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Effects of vitamin D3 stimulation of thioredoxin-interacting protein in hepatocellular carcinoma

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Abstract

Aim—Thioredoxin-interacting protein (TXNIP) promotes oxidative stress by inactivating thioredoxin (TXN). This protein is involved in diverse disease processes, including insulin resistance, atherosclerosis and carcinogenesis. The aim of the present study was to measure the expression and function of TXNIP in *in vitro* models of liver disease, as well as in primary human hepatocellular carcinoma (HCC) tissue specimens. In addition, we wanted to determine the effects of vitamin D3-induced TXNIP stimulation in HCC-derived cell lines.

Methods—TXNIP expression was measured by quantitative reverse transcription polymerase chain reaction and western blots. TXNIP expression was stimulated by vitamin D exposure and by transfection. Cell proliferation, apoptosis and reactive oxygen species were determined by standard assays.

Results—TXNIP expression levels were low in HCC cell lines, and vitamin D3 stimulated TXNIP expression *in vitro*. In HCC cells transfected with a TXNIP expression vector or treated with exogenous vitamin D3, there was a reduction in cell proliferation and an increase in apoptosis. Cells expressing TXNIP were markedly susceptible to oxidative injury induced by cobalt chloride or bacterial lipopolysaccharide. TXNIP expression was reduced or absent in a majority of primary human HCC specimens relative to matching, non-cancerous liver tissue.

Conclusion—TXNIP expression is low or absent in human HCC specimens and HCC-derived cell lines. Vitamin D3 stimulates TXNIP expression, resulting in diminished proliferation and enhanced apoptosis. Liver cells expressing TXNIP are primed for oxidative injury. These findings suggest that stimulation of TXNIP expression, by factors such as vitamin D3, may attenuate carcinogenesis in patients with chronic liver disease.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website:

Keywords

apoptosis; hepatocellular carcinoma; liver cancer; reactive oxygen species; thioredoxin-interacting protein; vitamin D

INTRODUCTION

The thioredoxin system is an important regulator of oxidative stress in nearly all cell types.¹ The components of this system include thioredoxin (TXN), thioredoxin-interacting protein (TXNIP), thioredoxin reductase and nicotinamide adenine dinucleotide phosphate (NADPH). TXNIP inactivates TXN,^{2,3} and is involved in many disease processes, such as insulin resistance, diabetes mellitus, atherosclerosis and carcinogenesis.^{1,4} TXNIP directly binds to the two cysteine residues in the active, catalytic center of TXN, thereby inhibiting the reducing ability of TXN.^{5,6} TXN ameliorates ethanol-induced oxidative stress and apoptosis in mice.⁷ In addition, overexpression of TXN prevents lipo-polysaccharide and thioacetamide (TAA)-induced hepatitis in mice, and inhibits the development of hepatic fibrosis in the TAA model.^{8,9} Increased expression of TXNIP has been shown to increase intracellular reactive oxygen species (ROS), increase apoptosis and reduce proliferation in non-hepatic cells.^{5,6} These findings have made the TXN–TXNIP axis an attractive target for disease prevention and treatment strategies.^{1,10}

The interactions between TXN and TXNIP also result in downstream consequences that are independent of the effects on oxidative stress. TXNIP binds to TXN, thereby abolishing TRX-mediated inhibition of apoptosis signal regulation kinase-1, which leads to downstream activation of the c-Jun N-terminal kinase/p38 mitogen-activated protein kinase pathway and apoptosis.⁴ TXNIP inhibits the transcription of cyclin A, causing cell cycle arrest.¹¹ TXNIP overexpression in human T-lymphotropic virus 1 positive T cells also results in increased p16 (CDKN2A) expression, and therefore prevention of these cells from entering S-phase.¹² Independent of TXN function, TXNIP stabilizes the nuclear form of p27^{kip1}, thus permitting p27^{kip1} to function as a cyclin-dependent kinase inhibitor.^{2,13} Taken together, when TXNIP is overexpressed *in vitro*, cell proliferation is decreased and apoptosis is increased.^{2,3} These findings regarding the nature and function of TXNIP have not yet been determined in models of liver disease.

