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Apoptosis of cholangiocytes modulated by thioredoxin of carcinogenic liver fluke

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ABSTRACT

Chronic infection with the food-borne liver fluke, *Opisthorchis viverrini*, frequently induces cancer of the bile ducts, cholangiocarcinoma. Opisthorchiasis is endemic in Thailand, Lao PDR, Cambodia and Vietnam, where eating undercooked freshwater fish carrying the juvenile stage of this pathogen leads to human infection. Because inhibition of apoptosis facilitates carcinogenesis, this study investigated modulation by thioredoxin from *O. viverrini* of apoptosis of bile duct epithelial cells, cholangiocytes. Cells of a cholangiocyte line were incubated with the parasite enzyme after which they were exposed hydrogen peroxide. Oxidative stress-induced apoptosis was monitored using flow cytometry, growth in real time and imaging of living cells using laser confocal microscopy. Immunolocalization revealed liver fluke thioredoxin within cholangiocytes. Cells exposed to thioredoxin downregulated apoptotic genes in the mitogen activated protein kinases pathway and upregulated anti-apoptosis-related genes including apoptosis signaling kinase 1, caspase 9, caspase 8, caspase 3, survivin and others. Western blots of immunoprecipitates of cell lysates revealed binding of thioredoxin to apoptosis signaling kinase 1. Together the findings indicated that thioredoxin from *O. viverrini* inhibited oxidative stress-induced apoptosis of bile duct epithelial cells, which supports a role for this liver fluke oxidoreductase in opisthorchiasis-induced cholangiocarcinogenesis.

1. Introduction

Infection with the liver fluke *Opisthorchis viverrini* is endemic in Thailand, Lao PDR, Cambodia and central Vietnam (Petney et al., 2013; Sithithaworn et al., 2012; Sripa et al., 2011). Infection follows the ingestion of uncooked/undercooked cyprinoid, fresh-water fish infected with the metacercariae of the parasite. Opisthorchiasis causes a spectrum of biliary system disease, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis (Mairiang et al., 2012; Mairiang and Mairiang, 2003). More problematically, chronic opisthorchiasis infection frequently causes cholangiocarcinoma, bile duct cancer (IARC, 2012; Sripa et al., 2012, 2007). Carcinogenesis of opisthorchiasis-induced bile duct cancer likely arises from several, interrelated molecular insults. These factors include inflammation associated reactive oxygen species (ROS) and reactive nitrogen species (RNS), lesions caused by the feeding and other mechanical activities of the worms within the bile ducts, and soluble mediators released by the parasites (IARC, 2012;

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Porta et al., 2011; Sripa et al., 2012, 2007). They also may include perturbation of the biliary and intestinal microbiome due to liver fluke infection (Plieskatt et al., 2013), and reactive oxysterol- and catechol estrogen quinone-like metabolites released by the fluke that can form depurinating adducts with chromosomal DNA of epithelial cells of the infected persons (Correia da Costa et al., 2014; Jusakul et al., 2012). The fluke secretes and/or excretes metabolic products, immunogens and other mediators (Mulvenna et al., 2010; Smout et al., 2009; Sripa and Kaewkes, 2000; Wongratanacheewin et al., 1988) including proteins like thioredoxin peroxidase (Suttiprapa et al., 2008) and thioredoxin (Suttiprapa et al., 2012).

Thioredoxin (Trx) is an inflammation-inducible oxidoreductase of 12 kDa that is expressed ubiquitously in prokaryotes and eukaryotes. This enzyme is involved in numerous physiological roles. Within the cell, Trx exerts cyto-protective effects: it scavenges ROS and thereby relieves oxidative stress, and it regulates redox-sensitive signaling pathways as well as ROS-independent genes. Outside the cell, Trx has cytokine and growth factor like properties, it promotes cell and tissue growth, and exhibits activities involved with protein disulfide reduction, protein repair, molecular chaperone tasks, structural components of enzymes, redox regulation of transcription factors, immunomodulation, and apoptosis (Carvalho et al., 2006; Holmgren and Lu, 2010; Nakamura et al., 1997; Powis and Kirkpatrick, 2007). Moreover, Trx has been implicated in carcinogenesis. It is encoded by a proto-oncogene that is overexpressed in tumors and correlates with poor prognosis (An and Kang, 2014). Trx stimulates survival of cancer cells, promotes angiogenesis, and inhibits apoptosis; evasion of apoptosis is a cardinal hallmark of cancer (Hanahan and Weinberg, 2000). Anti-apoptotic activities of thioredoxin, in particular interaction with apoptosis signal-regulating kinase-1 (ASK-1), are well described (Zhang et al., 2004). ASK-1 is a member of the mitogen activated protein kinase kinase kinase family (MAP3Ks); this enzyme activates c-Jun *N*-terminal kinase and p38K in response to oxidative and other stresses, leading to apoptosis, inflammation and other responses (Hattori et al., 2009; Zhang et al., 2004).

