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

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ABSTRACT

Steatotic grafts are excluded for use in partial liver transplantation (LT) because of the increased risk of primary nonfunction. 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, intragastric administration), reduced in size to $\sim 1/3$, and transplanted. Serum alanine aminotransferase (ALT) and total bilirubin levels as well as 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 $\sim 60\%$, 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 hours, and the mitotic index increased to 7% in LPG but was only 0.2% in FPG, indicating suppressed regeneration in FPG. Suramin increased BrdU incorporation and the 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 (TNF- α) mRNA was 2.2-fold higher in FPG than in LPG and was associated with activation of caspase-8 and caspase-3 in FPG. Suramin decreased TNF- α 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 TNF- α and TGF- β formation.

Introduction

Use of partial liver grafts and “extended criteria” organs is increasing due to the critical shortage of donor livers (Busuttill and Tanaka, 2003; Wertheim et al., 2011). 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 with nonsteatotic livers after transplantation (Sun et al., 2003;

Vetelainen et al., 2007; McCormack et al., 2011). 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, 2003). Alcohol consumption is also a well-known risk factor for causing steatosis, and many organ donors are accident victims where alcohol consumption was 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 (Kiuchi et al., 1999; Sugawara et al., 2001). 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). Because liver steatosis is highly associated

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ABBREVIATIONS: ALT, alanine aminotransferase; AU, arbitrary units; 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; 4-HNE, 4-hydroxynonenal adducts; HPRT, hypoxanthine phospho-ribosyl-transferase; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; LPG, lean partial grafts; LPS, lipopolysaccharide; LT, liver transplantation; NF- κ B, nuclear factor- κ B; p-EGFR, phospho-epidermal growth factor receptor; p-JNK, phospho-c-Jun N-terminal kinase; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α ; TUNEL, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling; UW solution, University of Wisconsin cold storage solution.

with obesity, potential donors with high body mass index are usually not evaluated for living liver donation (Trotter, 2001), and Rinella et al. (2001) have proposed that donors with more than 10% steatosis be excluded in living donor LT. 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 human immunodeficiency virus (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 a new use for this old drug. For example, recent studies have shown 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- κ B (NF- κ B) activation, and decrease toxic/proinflammatory cytokine formation (e.g., TNF- α and interleukin-6 [IL-6]) (Kharlamov et al., 2002; Eichhorst et al., 2004; Goto et al., 2006; Zhuang et al., 2009). Further, suramin has an antifibrotic effect in liver, kidney, and muscle (Li et al., 2009; Liu and Zhuang, 2011; 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 TGF- β formation (Korrapati et al., 2012b). Because 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 liver FPG.

Materials and 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 hours 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 elsewhere (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 hours.

Implantation was performed using the rearterialized 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 minutes during surgery, and implantation required less than 50 minutes total time. For sham operation rats, the abdominal wall was closed with a running suture 50 minutes 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 hours 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 ALT and Total Bilirubin. Blood samples were collected at 48 hours after implantation and serum was obtained by centrifugation and stored at –80°C until measurement. Serum ALT and total bilirubin levels 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.), and frozen sections were prepared and stained with Oil-Red-O 16 hours after ethanol treatment or 48 hours after implantation. At 48 hours 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 and 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 10 $\times$ objective lens. Necrotic areas were quantified by image analysis using an IPlab 3.7v software (BD Biosciences, Rockville, MD), as previously described elsewhere (Liu et al., 2012c). Mitotic cells were counted in 10 randomly selected fields under the light microscope, and the mitotic index was calculated.