Thioredoxin-interacting protein expression can be induced by a variety of environmental stimuli, including oxidative stress, high glucose and, importantly, the administration of 1,25 dihydroxyvitamin D3 (vitamin D3). TXNIP expression is reduced or absent in many cancers.^{2,3} Furthermore, a recombinant, inbred, congenic strain of HcB-19 mice carrying a *TXNIP* mutation spontaneously develops hepatocellular carcinoma (HCC).¹⁴

Based on the aforementioned functions of TXN and TXNIP in oxidative stress, apoptosis and cell proliferation, we hypothesized that TXNIP plays an important role in the pathogenesis of hepatitis, chronic liver disease and HCC. Moreover, we theorized that TXNIP contributes to the anti-neoplastic effects of vitamin D3. To test these hypotheses, we determined the expression and function of TXNIP in cell culture models of liver disease as

well as in human HCC specimens. Furthermore, we measured the effects of vitamin D3 stimulation of TXNIP in HCC-derived cell lines.

METHODS

Cell culture and vitamin D3 treatment

The human hcc cell lines Hep3B and HepG2 were obtained from the American Tissue Type Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose; Mediatech, Manassas, VA, USA), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antifungal (Sigma, St Louis, MO, USA), at 37°C and 5% CO₂. The HCC cell line Huh7 was a gift of Dr Andrew Cameron (at our institution) and maintained in the identical conditions. Cells were treated with 10 nM, 100 nM or 500 nM 1 α , 25-dihydroxyvitamin D3 (VitD3; Sigma) for 24–48 h. A 100- μ M stock solution of vitamin D3 (in 100% ethanol) was further diluted in 10% ethanol/DMEM and then added to the cells. Media and vitamin D3 was replaced every 24 h. Control cells were treated with equal volumes DMEM and 1% ethanol. Cells were cultured in six-well plates, and protein or RNA was extracted from each well.

Human tissues

Human HCC and adjacent, non-neoplastic tissues were obtained from the pathology suite or from the operating theater of The Johns Hopkins Hospital. All subjects signed an approved consent from The Johns Hopkins University Institutional Review Board. Samples were immediately placed in RNA Later Ice (Invitrogen, Carlsbad, CA, USA) or snap frozen in liquid nitrogen and stored at –80.0°C. Aliquots of normal hepatocytes were obtained from Cellz Direct (Durham, NC, USA).

Quantitative reverse transcription polymerase chain reaction (qRT–PCR)

Cell RNA was isolated with TRIzol (Invitrogen) via the manufacturer's instructions, then transcribed into cDNA using the SuperScript III first strand synthesis system (Invitrogen). RNA quantity was determined by an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Quantitative RT–PCR for *TXNIP*, *TXN*, *CDKN1A*, *CDKN1B* and *GAPDH* was performed with sequence-specific primers and probes using TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA). Samples were run in triplicate and performed on a 7900 HT machine (Applied Biosystems) and analyzed with the SDS version 2.3 software. Values for a gene of interest were normalized to either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β -actin. RNA from human tissues was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA). RNA quality was verified by agarose-formaldehyde gel electrophoresis with ethidium bromide staining. One-step qRT–PCR was performed on an iQ5 thermal cycler using a Quantitect SYBR green RT–PCR kit (Qiagen). Purified normal human liver RNA (Stratagene, La Jolla, CA, USA) was used to generate standard curves for the PCR reactions. Samples were run in triplicate and *GAPDH* served as the control to which gene of interest expression was normalized.

Transfection experiments

A full-length, human TXNIP plasmid was obtained from Dr Richard T. Lee (Harvard Medical School, Boston, MA, USA) and cloned into a mammalian expression vector (pcDNA3.1; Invitrogen) between the NheI and EcoRI restriction sites. The plasmid was then propagated in DH5 α (Invitrogen) competent cells grown overnight in LB broth (Quality Biological, Gaithersburg, MD, USA) and 50 μ g/mL ampicillin (Sigma), and then purified using the HiSpeed plasmid mini-kit (Qiagen). Transformants were analyzed for the presence of the insert with restriction enzyme digestion (New England Biolabs, Ipswich, MA, USA). Exponentially growing Huh7 cells were transfected using Lipofectamine 2000 (Invitrogen), and stably transfected cell lines were selected for in 500 μ g/mL Genetecin (Invitrogen), and maintained in 250 μ g/mL Genetecin. Expression of *TXNIP* was confirmed by PCR and immunoblotting. Empty vector was used as a control.

