Suramin Decreases Injury and Improves Regeneration of Ethanol-Induced Steatotic Partial Liver Grafts

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d) Abbreviations:
ALT: alanine aminotransferase
BrdU: 5-bromo-2’-deoxyuridine
CC: cleaved caspase
CDK: cyclin dependent kinase
CDKI: cyclin dependent kinase inhibitor
CyD1: cyclin D1
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
FPG: fatty partial grafts
HGF: hepatic growth factor
hpf: high power field
IL-6: interleukin-6
I/R: ischemia/reperfusion
JNK: c-Jun N-terminal kinase
LPG: lean partial grafts
LPS: endotoxin
LT: liver transplantation
NF-κB: nuclear factor-κB
p-EGFR: phospho-epidermal growth factor receptor
TGF-β: transforming growth factor-β
TNFα: tumor necrosis factor-α
UW solution: University of Wisconsin cold storage solution
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ABSTRACT

Steatotic grafts are excluded for use in partial liver transplantation (LT) due to increased risk of primary non-function. This study investigated the effects of suramin, a polysulfonated naphthylurea, on the outcome of steatotic partial LT. Rat livers were harvested after acute ethanol treatment (6 g/kg, i.g.), reduced in size to ~1/3, and transplanted. Serum alanine aminotransferase (ALT) and total bilirubin, and hepatic necrosis and apoptosis were significantly higher after transplantation of fatty partial grafts (FPG) than lean partial grafts (LPG). Suramin (5 mg/kg, i.p.) decreased ALT by ~60%, hyperbilirubinemia by 75%, necrosis by 83%, and apoptosis by 70% after FPG transplantation. Hepatic cellular 5-bromo-2'-deoxyuridine (BrdU) incorporation increased to 28% in LPG but was only 2% in FPG at 48 h and mitotic index increased to 7% in LPG but was only 0.2% in FPG, indicating suppressed regeneration in FPG. Suramin increased BrdU incorporation and mitotic index to 43% and 9%, respectively, in FPG. All FPG recipients died within 5 days. Suramin recovered survival of FPG to 62%. Tumor necrosis factor-α (TNFα) mRNA was 2.2-fold higher in FPG than in LPG and was associated with activation of caspase-8 and -3 in FPG. Suramin decreased TNFα and caspase activation in FPG. Transforming growth factor-β (TGF-β), phospho-Smad2/3 and p21Cip1 were significantly higher in FPG than in LPG and suramin blocked TGF-β formation and its down-stream signaling pathway. Taken together, suramin improves the outcome of FPG transplantation, most likely by inhibition of TNFα and TGF-β formation.
INTRODUCTION

Use of partial liver grafts and “extended criteria” organs is increasing due to critical shortage of donor livers (Wertheim, et al., 2011; Busuttil and Tanaka, 2003). Steatotic livers are among the most common types of marginal organs for transplantation. However, it is well known that steatotic livers are more susceptible to ischemia/reperfusion (I/R) injury and often lead to poorer outcomes compared to nonsteatotic livers after transplantation (Vetelainen, et al., 2007; McCormack, et al., 2011; Sun, et al., 2003). Many factors, such as obesity, high fat diet, immobility, insulin insensitivity, and alcohol consumption cause hepatic steatosis. Unfortunately, an estimated 65% of American adults are overweight or obese (MacDonald, Jr., 2003). Alcohol consumption is also a well-known risk factor for causing steatosis and many organ donors are accident victims where alcohol consumption is frequently involved.

Partial liver grafting, such as living donor liver transplantation (LT) and split LT, is also performed frequently to alleviate the severe shortage of donor livers. However, small-for-size grafts are also associated with increased graft failure (Sugawara, et al., 2001; Kiuchi, et al., 1999). Steatotic livers are currently excluded for use in partial LT clinically because of the fear that partial liver grafting and hepatic steatosis would additively or synergistically increase the risk of graft failure (Heaton, 2003). Since liver steatosis is highly associated with obesity, potential donors with high body mass index are usually not evaluated for living liver donation (Trotter, 2001) and Rinella et al. proposed that donors with more than 10% steatosis be excluded in living donor LT (Rinella, et al., 2001). Therefore, steatosis is also an important limiting factor of the usable donor pool for partial LT. In animal studies, high-fat diet-induced steatosis decreased survival in association with increased graft injury and suppressed liver
regeneration after partial LT in rodents (Morioka, et al., 2003; He, et al., 2010; Cheng, et al., 2010). Effective therapies are therefore needed to improve the outcomes of partial LT and expand the usable donor pool.

