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Aberrant expression of redox regulatory proteins in patients with concomitant primary Sclerosing cholangitis/inflammatory bowel disease

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ABSTRACT

Objective: Primary Sclerosing Cholangitis (PSC) is a severe cholestatic liver disease characterized by progressive peri-biliary tract inflammation, elevated oxidative stress and hepatocellular injury. A hallmark of PSC patients is the concurrent diagnosis of Inflammatory Bowel Disease occurring in approximately 70%–80% of PSC patients (PSC/IBD). We previously reported dysregulation of key anti-oxidant pathways in PSC/IBD. The objective of this study was to expand previous data by examining the abundance of thioredoxins (Trx) in PSC/IBD.

Methods: Using hepatic tissue and whole cell extracts isolated from age-matched healthy humans and patients diagnosed with end stage PSC/IBD, the protein abundance of thioredoxin, thioredoxin reductase (TrxR1), and their downstream substrates peroxiredoxins was assessed.

Results: Western blot analyses of thioredoxin and peroxiredoxin abundance revealed significant increases in abundance of Trx1 and TrxR1 whereas expression of thioredoxin-interacting protein was significantly decreased in PSC/IBD. Concurrently, abundance of cytosolic peroxiredoxins was not significantly impacted. The abundance of mitochondrial Trx2, along with peroxiredoxins 3, 5 and 6 were significantly decreased by concurrent PSC/IBD. Histological staining of Trx1/TrxR1 revealed elevated nuclear Trx1 and TrxR1 staining within cholangiocytes as well as an overall periportal increase in expression in PSC/IBD. An examination of additional anti-oxidant responses reveal suppression of gamma-glutamylcysteine synthetase and heme oxygenase (HO-1) whereas expression of the protein chaperone glucose regulated protein 78 increased suggesting elevated cellular stress in PSC/IBD.

Conclusions: Results herein suggest that in addition to severe dysregulation of anti-oxidant responses, cholestasis impacts both cytosolic/nuclear (Trx1) as well as mitochondrial (Trx2) redox signaling and control pathways.

1. Introduction

The orphan disease, Primary Sclerosing Cholangitis (PSC) is a progressive cholestatic liver disease of unknown etiology characterized by biliary inflammation, fibrosis, and stricturing of the intra and/or extrahepatic bile ducts (Eaton et al., 2013). The incidence rate is ~1:100,000 person-years with no medical therapies currently available. Long-term disease progression leads to biliary obstruction, repeated bouts of cholangitis, and secondary biliary cirrhosis with a median time of survival following diagnosis of 12–18 years (Eaton et al., 2013). In the absence of liver transplant, patients carry a 10–20% lifetime risk of hepatobiliary malignancy. Furthermore, 20–40% develop recurrent disease even after liver transplant (Hirschfield et al., 2013; Kugelmas et al., 2003; Tamura et al., 2007). A primary risk factor for PSC is inflammatory bowel disease (IBD) which is present in at least 70% of PSC patients but has been reported in upwards of 80% making PSC/IBD the primary subtype of PSC (Jiang and Karlsen, 2017; Lunder et al., 2016; Weismuller et al., 2017).

By its ability to reduce oxidized cysteine residues within proteins, the thioredoxin/thioredoxin reductase (Trx/TrxR) redox system plays a critical role in regulating cellular redox status and is directly regulated by changes in cysteine oxidation as well as Nrf2 dependent pathways (Jones, 2008; Jones and Go, 2010). There are two primary isoforms of thioredoxin (Trx1/Trx2) and Thioredoxin reductase (TrxR1/TrxR2). Of these, Trx1 has been identified in both the cytosol and nuclear fractions whereas Trx2/TrxR2 localize to the mitochondria. Thioredoxins