Immunoblotting

Cell protein was extracted from lysates prepared in NP-40 buffer supplemented with ethylenediamine-tetraacetic acid (EDTA)-free protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA) and 100 μ M Pefabloc (Roche). Human tissues were homogenized in the same buffer. Samples were subjected to one freeze-thaw, protein quantified by the Bradford method, and stored at -80°C . The proteins were separated on Mini-protean TDX pre-cast gels (Bio-Rad, Hercules, CA, USA) at 90 V for 90 min, and electrotransferred to nitrocellulose transblot membranes (Bio-Rad). Membranes were washed with Tris-buffered saline containing 1% Tween 20 (TBST), blocked for 1 h in 5% non-fat milk, rinsed in TBST, and then incubated overnight with either human antimouse antibodies to TXNIP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Actin (Sigma), at a concentration of 1:100. After repeated washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated mouse antihuman immunoglobulin G (1:15 000 dilution; Amersham Biosciences, Piscataway, NJ, USA) for 1 h. The membranes were washed again and visualized by enhanced chemiluminescence reaction (ECL plus; Amersham). Densitometry was determined using Image J software version 1.30 obtained from the National Institutes of Health.

Cell counts and proliferation

Cell counts and cell viability were measured by Trypan blue staining and read on a Countess automated cell counter (Invitrogen). Cell propagation was determined by the CellTiter96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) per the manufacturers' protocol. This assay is based upon the ability of living cells to convert a tetrazolium salt in the reaction mixture to a formazan product that can be detected by fluorometry. HepG2 or Huh7 (5×10^3 cells/well) were added to 96-well optical plates. Cells were treated with vitamin D3 as described above. There were six wells for each condition and assayed in duplicate. The absorbance was determined on a Perkin-Elmer (Waltham, MA, USA) fluorimeter at 570 nm.

Caspase 3 activity

HepG2 or Huh7 (1×10^6 cells/well) were added to six-well tissue culture plates. Cells were treated with vitamin D3 as described above, with four wells per condition. Untreated cells were used as controls. TXNIP-transfected Huh7 cells or empty vector controls were also used for this assay. Cell lysates were extracted in the provided reaction buffer using a kit that measures proteolytic cleavage of the specific fluorogenic substrate DEVD (Asp-Glu-Val-Asp)-AFC by Caspase 3 (Biovision, Mountain View, CA, USA). Protein concentration was determined by the Bradford method. Samples were analyzed on a Perkin-Elmer fluorimeter with a 400-nm excitation filter and 505-nm emission filter.

Cell death detection enzyme-linked immunoassay (ELISA)

Apoptosis was detected in cell lines by the Cell Death Detection ELISA (Roche). This assay detects histone-associated DNA fragments, which are found in apoptosis but not necrosis. TXNIP and empty vector-transfected Huh7 cells were grown on six-well plates, mobilized with trypsin, counted and the absorbance measured on a Perkin-Elmer plate reader at 405 nm. Readings were also taken at 490 nm as a reference wavelength.

Measurement of ROS and oxidative stress

Hydrogen peroxide concentration (H_2O_2) was determined by Amplex Red Assay (Invitrogen). Cells were cultured in six-well plates as above, treated with vitamin D3 and 0 or 100 μ M cobalt chloride for 24 h, harvested per the protocol, and the fluorescence was measured on a Perkin-Elmer plate reader at an excitation wavelength of 545 nm and emission wavelength of 590 nm. Each sample was run in triplicate, and the concentration of H_2O_2 was calculated from comparison to the standard curve. Lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) using the OxiSelect TBARS Assay kit (Cell Biolabs, San Diego, CA, USA). TXNIP and empty vector-transfected Huh7 cells were cultured in six-well plates and the cells were treated with 1 μ M lipopolysaccharide or vehicle for 24 h. Cells were harvested and processed per the manufacturer's protocol, and the absorbance was measured in triplicate on a Perkin-Elmer plate reader at 535 nm. The concentration of MDA was calculated via the generated standard curve.

Thioredoxin activity assay

Total available thioredoxin in cell lysates was measured by the insulin-disulfide reducing assay as described by Holmgren and Bjornstedt¹⁵ with minor modifications. Briefly, 90 μ g of protein was prepared in 34 μ L NP-40 buffer. The solution was activated with 1 μ L of dithiothreitol (DTT) buffer (50 mM HEPES, 1 mM EDTA, 1 mg/mL bovine serum albumin and 2 mM DTT) and incubated at 37°C for 15 min. Then, 20 μ L of a reaction mixture containing 1 M HEPES, 0.2 M EDTA, 40 mg/mL NADPH and 10 mg/mL insulin was added, followed by either 5 μ L of rat thioredoxin reductase (Sigma) or water to controls and this mixture was incubated at 37°C. The reaction was terminated after 20 min by the addition of 6 M guanidine HCl and 0.4 mg/mL DTNB in 0.2 M Tris-HCl at a pH of 8. The absorbance was read at 412 nm.