Suramin, a polysulfonated naphthylamine derivative of urea, has been widely used clinically for the treatment of human trypanosomiasis as well as infections of *Onchocerca volvulus* (Voogd, et al., 1993; Liu and Zhuang, 2011). Moreover, it blocks both the infectivity and the pathogenic effects of HIV in *vitro* and in humans (Mitsuya, et al., 1984). It was also tested for efficacy in selected malignancies and metastatic diseases (Liu and Zhuang, 2011). In recent years, research interest has turned to the new use of this old drug. For example, recent studies showed that suramin protects against liver injury after D-galactosamine and lipopolysaccharide (LPS) exposure in mice (Eichhorst, et al., 2004; Liu and Zhuang, 2011). Suramin also reduces ischemia/reperfusion-induced brain and kidney injury in rodents (Kharlamov, et al., 2002; Zhuang, et al., 2009). The mechanisms by which suramin protects these organs from injury are associated with inhibition of apoptosis, suppression of nuclear factor-κB (NF-κB) activation and decrease toxic/proinflammatory cytokine formation (e.g. tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6)) (Eichhorst, et al., 2004; Kharlamov, et al., 2002; Zhuang, et al., 2009; Goto, et al., 2006). Further, suramin has an anti-fibrotic effect in liver, kidney and muscle (Liu and Zhuang, 2011; Li, et al., 2009; Korrapati, et al., 2012a). Interestingly, suramin also promotes renal proximal tubular cell proliferation in vitro and in vivo and accelerates renal function recovery after I/R (Zhuang and Schnellmann, 2005; Zhuang, et al., 2009). Stimulation of cell proliferation after renal injury by glycerol appears to be due to suppression of transforming growth factor-β (TGF-β) formation (Korrapati, et al., 2012b). Since I/R injury, toxic cytokine formation, increased cell death and suppressed liver regeneration have
been shown to play important roles in fatty graft injury and/or small-for-size liver graft failure, we investigated whether suramin improves the outcome after transplantation of fatty partial liver grafts.
METHODS

Animals and Partial Liver Transplantation. Female Lewis rats (230-250 g) were gavaged with one inebriating dose of ethanol (6 g/kg) or an equal volume of saline. At 16 h after saline or ethanol treatment, partial LT was performed under ether anesthesia. The median lobe and the left lateral lobe were removed after ligation with 4-0 suture, resulting in a 66% decrease in liver mass. The remnant liver was rinsed with ~5 ml of ice-cold University of Wisconsin (UW) cold storage solution (Abbott Laboratories, North Chicago, IL) and explanted as previously described (Zhong, et al., 2005). Venous cuffs prepared from 14-gauge intravenous catheters were placed over the subhepatic vena cava and the portal vein in vitro. Reduced-size liver explants were stored in UW solution at 0–1°C for 3 h.

Implantation was performed using the re-arterialized two-cuff technique described elsewhere (Zhong, et al., 2005). The hepatic artery and the bile duct were connected with intraluminal splints (PE-10 and PE-50, respectively). Blood vessels were clamped for 18 to 20 min during surgery, and implantation required less than 50 min total time. For sham operation rats, the abdominal wall was closed with running suture 50 min after opening of the abdomen without transplantation. Fatty livers were randomly assigned to the suramin or vehicle treatment groups. Suramin (5 mg/kg) or an equal volume of vehicle (saline) was injected intraperitoneally 3 h after transplantation. Rat survival was observed for 7 days after surgery. All animals were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

Measurement of Serum Alanine Aminotransferase (ALT) and Total Bilirubin. Blood samples were collected at 48 h after implantation and serum was obtained by centrifugation and
stored at -80°C until measurement. Serum ALT and total bilirubin were determined by analytical kits from Pointe Scientific (Canton, MI) to evaluate liver injury and function, respectively.

**Histology.** To assess hepatic steatosis, livers were harvested under pentobarbital anesthesia (50 mg/kg, *i.p.*), frozen-sections were prepared and stained with Oil-Red-O 16 h after ethanol treatment or 48 h after implantation. At 48 h after sham or implantation surgery, livers were perfusion-fixed and harvested for histology as described elsewhere (Zhong, et al., 2005; Liu, et al., 2012c). Tissue blocks were imbedded in paraffin, and histology was examined after hematoxylin-eosin (H&E) staining (Zhong, et al., 2006). For quantification of necrosis, 10 images were captured randomly per slide using a Universal Imaging Image-1/AT image acquisition and analysis system (West Chester, PA) with an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) and a 10x objective lens. Necrotic areas were quantified by image analysis using an IPlab 3.7v software (BD Biosciences, Rockville, MD) as described elsewhere (Liu, et al., 2012c). Mitotic cells were counted in 10 randomly selected fields under the light microscope and mitotic index was calculated.

