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CD14 and Lipopolysaccharide Binding Protein Expression in a Rat Model of Alcoholic Liver Disease

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Lipopolysaccharide-binding protein (LBP) and CD14 play key intermediary roles in the activation of cells by endotoxin. As endotoxin has been postulated to participate in promoting pathological liver injury in alcoholic liver disease, we investigated the role of LBP and CD14 in alcoholic liver injury. Rats were fed intragastrically ethanol or dextrose and either medium-chain triglycerides, corn oil, or fish oil for 4 weeks. Kupffer cells, endothelial cells, and hepatocytes were isolated. LBP and CD14 mRNA levels were measured in liver and individual cell types. The highest levels of LBP and CD14 mRNA levels in the liver were found in the fish oil/ethanol group, which was also the group with the greatest degree of pathological injury and inflammation. CD14 mRNA levels were also significantly elevated in groups fed unsaturated fatty acids with dextrose. CD14 expression was localized to the Kupffer cells and LBP expression to the hepatocytes. Expression of CD14 mRNA was also found in nonmyeloid cells in the two experimental groups (fish oil/ethanol and corn oil/ethanol) that had liver necrosis and inflammation. Our results suggest that enhanced LBP and CD14 expression correlates with the presence of pathological liver injury in alcoholic liver injury. Furthermore, unsaturated fatty acids may prime cells to respond to endotoxin by enhancing CD14 expression. ***(Am J Pathol 1998, 152:841–849)***

The mechanism(s) contributing to alcohol-induced liver injury remain uncertain. There appears to be increasing evidence, both in humans and experimental animals, that alcohol toxicity is associated with increased levels of endotoxin in plasma.[1–4] In rats fed ethanol by intragastric cannula, the levels of endotoxin in plasma correlate with the severity of liver injury.[2] Moreover, direct evidence for a pathophysiological role for endotoxin in alcohol-induced liver injury is provided by studies that show that a reduction in endotoxin levels ameliorates liver injury.[5,6] Endotoxin (lipopolysaccharide, LPS) is believed to exert many of its effects in the liver via interaction with the Kupffer cell. Support for this hypothesis is provided in studies in which inactivation of Kupffer cells with gadolinium chloride treatment ameliorates liver injury associated with alcohol ingestion.[7] Although the molecular mechanisms responsible for activation of Kupffer cells have yet to be defined, it is known that stimulation of the Kupffer cell by endotoxin triggers the release of a variety of mediators of inflammation such as tumor necrosis factor (TNF)-α, other pro-inflammatory cytokines, and reactive oxygen intermediates.[8,9]

Two glycoproteins are clearly implicated in the molecular and cellular basis of the interaction between endotoxin and macrophages.[10,11] The first, lipopolysaccharide-binding protein (LBP), present in normal serum, recognizes and binds LPS with high affinity through its lipid A moiety.[12,13] LPS-LBP complexes then activate cells through the second glycoprotein, membrane-bound CD14 (mCD14) to produce inflammatory mediators.[11,14,15] An important role for CD14 is suggested by studies in which transfection of human CD14 into 70Z/3 B cells (normally CD14 negative) increases sensitivity to LPS in the presence of LBP by 10,000-fold compared with 70Z/3 control, nontransfected cells.[16] Second, several reports have shown CD14 to be critical to the response of macrophages to low concentrations of LPS in the presence of LBP.[17–19] Furthermore, CD14 transgenic mice are extremely sensitive to the toxic effects of LPS,[20] whereas CD14 knock-out mice are much less sensitive.[21]

To evaluate the importance of LBP and CD14 in alcoholic liver injury, we used the intragastric feeding rat model for alcoholic liver disease.[22,23] This is an extremely useful model in which biochemical changes can be related to the severity of pathological liver injury. Based on epidemiological observations relating the type of dietary fat to alcoholic liver disease,[24] studies in the intragastric feeding rat model have shown that none of the histological features of alcoholic liver injury develop in rats fed ethanol and saturated lipid,[25] whereas fatty liver, necrosis, inflammation, and fibrosis are seen in rats fed ethanol and lipid enriched in polyunsaturated fatty acids.[26] Fur-

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Table 1. Fatty Acid Composition (Percentage by Weight) of Medium-Chain Triglyceride, Corn Oil, and Fish (Menhaden) Oil Diets

Fatty acid	MCT	Corn Oil	Fish Oil
Octanoic (C8:0)	52.5	0	0
Decanoic (C10:0)	33.2	0	0
Lauric (C12:0)	0.5	0	0
Myristic (C14:0)	0	0.1	11.6
Palmitic (C16:0)	1.4	11.3	13.0
Palmitoleic (C16:1 w7)	0	0	13.3
Stearic (C18:0)	1.0	1.9	2.1
Oleic (C18:1 w9)	1.0	25.5	6.7
C18:1 w7	0	0	3.3
Linoleic (C18:2 w6)	1.5	59.0	1.1
Arachidonic acid (C20:4 w6)	0	0	0.7
(C20:4 w3)	0	0	1.9
Eicosapentaenoic acid (C20:5 w3)	0	0	17.3
(C22:5 w6)	0	0	0.4
(C22:5 w3)	0	0	2.0
Docosahexenoic acid (C22:6 w3)	0	0	8.2

MCT, medium-chain triglyceride.

thermore, rats fed ethanol with fish oil develop more severe liver injury than rats fed ethanol with corn oil.[27,28] Thus, the use of these different types of dietary fats (saturated fat, corn oil, and fish oil) allowed us to study the relationship between the severity of pathological changes in ethanol-fed rats and the expression of LBP and CD14, two key intermediary proteins, which participate in the activation of cells by LPS.