Statistical analysis

Data were analyzed with Student's *t*-test and presented as mean $\pm$ standard error. $P < 0.05$ was considered significant.

RESULTS

TXNIP mRNA expression is low in HCC cell lines

We determined A reference value for *TXNIP* mRNA expression in normal, non-diseased liver. In our tumor bank, we had five normal liver specimens. In addition to these samples, we procured five consecutively obtained batches of normal purified human hepatocytes as an additional non-diseased liver control. Tissue specimens contain hepatocytes and other cell types, such as biliary epithelium, vascular epithelium and peripheral blood cells. Therefore, we performed qRT-PCR on both normal liver tissues and purified normal hepatocytes to determine whether there was any difference in *TXNIP* expression between these two sources. The relative *TXNIP* expression level in the purified hepatocytes and the normal tissues was similar (1.34 ± 0.06 and 1.23 ± 0.25 , respectively). We also measured *TXNIP* mRNA expression in different types of immortalized liver cell lines. The results show that *TXNIP* expression is reduced in liver cancer cells compared to normal cells (Fig. 1). This data support our hypothesis that *TXNIP* expression is reduced in HCC and may be involved in tumor suppression.

Vitamin D3 stimulates TXNIP expression *in vitro*

We treated Huh7, HepG2 and Hep3B cells with escalating doses of vitamin D3 and at increasing time intervals. There was more than a twofold increase in *TXNIP* expression in Huh7 cells treated with 10 nM vitamin D3 for 24 h when compared to untreated cells ($P < 0.001$, Fig. 2a). In HepG2 cells treated with 10 nM vitamin D3 for 24 h, there was a similar increase in *TXNIP* expression ($P < 0.05$, Fig. 2a). In Hep3B cells, the *TXNIP* mRNA level was also significantly increased in vitamin D3-treated cells when compared to untreated cells ($P < 0.01$, Fig. 2a). *TXNIP* protein was also significantly increased in Hep3B cells treated with 10 nM vitamin D3 and for 72 h ($P < 0.005$, Fig. 2b). Similar results were found in HepG2 and Huh7 cells. Vitamin D3 had no effect on *TXNIP* protein expression in cells treated for less than 72 h (data not shown).

Vitamin D3 does not affect *TXN* or *CDNK1B* and reduces *CDNK1A* *in vitro*

In HepG2 cells treated with 0, 10, 100 or 500 nM vitamin D3 for 24 or 48 h, there were no significant changes in *TXN* mRNA level. The results for *TXN* mRNA in Huh7 and Hep3B cells were similar (data not shown). In HepG2 cells, vitamin D3 treatment resulted in a significant reduction in *CDNK1A* mRNA ($P < 0.01$, Fig. 3a). Nearly a threefold reduction in *CDNK1A* mRNA was seen in vitamin D3-treated Hep3B cells ($P < 0.0005$, Fig. 3b). *CDNK1A* mRNA was also reduced in vitamin D3-treated Huh7 cells (data not shown). Vitamin D3 treatment did not effect *CDNK1B* expression in all cell lines (data not shown).

TXNIP transfection reduces TRX activity

In order to test the effects of TXNIP expression on cell proliferation and apoptosis, a full-length human TXNIP plasmid or empty vector control was stably transfected into Huh7 cells. TXNIP protein in the transfected cells was significantly increased compared to the empty vector cells ($P < 0.05$, Supporting information Fig. S1.) Thioredoxin activity was significantly reduced in the TXNIP-transfected cells, indicating that the increased expression of TXNIP in these transfected cells binds to and inactivates thioredoxin, and is functionally relevant ($P < 0.05$, Fig. 4). Transfection of Hep3B and HepG2 cells was not efficient enough to create a stable, TXNIP-expressing model.

