Inhibition of tumor necrosis factor alpha secretion and prevention of liver injury in ethanol-fed rats by antisense oligonucleotides

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Abstract

Elevated serum tumor necrosis factor alpha (TNF-α) levels predict mortality in patients with alcoholic liver disease. Administration of anti-TNF-α antibodies, obliteration of Kupffer cells or gut sterilization protect against ethanol-induced hepatocellular injury in animal models. In this study, we evaluated the in vivo efficacy of an antisense phosphorothioate oligodeoxynucleotide (S-ODN) targeted against TNF-α mRNA (TJU-2755). Naïve rats that were administered TJU-2755 (10 mg/(kg body weight (BW)/day) for 2 days) in the free form were challenged with LPS to induce TNF-α secretion. Antisense TJU-2755 treatment reduced serum TNF-α levels by 62%. A comparison of the efficacies of mismatched and random S-ODNs with that of TJU-2755 showed that some non-specific inhibition might accompany the sequence-specific effects of TJU-2755. To optimize the targeting of the S-ODN, TJU-2755 was encapsulated in pH-sensitive liposomes for in vivo delivery to macrophages. The efficacy of liposome-encapsulated TJU-2755 was assessed in ethanol-fed animals that were administered LPS to induce liver injury. Liposomal delivery of TJU-2755 allowed a much lower dose (1.9 mg/kg BW/day, for 2 days) of the S-ODN to reduce LPS-induced serum TNF-α (by 54%) and liver injury (by 60%) in ethanol-fed rats. These data indicate that liposome-encapsulated S-ODNs targeted against TNF-α have therapeutic potential in the treatment of alcoholic liver disease.

Keywords: Antisense; Liposomes; TNF-α; Ethanol; Lipopolysaccharide; Liver

1. Introduction

Tumor necrosis factor alpha (TNF-α) has been proposed as a mediator of liver injury in the setting of chronic alcoholism [1–3]. Increases in serum TNF-α levels are predictive of mortality in patients with alcoholic hepatitis [4]. Monocytes of patients with alcoholic hepatitis show NF-κB activation, higher TNF-α mRNA levels and increased TNF-α release [5]. Knockout mice for the TNF-α receptor-1 (TNFR1) do not develop alcohol-induced liver injury when fed alcohol chronically [6]. Further, anti-TNF-α antibodies reduce alcohol-induced liver damage in rats [7].

Isolated hepatocytes are not sensitive to the cytotoxic actions of TNF-α [8]. However, after ethanol exposure, primary cultures of rat hepatocytes and human HepG2 cells exhibit marked TNF-α-induced cytotoxicity [9]. The sensitization induced by chronic ethanol exposure is enhanced by transfection of CYP2E1 in HepG2 cells [9], most likely the consequence of an increased oxidative stress and activation of NF-κB. Sensitization of hepatocytes to TNF-α by ethanol may also result from marked increases in TNFR1 expression after ethanol exposure [10].

Yet another mechanism to account for the actions of TNF-α in ethanol-induced hepatic damage, is a TNF-α mediated increase in leukocyte adhesion receptor ICAM-1 [11], which facilitates the sequestration of leukocytes from sinusoids [12,13]. The livers of ethanol-fed rats show

increased levels of ICAM-1 [14,15]. TNF-α also stimulates collagen deposition by activation of stellate cells via TGF-β, a process that is associated with liver fibrosis [16].

TNF-α production in alcoholics is also enhanced by endotoxemia, leading to the stimulation of Kupffer cells [17] and monocytes [18] by gut-derived bacterial LPS. The latter enters the portal circulation due to an increased intestinal leakiness [19–22]. In rats, destruction of Kupffer cells, and possibly other macrophages, with gadolinium chloride prevents liver damage induced by chronic alcohol feeding [23]. Similar effects have been documented by treatment of rats with antibiotics, which are known to reduce intestinal microflora and endotoxin levels [24]. Recently, Zhou et al. [25] showed in a mouse model that oxidative stress is a key factor in the hepatic production of TNF-α induced by acute ethanol administration. An increase in the stability of TNF-α mRNA as a cause for LPS-induced increase in TNF-α production in Kupffer cells following chronic ethanol exposure has also been reported [26].

An approach to regulate TNF-α actions might be of therapeutic value in the treatment alcoholic liver disease. In recent years, antisense oligonucleotide technology has emerged as a therapeutic approach in the treatment of a wide variety of disorders [27–29]. An antisense oligodeoxynucleotide drug, Vitravene®, (ISIS Pharmaceuticals, Carlsbad, CA) has been approved by the FDA for the treatment of cytomegalovirus (CMV) retinitis and there are over 20 antisense clinical trials undergoing for a variety of disorders (see www.clinicaltrials.gov). Our laboratory has developed a series of phosphorothioate oligodeoxynucleotides (S-ODNs) targeted against TNF-α mRNA which suppress LPS-induced production of TNF-α in rat Kupffer cells in vitro [30]. In the current study, we have assessed the in vivo efficacy of TJU-2755, the most effective S-ODN in vitro [30], in reducing plasma TNF-α levels in response to LPS administration, and the ex vivo release of TNF-α by liver and spleen tissue. We show that TJU-2755 is a potent inhibitor of TNF-α production in vivo. We also report that some sequence independent inhibitory effects of S-ODNs on TNF-α synthesis in vivo may add to the therapeutic effectiveness of the antisense oligonucleotide.