Materials and Methods

Animals

The experimental animals were male Wistar rats weighing between 225 and 250 g. Six groups of rats (five rats/group) were studied. Three groups of rats were fed ethanol (E) and a diet containing either medium-chain triglyceride (MCT), corn oil (CO), or fish oil (FO) as the source of fat (25% of calories). The fatty acid composition of the diets is shown in Table 1.[29] Control animals received the same diet but with isocaloric amounts of dextrose (D) instead of ethanol (E). The rest of the diet was composed of 25% protein and variable concentrations of carbohydrates depending on the dose of ethanol fed. In the ethanol-fed rats, an initial dose of 8 g/kg/day was administered to the rats, representing an initial contribution of approximately 32% of the total calories. The ethanol concentration within the diet was gradually increased up to 16 g/kg/day to maintain blood alcohol levels between 150 and 350 mg/dl. All animals were fed by continuous infusion of liquid diet through permanently implanted gastric tubes as previously described.[22,24] All diets were prepared fresh daily. In particular, the fish oil diet was stored in airtight containers, filled with nitrogen, in a cold room at 4°C. Ethanol levels were measured after the maximal dose of alcohol was reached for each rat. Ethanol levels were measured twice per rat. The first blood draw occurred after peak ethanol dosing was achieved, and blood was withdrawn from the tail vein. Approximately 200 μl was withdrawn. The second blood draw occurred at the time of sacrifice. The animals were sacrificed after 1 month.

Eight- to nine-week-old Balb-c mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were injected intraperitoneally with either saline or LPS (*Escherichia coli* 0111; Sigma Chemical Co., St. Louis, MO), 20 ng/mouse. Whole livers were harvested for RNA and protein analysis at 3, 6, and 9 hours after injection. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

Histological Analysis

At the time of killing, a small sample of liver was obtained for histological examination. Liver tissue was fixed in formalin and stained with hematoxylin and eosin (H&E). The samples were examined under light microscopy by a pathologist who had no prior knowledge of the treatment groups. The pathology was evaluated as previously described[29]: for steatosis, 1+ = <25% of cells containing fat, 2+ = 26 to 50%, 3+ = 51 to 75%, and 4+ = >75%; for inflammation and necrosis, 1+ = one focus/lobule and 2+ = two or more foci/lobule. In addition, the number of inflammatory cells/mm^2 was also determined.

Isolation of Hepatocytes, Kupffer Cells, and Endothelial Cells

Liver cells were purified as previously described.[30,31] Briefly, after intravenous administration of sodium heparin, the livers were perfused with Ca^{2+}-free buffer containing 0.1 mol/L HEPES with 0.83% NaCl/0.05% KCl, pH 7.4. Livers were then excised, minced, and incubated with 0.05% collagenase in buffer (0.1 mol/L HEPES with 0.39% NaCl, 0.05 mol/L $CaCl_2$, pH 7.6) at 37°C for 60 minutes. The resulting suspension was pelleted and reincubated with fresh collagenase buffer for 30 minutes. The cell suspension was again pelleted and resuspended. Hepatocytes were removed by low-speed centrifugation (50 × *g* for 10 minutes, three times). The remaining cells were washed with Gey's balanced salt solution. Final purification was achieved by centrifugation in a 17.5% solution of metrizamide in Gey's balanced salt solution. This fraction contained approximately 65% Kupffer cells with the balance being liver endothelial and stellate cells. Kupffer cells were additionally purified by inculation in a 48-well tissue culture dish at 37°C in a humid atmosphere of 5% carbon dioxide for 2 hours. Adherent cells formed a monolayer on the culture dish; >85% of the cells were macrophages. The different cell types were identified by morphology and immunohistochemical markers that included peroxidase, acid phosphatase, α_1-antichymotrypsin, ED1, and cytokeratins. Endothelial cells were the nonadherent cells from the Kupffer cell preparation and were plated onto type I collagen-coated dishes. In the endothelial fraction, other inflammatory cells such as neutrophils and lymphocytes as well as stellate cells may cause contamination of this

fraction, but the majority of the cells are endothelial cells as assessed by immunohistochemical markers. The cells were stored at −70°C.

RNA Extraction, Polymerase Chain Reaction (PCR), and Northern Blot Analysis

Total RNA was isolated by standard methods with Trizol Reagent (Life Technologies, Inc., Grand Island, NY).[32] Reverse transcription (RT)-PCR was carried out as previously described.[33] The conditions for amplification were as follows: denaturation at 94°C for 1 minute, annealing at 57°C for 2 minutes, and extension at 72°C for 3 minutes for 30 cycles. The primers for rat LBP and β-actin were as previously published[34]: LBP, 5′ CAA ACT CTG CCA GTC ACA and 3′ GGA CAT TGG CAC CCA AGT; β-actin, 5′ GAT GGT GGG TAT GGG TCA GAA GGA and 3′ GCT CAT TGC CGA TAG TGA TGA CCT.