Cell proliferation is reduced in cancer cells expressing TXNIP

After 48 h of treatment with 10 nM vitamin D3, cell proliferation in HepG2 cells was reduced to 78% of control cells ($P < 0.05$). In addition, proliferation in HepG2 cells treated with 100 nM VitD3 for 48 h was only 71% of control cells ($P < 0.05$, Fig. 5). There was no change in proliferation after 24 h of treatment. Similar results were found in vitamin D3-treated Huh7 and Hep3B cells.

Apoptosis is increased in cancer cells expressing TXNIP

The amount of histone-associated DNA fragments as measured by immunoassay was nearly twofold higher in unstimulated, TXNIP-transfected Huh7 cells when compared to Huh7 cells transfected with the empty vector. This result indicates that there is a significantly increased amount of apoptosis in Huh7 cells overexpressing TXNIP ($P < 0.005$, Fig. 6a). Activated caspase 3 is an essential component in the extrinsic pathway of apoptosis. In the TXNIP-transfected Huh7 cells, the level of caspase 3 was also higher than in the control cells (Fig. 6d). In vitamin D3-stimulated Huh7 cells, there was a dose-dependent increase in caspase 3 activity. In cells treated with 100 nM vitamin D3 for 24 h, there was a 2.5-fold increase in caspase 3 activity ($P < 0.005$), and a 3.5-fold increase in cells treated with 500 nM vitamin D3 for 24 h ($P < 0.005$). In cells treated with only 10 nM vitamin D3, there was no change in caspase 3 activity after 24 h (Fig. 6b).

TXNIP expressing cells are primed for oxidative stress

The concentration of intercellular H_2O_2 was measured in HepG2 cells treated for 24 h with 10 nM vitamin D3. Interestingly, the level of H_2O_2 was higher in the control cells (209 ± 0.56 nM) when compared to the vitamin D3-treated cells (71 ± 15 nM; Fig. 7a). In addition, the concentration of H_2O_2 was lower in the TXNIP-transfected Huh7 cells (122 ± 0.01 nM) when compared to cells transfected with an empty vector (479 ± 0.04 nM). We then stimulated the vitamin D-treated cells with cobalt chloride to induce oxidative stress. The hydrogen peroxide concentration in the HepG2 cells treated with 100 μ M cobalt chloride for 24 h was 317 ± 11 nM. When cells were treated with a combination of 10 nM vitamin D3 and 100 μ M cobalt chloride for 24 h, the concentration of H_2O_2 more than doubled when compared to treatment with vitamin D3 alone (188 ± 31 vs 71 ± 15 nM, $P < 0.005$; Fig. 7a). We then measured the concentration of H_2O_2 in the Huh7 cells transfected with the TXNIP expression vector, an empty vector, and with or without 100- μ M cobalt chloride stimulation for 24 h. There was a significant increase in the concentration of H_2O_2 in the TXNIP-

transfected, cobalt chloride-treated cells when compared to the untreated TXNIP-transfected cells (210 ± 14 vs 154 ± 9 nM, respectively, $P < 0.005$; Fig. 7b). There was a significant increase in H_2O_2 in the TXNIP-transfected, cobalt chloride-treated cells (210 ± 14 nM) compared to cells transfected with an empty vector and given cobalt chloride (134 ± 7 nM, $P < 0.001$).

The concentration of MDA, a marker of lipid peroxidation and oxidative stress, was not different between TXNIP-transfected and cells transfected with an empty vector. However, upon stimulation with $1 \mu\text{M}$ lipopolysaccharide (LPS) for 24 h, there was a marked increase in the concentration of MDA in the TXNIP-transfected, LPS-challenged cells ($68 \pm 14 \mu\text{M}$) as compared to the MDA concentration of $4.3 \pm 0.09 \mu\text{M}$ in the untreated TXNIP-transfected cells. The values in the LPS-treated, empty vector cells and untreated empty vector cells were $4.5 \pm 0.27 \mu\text{M}$ and $4.6 \pm 0.08 \mu\text{M}$, respectively (Fig. 7c).

TXNIP expression is decreased in human HCC

Thioredoxin-interacting protein mRNA was measured in 15 human HCC and matched, non-tumor, adjacent, control tissues. The level of TXNIP mRNA was lower in 11 out of 15 HCC (73%) when compared to the control tissue (Fig. 8a). TXNIP protein expression was measured by immunoblot in 10 HCC and in 10 matched, non-tumor, adjacent, control tissues. TXNIP protein was less in the HCC lysates than in the controls ($P < 0.05$, Fig. 8b,c).