A concern relating the use of S-ODNs in humans is their anti-clotting activity promoted by the multi negatively charged phosphate groups. In primates, these effects start at doses of 10 mg/kg but are absent at lower doses [31]. Therefore, an approach to preventing S-ODN interaction with anticlotting factors (e.g. antithrombin III) may be beneficial. We have previously shown that TJU-2755 can be encapsulated in pH-sensitive liposomes, a formulation which primarily targets Kupffer cells and other macrophages [32,33]. The present studies show that pretreatment of rats fed alcohol chronically with liposome-encapsulated TJU-2755 markedly reduced LPS-induced liver damage when administered at 1.9 mg/kg, thus indicating a therapeutic potential of liposome-encapsulated S-ODNs against alcoholic liver disease.

2. Material and methods

2.1. Chemicals

Cholesterol and cholesteryl hemisuccinate (CHEMS) were purchased from Sigma Chemicals Co. Phosphatidyl ethanolamine (PE) (transphosphatidylated from egg lecithin) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). HPLC-purified S-ODN TJU-2755 (5′-TGATCCACTCCCCCCTCCACT-3′; $T_m = 68$ °C against its mRNA target) was synthesized by Avecia LSM (Milford, MA). Three other 'control' S-ODNs were also synthesized: *M4*, four mismatches of TJU-2755 (5′-T*C*ATCC*T*CT*G*CCCCC*A*CCACT-3′; maximum relative $T_m = 56$ °C); *M5*, five mismatches of TJU-2755 (5′-TGA*GG*CACT*ACC*A*G*CTCCACT-3′; maximum relative $T_m = 50$ °C) and *MX*, a random S-ODN (5′-CCTTGTTCCCTTCTCCAGCTG-3′; maximum relative $T_m = 18$ °C); i.e. non-hybridizing at body temperature. Control S-ODNs were synthesized either by Avacia LSM or by TriLink BioTechnologies. LPS was purchased from the Sigma Chemical Company. All other chemicals used were of reagent grade, purchased either from Sigma or from Fisher Scientific.

2.2. Animals

Male Lewis rats were purchased from Harlan Sprague–Dawley Inc. All animals were acclimatized for at least 1 week following their arrival at our facility. For testing the in vivo efficacy of S-ODNs in naive animals, male rats in the body weight (BW) range of 300–325 g were routinely used. For studies on ethanol-fed animals, male rats (starting at 125–130 g) were pair-fed control and ethanol-containing Lieber-DeCarli liquid diets (BioServ) as described elsewhere [34,35]. The relative proportion of total calories in the ethanol-containing diet was 18% protein, 35% fat, 11% carbohydrate and 36% ethanol. The control group had a similar diet composition except that carbohydrates (maltose-dextrin) replaced ethanol. Littermate rats were maintained on the respective diets for 8–10 weeks. The daily intake of ethanol averaged 12–14 g/kg BW. At the end of the feeding period, the body weights of ethanol-fed rats averaged between 275 and 325 g and were within 90–95% of their pair-fed control animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University, Philadelphia which is an AAALAC-accredited facility.

2.3. In vivo administration of S-ODN and LPS treatment in naïve rats

The in vivo efficacy of the S-ODNs was initially tested in naive animals. Unless indicated otherwise, two daily doses (10 mg/(kg BW/day) on two consecutive days) of the S-ODN dissolved in PBS were injected intravenously via the tail vein.

After 24–48 h of the second dose of S-ODN, the rats were intravenously administered a test dose of LPS (50 μg/kg BW in 0.7–1 ml of saline) and sacrificed 90 min later. At the time of sacrifice, animals were anesthetized (Ketamine, 100 mg/kg BW; Xylazine, 6 mg/kg BW). Venous blood was then collected in a tube and later centrifuged to obtain serum, and stored at −80 °C until analysis of TNF-α. Where applicable, the liver and spleen were removed for the preparation of slices as described below.