Immunohistochemistry. Previous studies had shown that after partial LT the cell proliferation increased gradually over 24 hours and peaked at between 38 to 48 hours (Zhong et al., 2006). Therefore, in the present study we compared cell proliferation at 48 hours after partial LT. We injected BrdU (100 mg/kg, i.p.) 1 hour before liver harvest, and performed immunohistochemical analysis of BrdU to detect the 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 40 $\times$ objective lens, and the percentage of BrdU-positive cells were calculated. Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (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 Polymerase Chain Reaction. Quantitative polymerase chain reaction (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 μ g) using a Bio-Rad iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). We conducted qPCR using the primer sequences in Table 1 and a CFX96 Real Time-PCR Detection System (Bio-Rad Laboratories). The abundance of mRNA was normalized against hypoxanthine phospho-ribosyl-transferase (HPRT) using the $\Delta\Delta C_t$ method.

Immunoblotting. Proteins were detected by immunoblot analysis as previously described elsewhere (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), epidermal growth factor receptor (EGFR), phospho-EGFR (p-EGFR; GenWay Biotech, San Diego, CA), c-Jun N-terminal kinase-1/2

TABLE 1
Real-time PCR primers

mRNA	Primers
TNF- α	
Forward	5'-CAGACCCTCAGCTCAGATCATCTT-3'
Reverse	5'-CAGAGCAATGACTCCAAAGTAGACCT-3'
HPRT	
Forward	5'-TCGAAGTGTGGATACAGGCCAGA-3'
Reverse	5'-TACTGGCCACATCAACAGGACTCT-3'

TNF- α , tumor necrosis factor; HPRT, hypoxanthine phospho-ribosyl-transferase.

(JNK1/2), phospho-JNK (p-JNK), extracellular signal-regulated kinases-1/2 (ERK1/2), phospho-ERK (p-ERK), Smad2/3, phospho-Smad2/3, p21Cip1 (Santa Cruz Biotechnology, 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 overnight, respectively. Horseradish peroxidase-conjugated secondary antibodies were applied, and detection was by chemiluminescence (Pierce Biotechnology, Rockford, IL).

Statistical Analysis. Groups were compared using Kaplan-Meier test or analysis of variance plus a Student-Newman-Keuls post hoc test, as appropriate. There were eight transplantations per group in survival experiments and four transplantations per group for all other parameters. Data shown are mean $\pm$ S.E.M. $P < 0.05$ was considered statistically significant.

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 hours 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 pathologic 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 hours after partial LT decreased necrosis in FPG to control levels (Fig. 1, B and C).

Apoptosis was evaluated by TUNEL staining (Fig. 2, A 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 increased 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

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, i.p.) was injected 3 hours after partial liver transplantation (PLT). (A) Livers were collected at 16 hours after saline or ethanol treatment (first and second panels) or at 48 hours after transplantation of FPG with or without suramin treatment (third and fourth panels) for Oil-Red-O staining to detect steatosis. Bar is 50 μ m. (B) Livers were collected 48 hours after sham-operation (Sham) or transplantation of LPG and FPG for H&E staining. Bar is 100 μ m. (C) Necrotic areas were quantified by computerized image analysis of 10 random fields per slide. (D) Blood was collected at 48 hours after transplantation for ALT measurement. ^a $P < 0.05$ versus sham-operated lean livers; ^b $P < 0.05$ versus sham-operated fatty livers; ^c $P < 0.05$ versus LPG; ^d $P < 0.05$ versus FPG ($n = 4$ per group).

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

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 ~920 U/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. 3, A and B). In contrast, BrdU labeling was only 2% in FPG, and suramin increased BrdU incorporation in FPG to ~43% (Fig. 3, A and B).

The mitotic index, the number of hepatocytes undergoing mitosis, was 0.1% in the livers from sham-operated rats. The mitotic index increased to 7% in LPG but remained at 0.2% in FPG (Fig. 3C). Suramin increased the mitotic index 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 hours 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).

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

Suramin Decreases TNF- α Formation and Caspase-8 Activation after Transplantation of FPG.

