1-2 \times 10^6$ cells and suspended them in 100 µl of solution V (Amata, Cologne, Germany). Next, we added 2 µl of 20 µM TXN siRNA (S1), 20 µM negative control siRNA (#1 or #2), or H₂O (no-siRNA control) to the cell suspensions, which were immediately nucleofected with program T-020 (Amata) according to the manufacturer's instructions.

Fluorescence immunohistochemistry

Cells were plated on glass slides and allowed to attach overnight. After the cells were fixed with 100% methanol and blocked with 5% bovine serum albumin, anti-phospho-H2A.X (Ser139) (anti-γ-H2A.X) was applied to the cells (1:100 dilution), which were incubated overnight. The antibody was then detected by incubation with Alexa Fluor 594 immunoglobulin (Thermo Fisher Scientific, Waltham, MA). Cell DNA was counterstained with DAPI to define the nuclear area. Cells were analyzed and photographed on an Olympus IX81 DSU confocal microscope with SlideBook software.

Cellular senescence assay

Cells were plated in 6-well plates after nucleofection and grown under normoxia and hypoxia. After the indicated period, the cells were fixed with fixative solution and stained for β-galactosidase activity according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA).

Statistical analysis

Half maximal inhibitory concentration (IC₅₀) values were calculated with SigmaPlot version 10.0.1 (Systat Software, San Jose, CA). The two-tailed Student's *t* test was used for unpaired comparisons. Data are presented as mean ± SD of at least three replicate experiments.

Results and Discussion

TXN functions as a hydrogen donor but does not affect sensitivity of MHCC97H cells to PEITC

Through proteomic profiling, Xia's group found that, together with other invasion-associated proteins, TXN was markedly upregulated in the high-metastatic-potential

MHCC97H cell line compared with its low-metastatic-potential counterpart, MHCC97L.⁷ As a component of cellular redox systems, TXN is involved in a plethora of physiologic and pathologic processes. Elevated TXN levels were also found to be associated with resistance to several commonly used chemotherapeutic agents.⁹ TXN may provide regenerative thiol (-SH) to cytosolic proteins to keep them in an activated state;¹⁰ such proteins include matrix metalloproteinases and their inhibitors. In this way, TXN indirectly enhances invasion by malignant cells.⁶

A natural compound, PEITC, has been found to effectively disrupt the glutathione antioxidant system and kill cancer cells by increasing ROS production.¹¹ We found that treatment of MHCC97H cells with increasing concentrations of PEITC indeed resulted in increasing levels of oxidized TXN and, correspondingly, decreasing levels of reduced TXN (Fig. 1A), indicating that TXN is one of the major redox proteins in MHCC97H cells. We then tested the effects of silencing TXN expression on the sensitivity of MHCC97H cells to PEITC. TXN expression was successfully silenced by nucleofection with TXN siRNA (S1) (Fig. 1B). However, silencing TXN expression had no significant effects on the sensitivity of MHCC97H cells to PEITC, according to IC₅₀ values ($P > 0.05$) (Fig. 1C), indicating the redundancy of cellular mechanisms for coping with oxidative stress. Although the TXN levels were not further increased under hypoxia (Fig. 4C), the TXN levels in two pancreatic cell lines, MIAPaCa2 and Panc-1, were dramatically induced by hypoxia (Fig. 1D), suggesting that after a series of metastatic selections, MHCC97H cells constitutively expressed high levels of TXN.

TXN knockdown inhibits cell proliferation and colony formation

Because high levels of TXN in MHCC97H cells were not associated with their resistance to PEITC, we wondered what unique functions high levels of TXN might have in MHCC97H cells. Clinically, high TXN expression levels have been found to correlate with cancer progression and poor prognosis.¹² Therefore, we investigated the effects of silencing TXN expression on the proliferation of MHCC97H cells by using the MTT assay and the Matrigel/3D colony formation assay.

When TXN expression was knocked down with siRNA (Fig. 1B), we observed significant declines in the proliferation rate under both normoxia and hypoxia ($P < 0.01$, Fig. 2A) compared with negative control siRNA (#1) and no-siRNA control. The ability of TXN-silenced cells to form colonies was also substantially decreased under both normoxia and hypoxia, as indicated by the size and number of cell colonies formed in Matrigel (Fig. 2B). Thus, high levels of cellular TXN in MHCC97H cells must be important for cell proliferation and survival. This finding is consistent with clinical observations that increased TXN levels are associated with greater cell proliferation, and the upregulation of TXN by tumor cells might be an adaptation to the tumor microenvironment to increase proliferation.¹³