2.4. Preparation and incubation of liver and spleen slices

In order to determine the ex vivo output of TNF-α from liver and spleen, the organs of PBS- or TJU-2755-treated rats were taken 90 min after LPS administration. Liver slices were prepared using a recessed glass guide and cultured according to the procedure described by Videla and Israel [36] with modifications. Briefly, liver slices (approximately, 10 mm × 4 mm × 0.4 mm; middle lobe) or splenic slices (6 mm × 4 mm × 0.4 mm) were quickly prepared and rinsed in ice-cold William's E medium to remove residual blood components. Slices (two slices per dish) were transferred to polycarbonate flasks containing 8 ml of fresh medium (90% William's E medium equilibrated with 18% oxygen, 5% CO_2 and balance N_2, and containing 10% fetal bovine serum, 2 mM bezamidine, 2 mM glutamine and 10 mM Hepes buffer, pH 7.4), and incubated at 37 °C for 60 min. Aliquots of the medium were taken at the beginning and at end of 60 min incubation. The media were stored at −80 °C for assay of TNF-α.

2.5. Preparation of S-ODN-encapsulated pH-sensitive liposomes

Liposomes encapsulated with S-ODN were prepared by the reverse phase method originally described by Szoka and Papahadjopolous [37] with modifications [32]. Briefly, a mixture of lipids (PE, CHEMS and cholesterol in a molar ratio of 7:4:2) were dissolved in 4.5 ml of chloroform. The total lipid content of the mixture was 25 mg (16.23 mg PE; 6.27 mg CHEMS and 2.5 mg cholesterol). For S-ODN encapsulation, 25 mg of TJU-2755 were dissolved in 0.4 ml of Tris–EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8) and diluted to 1.5 ml with a hypotonic buffer (made up of 1:9 diluted phosphate buffered saline (PBS) supplemented with 25 mM sodium phosphate, pH 7.4). The aqueous S-ODN solution was added to the lipid mixture in chloroform and sonicated in a bath-type sonicator for 5 min. The organic solvent was evaporated at room temperature using a rotary-type evaporator attached to an automatic pressure gauge (BUCHI Rotovapor R-134, Flawil, Switzerland). The resultant liposomal suspension was diluted with the hypotonic buffer and centrifuged at 100,000 × g for 45 min to separate the liposomes from the medium. The pellet was washed twice with PBS and resuspended in a volume not exceeding 1 ml of PBS.

Control ("empty") liposomes were prepared without S-ODN but using the same proportion of buffers and lipid mixture. To determine the concentration of encapsulated S-ODN, an aliquot of the liposomal preparation was first treated with a mixture of chloroform and methanol (1:1, v/v) and then diluted with chloroform to extract the lipid. An equal volume of TE buffer was added, vortexed and centrifuged to separate the upper aqueous layer. The amount of S-ODN that was extracted in the aqueous layer was quantified spectrophotometrically at 260 nm. The amount of S-ODN encapsulated by the liposomes ranged from 10 to 14% of the total S-ODN taken for encapsulation.

2.6. LPS administration and assessment of liver damage in ethanol-fed rats

Prior to testing the in vivo efficacy of liposomal TJU-2755, the model of LPS-induced liver damage in ethanol-fed rats was established as described by Pennington et al. [38] with modifications. Briefly, rats fed the control and ethanol-containing liquid diets for 8–10 weeks were injected with sub-lethal doses of LPS (mixture of *Escherichia coli* Serotype 026:B6 and *Salmonella enteridities* 1:1, w/w) to induce liver damage. Animals were injected with the LPS mixture via the tail vein in doses ranging from 0.5 to 3.5 mg/kg BW, in a total volume of PBS not exceeding 1 ml as described above for naive rats. Diets were removed 4 h prior to injection of LPS and restored 2 h later. Animals were anesthetized as described earlier and sacrificed 24 h after LPS administration. Venous blood was collected for the determination of serum alanine aminotransferase (ALT) and a section of the liver from the main lobe of each animal was processed for histology as described below.

2.7. Liposomal TJU-2755 administration and assessment of LPS-induced liver damage in ethanol-fed animals

The in vivo efficacy against liver damage of liposome encapsulated TJU-2755 was assessed following intravenous injections of liposomes containing the S-ODN, or "empty" liposomes. Since the amount of the injected lipid (5 mg/300 g rat) present in "empty" liposomes does not alter LPS-induced TNF-α production [32], animals treated with "empty" liposomes were considered as the 'control' group. Where indicated, male Lewis rats maintained on the ethanol-containing diet for 8–10 weeks were injected with two daily doses of either empty or TJU-2755-encapsulated liposomes, in a volume of PBS not exceeding 1.0 ml. The intended dose of the S-ODN was 2 mg/kg BW. However, since the amount of the liposomal lipid injected was maintained nearly constant (15–17 mg/kg BW), and the encapsulation of the S-ODN varied from one preparation of liposomes to another (as indicated above, varying from 10 to 14%), the actual amount of TJU-2755 injected varied

slightly between animals (1.67–2.2 mg/kg BW averaging 1.9 mg/kg). After 48 h of the last liposomal injection, the animals were challenged with LPS (2 mg/kg BW). Diets were removed 4 h prior to injection of LPS and restored 2 h later as described above. Blood was drawn from tail vein 2 h after LPS injection for the determination serum TNF-α. Animals were anesthetized and sacrificed 24 h after LPS administration. Venous blood was collected for the determination of serum ALT levels and a section of the liver was processed for histology as described below.