**Immunohistochemistry.** Previous studies showed that after partial LT, cell proliferation increased gradually over 24 h and peaked at between 38 to 48 h (Zhong, et al., 2006). Therefore, in the present study we compared cell proliferation at 48 h after partial LT. 5-Bromo-2’-deoxyuridine (BrdU, 100 mg/kg *i.p.*) was injected 1 h prior to liver harvest, and immunohistochemistry of BrdU was performed to detect cells synthesizing DNA as described elsewhere (Zhong, et al., 2005; Liu, et al., 2012a). BrdU-positive and negative hepatocytes were counted in 10 randomly selected fields using a light microscope with a 40x objective lens and
the percentage of BrdU-positive cells were calculated. Apoptosis was detected by TUNEL staining using an in situ cell death detection kit from Roche Diagnostics (Penzberg, Germany).

**Detection of Tumor Necrosis Factor-α mRNA by Quantitative Real-Time PCR.** qPCR was performed as described elsewhere (Liu, et al., 2012c). After total RNA isolation from liver tissue with Trizol (Invitrogen, Grand Island, NY), single stranded cDNAs were synthesized from RNA (2 μg) using a Bio-Rad iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). qPCR was conducted using the primer sequences in Table 1 and a CFX96 Real Time-PCR Detection System (Bio-Rad, Hercules, CA). The abundance of mRNAs was normalized against hypoxanthine phospho-ribosyl-transferase (HPRT) using the ΔΔCt method.

**Immunoblotting.** Proteins were detected by immunoblot analysis as previously described (Liu, et al., 2012c) with primary antibodies specific for cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), cleaved caspase-8, TGF-β (Abcam, Cambridge, MA), phospho-epidermal growth factor receptor (p-EGFR, GenWay Biotech, San Diego, CA), EGFR, phospho-c-Jun N-terminal kinase (JNK)-1/2, JNK1/2, phospho-extracellular signal-regulated kinases (ERK)-1/2, ERK1/2, phospho-Smad2/3, Smad2/3, p21Cip1 (Santa Cruz Biotech., Santa Cruz, CA) and 4-hydroxynonenal adducts (4-HNE, Alpha Diagnostic, San Antonio, TX) at concentrations of 1:100 to 1000, and actin (ICN, Costa Mesa, CA) at a concentration of 1:3000 at 4°C over night, respectively. Horseradish peroxidase-conjugated secondary antibodies were applied, and detection was by chemiluminescence (Pierce Biotec., Rockford, IL).

**Statistical Analysis.** Groups were compared using Kaplan-Meier test or ANOVA plus a Student-Newman-Keuls posthoc test, as appropriate. There were 8 transplantations per group in
survival experiments and 4 transplantations per group for all other parameters. Data shown are means ± S.E.M.. Differences were considered significant at p<0.05.
RESULTS

Acute Ethanol Treatment Causes Hepatic Steatosis. Previous studies showed that one inebriating dose of ethanol causes overt steatosis (Zhong, et al., 1997; Liu, et al., 2012b). Consistent with these previous reports, livers from rats given saline revealed sparse distribution of small red fat droplets within hepatocytes (Fig. 1A). In contrast, livers from rats treated with ethanol showed red fat droplets occurring in 60-70% of hepatocytes. The steatosis is mainly microvesicular mixed with scattered macrovesicular fat droplets (Fig. 1A). These results confirm that acute ethanol treatment causes overt hepatic steatosis. At 48 h after transplantation, red fat globules still existed in fatty partial grafts (FPG) regardless with or without suramin treatment (Fig. 1A). Therefore, suramin did not affect steatosis after transplantation.

Suramin Attenuates Injury of FPG after Transplantation. No pathological changes were observed in lean livers after sham operation (Fig. 1B). Ethanol treatment alone did not cause necrosis (not shown). After transplantation of LPG, small necrosis was observed (Fig. 1B). In contrast, transplanted FPG exhibited overt focal necrosis, primarily occurring in the periportal and midzonal regions of the liver lobules (Fig. 1B). Necrotic areas were ~3% in LPG and increased to ~14% in FPG (Fig. 1C). Suramin (5 mg/kg) given 3 h after partial LT decreased necrosis in FPG to control levels (Fig. 1 B & C).

Apoptosis was evaluated by TUNEL staining (Fig. 2A and B). TUNEL-positive hepatocytes were 0.13% and 0.4%, respectively in lean livers and after acute ethanol treatment without transplantation. TUNEL-positive cells increased to 1% in LPG and 3% in FPG. This small increase in apoptosis in FPG was attenuated by suramin. Cleaved caspase-3 was barely detectable after sham-operation, increased modestly in LPG, and overtly in FPG, confirming the
occurrence of apoptosis (Fig. 2C). Suramin-treatment blunted the activation of caspase-3. Together, these data demonstrate that cell death increased more in FPG, with necrosis being the predominant form of cell death over apoptosis. Suramin attenuated both necrosis and apoptosis.