Although silencing TXN inhibited MHCC97H cell proliferation and colony formation, it did not have any effects on cell cycle phases (G₁, S, and G₂/M) at normoxia and hypoxia (Fig. 3A and B) compared with negative control siRNA. Overall cell growth was significantly slower under hypoxia (percentage of S-phase cells, $P < 0.05$). Moreover, silencing TXN did not induce apoptosis (percentage of pre-G₁ cells, $P > 0.05$), consistent with the results of western blotting (Fig. 3C) and TUNEL assay (data not shown). Then, we speculated that there must be other underlying mechanisms for inhibition of cell survival and proliferation of MHCC97H cells, especially under hypoxia.

TXN knockdown induces cellular senescence under hypoxia

Kim's group¹⁴ reported that overexpression of TXN by transfection induces cellular senescence in the human sarcoma cell line HT-1080. Paradoxically, suppression of TXN has also been shown to induce premature senescence in normal human fibroblasts.^{14, 15} We expected that silencing TXN in MHCC97H cells would lead to cellular senescence and growth inhibition under normoxia and hypoxia. One of the commonly used markers to identify senescent cells is senescence-associated β -galactosidase.¹⁶ There were many blue-stained spots, indicating β -galactosidase activity, in the TXN-silenced MHCC97H cells under hypoxia, whereas there were only a few such spots in both the control cells and the TXN-silenced MHCC97H cells under normoxia (Fig. 4A). The results clearly showed that silencing TXN led to cellular senescence, thus inhibiting proliferation of MHCC97H cells under hypoxia. However, the mechanism by which silencing TXN inhibited cellular proliferation in MHCC97H cells under normoxia remains elusive.

Our results that high TXN expression in MHCC97H cells prevented cellular senescence under hypoxia may be a landmark for their high metastatic potential. Hypoxia is one of the most common microenvironmental stresses in the progression of human solid tumors from microinvasive to metastatic cancers *in vivo*. In several reported studies of human cancer cells under hypoxia, TXN levels were elevated, enabling the dynamic redox balance to be maintained.⁵ Thus, intrinsic TXN expression provides MHCC97H cells with an advantage to survive hypoxic conditions and potentially allows the development of a more metastatic phenotype.

TXN knockdown induces the DNA damage response under hypoxia

Our study was complicated by the multiplicity of causes for cellular senescence, such as telomere shortening, DNA damage, chromatin perturbation, oncogene overstimulation, and various stresses.⁴ On the basis of the known TXN functions, we assumed that the cellular senescence induced by silencing TXN in MHCC97H cells would be telomere-independent and might result from internal and/or microenvironmental oxidative stress. These stresses most often result in DNA damage and response. H2A.X is a histone variant that is phosphorylated (γ -H2A.X) at Ser139 upon DNA damage and recruits multiple proteins to the damage sites for repair.¹⁷ As an initial step in studying the molecular mechanisms underlying such TXN function, we determined the expression of γ -H2A.X in MHCC97H cells.

Our results demonstrated that TXN silencing indeed elicited DDR, as indicated by elevated expression of γ -H2A.X, under hypoxia. Without nucleofection, we found moderate levels of DDR in MHCC97H cells under normoxia but minimal levels of DDR in MHCC97H cells under hypoxia. Silencing TXN expression strongly stimulated DDR in MHCC97H cells under hypoxia but not under normoxia (Fig. 4B and C). These results clearly demonstrate that highly expressed TXN in MHCC97H cells protects the cells from DNA damage, and this protection may account for the prevention of cellular senescence under hypoxia. On the basis of the functions of TXN as an oxidizing-agent scavenger, we speculate that TXN knockdown is likely to induce cellular senescence under hypoxia by increasing intracellular ROS and DNA damage.

In addition, we extracted the p53 gene from MHCC97H cells and detected a missense mutation in the DNA binding domain (data not shown). This point mutation abolishes p53 DNA binding and transcription functions.¹⁸ The DDR and cellular senescence under hypoxia in MHCC97H cells after TXN knockdown is p53-independent and may engage the p16-pRB pathway.¹⁹ Although much work is needed to elucidate this pathway, our research has opened a new and important avenue in this area.

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Abbreviations

DDR	DNA damage response
HCC	hepatocellular carcinoma
IC₅₀	half maximal inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PEITC	β-phenylethyl isothiocyanate
ROS	reactive oxygen species
TXN	thioredoxin 1

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Fig. 1.