Abbreviations: ALT, Alanine aminotransferase; BDL, Bile duct ligation; GCLC, Gamma-glutamylcysteine; GSH, Reduced glutathione; GST, Glutathione S-Transferase; HO-1, Heme Oxygenase 1; 4-HNE, 4-hydroxy-2-nonenal; IBD, Inflammatory Bowel Disease; LTx, Liver transplant; PBC, Primary Biliary Cholangitis; Prdx, Peroxiredoxin; PSC, Primary Sclerosing Cholangitis; ROS, Reactive oxidative species; Trx, Thioredoxin; TrxR, Thioredoxin reductase; TxNIP, Thioredoxin interacting protein; UDCA, Ursodeoxycholic acid; LE, Liver extracts

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contain reactive cysteines and are potent protein-disulfide reductases that modulate redox signaling and control via regulation of protein dithiol/disulfide status and delivering reducing power to key antioxidant enzymes, including peroxiredoxins (Prdx) (Jones, 2008; Jones and Go, 2010). This yields an oxidized Trx-disulfide, which is then reduced by the NADPH-dependent TrxR1/2, thereby regenerating Trx activity (Jones, 2008). Within the cell, Prdx's function is to regulate peroxide concentrations and to modulate redox status of client proteins via mechanisms termed "redox relays" (Selvaggio et al., 2018). With the exception of Prdx6, which only contains one cysteine, Prdx1-5 contain two cysteine residues that once oxidized can be reduced by thioredoxins (Rhee et al., 2005).

Thioredoxins have been shown to be important in the pathogenesis of chronic liver diseases. In murine chronic alcohol models, Trx1 expression is decreased and injection of recombinant Trx1 mitigates EtOH induced damage (Cohen et al., 2009). In transgenic models, overexpression of Trx1 can exert an opposing effect by increasing NF-κB activity and TNFα production supporting a possible contribution of Trx1 to elevation of inflammation (Go et al., 2011; Trevelin et al., 2016). Data examining thioredoxins in cholestatic liver disease is scant. In human Primary Biliary Cholangitis (PBC), levels of Trx1 and TrxR1 were normal; however, treatment with ursodeoxycholic acid (UDCA) activated Nrf2, causing increased expression of Trx1 and TrxR1 (Kawata et al., 2010). In another study, serum and tissue extracts from PBC patients and from rodent bile duct ligation (BDL) models suggested that total Trx activity is initially elevated, but is then diminishes as disease severity increases (Grattagliano et al., 2014; Grattagliano et al., 2008). In the BDL model, increasing Trx1 nuclear localization assisted in mitigating cholangiocyte proliferation, but its impact was not examined with respect to cholestatic injury and Trx1 expression following BDL was not compared to normal liver (DeMorrow et al., 2008). Finally, although not directly examining Trx1, increasing biliary flow in acute human obstructive cholestasis decreased ALT, elevated GSH and decreased lipid peroxidation supporting the critical contribution of cellular redox status and injury (Vendemiale et al., 2002).

We have previously reported that tissue obtained from PSC/IBD patients exhibits elevated production of products of lipid peroxidation and significant dysregulation of cellular antioxidant responses (Shearn et al., 2017). In the present study, we sought to expand the original report by examining the expression of Trx1/TrxR1/Prdx2 and Trx2/TrxR2/Prdx3 pathways in hepatic tissue isolated from PSC/IBD patients. Data herein suggest that, when compared to previously published reports regarding PBC, patients with PSC/IBD may possess distinct dysregulation of hepatocellular redox responses further supporting the uniqueness of PSC/IBD.

2. Methods

2.1. Sample procurement

As previously reported, paraffin embedded and frozen hepatic tissue from normal and end stage PSC/IBD patients (N = 9 PSC/IBD, 8 Normal) were procured during transplantation (ages 25–62, Male/Female) and obtained from the University of Minnesota Liver Tissue Cell Distribution Center NIH Contract #HHSN276201200017C Exempt. A complete biochemical description of each patient is reported in (Shearn et al., 2017). Liver extracts (LE) of each sample was prepared by dounce homogenization (10×) of tissue resuspended in 50 mM tricine pH 8.0, 1 mM NaCl plus phosphatase and protease inhibitors (SIGMA ALDRICH, St. Louis, MO) followed by sonication (3 × 15 seconds@ 4 °C) and processing as previously described (Shearn et al., 2017).