Graft injury was also assessed by the ALT release into the blood. Serum ALT levels were ~90 U/L in rats after sham operation (Fig. 1D). ALT increased to ~890 U/L after transplantation of LPG and further increased to ~2200 U/L after transplantation of FPG, indicating more severe liver injury. Suramin treatment decreased ALT in rats with FPG to ~920U/L (Fig. 1D).

**Suramin Improves Regeneration of FPG after Transplantation.** Liver regeneration is crucial for graft survival and recovery of liver function after partial LT. BrdU incorporation was used to evaluate the entry of hepatocytes into the S-phase of the cell cycle. BrdU-positive cells were ~0.1% in sham-operated lean livers and increased to 28% in LPG (Fig. 3A and B). In contrast, BrdU labeling was only 2% in FPG and suramin increased BrdU incorporation in FPG to ~43% (Fig. 3A and B).

Mitotic index (MI), the number of hepatocytes undergoing mitosis, was 0.1% in the livers from sham-operated rats. MI increased to 7% in LPG but remained at 0.2% in FPG (Fig. 3C). Suramin increased MI in FPG to 9% (Fig. 3C). Together, these data indicate that steatosis suppressed liver regeneration after partial LT and this effect was reversed by suramin.

**Suramin Improves Function and Survival of FPG after Transplantation.** Total bilirubin was 0.06 mg/dL in rats that received saline and underwent sham operation (Fig. 4A). In rats that received LPG, bilirubin increased to 2.0 mg/dL at 48 h after transplantation. In FPG recipients, however, bilirubin increased to 9 mg/dL and suramin decreased serum bilirubin to 2.3 mg/dL (Fig. 4A).
All rats survived after sham operation (data not shown). In contrast, all FPG recipients died in the first 5 days after transplantation. Suramin increased survival of FPG recipients to 62% (Fig. 4B).

**Suramin Decreases TNFα Formation and Caspase-8 Activation after Transplantation of FPG.** TNFα activates death receptor-mediate signaling pathways and causes mitochondrial dysfunction, leading to cell death (Malhi, et al., 2006). On the other hand, TNFα is a mitogen for cultured hepatocytes (Riehle, et al., 2011). Therefore, we investigated TNFα expression after transplantation. TNFα mRNA was 0.003 and 0.005 A.U. in lean and fatty livers, respectively, without transplantation (Fig. 5A). TNFα mRNA increased to 0.013 A.U. in LPG and 0.028 A.U. in FPG after transplantation. Suramin treatment decreased TNFα mRNA in FPG to 0.015 A.U. (Fig. 5A).

TNFα causes caspase-8 activation via death receptors (Malhi, et al., 2006). Cleaved caspase-8 was barely detectable in sham-operated lean livers and remained low in sham-operated fatty livers and LPG after transplantation (Fig. 5B). Cleaved caspase-8 increased in FPG after transplantation and suramin blunted caspase-8 activation in FPG (Fig. 5B).

**Suramin Does not Increase EGFR Activation and Only Very Modestly Increases JNK Activation in FPG.** Suramin prevents suppression of liver regeneration in FPG (Fig. 3). Therefore, we investigated the effects of suramin on EGFR, ERK and JNK activation. Low levels of phospho-EGFR was detected in lean and fatty livers without transplantation. EGFR expression was similar in all groups before and after transplantation (Fig. 6A). Phospho-EGFR increased in LPG after transplantation (Fig. 6A and B), consistent with cell proliferation in these
grafts. Phospho-EGFR was slightly lower in FPG than in LPG and suramin did not increase EGFR activation in FPG (Fig. 6A and B).

JNK activation was shown to be involved in liver regeneration after liver resection and partial LT (Schwabe, et al., 2003; Zhong, et al., 2006). Therefore, we explored the effects of suramin on JNK activation. JNK1/2 expression was similar in all groups with or without transplantation (Fig. 6A). Low levels of phospho-JNK, mainly JNK2, was detected in lean livers without transplantation. Ethanol treatment increased JNK activation slightly. After transplantation, phospho-JNK2 increased markedly (~6-fold) in LPG but remained at low levels in FPG (Fig. 6A and C). Suramin did not statistically increase phospho-JNK2 (Fig. 6A and C). ERK1/2 is another signaling molecule that promotes liver regeneration. Phospho-ERK was barely detectable in FPG and suramin did not increase ERK phosphorylation (data not shown).

**Suramin Decreased TGF-β1/Smad Signaling in FPG.** TGF-β is a potent inhibitor of cell proliferation (Barnard, et al., 1990). Therefore, we investigated the effects of suramin on TGF-β. TGF-β1 was barely detectable in sham-operated lean or fatty livers (Fig. 7). After transplantation, TGF-β1 increased slightly in LPG and markedly in FPG. Suramin inhibited the increase of TGF-β1 in FPG (Fig. 7).