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

TNF- α 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 p-EGFR was detected in lean and fatty livers without transplantation. EGFR expression was similar in all groups before and after transplantation (Fig. 6A). In LPG, p-EGFR increased after transplantation (Fig. 6, A and B), consistent with cell

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

proliferation in these grafts. In FPG, p-EGFR was slightly lower than in LPG, and suramin did not increase EGFR activation in FPG (Fig. 6, A 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 p-JNK, mainly JNK2, was detected in lean livers without transplantation. Ethanol treatment increased JNK activation slightly. After transplantation, p-JNK2 increased markedly (~6-fold) in LPG but remained at low levels in FPG (Fig. 6, A and C). Suramin did not statistically significantly increase p-JNK2 (Fig. 6, A and C). ERK1/2 is another signaling molecule that promotes liver regeneration. We found that p-ERK was barely detectable in FPG, and suramin did not increase ERK phosphorylation (data not shown).

Suramin Decreased TGF- β ₁/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- β ₁ was barely detectable in sham-operated lean or fatty livers (Fig. 7). After transplantation, TGF- β ₁ increased slightly in LPG and markedly in FPG. Suramin inhibited the increase of TGF- β ₁ in FPG (Fig. 7).

Biologic effects of TGF- β are predominantly mediated by Smad signaling (Lonn et al., 2009). Expression of Smad2/3

Fig. 5. Suramin blunts TNF- α formation and caspase-8 activation in FPG. Suramin (Sura, 5 mg/kg, i.p.) was injected 3 hours after partial liver transplantation (PLT). Livers were harvested 48 hours after sham-operation (Sham) or PLT. (A) TNF- α mRNA was detected by qPCR. ^a*P* < 0.05 versus sham-operated lean livers; ^b*P* < 0.05 versus sham-operated fatty livers; ^c*P* < 0.05 versus LPG, and ^d*P* < 0.05 versus FPG (*n* = 4 per group). (B) Cleaved caspase-8 (CC8) and actin were detected by immunoblot analysis, and representative images are shown (*n* = 4 per group).

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 (Robson et al., 1999; Yue and Mulder, 2001). 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, 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 (Garcia Urena et al., 1998; Rinella et al.,

Fig. 6. Suramin does not increase EGFR activation and only very modestly increased JNK activation in FPG. Suramin (Sura, 5 mg/kg, i.p.) was injected 3 hours after partial liver transplantation (PLT). Livers were harvested 48 hours after sham-operation (Sham) or PLT. (A) 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 ($n = 4$ per group). (B, C) Quantification of p-EGFR and p-JNK2 by densitometry is shown in B and C, respectively. ^a $P < 0.05$ versus sham-operated lean livers; ^b $P < 0.05$ versus sham-operated fatty livers; ^c $P < 0.05$ versus FPG. Differences of p-EGFR and p-JNK2 are not statistically significant between FPG with and without suramin-treatment ($n = 4$ per group).

2001), increases primary graft nonfunction after LT. Partial LT has been frequently performed in recent years to alleviate the severe shortage of donor organs (Strong, 2006; Hashimoto and Miller, 2008), but steatotic grafts are excluded for use in partial LT because of 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 overnutrition, obesity, diabetes mellitus, metabolic disorders, alcohol consumption, drugs, and viral hepatitis (Teli et al., 1995). Westernized diets have a high-fat content. Previous studies have shown that high-fat diet-induced steatosis compromises the survival of partial LT (Morioka et al., 2003; He et al., 2010).

Fig. 7. Suramin decreases TGF- β /Smad/CDKI signaling in FPG. Suramin (Sura, 5 mg/kg, i.p.) was injected 3 hours after partial liver transplantation (PLT). Livers were harvested 48 hours after sham-operation (Sham) or PLT. Transforming growth factor- β 1 (TGF- β 1), Smad2/3, phospho-Smad2/3 (p-Smad2/3), p21Cip1, and actin were detected by immunoblotting. Representative images are shown ($n = 4$ per group).

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. Because suramin is a U.S. Food and Drug Administration (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 with other new therapeutic reagents that require extensive preclinical studies and FDA approval.

