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Original Contribution

High levels of thioredoxin reductase 1 modulate drug-specific cytotoxic efficacy

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ABSTRACT

The selenoprotein thioredoxin reductase 1 (TrxR1) is currently recognized as a plausible anticancer drug target. Here we analyzed the effects of TrxR1 targeting in the human A549 lung carcinoma cell line, having a very high basal TrxR1 expression. We determined the total cellular TrxR activity to be 271.4 ± 39.5 nmol min^{-1} per milligram of total protein, which by far exceeded the total thioredoxin activity (39.2 ± 3.5 nmol min^{-1} per milligram of total protein). Knocking down TrxR1 by approx 90% using siRNA gave only a slight effect on cell growth, irrespective of concurrent glutathione depletion (≥98% decrease), and no increase in cell death or distorted cell cycle phase distributions. This apparent lack of phenotype could probably be explained by Trx functions being maintained by the remaining TrxR1 activity. TrxR1 knockdown nonetheless yielded drug-specific modulation of cytotoxic efficacy in response to various chemotherapeutic agents. No changes in response upon exposure to auranofin or juglone were seen after TrxR1 knockdown, whereas sensitivity to 1-chloro-2,4-dinitrobenzene or menadione became markedly increased. In contrast, a virtually complete resistance to cisplatin using concentrations up to 20 μM appeared upon TrxR1 knockdown. The results suggest that high overexpression of TrxR has an impact not necessarily linked to Trx function that nonetheless modulates drug-specific cytotoxic responses.

To protect from oxygen radical-induced damage, cells have developed multifaceted antioxidant systems [1]. The thioredoxin (Trx) system is one important enzymatic network for antioxidant defense and has also a number of additional redox regulatory functions [1,2]. Trx reduces and thereby supports the activity of several proteins, e.g., antioxidant peroxiredoxins or methionine sulfoxide reductase and redox-regulated transcription factors or other signaling molecules [2,3]. The Trx system also supports deoxyribonucleotide synthesis, with reduced Trx regenerating a dithiol in ribonucleotide reductase oxidized upon each cycle of catalysis [4]. For its enzymatic function, Trx must be reduced by TrxR (EC 1.8.1.9). In human, three TrxR-encoding genes are found, i.e., *TXNRD1,* encoding TrxR1, which is the major TrxR form in most cells [5,6]; *TXNRD2,* encoding TrxR2, which is predominantly found in mitochondria [7]; and *TXNRD3,* for thioredoxin glutathione reductase (TGR) mainly expressed in testis [8]. Both TrxR1 and TrxR2 are essential for embryonic development as shown in knockout mouse models [9,10], as are their principal substrates Trx1 and Trx2 [11,12]. TrxR proteins are the only enzymes known to reduce Trx and are thereby believed to be essential for all Trx-dependent reduction processes. Being selenoproteins, mammalian TrxR's and thereby the complete Trx systems are fully dependent upon selenium [13,14]. Increasing selenium availability generally results in increased TrxR1 activity, until saturation levels are reached [15–18].

With regard to cancer development and treatment, most studies have focused on the potential importance of TrxR1 as a drug target, as reviewed elsewhere [19–22] and as shall briefly be introduced here. Several observations show that both TrxR1 and Trx1 are often overexpressed in various cancer forms under basal selenium supply [21]. Furthermore, TrxR1 has been shown to promote and even be essential for tumor growth in xenograft cancer models [23,24]. Cancer cells with lowered TrxR1 expression have also been shown to be more sensitive to UV irradiation or to low doses of cadmium [25,26]. It is not only for its growth-promoting properties that TrxR1 has been identified as a potential target for anticancer therapy, but also because of its selenium-dependent enzymological properties. The Sec residue is an integral part of the TrxR1 active site [27,28]. With a high reactivity of Sec and an easily accessible active site, TrxR1 has a broad substrate specificity, reducing not only Trx1, but also other agents, e.g., 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB),