2.8. Histology

After anesthesia and venous blood collection, a section of the liver from the main lobe was taken and fixed in 4% buffered-formalin for staining with hematoxylin and eosin and read blindly with regard to the treatment group. The severity of liver injury was determined semi-quantitatively using histology scores: (0) normal; (1) trace necrosis; (2) mild necrosis and (3) 5% necrosis.

2.9. TNF-α assay

TNF-α in serum samples and in media incubated with liver and spleen slices, were determined by ELISA using a Cytoscreen KRC3012 kit (Biosource) according to manufacturer's specifications.

2.10. ALT assay

Serum ALT levels were determined using the kit (Sigma Kit # 52) as per manufacturer's instructions.

2.11. Statistical analysis

For continuous measures, independent group *t*-tests were used for mean comparisons. For categorical measures (histology), Wilcoxon rank sum tests were used. Measures of ALT were transformed to the natural logarithm scale prior to analysis due to skewed data. For the LPS dose–response data, an adjustment for multiple comparisons was made based on methods of Sidak [39]. Two-sided tests are reported in all cases. *P*-values of <0.05 were considered significant.

3. Results

3.1. In vivo efficacy of S-ODN, TJU-2755

Fig. 1 shows the effect of TJU-2755 on LPS-induced TNF-α production in vivo. Rats were injected with two daily doses of TJU-2755 or PBS and serum TNF-α levels were measured 90 min after LPS, which was administered 24 or 48 h after the second dose of the S-ODN. In the absence of LPS treatment, TNF-α was not detectable in the
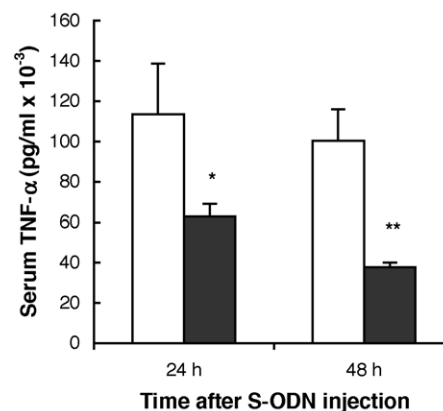

Fig. 1. Effect of TJU-2755 pretreatment on LPS-induced TNF-α secretion. Male Lewis rats were injected (i.v. via tail vein) two daily doses (10 mg/(kg BW/day) for two consecutive days) of TJU-2755 (S-ODN) (shaded bars) or an equivalent volume of PBS as control (open bars). After 24 and 48 h of the last injection, rats were intravenously administered LPS (50 μg/kg BW) and sacrificed 90 min later. Serum was collected and TNF-α assayed by ELISA. Bars indicate serum TNF-α levels (mean ± S.E.) from four to six pairs of animals. Animals of similar body weights receiving PBS (control) or S-ODN constituted a pair. *P < 0.05 and **P < 0.01 between control and S-ODN-treated animals. There was no detectable TNF-α in the serum in the absence of LPS treatment (not shown).

serum (not shown). Data in Fig. 1 show that pre-treatment of animals with TJU-2755 significantly reduced the LPS-induced production of TNF-α after TJU-2755 (10 mg/kg) administration; 45% inhibition (*P* < 0.05) at 24 h and 62% inhibition (*P* < 0.01) at 48 h. The effect of TJU-2755 at 5 mg/kg BW was less marked (37% inhibition at 48 h) and did not reach significance (data not shown). Therefore, in subsequent experiments with naive animals, "naked" TJU-2755 was used at the dose of 10 mg/kg BW.

3.2. S-ODN administration and TNF-α secretions by liver and spleen

Data in Fig. 2 show that pretreatment of rats with two daily doses (10 mg/kg) of TJU-2755 inhibited LPS-induced TNF-α secretion from both liver and spleen. In the liver, the inhibition was 37% (*P* < 0.05) and 48% (*P* < 0.01) at 24 and 48 h, respectively, after S-ODN treatment. In the spleen, the inhibition was 55% (*P* < 0.01) at 24 h and 25% not significant (N.S.) at 48 h. On a unit tissue weight basis, the amount of TNF-α secreted by spleen slices was four- to five-fold higher than by liver slices, reflecting the greater abundance of macrophages in the spleen.