TXN in MHCC97H cells acts as a reducing agent but does not affect sensitivity to PEITC. (A) Redox western blot of MHCC97H cells after 24 h of PEITC treatment. Whole-cell lysates were treated with either 4 mM dithiothreitol (DTT) or 4 mM diamide for reduced TXN and oxidized TXN, respectively. The free thiols were then carboxymethylated with 10 mM iodoacetic acid in the presence of 5 M guanidine. The proteins were separated on a 12.5% native polyacrylamide gel, and the membrane was probed with anti-TXN antibody. The proportion of oxidized TXN increased with increasing concentration of PEITC, whereas the proportion of reduced TXN decreased correspondingly. (B) Western blot showing the successful silencing of TXN in MHCC97H cells by different amounts of TXN siRNA (S1, 20 mM) after nucleofection for up to 6 days. The negative control siRNA (#1) and no-siRNA control had no effect on the expression of TXN. (C) About 5×10^3 nucleofected cells were seeded in 96-well plates. The cells were treated with various concentrations of PEITC one day later. MTT was added after 72 h of PEITC treatment and the purple formazan product was dissolved in 200 μ l of DMSO and absorbance was read at 570 nm with a microplate reader. Each determination was performed in quadruplicate. We found no significant difference in the sensitivity (IC_{50}) of MHCC97H cells to PEITC between the S1 siRNA and control groups ($P > 0.05$). Data are expressed as mean $\pm$ SD. (D) MIAPaCa2 and Panc-1 cells were cultured under either normoxia or hypoxia (1% O_2) for three days and harvested for western blot. The membrane was probed with TXN antibody and β -actin. The levels of TXN were remarkably elevated under hypoxia.

Fig. 2.

TXN silencing inhibits cell proliferation and colony formation. (A) About 5×10^3 nucleofected cells were plated in 96-well plates and incubated for 5 days. The proliferation rate was determined with MTT assays. TXN silencing significantly decreased the proliferation rate under normoxia and hypoxia compared with negative control siRNA (#1) and no-siRNA control (*, $P < 0.05$; **, $P < 0.01$). Data are expressed as mean $\pm$ SD. (B) About 5000 nucleofected MHCC97H cells were plated on Matrigel and incubated for 8–10 days. The number and size of the colonies were recorded photographically. TXN silencing dramatically decreased both the number and the size of the colonies under normoxia and hypoxia.

Fig. 3.

Cells grow significantly slowly under hypoxia, but TXN silencing does not have effects on apoptosis and cell cycle phases. (A) After two days recovery from nucleofection, the cells were incubated at normoxia and hypoxia, respectively, for three days. Subsequently the cells were subject to cell cycle analyses. Representative cell cycle histograms were shown with different treatments and culture conditions indicated above and on the left. (B) Cell cycle analyses indicated that the overall cell growth was significantly decreased at hypoxia compared to at normoxia (the percentages of S and G2/M cells significantly decreased, $p < 0.05$; the percentage of G1 cells significantly increased, $p < 0.05$). However, there was no significant difference of the percentages of pre-G1 cells (apoptotic cells) between TXN and #2 siRNA nucleofected cells. (C) No cleaved fragment of PARP was detected by Western blot, indicating that silencing TXN didn't induce apoptosis at normoxia and hypoxia.

Fig. 4.

TXN silencing induces cellular senescence and the DNA damage response in MHCC97H cells under hypoxia. (A) About 2×10^4 nucleofected cells were plated in 6-well plates in duplicate. The plates were incubated at normoxia and hypoxia respectively for 5 days. The cells were fixed with fixative and stained for β -galactosidase activity. Representative images were shown. We detected strong β -galactosidase activity under hypoxia in MHCC97H cells after TXN silencing (S1 siRNA) compared with negative control siRNA (#1) and no-siRNA control. The scattered weakly blue-stained spots in the control cells and the cells under normoxia might represent either naturally occurring senescence or background β -galactosidase activity in the cultures. (B) Cells were treated and cultured as in the senescence assay. Then, cells were fixed with methanol, and γ -H2A.X was detected by immunohistochemical staining (red). The nuclei were counterstained with DAPI (blue). Representative images were shown. Under hypoxia, the level of γ -H2A.X in MHCC97H cells was almost undetectable (negative control #1 siRNA) but markedly increased after TXN silencing (S1 siRNA). Under normoxia, the level of γ -H2A.X mildly increased after TXN silencing (S1 siRNA) compared with negative control #1 siRNA. Images for no-siRNA controls were similar to those for #1 negative control siRNA. (C) Western blot results showed that silencing TXN induced DDR by the elevated γ -H2A.X and p-Chk1 levels.