Because the sequence for rat CD14 has not been published, PCR primers were designed based on the sequence of mouse CD14 obtained from GenBank (accession number X13333): CD14, 5′ TGA GTA TTG CCC AAG CAC ACT and 3′ GTA ACT GAG ATC CAG CAC GCT.

The validity of the CD14 primer was tested on cDNA from normal whole rat liver. Using RT-PCR and 1 μg of starting total rat liver RNA as a template, RT and PCR were carried out as described above. The PCR product was approximately 373 bp in size, which is similar to that found in mouse tissues. The PCR product was subcloned into the TA cloning vector (Invitrogen Corp., San Diego, CA) and sequenced. Sequence comparison with mouse CD14 showed greater than 90% homology. The insert of this plasmid was later used as a cDNA probe for Northern blot analysis.

For the Northern blot analysis, 20 μg of total RNA was electrophoresed on a 1% agarose gel containing 3% formaldehyde before transfer to a Gene Screen membrane (Dupont NEN, Boston, MA). All of the cDNA probes were labeled with [^{32}P]dCTP with random priming. The cDNA probe for LBP was cloned from a rat acute-phase reaction liver library.[34] The cDNA probe for rat CD14 was obtained as described above. Hybridizations were carried out overnight at 43°C in a solution containing 50% deionized formamide, 0.25 mol/L sodium phosphate (pH 7.2), 0.25 mol/L sodium chloride, 1 mmol/L EDTA, 7% SDS, and 100 μg/ml denatured salmon sperm DNA. The hybridized filters were washed successively at 53°C in 2X SSC/0.1% SDS, $NaHPO_4$/1 mmol/L EDTA/0.1% SDS, and 25 mmol/L $NaHPO_4$/1 mmol/L EDTA/1% SDS. The blots were analyzed with autoradiography as well as a radioanalytic scanner (AMBIS 4000, Scanalytics Corp, Billerica, MA). All blots were stripped and reprobed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. The relative levels of steady-state mRNA was quantitated with the radioanalytic scanner and expressed as a ratio of LBP/GAPDH and CD14/GAPDH.

For the PCR reactions, PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining. A β-actin control was used concurrently with each cDNA template to validate the quality of the cDNA. An estimate of the signal size was determined with scanning densitometry, and the size of the signal was expressed as a ratio of either LBP or CD14 to β-actin. The value of the smallest signal for CD14 in the cell types was in the MCT/dextrose-fed rats; the signal intensity was arbitrarily set at 1.0. For endothelial cells and hepatocytes, the signal was present only in corn oil/ethanol- and fish oil/ethanol-fed rats, and therefore the signal intensity in corn oil/ethanol-fed rats was set at 1.0.

Western Blot Analysis

Livers from mice and RAW 264.7 cells (a mouse macrophage cell line used as a positive control for CD14 expression) were lysed with lysis buffer containing 1% Nonidet P-40, 5 mmol/L phenylmethyl sulfonyl fluoride, and 10 μg/ml leupeptin. The samples were separated by SDS-polyacrylamide gel electrophoresis with a 10 to 12.5% gel under reducing conditions using the methods of Laemmli.[35] Transfer was carried out electrophoretically by the methods of Towbin et al[36] to nitrocellulose (Schleicher and Schuell, Keene, NH). The membrane was probed with monoclonal rat anti-mouse CD14, rmC5-3, purchased from Pharmingen (San Diego, CA) followed by a horseradish-peroxidase-linked anti-rat Ig (Pharmingen, San Diego, CA). Detection was carried out with the ECL western blotting kit (Amersham International, Little Chalfont, UK).

Statistical Methods

Data were analyzed using analysis of variance and Fisher's protected least significant difference.

Results

Pathological Changes

In each of the groups studied, rats increased their body weight at a constant rate. Although the average weight gain was lower in ethanol-fed rats compared with controls, the differences between the various groups were not significant. There was also no significant difference in blood ethanol levels between the different ethanol-treated groups (range, 150 to 350 mg/dl). Each rat had two separate ethanol measurements. These measurements were made at two different time points after the maximal dose of alcohol was achieved.

The mean ± SE for the blood alcohol levels (mg/dl) in the different groups were MCT-E, 231 ± 46; CO-E, 219 ± 53; and FO-E, 229 ± 38. The pathological changes in the animals in the various groups are shown in Table 2. None of the rats in the dextrose-fed control groups or in the group of rats fed MCTs and ethanol developed pathological changes in the liver (Figure 1). The most severe liver injury was seen in fish oil/ethanol-fed rats (Figure 2). The number of inflammatory cells was significantly higher in the fish oil/ethanol group (mean ± SD, 24.4 ± 3.8) compared with the corn oil/ethanol group (5.0 ± 0.8; $P < 0.001$).