Suramin Prevents FPG Injury: The Role of TNF- α . 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 the loss of mitochondrial membrane potential (Eichhorst et al., 2004). Suramin also prevents TNF- α -mediated fulminant liver damage caused by LPS and D-galactosamine (Eichhorst et al., 2004). Another study showed that suramin suppresses NF- κ B 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 TNF- α production increases in fatty livers and obesity (Diehl, 2004; Byrne et al., 2009). Kupffer cells, the hepatic resident macrophages, produce cytotoxic/

Fig. 8. Suramin did not decrease 4-hydroxynonenal (4-HNE) adduct formation in FPG. Suramin (Sura; 5 mg/kg, i.p.) was injected 3 hours after partial liver transplantation (PLT). Livers were harvested 48 hours after sham-operation (Sham) or PLT. 4-HNE adducts and actin were detected by immunoblot analysis. Representative images are shown ($n = 4$ per group).

inflammatory cytokines after I/R and LT (Thurman et al., 1995; Boros and Bromberg, 2006). Activities of Kupffer cells increased in alcohol-induced fatty livers compared with lean livers after full-size LT (Zhong et al., 1997). TNF- α 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, TNF- α 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.

TNF- α causes cell injury/death in multiple ways. TNF- α activates death receptors, leading to activation of caspases and apoptosis (Malhi et al., 2006). Moreover, TNF- α up-regulates cytotoxic iNOS expression through a NF- κ B 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; Shi et al., 2010; Liu et al., 2012b). Energy supply is essential for cell survival and function. TNF- α 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 nonalcoholic FPG than in LPG (He et al., 2010).

In this study, TNF- α formation increased much higher in FPG than in LPG, which was associated with higher caspase-8 activation (Fig. 5). Suramin blunted TNF- α 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 TNF- α formation.

Suramin Improves Regeneration of FPG: The Role of TGF- β /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 nonalcoholic FPG (He et al., 2010). Suramin increased regeneration of FPGs 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., TNF- α and IL-6) activate transcription factors and provide early signals triggering regeneration (Streetz et al., 2000; Riehle et al., 2011). However, suramin decreased mitogenic TNF- α formation in FPG (Fig. 5). Growth factors, such as the epidermal growth factor (EGF) family, stimulate cell-cycle progression (Michalopoulos and Khan, 2005; Riehle et al., 2011). 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 proregenerative 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). TGF- β_1 is increased in small-for-size liver grafts (Zhong et al., 2010), and suramin was recently shown to decrease renal TGF- β_1 formation after glycerol-induced acute kidney injury (Korrapati et al., 2012b). Therefore, we investigated the effects of suramin on TGF- β_1 formation in FPG. Indeed, TGF- β_1 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 biologic effects primarily by the Smad signaling pathway. Activation of TGF- β receptors leads to phosphorylation of Smad2/3, which translocates into the nucleus and activates 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 that 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 TGF- β_1 /Smad/CDK inhibitor signaling. Whether suramin also affects other proteins, microRNAs, hormones, or

transcription factors that regulate cell proliferation remains to be studied. Because TGF- β also enhances apoptosis, suppressed TGF- β 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). TNF- α inhibits mitochondrial function (Zoratti and Szabo, 1995; Zamzami et al., 1996). Therefore, protection of mitochondria (e.g., by decreasing TNF- α) 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 increase liver injury and suppress liver regeneration. Suramin decreases injury and improves regeneration of alcoholic FPG, most likely by suppression of TNF- α and TGF- β 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 nonalcoholic liver diseases. This study and our previous studies have shown that both alcoholic and nonalcoholic steatosis compromises the outcome of partial liver transplantation; whether suramin also improves the outcome of nonalcoholic FPG transplantation remains to be investigated.

Authorship Contributions

Participated in research design: Zhong, Schnellmann, He.

Conducted experiments: He, Rehman, Shi, Krishnasamy.

Contributed new reagents or analytic tools: Zhong, Lemasters.

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

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

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