3.3. Specificity of TJU-2755: effect of control S-ODNs on TNF-α secretion

To determine the specificity of TJU-2755, which targets the 3′-untranslated region of rat TNF-α mRNA [30], S-ODNs with increasing mismatches versus TJU-2755 were constructed as 'control' S-ODNs and tested for their in vivo
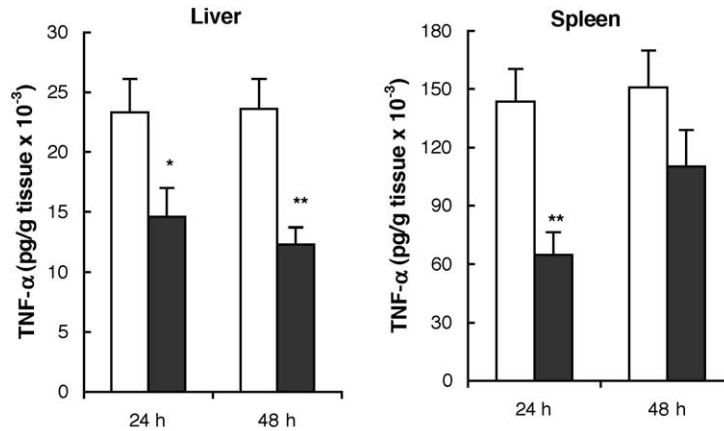
Fig. 2. Effect of TJU-2755 pretreatment on LPS-induced TNF-α secretion by liver and spleen ex vivo. Male Lewis rats were injected with two daily doses (10 mg/kg BW) of TJU-2755 (shaded bars) or an equivalent volume of PBS (open bars) and treated with LPS as described in legend to Fig. 1. Upon animal sacrifice (90 min post-LPS), liver and spleen slices were promptly prepared and incubated at 37 °C for 60 min as detailed in Section 2. TNF-α secreted into the medium was measured by ELISA. Bars represent TNF-α values (mean ± S.E.) from four to six pairs of control and TJU-2755-treated animals ([*]$P < 0.05$ and [**]$P < 0.01$).

effects. Data in Fig. 3 also show inhibition of LPS-induced TNF-α production by the 'control' S-ODNs. Among the 'control' S-ODNs, the highest inhibition (49%) was seen with the S-ODN with four mismatches. Intermediate inhibition (38%, N.S.) was observed with five mismatched bases and the lowest inhibition (25%, N.S.) occurred with a random S-ODN. The data are indicative of some non-specific effects of S-ODNs similar to that reported for other S-ODNs [40]. Nevertheless, since TJU-2755 was the most effective of the S-ODNs tested (62% inhibition), in the subsequent studies, TJU-2755 was used for testing the in
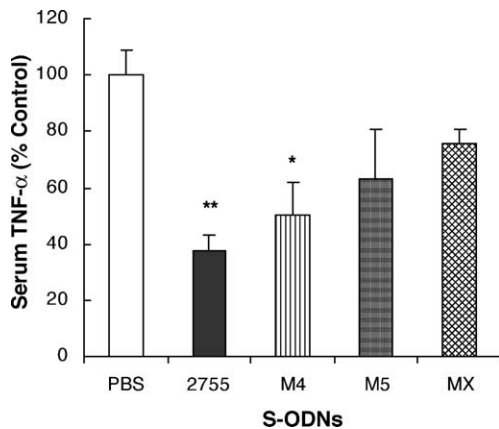

Fig. 3. Effect of mismatched- and random S-ODN-pretreatment on LPS-induced TNF-α secretion in vivo. Male Lewis rats were injected (i.v.) two daily doses (10 mg/kg BW) of TJU-2755 or various (21-mer) S-ODNs, M4 (four mismatches vs. TJU-2755), M5 (five mismatches vs. TJU-2755) and MX (random S-ODN) along with PBS controls. After 48 h of the second injection, rats were administered LPS (50 μg/kg BW) and sacrificed after 90 min. Serum was assayed for TNF-α by ELISA. Bars indicate serum TNF-α levels (mean ± S.E.) expressed as percent of the values from corresponding control animals of similar body weights that were treated identically except that they received an equal volume of PBS in place of S-ODN. With the exception of TJU-2755 ($n = 6$), data for all of the control S-ODNs are (mean ± S.E.) of three experiments, using a pair of (PBS and S-ODN-treated) rats per experiment ([*]$P < 0.05$ and [**]$P < 0.01$).

vivo efficacy against LPS-induced liver damage in ethanol-fed rats.

3.4. LPS-induced liver injury in ethanol-fed rats

In order to test the in vivo efficacy of TJU-2755, we first established the LPS-induced liver injury model in ethanol-fed rats. Rat's pair fed with the control or ethanol-containing liquid diets were injected with increasing doses of LPS and sacrificed 24 h later. Although test doses (50 μg/kg BW) of LPS do induce TNF-α secretion, higher doses are needed to induce liver injury [38]. Tissue injury was assessed by serum ALT determination and by liver histology. Fig. 4 shows the concentration dependence of LPS on serum ALT levels in ethanol-fed rats and pair-fed controls. As shown in Fig. 4, significant ($P < 0.01$) increases in serum ALT levels were observed in ethanol-fed rats at an LPS dose of 1 mg/kg; serum ALT activity increased with higher doses of LPS. Among the pair-fed control animals, there was a mild increase in ALT activity at the highest dose (3.5 mg/kg BW) of LPS tested. Liver histology for ethanol-fed animals that received LPS (2 mg/kg) showed foci of parenchymal necrosis with infiltration of polymorphonuclear leukocytes (Fig. 5A). In the severely affected animals, approximately 5% of the liver was necrotic. There was no necrosis in the livers of pair-fed control rats receiving an identical dose of LPS (Fig. 5B).