Table 2. Pathological Changes in the Different Experiment Groups

Group	Rat number	Fatty liver (0–4)	Necrosis (0–2+)	Inflammation (0–2+)	Inflammatory cells/mm^2
Corn oil and ethanol	1	4	1	1	4.9
	2	3	1	1	4.6
	3	2	0	1	4.0
	4	4	1	2	8.2
	5	2	1	1	3.4
Fish oil and ethanol	1	4	1	2	13.1
	2	4	2	2	36.1
	3	4	2	2	27.2
	4	3	2	2	19.8
	5	4	1	2	25.9

None of rats in the dextrose-fed control groups or rats fed medium-chain triglycerides and ethanol developed pathological changes in the liver.

Enhanced Expression of CD14 and LBP Correlates with Inflammatory Changes in the Liver

The livers of rats in the individual groups were examined for CD14 and LBP mRNA expression by Northern blot analysis (Figure 3). A fair amount of variation in LBP mRNA signal was observed on the Northern blot film, which represents both actual differences between the experimental animals as well as differences in loading. To correct for loading differences, the blots were all probed for GAPDH. As evidenced by the accompanying photograph of the ethidium-bromide-stained gel, the variations in GAPDH mRNA levels reflect variability in the loading of the gel. To correct for differences due to loading, the results in Figures 4 and 5 are expressed as a ratio of LBP or CD14 over GAPDH signal. The mRNA signals were measured by direct measurement of radioactivity using a radioanalytic scanner rather than by a relatively indirect method of measuring optical density of exposed film. The quantitative range of the former method is greater than the latter.

In the dextrose-fed rats, there was no significant difference in the levels of LBP mRNA between the different dietary groups (Figure 4). In contrast to the effects of fatty acids on LBP mRNA in dextrose-fed animals, the degree of saturation of fatty acids significantly influenced whole-liver CD14 mRNA levels. The levels of CD14 mRNA were significantly higher in the CO-D and FO-D groups compared with the MCT-D group ($P < 0.01$). This increase CD14 mRNA levels in the CO-D and FO-D groups was not associated with any pathological changes. Our own previous work as well as that of others have shown almost nondetectable levels of LBP and CD14 mRNA levels using Northern blot analysis in control animals fed a normal diet and sham-operated animals.[37–39]

Ethanol administration led to a significant increase ($P < 0.01$) in LBP mRNA levels only in the rats fed the fish oil diet. The levels of LBP mRNA in the FO-E group were significantly higher ($P < 0.01$) than the levels in MCT-E and CO-E groups. The highest level of CD14 mRNA was also seen in the fish oil/ethanol (FO-E)-fed rats (Figure 5). The levels of CD14 mRNA and LBP mRNA in general correlated with the number of inflammatory cells in the livers of the different ethanol-fed groups.

Increased Expression of CD14 mRNA in Kupffer Cells Correlated with the Presence of Necro-Inflammatory Changes

In a second set of experiments, different cell populations (Kupffer cells, endothelial cells, and hepatocytes) within the livers of each of the animal groups were isolated and examined for LBP and CD14 mRNA using PCR. LBP mRNA by PCR was identified in the hepatocytes of all the different animal groups. There was no difference in LBP mRNA in the dextrose-fed groups. The level of LBP

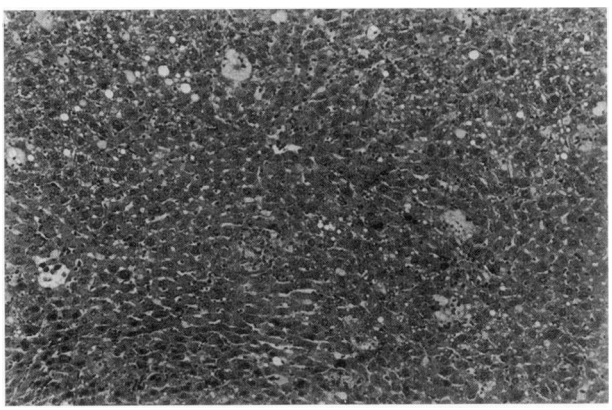

Figure 1. Liver from a rat fed medium-chain triglycerides and ethanol showing absence of pathological changes. H&E; magnification, ×155.

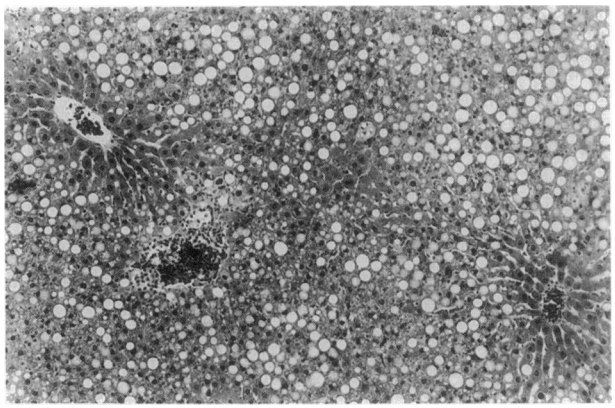

Figure 2. Liver from a rat fed fish oil and ethanol showing fatty liver, necrosis, and inflammation. H&E; magnification, ×155.