Abbreviations: Trx, thioredoxin; TrxR, thioredoxin reductase; DTNB, 5,5′-dithiobis [2-nitrobenzoic acid]; Sec, selenocysteine; DNCB, 1-chloro-2,4-dinitrobenzene; cDDP, cisplatin; Oxa, oxaliplatin; SecTRAP, selenium-compromised thioredoxin reductase-derived apoptotic protein; A549, human lung carcinoma cell line; PEST, penicillin and streptomycin; siRNA, small inhibitory RNA; FCS, fetal calf serum; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; PI, propidium iodide; BSO, L-buthionine sulfoximine; Grx, glutaredoxin; ROS, reactive oxygen species; PBS, phosphate-buffered saline; NADPH, nicotinamide adenine dinucleotide phosphate.

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lipoic acid, lipoamide, selenite, menadione (vitamin K_3), ubiquinone, and vitamin C [1,5,29–33]. Moreover, the N-terminal CVNVGC motif can reduce certain substrates directly [34]. In addition, the Sec residue in reduced (but not oxidized) TrxR can also be rapidly targeted by electrophilic agents. Several gold compounds are potent inhibitors of TrxR1, e.g., auranofin used in treatment of rheumatoid arthritis, which inhibits the enzyme even in the nanomolar range [7,35,36]. Recent results also show that auranofin inhibits overall selenoprotein synthesis [37]. The first identified inhibitor of TrxR1 was 1-chloro-2,4-dinitrobenzene (DNCB), which also concomitantly induces an inherent superoxide-producing NADPH oxidase activity in the derivatized enzyme [38]. Other inhibitors of TrxR1 include naturally occurring electrophiles such as quinones [39], isothiocyanates [40], mercury [41], or various flavonoids [42,43], as well as several clinically used anticancer drugs including arsenicals [44] and cisplatin (cDDP) [45–47]. TrxR1 derivatized with cDDP generates an enzyme inactive for its normal function, but which may gain a new pro-oxidant function within a cellular context, capable of inducing a rapid cell death in the form of selenium-compromised thioredoxin reductase-derived apoptotic proteins, SecTRAPs [48,49]. All of these properties combined have contributed to the commonly held view that TrxR1 may be a prime molecular target for anticancer chemotherapy [19–22].

The purpose of this study was to investigate the impact of an endogenously high expression of the selenium-dependent TrxR1 in cancer cells, using knockdown of the enzyme in a human lung carcinoma cell line (A549) that has among the highest known overexpression of TrxR1 of all studied cancer cells (www.proteinatlas.org). By a thorough analysis of the phenotype of these cells upon TrxR1 knockdown, we reasoned that insights could be gained regarding the functional consequences of overexpression of the enzyme in cancer cells and of its targeting in such cells by chemotherapy. The results reveal a surprising complexity in the roles of TrxR1 for modulating cellular responses to different cytotoxic agents.

Experimental procedures

Chemicals and reagents

Recombinant rat TrxR1 was produced as described [50] and human wt Trx [51] was kindly provided by Arne Holmgren (Karolinska Institutet, Stockholm, Sweden). Yeast glutathione reductase (GR) (Cat. No. G3664), reduced glutathione (GSH), and oxidized glutathione (GSSG) were obtained from Sigma–Aldrich Chemicals (Steinheim, Germany). Chemicals used in the drug sensitivity assays were as follows: auranofin from Alexis Biochemicals (San Diego, CA, USA), cDDP (Platinol) from Bristol–Myers Squibb Pharmaceuticals (New York, NY, USA), and DNCB, juglone (5-hydroxy-1,4,-naphthoquinone), menadione (2-methyl-1,4-naphtoquinone, vitamin K_3), and oxaliplatin (Oxa) all from Sigma–Aldrich Chemicals. All other regular chemicals or reagents were of high purity and purchased from Sigma–Aldrich Chemicals, unless otherwise specified.

Cell cultures

Human lung carcinoma cells (A549 cells) were obtained from the American Tissue Culture Collection (CCL-185) and cultivated in Dulbecco's modified Eagle medium with 4.5 g/l glucose content (GIBCO/Invitrogen, Carlsbad, CA, USA). The medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin (PEST), all from PAA Laboratories (Pasching, Austria). Selenium was added to the medium in the form of sodium selenite at concentrations as described in the text. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2 and kept under logarithmic growth phase for all experiments unless stated otherwise.