3.5. Liposomal-S-ODN and prevention of liver damage

In non-human primates, doses of S-ODN starting at 10 mg/kg inhibit blood coagulation [31]. Therefore, it was necessary to undertake studies aimed at specifically targeting macrophages, including Kupffer cells, to lower the dose as well as to avoid contact of the S-ODN with clotting factors present in blood. For this purpose, TJU-2755 was encapsulated in liposomes and administered
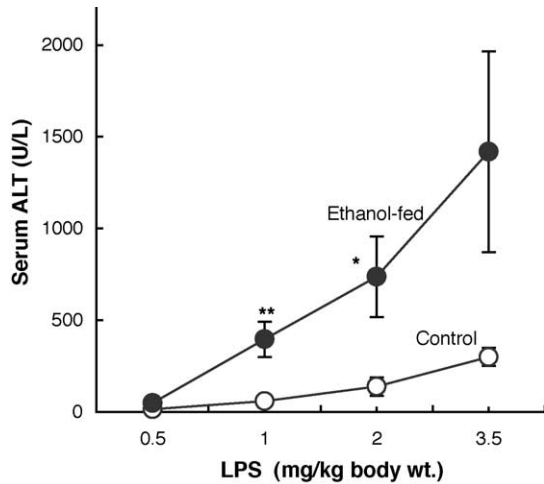
Fig. 4. Effect of LPS administration on serum ALT levels in control and ethanol-fed rats. Ethanol-fed (●) and pair-fed control (○) rats, maintained on respective diets for 8–10 weeks, were injected intravenously with various doses (0.5–3.5 mg/kg) of LPS as described in Section 2. Animals were sacrificed 24 h after LPS injection and serum ALT levels were determined. Each data point represents ALT values (mean ± S.E.) from three to six animals ($^*P < 0.05$ and $^{**}P < 0.01$).

Fig. 6. Effect of liposomally delivered TJU-2755 (S-ODN) on LPS-induced liver damage in ethanol-fed rats: rats fed the ethanol-containing liquid diet for 8–10 weeks were injected intravenously with two daily doses of liposomal preparations containing either TJU-2755 (average 1.9 mg/kg BW) or an equivalent volume of empty liposomes (control, see Section 2). After 48 h of the second dose, the diet was withdrawn for 4 h and the animals were injected intravenously with 2 mg/kg BW of LPS. The diet was reinstated 2 h post-injection and animals were sacrificed 24 h after the LPS challenge. Serum was collected for ALT determination (panel A) and liver was processed for histology (panel B). Data represent ALT values (mean ± S.E.) from $n = 10$ animals in (A) and $n = 6$ in (B) ($^*P < 0.05$ and $^{**}P < 0.01$).

intravenously [32,33]. Since pair-fed control rats did not show liver damage with LPS (Fig. 4), the protective effect of TJU-2755 was evaluated only in ethanol-fed rats. Liposome-encapsulated TJU-2755 was administered prior to the induction of liver damage with LPS as described in Section 2. The average dose of the S-ODN injected was 1.9 mg/kg BW (two daily doses). Animals of equivalent body weights that received ''empty'' liposomes were treated as the control group. As shown in Fig. 6, pre-treatment with TJU-2755 in the encapsulated form reduced LPS-induced liver damage by 60% ($P < 0.02$), as indicated by both reductions in ALT levels (Fig. 6A) and histology scores (Fig. 6B). Further, there was a proportional reduction in the levels of serum TNF-α in the TJU-2755-treated group compared to the empty liposome control group (Fig. 7).