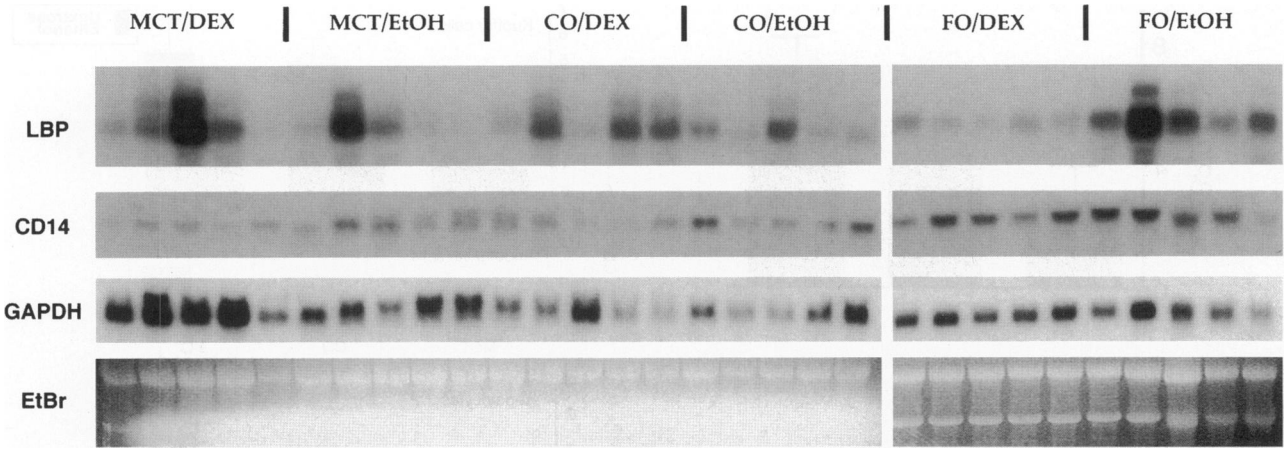

Figure 3. Northern blot analysis of LBP and CD14 mRNA in ethanol-fed rats. Total RNA (20 μg) from livers of rats in the different experimental groups was used to perform Northern blot analysis (see Materials and Methods). The blots were consecutively probed for LBP, CD14, and GAPDH. The first three panels represent the Northern blots after hybridization with the respective probes, and the fourth panel is the picture of the ethidium bromide (EtBr)-stained gel before transfer. The large area of ethidium staining in the upper blot is an artifact of the movement of ethidium from the bottom gel to the top gel, as the ethidium was added to the samples within each gel before electrophoresis. The variable amount of GAPDH mRNA within the different samples is a reflection of unequal loading as demonstrated by its correlation with the ethidium staining of the 25 S rRNA. To equalize for loading, all of the bands were analyzed with a radioanalytic scanner and results equalized for GAPDH. The quantitative data are shown in Figures 4 and 5.

mRNA in the hepatocytes reflected the levels seen in the whole liver by Northern blot analysis. CD14 mRNA was found in the Kupffer cells of all the dextrose- and ethanol-treated animals. Rats showing necro-inflammatory changes had relatively higher levels of CD14 expression in Kupffer cells. Interestingly, however, CD14 mRNA expression was also detected in the endothelial cells and hepatocytes of the animals exhibiting pathological injury, ie, CO + E and FO + E (Figure 6).

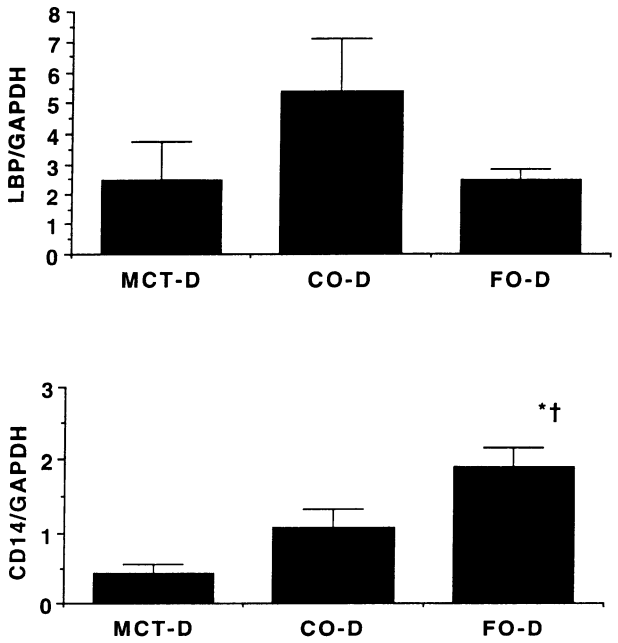

Figure 4. Expression of LBP mRNA and CD14 mRNA in the livers of rats fed dextrose and different dietary fats. There was no significant difference in LBP mRNA expression between the different groups. The highest level of CD14 mRNA was seen in the fish oil/dextrose-fed (FO-D) group (1.9 ± 0.24); this was significantly higher than that in corn oil/dextrose-fed rats (CO-D) (1.07 ± 0.28; $P < 0.05$) and medium chain triglycerides/dextrose-fed (MCT-D) rats (0.42 ± 0.14; $P < 0.01$). *$P < 0.01$ *versus* MCT-D; †$P < 0.05$ *versus* CO-D.