DISCUSSION

This study demonstrates that TXNIP expression at the mRNA and protein levels is upregulated by vitamin D3 stimulation in Huh7, HepG2 and Hep3B liver cancer cells. Although this is a novel finding, it is expected given that TXNIP was originally described as being a protein upregulated by vitamin D3 treatment in HL-60 cells.⁵ We chose to use a minimal dose and duration of vitamin D3 treatment based on our previous studies.¹⁶ Through our manipulation of TXNIP expression, we were able to demonstrate several important downstream functions of TXNIP in these cells. One key finding in cells treated with vitamin D3 was a reduction in cell proliferation as compared to untreated cells. Vitamin D3 has been credited for its beneficial effects in terms of cancer prevention and as an antineoplastic agent.¹⁷ The mechanisms underlying the effects of vitamin D3 on cancer growth are not well understood. In the vitamin D3-treated cells, in addition to finding an increase in TXNIP expression, we found a significant reduction in *CDKN1A* expression. *CDKN1A*, by way of its cognate protein, p21(WAF1/CIP1), is a well-recognized tumor suppressor that binds to and inactivates cyclin-dependent kinases and regulates the cell cycle at G1/S. Our results in both HepG2 and Hep3B cells indicate that a reduction in cell proliferation in vitamin D3-treated cells is not due to an increase in *CDKN1A* expression. We then examined the expression of another regulator of the cell cycle at G1, *CDKN1B*. This gene encodes p27^{kip1}, and in lung fibroblasts, TXNIP was previously shown to interact with the C-Jun activation domain binding protein-1 (*JAB-1*) and inhibit JAB-1 mediated translocation of p27^{kip1} from the nucleus to the cytoplasm, thus stabilizing p27^{kip1} function as a cyclin-dependent kinase inhibitor.^{2,13} Similarly, in our studies, we found no reduction in *CDKN1B* expression in the vitamin D3-treated cells. These findings suggest that the

increased TXNIP expression in our vitamin D-treated cells may lead to stabilization of p27^{kip1}, resulting in reduced cell proliferation.

A second finding is that increased TXNIP expression induced by vitamin D3 is associated with increased apoptosis in HCC-derived cells. Vitamin D3 has been reported to regulate the expression of up to 3% of the genome.¹⁸ We propose that the antineoplastic effects of vitamin D3 are in part related to the upregulation of TXNIP expression. In order to specifically test the effects of increased TXNIP expression, we created a stable *in vitro* model. A significant increase in histone-associated DNA fragments was found in cells transfected with TXNIP as compared to cells transfected with an empty vector. This finding indicates that increased TXNIP expression is associated with a concomitant increase in cancer cell death from apoptosis and not necrosis. Furthermore, caspase 3 activity was also increased in the TXNIP-transfected cells as compared to cells transfected with an empty vector. Caspase 3 activation is a critical step in the extrinsic death receptor pathway of apoptosis. To further corroborate the link between vitamin D3, TXNIP and apoptosis, our results demonstrate significant increases in caspase 3 activity in vitamin D3-treated HCC cell lines.

A third finding of our study concerns oxidative stress. Oxidative stress is a fundamental component in the pathogenesis of hepatitis, activation of hepatic stellate cells to produce fibrosis and hepatic carcinogenesis.^{19,20} We theorized that the manipulation of TXNIP expression, with subsequent reduction in TRX function, would result in changes in intracellular ROS and markers of oxidative stress. In the vitamin D3-treated cells, there was less measured hydrogen peroxide than in the untreated cells. Similarly, in the TXNIP-transfected cells, there was less measured hydrogen peroxide than in cells transfected with an empty vector. It is not clear why there is less basal hydrogen peroxide in the transfected cells, but it may be related to compensatory changes in glutathione activity. However, when the vitamin D3 and untreated control cells were challenged with cobalt chloride, a chemical that induces ROS and mimics hypoxic cell injury,²¹ the level of hydrogen peroxide in the vitamin D3-treated cells more than doubled. This result indicates that with vitamin D3 treatment, cancer cells are unable to effectively scavenge ROS, and are primed for oxidative injury. The TXN expression in these cells is unchanged; therefore, we think that the increase in ROS may be the result of vitamin D3-stimulated TXNIP expression, with subsequent decrease in TXN-reducing function. This is supported by the result that cobalt chloride-treated, TXNIP-transfected cells also have significantly more hydrogen peroxide than untreated controls. In these transfected cells, we have demonstrated reduced TXN activity. Additional evidence to support the role of TXNIP and its impact upon oxidative injury in liver cancer cells was demonstrated in experiments measuring lipid peroxidation. There was no difference in lipid peroxidation among TXNIP-transfected cells, cells transfected with an empty vector, and cells transfected with an empty vector treated with 1 μ M LPS for 24 h. However, in the TXNIP-transfected cells treated with LPS, there was a marked increase in measured lipid peroxidation.