4. Discussion

Liver damage resulting from chronic alcohol abuse may involve secondary insults such as endotoxemia and viral infections. The role of endotoxemia (LPS) in promoting alcohol-related liver injury has been demonstrated in a number of experimental models [12,19,20,24,41–43]. Among the proinflammatory stimuli, TNF-α is a key player in the cascade of events that results in liver injury [44]. Although antibodies against TNF-α have been shown to reduce TNF-α mediated liver damage [7] toxicity following chronic antibody administration limits the long-term use of antibodies [44,45]. Against that background, we undertook a study to test the in vivo efficacy of a new class of drugs, the S-ODNs. The potential of S-ODNs as future
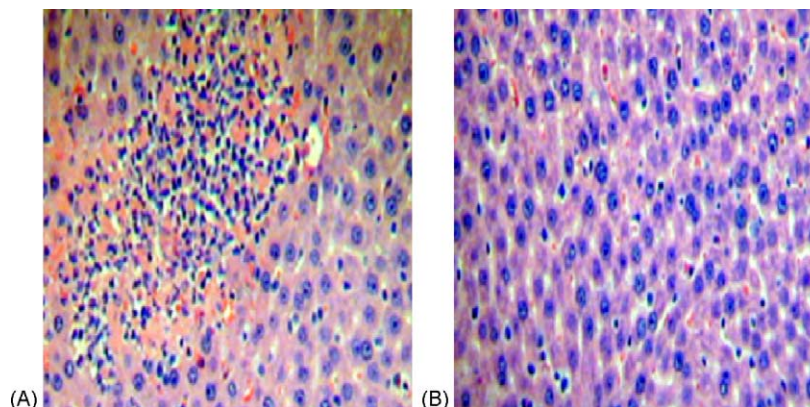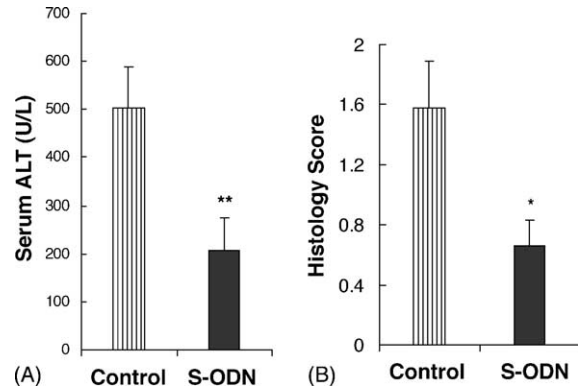

Fig. 5. Effect of ethanol feeding on LPS-induced liver damage; histology. Ethanol- and pair-fed control rats were challenged with LPS (2 mg/kg) for 24 h as described in Section 2. Liver was processed for light microscopy. (A) Ethanol-fed rats and (B) pair-fed controls. Note focal necrosis with infiltration by polymorphonuclear leukocytes in panel A. H&E staining, magnification ×400.
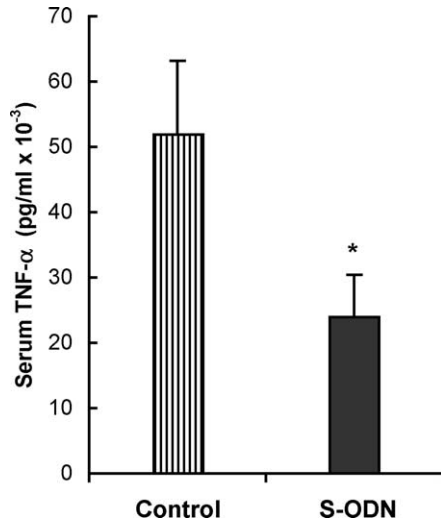
Fig. 7. Serum levels of TNF-α in ethanol-fed rats treated with TJU-2755. Rats were fed the ethanol-containing diet for 8–10 weeks were treated as described in legend to Fig. 6. After 2 h of LPS injections, blood was drawn from the tail vein and allowed to coagulate at room temp. Serum was collected and TNF-α determined by ELISA as described in Section 2. Data represent TNF-α values (mean ± S.E.) from four to six animals ($^*P < 0.05$).

therapeutic drugs against many of the diseases has been summarized elsewhere [27–29].

Our primary objective was to test the in vivo efficacy of the antisense TJU-2755, which had been shown to be most active in targeting TNF-α mRNA and in inhibiting TNF-α production by Kupffer cells in vitro [30]. Initially, we tested the efficacy of TJU-2755 in naive animals that were given a challenge dose of LPS after two daily doses of the S-ODN, injected in the free ('naked') form. TJU-2755 markedly reduced (62%) plasma TNF-α levels following LPS administration in vivo. Since Kupffer cells and splenic macrophages are the major producers of TNF-α following exposure to LPS [17], we compared the pattern of secretion of TNF-α by these organs ex vivo. As expected, TNF-α secretion from liver slices rather than spleen slices followed a similar pattern to that observed in serum. It should be noted that while there appears to be a higher density of macrophages in spleen, as shown by four- to five-fold higher TNF-α output per unit weight (Fig. 2) compared to liver, spleen is only a tenth of the liver weight. The fact that TNF-α production by spleen was also reduced by treatment with TJU-2755 suggests a possible role for the spleen in the pathogenesis of liver injury. Indeed, there are reports showing that splenectomy prevents LPS-induced liver damage in ethanol-fed rats [46], an observation confirmed by us (unpublished data).