CD14 mRNA Expression Correlates with Protein Expression in Mouse Liver

Because there are no available antibodies for rat LBP or CD14, we were unable to confirm protein expression in the rat model. However, previous studies with human cells have suggested that LBP and CD14 mRNA levels correlate with protein expression. To confirm this finding in a rodent model, we were able to obtain rat anti-mouse CD14 antibodies and studied mRNA and protein expression in a mouse model of low-grade endotoxemia. To mimic the low-level endotoxemia that accompanies alcohol ingestion, we injected mice (n = 2/group) with either saline (control) or 20 ng of LPS per mouse and harvested whole livers at 3, 6, and 9 hours after LPS injection for both CD14 mRNA and protein levels. CD14 mRNA, as analyzed by Northern blot analysis, showed minimal baseline CD14 mRNA steady-state levels, which increased markedly after LPS injection and peaked at 3 hours (Figure 7). CD14 protein expression was examined by Western blot analysis with rat anti-mouse CD14 antibodies. CD14 protein correlated with mRNA levels with minimal baseline CD14 expression that markedly increased after LPS injection and peaked later than mRNA expression at 6 and 9 hours (Figure 8).

Discussion

The present study confirms our previous findings[25–28] that show that the type of dietary fat plays a significant role in determining the pathological effects of ethanol on the liver. Rats fed a diet containing MCTs and alcohol developed none of the histological features of ethanol-induced liver injury, whereas rats fed diets rich in the unsaturated fat (fish oil and corn oil) developed fatty liver, necrosis, and inflammation. Control rats fed the dextrose-containing diets showed no liver pathology. In this report,

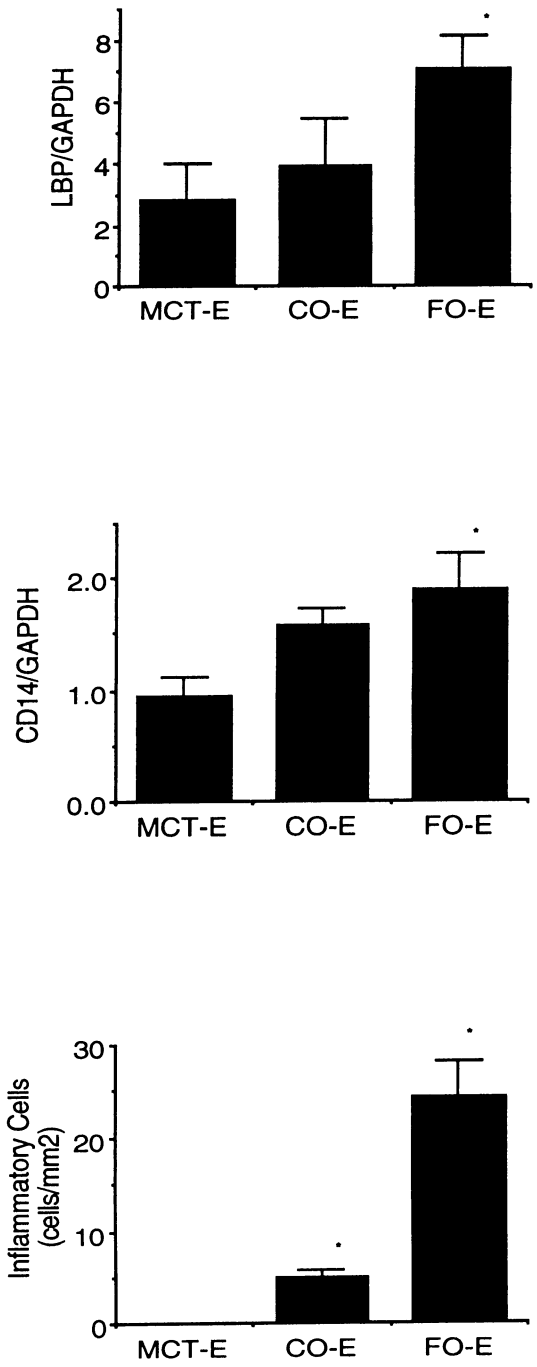

Figure 5. Expression of CD14 and LBP in relationship to the presence of inflammatory cells in the liver of rats fed ethanol and different fats. CD14 mRNA levels were highest in the fish oil/ethanol-fed (FO-E) rats (1.9 ± 0.32) and lowest in the MCT/ethanol-fed (MCT-E) rats (0.95 ± 0.16; $P < 0.01$). Similarly, the highest level of LBP mRNA was seen in the fish oil/ethanol-fed rats (FO-E; 7.1 ± 1.1). This level was significantly higher than the level in the MCT/ethanol-fed (MCT-E) rats (2.7 ± 1.2; $P < 0.05$). The levels of CD14 mRNA and LBP mRNA correlated with the number of inflammatory cells/mm^2 in the different groups. The greatest number of inflammatory cells were seen in the fish oil/ethanol (FO-E) group (24.4 ± 3.8), which was significantly higher than the number of cells in the corn oil/ethanol (CO-E) group (5.0 ± 0.8; $P < 0.001$) and MCT/ethanol (MCT-E) group (0.0; $P < 0.001$).