Biological effects of TGF-β are predominantly mediated by Smad signaling (Lonn, et al., 2009). Expression of Smad2/3 was not different among groups with or without transplantation (Fig. 7). Phospho-Smad2/3 was barely detectable in lean or fatty livers without transplantation and increased slightly in LPG. Phospho-Smad2/3 increased to a much higher level in FPG, indicating Smad2/3 activation (Fig. 7). Suramin decreased phospho-Smad2/3 in FPG to the level of LPG.
Antiproliferative effects of TGF-β in some cell types are mediated by cyclin-dependent kinase (CDK) inhibitors (Yue and Mulder, 2001; Robson, et al., 1999). p21Cip1, a universal inhibitor of CDKs, was undetectable in lean and fatty livers without transplantation (Fig. 7). After transplantation p21Cip1 increased slightly in LPG and markedly in FPG. Suramin blunted the increase of p21Cip1 in FPG.

**Suramin Did Not Decrease Oxidative Stress in FPG.** Oxidative stress plays an important role in fatty liver graft and small-for-size graft failure (Zhong, et al., 1997; Zhong, et al., 2005); therefore we investigated the effects of suramin on 4-HNE adduct formation, an indicator of lipid oxidation. In the livers of sham-operated rats, only weak 4-HNE-positive bands were detected (Fig. 8). After ethanol treatment alone or transplantation of LPG, multiple 4-HNE-positive bands increased, indicating formation of 4-HNE protein adducts. After transplantation of FPG, 4-HNE protein adducts increased to a markedly higher level than in LPG (Fig. 8). Suramin did not blunt the increases of 4-HNE adducts.
DISCUSSION

**Suramin Improves the Outcome of FPG Transplantation.** Hepatic steatosis, which is observed in 26% to 50% of potential liver donors (Rinella, et al., 2001; Garcia Urena, et al., 1998), increases primary graft non-function after LT. Partial LT is frequently performed in recent years to alleviate the severe shortage of donor organs (Hashimoto and Miller, 2008; Strong, 2006) but steatotic grafts are excluded for use in partial LT due to the fear of dual risks resulting from small graft size and steatosis (Heaton, 2003). Therefore, effective treatments are needed to improve the outcome of FPG transplantation. Steatosis is induced by multiple risk factors, including over-nutrition, obesity, diabetes mellitus, metabolic disorders, alcohol consumption, drugs, and viral hepatitis (Teli, et al., 1995). Westernized diets have a high fat content. Previous studies showed that high-fat diet-induced steatosis compromises the survival of partial LT (Morioka, et al., 2003; He, et al., 2010). In this study, we investigated the effects of acute alcohol-induced steatosis on the outcome of partial LT. Consistent with previous reports, acute ethanol treatment causes microvesicular steatosis mixed with macrovesicular fat droplets. Liver injury (ALT release, necrosis and apoptosis) was greater after transplantation of FPG than LPG (Figs. 1 and 2). Liver regeneration (BrdU and mitotic index) occurred in LPG but was suppressed in FPG (Fig. 3). Liver function (bilirubin) was poorer after transplantation of FPG than LPG (Fig. 4). Together, these data showed that acute alcoholic steatosis also compromises the outcomes of partial LT. Interestingly, suramin did not decrease steatosis but attenuated injury, stimulated regeneration and improved the function and survival of FPG (Figs. 1-4), providing a promising therapy for steatotic graft dysfunction after partial LT. Since suramin is a FDA approved drug and is currently used clinically for treatment of trypanosomiasis and
infections of *Onchocerca volvulus*, it could be easily translated for clinical use for transplantation compared to other new therapeutic reagents that require extensive preclinical studies and FDA approval.