Transient knockdown of TrxR1

Small interfering RNA (siRNA) molecules, specifically targeting the TrxR1 mRNA, were obtained from Qiagen (Valencia, CA, USA). For phenotypic confirmation, two different siRNA sequences were used (herein named Siseq1 and Siseq2), targeting different areas of the TrxR1 mRNA: Siseq1, sense 5′-(GCAAGACUCUCGAAAUUAU)dTdT-3′, antisense 5′-(AUAAUUUCGAGAGUCUUGC)dAdG-3′, and Siseq2, sense 5′-(CCUGGCAUUUGGUAGUAUA)dTdT-3′, antisense 5′-(UAUACUACCAAAUGCCAGG)dCdA-3′. Siseq1 targets an mRNA region encoding the N-terminal redox-active CVNVGC motif in the protein and Siseq2 targets a site in the exon covering the 3′-untranslated region (3′UTR) of the mRNA downstream of the selenocysteine insertion sequence element, needed for Sec incorporation [52]. Both siRNA constructs should thereby knock down all major TrxR1 splice forms (Supplementary Fig. S1). Two different nonsilencing siRNA controls, showing no apparent homology to any region of the human genome, were used, i.e., the Alexa 488-labeled AllStar negative control (Qiagen) as control for transfection efficiency and an unlabeled scramble control (mock) (Cat. No. 1022076; Qiagen) used for all other experiments. The sequences for the mock siRNA construct were sense 5′-(UUCUCCGAACGUGUCACGU)dTdT-3′ and antisense 5′-(ACGUGACACGUUCGGAGAA)dTdT-3′. Untransfected control cells as well as mock-treated cells were included in all siRNA experiments, as described in the text. For transfection experiments A549 cells were seeded in six-well plates at a density of about 30,000–32,000 cells per well 15–18 h before transfection. SiRNA transfection was then performed according to the manufacturer's protocol by mixing 9 μl transfection reagent (Hiperfect; Qiagen) and 10 nM siRNA duplexes in a total volume of 100 μl serum-free medium, per sample. Cells were incubated for 24 h with the transfection complexes in 2.4 ml medium without antibiotics, whereupon the medium was replaced with fresh PEST-containing medium and experiments were conducted as described.

Selenium content in medium

Selenium (m/z 78) content in the fetal calf serum utilized in this study was analyzed with inductively coupled plasma mass spectrometry as described elsewhere [53] and was determined to be 18.7 ng Se per gram of FCS, i.e., approx 18.7 μg/L. In the medium supplemented with 10% FCS, the total selenium concentration would thereby be in the range of 20–25 nM. In the experiments as described in the text, an additional 25 nM selenium in the form of sodium selenite was added to the cells during seeding to saturate the synthesis of TrxR1 in the cell cultures. Extensive control experiments verified that this was a required but sufficient selenium supplementation to reach TrxR1 saturation.

^{75}Se radioisotope labeling of cellular proteins

To verify knockdown of TrxR1, cells were seeded in six-well plates together with ^{75}Se-labeled selenite (Research Reactor Center, University of Missouri, Columbia, MO, USA) added to the medium, using 2 μCi/ml in a total volume of 1.5 ml. No additional unlabeled selenite was added. Using the specification from the distributors the total concentration of selenite added to the cells was calculated to be within the range of 20 to 100 nM. Fifteen to eighteen hours after seeding with [^{75}Se]selenite, siRNA was added to the cells in the presence of 2 μCi/ml ^{75}Se, according to the same procedures as described above. Cell extracts were subsequently prepared 48, 72, 96, and 144 h post-siRNA treatment and 25 μg total protein per sample was analyzed on 4–12% NuPAGE Bis–Tris reducing SDS–PAGE (equipment, gels, and buffers from Invitrogen). Gels were stained with Coomassie blue to visualize total protein. The gels were then dried and exposed on a phosphor screen. The autoradiography was