Additionally, our study also shows that TXNIP expression is reduced in a subset of human HCC. We found reduced mRNA expression in 73% of tumors when compared to matched, adjacent, control tissue. The TXNIP protein expression is also lower in the majority of the

HCC compared to the control tissue. This clinical data supports what has been previously published in other organ systems regarding TXNIP as a potential tumor-suppressor gene. The mechanisms by which TXNIP expression is reduced in HCC is not known, but mutations have not been described. Low vitamin D levels are associated with HCC.²² It is possible that vitamin D deficiency and loss of TXNIP expression in HCC are related. Furthermore, based on our findings, it is apparent that future studies investigating the regulation of TXNIP expression in HCC are justified.

In conclusion, our data indicates that vitamin D3-induced stimulation of TXNIP expression in liver cancer cells leads to a reduction in cell proliferation, an increase in apoptosis and a priming of these cancer cells for oxidative injury. Furthermore, we found that TXNIP expression is decreased in human HCC tissues. These findings suggest that stimulation of TXNIP expression, by factors such as vitamin D3, may attenuate the pathogenesis of HCC in patients with chronic liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. TXNIP mRNA in normal hepatocytes and hepatocellular carcinoma cell lines. Values are expressed as mean $\pm$ SE and are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Figure 2.

Effect of vitamin D3 on TXNIP mRNA and protein in liver cancer cell lines. (a) mRNA expression; Huh7 and HepG2 cells treated with 10 nM vitamin D3 for 24 h, Hep3B cells treated with 100 nM vitamin D3 for 48 h (* $P < 0.001$, ** $P < 0.05$, *** $P < 0.01$). (b) Protein and relative densitometry; Hep3B cells treated with 10 nM vitamin D3 for 72 h (* $P < 0.005$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 3.

Effect of vitamin D3 on *CDKN1A* mRNA expression in liver cancer cells. (a) HepG2 cells treated with 10 nM vitamin D3 for 24 h (* $P < 0.001$). (b) Hep3B cells treated with 100 nM vitamin D3 for 48 h (** $P < 0.0005$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 4. Thioresdoxin activity in empty vector and TXNIP-transfected Huh7 cells. The results displayed are the mean $\pm$ standard error of six samples for each cell type (* $P < 0.05$).

Figure 5.

Effect of vitamin D3 on cell proliferation in HepG2 cells. Cells were treated with 10 or 100 nM vitamin D3 for 24 (grey bars) or 48 h (black bars). The results are expressed as the percentage of the untreated control (* $P < 0.05$). □, 24 h; ■, 48 h.

Figure 6.

Effect of TXNIP expression on apoptosis in liver cancer cells. (a) Histone-associated DNA fragments in empty vector (EV) and TXNIP-transfected (TF) Huh7 cells (* $P < 0.01$). (b) Caspase 3 activity in Huh7 cells treated with escalating doses of vitamin D3 for 24 h (** $P < 0.005$). (c) Fold change in caspase 3 activity in vitamin D3 treated versus control Huh7 cells. (d) Caspase 3 activity in EV and TF Huh7 cells.

Figure 7.

Oxidative stress in cells expressing TXNIP. (a,b) Concentration of Hydrogen peroxide (H₂O₂) in HepG2 cells treated with 10 nM vitD3 (d) or 100 μM cobalt chloride (c), or both, for 24h (a) TXNIP-transfected (TF) or empty vector (EV) Huh7 cells, with or without cobalt chloride (b). (c) Concentration of malondialdehyde in TF cells, with or without lipopolysaccharide (* $P < 0.005$, ** $P < 0.001$).

Figure 8.

TXNIP in human liver tissues. (a) TXNIP mRNA in 15 HCC (T) and matched, non-tumor control tissues (N). (b) TXNIP protein relative to β-actin in human HCC and matched, non-tumor control tissue ($P < 0.05$). (c) representative immunoblot of TXNIP and β-actin intensity in control (N) and HCC (T) specimens.