**Suramin Prevents FPG Injury: the Role of TNFα.** The mechanism by which suramin decreases injury of FPG remains unclear. Although oxidative stress increases in FPG, suramin did not decrease oxidative stress (Fig. 8). Therefore, either suramin acts downstream of oxidant formation or through other mechanisms. Suramin inhibits death-receptor mediated apoptosis in vitro, possibly by decreasing caspase-8, 9 and 10 activation and preventing loss of mitochondrial membrane potential (Eichhorst, et al., 2004). Suramin also prevents TNFα-mediated fulminant liver damage caused by LPS and D-galactosamine (Eichhorst, et al., 2004). Another study showed that suramin suppresses NF-κB activation, toxic/proinflammatory cytokine production and liver damage including necrosis, apoptosis and inflammation after LPS and D-galactosamine exposure (Goto, et al., 2006). It is well known that TNFα production increases in fatty livers and obesity (Diehl, 2004; Byrne, et al., 2009). Kupffer cells (KCs), the hepatic resident macrophages, produce cytotoxic/inflammatory cytokines after I/R and LT (Boros and Bromberg, 2006; Thurman, et al., 1995). Activities of KCs increased in alcohol-induced fatty livers compared to lean livers after full-size LT (Zhong, et al., 1997). TNFα production also increases in small-for-size grafts after partial LT (Tian, et al., 2006). Steatosis and partial LT may additively or synergistically increase toxic/inflammatory cytokine formation. Indeed, TNFα formation increased to a markedly higher level in FPG than in LPG (Fig. 5) in this study. Suramin blocked TNF-α formation, and, in turn blocked downstream activation of TNF-α targets.
TNFα causes cell injury/death in multiple ways. TNFα activates death receptors, leading to activation of caspases and apoptosis (Malhi, et al., 2006). Moreover, TNFα upregulates cytotoxic iNOS expression through a NF-κB pathway (Holohan, et al., 2008). RNS modify proteins thus altering their functions. Increased RNS formation is associated with more severe graft injury, poorer graft function and decreased survival (He, et al., 2010; Liu, et al., 2012b; Shi, et al., 2010). Energy supply is essential for cell survival and function. TNFα and RNS are important triggers of onset of the mitochondrial permeability transition (MPT), a common pathway of cell necrosis and apoptosis due to failure of ATP production and mitochondrial cytochrome c release (Zoratti and Szabo, 1995; Zamzami, et al., 1996). Activation of caspase-8 leads to truncation and translocation of Bid to mitochondria which also triggers the MPT (Malhi, et al., 2006). Moreover, RNS inhibits cytochrome c oxidase and mitochondrial respiration (Anggard, 1994). Indeed, our recent study showed that iNOS expression and mitochondrial damage were markedly higher in non-alcoholic FPG than in LPG (He, et al., 2010). In this study, TNFα formation increased much higher in FPG than in LFG which was associated with higher caspase-8 activation (Fig. 5). Suramin blunted TNFα formation (Fig. 5) and decreased cell injury and death in FPG (Figs 1-3). Therefore, suramin attenuated FPG injury, at least in part, by decreasing TNFα formation.

**Suramin Improves Regeneration of FPG: The Role of TGFβ/Smad Signaling.** Liver regeneration is critical for recovery of liver mass and function after liver resection and partial LT. LPG retained the capability of regeneration (Fig. 3). By contrast, cell proliferation was suppressed in FPG (Fig. 4), similar to non-alcoholic FPG (He, et al., 2010). Suramin increased regeneration of FPG and improved their function (Fig. 3-4). These results are consistent with previous studies showing that suramin induces outgrowth, scattering, and proliferation of
cultured renal proximal tubular cells (Zhuang and Schnellmann, 2005) and enhances proliferation of renal tubular cells after I/R in vivo (Zhuang, et al., 2009).

Liver regeneration is tightly regulated by a variety of transcription factors, cytokines and growth factors (Riehle, et al., 2011). Cytokines (e.g., TNFα and IL-6) activate transcription factors and provide early signals triggering regeneration (Riehle, et al., 2011; Streetz, et al., 2000). However, suramin decreased mitogenic TNFα formation in FPG (Fig. 5). Growth factors, such as EGF family, stimulate cell-cycle progression (Riehle, et al., 2011; Michalopoulos and Khan, 2005). Binding of ligands to EGFR leads to activation of its downstream signaling pathways such as the ERK and PI3K pathways (Jorissen, et al., 2003). Inhibition of EGFR suppressed liver regeneration after partial LT (Liu, et al., 2012a). However, suramin did not increase EGFR activation in FPG (Fig. 6). Activation of JNK could increase expression of cyclin D1, which drives hepatocytes to enter the cell cycle (Nelsen, et al., 2001). However, JNK phosphorylation was lower in FPG and suramin only increased JNK activation very modestly (Fig. 6). Thus, it is unlikely that suramin improves regeneration in FPG primarily by increasing JNK activation.

Liver regeneration is controlled by a delicate balance of pro-regenerative and regenerative-inhibitory factors. TGF-β, the most potent growth inhibitory polypeptide of cell proliferation in many cell types (Barnard, et al., 1990), counterbalances the stimulatory effects of mitogens during liver regeneration (Bissell, et al., 1995). TGFβ1 is increased in small-for-size liver grafts (Zhong, et al., 2010) and suramin was recently shown to decrease renal TGF-β1 formation after glycerol-induced acute kidney injury (Korrapati, et al., 2012b). Therefore, we investigated the effects of suramin on TGF-β1 formation in FPG. Indeed, TGF-β1 increased to a much higher level in FPG than in LPG, and suramin blunted the TGF-β increases in FPG (Fig.
7). TGF-β elicits its biological effects primarily by the Smad signaling pathway. Activation of TGF-β receptors leads to phosphorylation of Smad2/3 which translocate into the nucleus and activate target genes encoding regulatory proteins for cell proliferation, differentiation and cell death (Lonn, et al., 2009). Indeed, p-Smad2/3 increased markedly in FPG and this effect was blunted by suramin treatment (Fig. 7).