we have extended our investigation of the mechanisms involved in alcohol-induced liver injury. Endotoxin has been proposed to be one of the initiating events in the

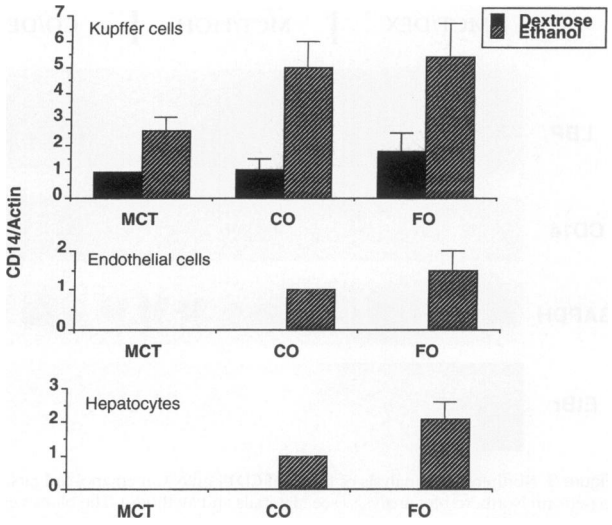

Figure 6. Evaluation of CD14 mRNA in the individual liver cell types in the different experimental groups. CD14 mRNA was measured by RT-PCR (see Materials and Methods) and the levels standardized using β-actin mRNA. CD14 mRNA was detected in Kupffer cells in all of the dextrose-fed groups and was significantly increased ($P < 0.01$) by ethanol in the corn oil and fish oil groups. CD14 mRNA was also detected in the endothelial cells and hepatocytes in the corn oil/ethanol and fish oil/ethanol-fed rats.

inflammatory response in alcoholic liver injury.[4] As several lines of evidence indicate that LBP and CD14 are important in mediating the effects of endotoxin,[11–13,15] the evaluation of these molecules in alcoholic liver injury was a logical extension of our previous studies, which show increased levels of plasma endotoxin and pro-inflammatory cytokines in the livers of ethanol-fed rats.[2,40] In the present study, the severity of pathological changes in ethanol-fed rats was mirrored by an increase in intrahepatic CD14 and LBP mRNA levels. We were unable to determine whether up-regulation of mRNA led to increased protein expression in this model, as antibodies to rat CD14 and LBP are not available. In this regard, it is important to note that previous studies in mice and humans have demonstrated evidence for transcriptional regulation of both these proteins.[37,41] Furthermore, we

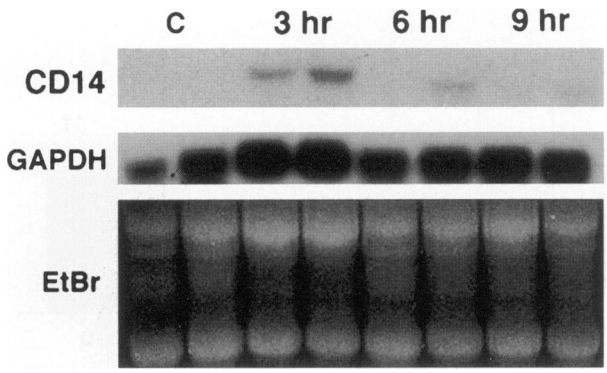

Figure 7. Northern blot analysis of CD14 mRNA from mice whole livers. Total RNA (20 μg) was extracted from mice 3 hours after saline injection (C) or at 3, 6, or 9 hours after LPS (20 ng/mouse, i.p.) injection. Each lane represents RNA from one mouse. Blots were probed sequentially with CD14 and GAPDH. The first two panels represent the Northern blots after hybridization with the respective probes, and the third panel is the ethidium-bromide-stained gel (EtBr) before transfer.

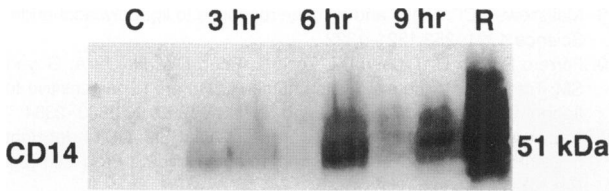

Figure 8. Western blot analysis of CD14 protein from mice whole livers. Whole livers from mice injected with either saline (C) or LPS and harvested at 3, 6, or 9 hours and RAW 264.7 cells (R) were lysed in lysis buffer before electrophoresis. RAW 264.7 cells, a mouse macrophage cell line, have previously been shown to express CD14 and are used as a positive control. Multiple isoforms of CD14 are detected around 50 kd in both the livers of LPS-injected mice and in RAW cells consistent with CD14 protein expression.

have demonstrated in another rodent model of low-grade endotoxemia that CD14 mRNA is increased in the liver after injection of extremely small doses of LPS and that this increase in CD14 mRNA levels is followed by an increase in CD14 protein expression. The increase in CD14 expression in whole mouse liver after LPS injection may represent either an increase in the expression of CD14 within cells that reside in the liver or may represent recruitment of inflammatory cells that have high expression of CD14. In either case, an increase of CD14 may result in greater sensitivity to LPS.