2.2. Histological evaluation of thioredoxin 1 and thioredoxin reductase 1 expression

Formalin fixed slides prepared from normal and end stage PSC/IBD patients (N = 9 PSC, 8 Normal) were used. Antigen retrieval was completed using citrate pH 6.0 and the Biocare heating system (Biocare, Medical, Pacheco, CA) at 100 °C for 10 min as previously described (Shearn et al., 2013a). Slides were blocked in 5% nonfat dry milk in TBST for 1 h followed by incubation with rabbit polyclonal anti-Trx1 (1:250 dilution Cat#14999-1-AP Proteintech, Rosemont, IL) or anti-TrxR1 (1:500 dilution Cat#11117-AP-1, Proteintech) overnight at 4 °C. The following morning, slides were washed three times for 6 min each wash in TBST. Following washing, a horse anti-rabbit HRP-conjugated secondary antibody was applied for 25 min (IMMPRESS HRP Horse anti-rabbit, Cat#MP-7401, Vector Labs, Burlingame, CA). Peroxidase development was performed using the ImmPACT DAB peroxidase substrate kit (Cat#SK-4104, Vector labs). Histologic images were captured on an Olympus BX51 microscope equipped with a four-megapixel Macrofire digital camera (Optronics; Goleta, CA) using the PictureFrame Application 2.3 (Optronics). All images were cropped and assembled using Photoshop CS2 (Adobe Systems, Inc.; Mountain View, CA).

2.3. Western blotting

Western blotting for Trx1 (Cat#14999-1-AP Proteintech), TrxR1 (Cat#LF-PA0023 AbFrontier, Seoul, Korea), TxNIP (Invitrogen Cat#403700, Trx2 (Cat#13089-1-AP, Proteintech), TrxR2 (Cat#LF-PA0024 AbFrontier), GCLC(Cat#NBP1-49762 Novus Biologicals, Littleton, CO), GSTκ (Cat# 14535-1-AP Proteintech), Prdx1 (Cat#PA3-750, Thermofisher, Waltham, MA), Prdx2 (Cat#ab-16748 ABCAM, Cambridge, MA), Prdx3(Cat#ab-16751, ABCAM), Prdx5 (Cat#17724-1-AP, Proteintech), Prdx6 (Cat#ab-16824 ABCAM)and anti-GAPDH (Millipore, Billerica, MA) was performed from 10 μg of whole cell liver extracts as previously described (Shearn et al., 2014; Shearn et al., 2011; Shearn et al., 2013a; Shearn et al., 2013b). Quantification of expression of each protein was performed using ImageJ (NIH) and normalized to overall GAPDH expression.

2.4. Statistical analysis

The data are presented as means ± Standard Error (SE) n = 6/condition. Comparisons between normal and PSC/IBD tissue was accomplished by Student's *t*-tests. Statistical significance was set at $P < 0.05$. Prism 5 for Windows (GraphPad Software, San Diego, CA) was used to perform all statistical tests.

3. Results

We have previously determined that tissues prepared from human end-stage PSC-IB-D patients display decreased expression of catalase as well as GSTμ and GSTA4 potentially contributing to increased concentrations of reactive oxidative species (Shearn et al., 2017). To ascertain the impact of chronic cholestasis on the Trx1 pathway, protein abundance of Trx1, TrxR1 and thioredoxin interacting protein (TxNIP) was examined in whole cell extracts (LE). From Fig. 1, abundance of Trx1 and TrxR1 was significantly increased by 2-fold in PSC/IBD sample. Abundance of TxNIP, however, was observed to be significantly decreased. Following oxidation by hydrogen peroxide, Prdx1 and 2 can serve as substrates for Trx1; therefore, the abundance of cytoplasmic Prdx1, 2 and 6 was examined in LE using standard Western blotting. From Fig. 1, abundance of Prdx1 and Prdx2 was not significantly changed but surprisingly expression of Prdx6 significantly decreased.

To further understand the impact of PSC/IBD on Trx1/TrxR1 abundance, hepatocellular localization of Trx1/TrxR1 was examined using paraffin embedded tissue sections and IHC. In Fig. 2, Normal