Cell cycle progression is controlled by cyclins and protein kinase complexes of CDKs (Roberts, et al., 1994). In some cells, TGF-β up-regulates the CDKIs, (e.g. p15Ink4B, p27Kip1 and p21Cip1) (Hannon and Beach, 1994) which inhibit cyclin/CDKs, leading to cell cycle arrest. In this study, p21Cip1, a potent universal growth inhibitor which binds to and inhibits the activities of cyclin D-Cdk4/6, cyclin E-Cdk2, and cyclin A-Cdk2 (Sherr and Roberts, 1999), was much higher in FPG than in LPG and suramin treatment blocked the increase in p21Cip1 in FPG (Fig. 7). These data suggest that suramin improves regeneration of FPG, at least in part, by inhibiting TGFβ1/Smad/CDK inhibitor signaling. Whether suramin also affects other proteins, microRNAs, hormones and transcription factors that regulate cell proliferation remains to be studied. Since TGF-β also enhances apoptosis, suppressed TGFβ1 formation by suramin would also contribute to suppression of apoptosis in FPG (Fig. 5).

Energy status also affects liver regeneration after partial LT (Zhong, et al., 2006). ATP not only serves as the energy supply for regeneration, but also affects the signal transduction (Thevananther, et al., 2004). TNFα inhibits mitochondrial function (Zoratti and Szabo, 1995; Zamzami, et al., 1996). Therefore, protection of mitochondria (e.g. by decreasing TNFα) may also contribute to prevention of defective regeneration of FPG by suramin.
In summary, this study shows that acute alcohol-induced hepatic steatosis and partial LT synergistically increases liver injury and suppresses liver regeneration. Suramin decreases injury and improves regeneration of alcoholic FPG, most likely by suppression of the TNFα and TGF-β1 formation. Therefore, suramin could be a promising therapy to improve the outcome of transplantation of FPG caused by alcohol. There are many similarities in pathophysiology in alcoholic and non-alcoholic liver diseases. This study and our previous studies showed that both alcoholic and non-alcoholic steatosis compromises the outcome of partial liver transplantation. Whether suramin also improves the outcome of non-alcoholic FPG transplantation remains to be investigated.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Zhong, Schnellmann, and He.

Conducted experiments: He, Rehman, Shi and Krishnasamy.

Contributed new reagents or analytic tools: Zhong and Lemasters.

Performed data analysis: He, Rehman, Shi, and Zhong.

Wrote or contributed to the writing of the manuscript: Zhong, Schnellmann, and Lemasters.

The authors have no conflict of interests to declare.
REFERENCES


FOOTNOTES

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*: SH and HR contributed equally to the work.
FIGURE LEGENDS

**Fig. 1. Suramin Attenuates Injury of FPG.** Rats were gavaged with one dose of saline or ethanol (6 g/kg). Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). A: livers were collected at 16 h after saline or ethanol treatment (1st and 2nd panels) or 48 h after transplantation of FPG with or without suramin treatment (3rd and 4th panels) for Oil-Red-O staining to detect steatosis. Bar is 50 μm; B: livers were collected 48 h after sham-operation (Sham) or transplantation of LPG and FPG for H&E staining. Bar is 100 μm. Necrotic areas (C) were quantified by computerized image analysis of 10 random field per slide. Blood was collected at 48 h after transplantation for ALT measurement (D). a, p<0.05 vs sham-operated lean livers; b, p<0.05 vs sham-operated fatty livers, c, p<0.05 vs LPG, and d, p<0.05 vs FPG (n=4 per group).

**Fig. 2. Suramin Decreases Apoptosis of FPG.** Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). Livers were collected 48 h after sham-operation (Sham) or transplantation of LPG and FPG. Apoptosis was detected by TUNEL staining (A, red nuclear staining) and quantified by counting TUNEL-positive hepatocytes in 10 random fields under microscope using a 40x objective lens (B). a, p< 0.05 vs sham-operated lean livers; b, p< 0.05 vs sham-operated fatty livers, c, p<0.05 vs LPG, and d, p<0.05 vs FPG (n=4 per group). Cleaved caspase-3 (CC3) and actin were detected by immuoblotting and representative images are shown in C (n=4 per group).