The increase in LBP and CD14 mRNA levels in the corn oil/ethanol- and fish oil/ethanol-fed rats is correlated with the degree of inflammation in the livers of these animals. The hepatic production of LBP, an acute-phase protein, is regulated by pro-inflammatory cytokines,[42,43] which are known to be increased in experimental models of alcoholic liver disease as well as in human alcoholic liver disease.[40,44,45] The role of LBP in the acute-phase response is unknown. Although it has been well established that the presence of LBP augments the response of cells to low, and thus physiologically important, levels of endotoxin (picogram to nanogram amounts), it is unclear what effects increased levels of LBP have on the systemic response to endotoxin. Furthermore, recent evidence suggests that LBP may have a dual role as a lipid transfer protein that can either augment the cellular response to LPS by transferring LPS to membrane CD14 or neutralize endotoxin by either transferring LPS to high-density lipoprotein or participating in the cellular clearance of LPS from the systemic circulation.[46–49] What role the increased levels of LBP plays in alcoholic liver injury is thus as yet unclear.

The increase in intrahepatic CD14 mRNA levels could be a result of either an increase in the number of infiltrating mononuclear cells or increased expression by Kupffer cells. Little information is available about the factors that regulate CD14 expression. Matsurra et al[37] demonstrated marked up-regulation of CD14 mRNA and protein expression by *in situ* hybridization and immunohistochemistry in the livers of mice injected with high doses of LPS. Others have shown that, in leukemia cell lines such as THP-1 and HL-60, CD14 expression is increased after differentiation is induced by vitamin D.[14]

In our studies using Northern blot analysis, CD14 mRNA was detected in the livers of rats fed a diet containing MCTs and dextrose; the CD14 mRNA level increased when rats were fed polyunsaturated fatty acids with dextrose. The livers of the animals fed dextrose were histologically normal. From previous studies, we have found that the dextrose-fed animals have no significant endotoxemia.[2,3] Thus, the inciting factor for CD14 up-regulation is unclear. By RT-PCR analysis, the CD14 mRNA in the dextrose-fed rats was localized to the Kupffer cells. In those rats demonstrating pathological liver injury (CO-E and FO-E), the increase in CD14 mRNA in the liver was localized to both Kupffer cells and non-myeloid cells, ie, endothelial cells and hepatocytes. Both groups of animals have previously been shown to have the highest levels of endotoxin in plasma.[50] Extramyeloid expression of CD14 has also been described by Fearns et al[51] in mice after LPS injection. Its functional significance is as yet unknown. We, however, wish to caution that part of the increased expression of CD14 in endothelial cells and hepatocytes may represent contamination of the cell fractions by Kupffer cells.

As CD14 expression can alter the cellular response to LPS, we would speculate that up-regulation of this LPS receptor by a diet of polyunsaturated fatty acids may contribute to ethanol-induced liver injury especially when ethanol administration leads to increased endotoxin levels. Thus, an increase in CD14 expression in Kupffer cells may be one mechanism by which sensitivity to endotoxin is increased in rats fed fish oil or corn oil. The mechanisms by which a diet rich in polyunsaturated fatty acids enhance the expression of CD14 within the liver is unknown and is currently being investigated. One proposed mechanism for endotoxin-mediated liver injury is that stimulation of cells by endotoxin through CD14 leads to NF-κB activation and production of pro-inflammatory cytokines.[52] An increase in pro-inflammatory cytokines has been found in rats fed ethanol and polyunsaturated fatty acids.[50] We have also recently shown that the highest level of NF-κB activation occurs in the livers of rats fed fish oil and ethanol.[53]

It is important to note that a CD14-independent pathway by which LPS activates Kupffer cells has been reported.[54] However, due to the lack of anti-rat CD14 and anti-rat LBP antibodies, the conclusion that a CD14-independent pathway exists is based on whether Kupffer cell activation by LPS is serum dependent or independent. Given that serum contains multiple factors, including sCD14 and high-density lipoprotein, which are known to antagonize LPS activation of macrophages, the results from these studies are somewhat difficult to interpret.[47,48,55] We have found in our laboratory that the effect of serum on Kupffer cell activation by LPS is very variable and depends very much on the source of serum (unpublished observations). Nevertheless, it is quite possible that a CD14-independent pathway of LPS activation of Kupffer cells does exist; however, the relative importance of CD14-independent and -dependent pathways in the cellular response of the liver to endotoxin remains to be defined.

In conclusion, we have shown that increased levels of LBP and CD14 mRNA are seen in rats demonstrating pathological liver injury. This observation provides one mechanism by which ethanol-induced endotoxemia in-

duces NF-κB activation and production of pro-inflammatory cytokines. The fact that polyunsaturated fatty acids can induce CD14 provides one additional explanation as to why these fatty acids promote alcoholic liver injury.

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