Although earlier in vitro studies showed that the inhibitory effect of TJU-2755 is highly sequence-specific [30], present data demonstrate that factors exist in vivo that lead to an additional sequence-non-specific reduction in TNF-α generation or secretion by S-ODNs. In order to examine the sequence specificity of TJU-2755 in vivo, we compared

the efficacy of TJU-2755 versus mismatched and random-matched S-ODNs. These included S-ODNs with four or five mismatches and a random S-ODN. Although TJU-2755 was the most effective S-ODN (62% inhibition), there was considerable inhibition of TNF-α production by the control S-ODNs, with the highest inhibitory activity (49%) shown by M4, which had the least number of mismatches while the lowest inhibitory activity (25%) was observed with MX which had a totally unrelated sequence. It should be noted that the relative T_m of M4 and M5 (if nucleotides were contiguous) would be 56 and 50 °C, respectively, thus able to partly hybridize to TNF-α mRNA at 37 °C and activate RNase H, which hydrolyzes the RNA moiety in RNA–DNA hybrids. On the other hand, MX with a maximum relative T_m of 18 °C could not hybridize to the mRNA target, indicating a clear sequence-non-specific effect. These results bear resemblance to other reports [40,47], which cite some non-specific effects of S-ODNs. Fortuitously in this case, these effects add (rather than counteract) to the antisense effects of TJU-2755 on TNF-α production and might be of therapeutic value.

As discussed above, one of the non-specific effects reported for S-ODNs is the inhibition of blood coagulation [31]. Such an effect would be problematic if used in patients with alcoholic liver disease, in which coagulation is already compromised. Therefore, to prevent the contact of S-ODN with clotting factors, we used a liposome-based delivery system that was recently developed in our laboratory to target macrophages [32,33]. Our studies showed that the liposome-based delivery system attains a 20-fold higher concentration of S-ODN in Kupffer cells than that attained in hepatocytes [33]. A review of various aspects of targeting S-ODNs to Kupffer cells has been recently published [48]. Since the in vivo efficacy of liposome-encapsulated TJU-2755 has already been tested against TNF-α production in naive animals [32], we set out to use such a formulation to test the efficacy of this S-ODN against LPS-induced liver damage in ethanol-fed rats.

In order to test the in vivo efficacy of liposome-encapsulated TJU-2755 against LPS-induced liver damage, we used inbred Lewis rats. Both Wistar and Sprague–Dawley rats are outbred animals. Chronic ethanol feeding sensitizes rat liver to injury in response to LPS [13–15,25,38,41,42]. Our initial studies with Sprague–Dawley rats showed marked resistance and variability to the hepatotoxic effects of LPS, whether fed ethanol-containing or isocaloric carbohydrate diets (not shown). Although several other groups have reported an enhanced sensitivity of ethanol-fed animals to sub-lethal doses of LPS, the extent of liver damage have varied considerably [13,38,42]. The differences may be attributed to the strain of the animal, duration of ethanol feeding, treatment of the animals before and after LPS injection, the dose of LPS and the type of LPS used. In our study, to reduce variability of LPS effects, we used the inbred Lewis rats and the LPS administered was a 1:1 (w/w) mixture of *E. coli* and

S. enteriditis as reported by Pennington et al. [38]. For Lewis rats, good reproducibility and acceptable variability were found. However, considerable variation in the sensitivity to LPS-induced TNF-α production was observed between batches and between seasons. Among ethanol-fed animals, the variability of liver damage (ALT levels) also increased with increasing doses of LPS, suggestive of varying threshold levels for individual animals at higher doses.

In our studies, rats injected with 'empty' liposomes were treated as the 'control' group, since it was previously shown that LPS-induced TNF-α secretions were not affected by 'empty' liposome administration [32]. Liposome-encapsulated TJU-2755 reduced LPS-induced liver injury by 60%. The proportional reduction (54%) in serum TNF-α levels of animals injected with liposome-encapsulated TJU-2755 further suggests that the S-ODN reduces liver damage by lowering TNF-α secretion. The effect of liposome-encapsulated TJU-2755 in reducing TNF-α mRNA levels in vivo has already been shown in our previous studies [32]. It should also be noted that the stability of TJU-2755 encapsulated in liposomes exceeds 1 month at 0–4 °C [32], thus constituting an efficient and stable pharmaceutical preparation for S-ODN delivery to macrophages. Furthermore, liposomes prepared from naturally occurring lipids, are unlikely to elicit any immune response unlike TNF-α antibodies [44,45]. Additionally, anionic liposomes have been approved by FDA, and thus are an attractive delivery vehicle for targeting S-ODNs to macrophages.

In summary, data presented show that (i) an antisense phosphorothioate oligodeoxynucletide directed against TNF-α mRNA is highly effective in reducing LPS-induced liver injury in ethanol-fed animals, (ii) phosphorothioate oligodeoxynucletides also contribute in a sequence-non-specific manner to inhibit LPS activated TNF-α synthesis or its release into the serum and (iii) encapsulation of phosphorothioate oligodeoxynucletides in liposomes allows macrophage directed delivery and permits the use of lower doses, to achieve therapeutic effects. Further studies on the effectiveness of liposome-encapsulated anti TNF-α antisense oligonucleotides may pave the way towards testing the efficacy of this preparation in human subjects suffering from alcoholic hepatitis.

Acknowledgments

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