**Fig. 3. Suramin Improves Regeneration of FPG.** Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). Livers were collected 48 h after sham-operation (Sham) or transplantation of LPG and FPG. 5-Bromo-2’-deoxyuridine (BrdU) incorporation was detected immunohistochemically (A). Bar is 100 μm. BrdU-positive and -
negative cells (B) as well as mitotic and non-mitotic cells (C) were counted in 10 randomly selected fields in a blinded manner. a, p<0.05 vs sham-operated lean livers; b, p<0.05 vs LPG, and c, p<0.05 vs FPG (n=4 per group).

**Fig. 4. Suramin Improves Function and Survival of FPG.** Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). Blood was collected at 48 h after sham-operation (Sham) or PLT for total bilirubin measurement (A). a, p<0.05 vs sham-operated lean livers; b, p<0.05 vs LPG, and c, p<0.05 vs FPG (n=4 per group). Rats were observed 7 days for survival after sham operation or transplantation of FPG (B). Difference is statistically significant as assessed by the Kaplan-Meier test (p<0.05, n=8 per group) between FPG with and without suramin-treatment.

**Fig. 5. Suramin Blunts TNFα Formation and Caspase-8 Activation in FPG.** Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). Livers were harvested 48 h after sham-operation (Sham) or PLT. TNFα mRNA was detected by qPCR (A). a, p<0.05 vs sham-operated lean livers; b, p<0.05 vs sham-operated fatty livers, c, p<0.05 vs LPG, and d, p< 0.05 vs FPG (n=4 per group). Cleaved caspase-8 (CC8) and actin were detected by immunoblotting and representative images are shown in B (n=4 per group).

**Fig. 6. Suramin does not Increase EGFR Activation and Only Very Modestly Increased JNK Activation in FPG.** Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). Livers were harvested 48 h after sham-operation (Sham) or PLT. Phospho-epidermal growth factor receptor (p-EGFR), epidermal growth factor receptor (EGFR), c-Jun N-terminal kinase 1/2 (JNK1/2), phospho-c-Jun N-terminal kinase 1/2 (p-JNK1/2), and actin were detected by immunoblotting. Representative images are shown in A (n=4 per group). Quantification of p-EGFR and p-JNK2 by densitometry is shown in B and C, respectively. a,
p<0.05 vs sham-operated lean livers; b, p<0.05 vs sham-operated fatty livers, and c, p<0.05 vs LPG. Differences of p-EGFR and p-JNK2 are not statistically significant between FPG with and without suramin-treatment (n=4 per group).

**Fig. 7. Suramin Decreases TGFβ/Smad/CDKI Signaling in FPG.** Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). Livers were harvested 48 h after sham-operation (Sham) or PLT. Transforming growth factor-β1 (TGF-β1), Smad2/3, phospho-Smad2/3 (p-Smad2/3), p21Cip1 and actin were detected by immunoblotting. Representative images are shown (n=4 per group).

**Fig. 8. Suramin Did Not Decrease 4-Hydroxynonenal Adduct Formation in FPG.** Suramin (Sura; 5 mg/kg, ip) was injected 3 h after partial liver transplantation (PLT). Livers were harvested 48 h after sham-operation (Sham) or PLT. 4-Hydroxynonenal (4-HNE) adducts and actin were detected by immunoblotting. Representative images are shown (n=4 per group).
Table 1: Real-Time PCR Primers

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>Primers</th>
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| TNF-α | Forward: 5'-CAGACCCTCACACTCAGATCATCTT-3'  
        | Reverse: 5'-CAGAGCAATGACTCCAAAGTAGACCT-3'  |
| HPRT  | Forward: 5'-TCGAAGTGTTGGATACAGGCCAGA-3'  
        | Reverse: 5'-TACTGGCCACATCAACAGGACTCT-3'  |

TNF-α, tumor necrosis factor; HPRT, hypoxanthine phospho-ribosyl-transferase
Fig. 1
Fig. 2
Fig. 3

(A) Images showing different treatments: Saline+Sham, Saline+PLT, Ethanol+PLT, Ethanol+PLT+Sura.

(B) Bar graph showing BrdU-positive cells:
- Sham: a
- PLT: a, c

(C) Bar graph showing Mitotic Index:
- Saline: a
- Ethanol: a, c
- Ethanol+Sura: b

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A

Total Bilirubin (mg/dL)

Saline  Saline  Ethanol  Ethanol+Sura

Sham

PLT

a,b

a,c

B

Survival Probability

Ethanol+PLT+SurA

Ethanol+PLT

Days

0  2  4  6

Fig. 4
Fig. 5
Fig. 6
<table>
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<tr>
<th></th>
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**Fig. 7**

- TGF-β1
- p-Smad2/3
- Smad2/3
- p21Cip1
- Actin
Fig. 8