We have speculated that *O. viverrini* Trx-1 may contribute to the tumorigenic nature of chronic opisthorchiasis in the biliary tree (Sripa et al., 2012; Suttiprapa et al., 2012). Recombinant *Ov*-Trx-1 was produced in *E. coli* and its activity as an oxidoreductase characterized. Expression of *Ov*-Trx-1 in developmental stages of the liver fluke has been reported, and antibodies against the enzyme revealed its presence in cholangiocytes of hamsters adjacent to the liver fluke (Suttiprapa et al., 2012). Inflammatory cells secrete ROS/RNS that are potentially genotoxic to fluke and to bystander cells. Nonetheless, *O. viverrini* flukes can survive for years, bathed in bile (Kaewkes, 2003). Thioredoxin from *O. viverrini* may be involved as a key main redox protein that protects this trematode from ROS (Suttiprapa et al., 2012). Given that inhibition of homeostatic apoptosis is obstructed in carcinogenesis, here we investigated anti-apoptotic effects of liver fluke thioredoxin on human normal cholangiocytes under oxidative stress, and on the interaction with apoptosis signal-regulating kinase-1, ASK-1.

2. Materials and methods

2.1. Recombinant thioredoxin-1 of O. viverrini

The nucleotide sequence encoding *Ov*-Trx-1 was amplified from a cDNA library constructed from adult *O. viverrini* liver flukes (Laha et al., 2007). The nucleotide sequence of the amplicon was verified by sequencing, after which it was ligated into the expression vector pET-15b (Novagen, CA, USA). BL21 (DE3) strain *Escherichia coli* cells were transformed with the ligation products, and recombinant clones obtained by antibiotic selection of transformed bacteria. Expression of recombinant *Ov*-Trx-1 was induced with isopropyl β-D-1-thiogalactopyranoside; expression was monitored by SDS-PAGE (20% separating gel and 5% stacking gel)/Coomassie Brilliant Blue staining. Recombinant *Ov*-Trx-1 was affinity purified on Ni-NTA resin (QIAGEN Inc., Valencia, CA, USA), dialyzed against 1× PBS, absorbed with Triton-X114 to remove residual lipopolysaccharide (Aida and Pabst, 1990; Suttiprapa et al., 2012) and Bio-Beads SM2 (Bio-Rad, Hercules, CA, USA) to remove Triton-X114, and then filtered through a 0.2 μm pore size membrane. Yields were quantified by Bradford assay, after which *Ov*-Trx-1 was stored in aliquots at −80 °C.

2.2. Anti-Ov-Trx-1 sera

Mice were vaccinated subcutaneously with purified *Ov*-Trx-1, 25 μg per immunization. The first immunization with *Ov*-Trx-1 was formulated with Freund's complete adjuvant and second and third immunizations with recombinant protein in Freund's incomplete adjuvant. Blood and sera were collected two weeks after the third immunization. Protocols for these experiments were approved by the Animal Ethics Committee of Khon Kaen University, approval number AEKKU25/2554, according to the Ethics of Animal Experimentation of the National Research Council of Thailand.

2.3. Uptake of liver fluke thioredoxin by human cholangiocytes

H69 is a cell line derived from an immortalized human cholangiocyte (Grubman et al., 1994; Park et al., 1999). H69 cells seeded at 20,000 cells on glass cover slips were cultured in 10% fetal bovine serum (FBS) in H69 medium (Grubman et al., 1994; Smout et al., 2009) for 48 h, and thereafter in H69 medium supplemented with 1% FBS and *Ov*-Trx-1 at 50 μg/ml for 30, 60 and 120 min. Cells were washed in cold 1× PBS, fixed in 2% paraformaldehyde for 10 min, and re-washed in PBS. Cells were permeabilized with 0.5% Triton X-100/1× PBS for 5 min, washed with cold PBS, and probed with anti-*Ov*-Trx-1 sera, diluted 1:300 at 4 °C for 18 h in a humidified atmosphere. After washing, cells were stained with goat anti-mouse IgG-Alexa Fluor 568 diluted 1:500. The cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI) for 2 h at 4 °C. Last, the cells were washed with 1× PBS and fluorescence investigated using confocal laser scanning microscopy (CLSM) (Zeiss LSM 510 system, which includes an Axio Examiner Z1 microscope and a Quasar 32-channel spectral detector, Carl Zeiss, Oberkochen, Germany). Samples were scanned sequentially using a Plan-Apochromat 63×/1.40 Oil DIC objective (Zeiss). For acquisition of signals from the DAPI channel, a 405 diode laser line was used for excitation and emission was filtered in a band between 410 and 585 nm. Immunolabeling (Alexa Fluor 633) (Life Technologies, Pittsburgh, PA) was revealed by excitation with a diode 633 laser line, with emission recorded between 638 and 747 nm. Optical confocal sections were generated by adjusting the pinhole to one Airy unit using the most red-shifted channel, producing an optical section of about 0.7 μm in all channels. Confocal images were captured in sequential acquisition mode to avoid excitation bleed-through, particularly apparent with DAPI. Image frames measured 1024 × 1024 pixels with a pixel dimension of 0.132 μm. Image manipulation was undertaken with the assistance of Zen 2009 software (Zeiss); manipulations were limited to adjustment of brightness, cropping, insertion of scale bars and the like; image enhancement algorithms were applied in linear fashion across the image and not to selected aspects. Control images were adjusted similarly.