Lactoferrin protects against chemical-induced rat liver fibrosis by inhibiting stellate cell activation

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ABSTRACT

Liver diseases, which can be caused by alcohol abuse, chemical intoxication, viral hepatitis infection, and autoimmune disorders, are a significant health issue because they can develop into liver fibrosis and cirrhosis. Lactoferrin (LF), a siderophilic protein with 2 iron-binding sites, has been demonstrated to possess a multitude of biological functions, including antiinflammation, anticancer, and antimicrobial effects, as well as immunomodulatory-enhancing functions. In the current study, we induced hepatotoxicity in rats with dimethylnitrosamine (DMN) to establish a situation that would enable us to evaluate the hepatoprotective effects of LF against hepatic injury. Our results showed that DMN-induced hepatic pathological damage significantly decreased the body weight and liver index, increased the mRNA and protein levels of collagen α-1(I) (ColIα-1) and α-smooth muscle actin, and increased the hydroxyproline content. However, treatment with LF significantly increased body weight and liver index, decreased the mRNA and protein levels of Collα-1 and α-smooth muscle actin, and suppressed the hydroxyproline content when compared with the DMN-treated group. Liver histopathology also showed that low-dose LF (100 mg/kg of body weight) or high-dose LF (300 mg/kg of body weight) could significantly reduce the incidences of liver lesions induced by DMN. These results suggest that the LF exhibits potent hepatoprotection against DMN-induced liver damage in rats and that the hepatoprotective effects of LF may be due to the inhibition of collagen production and to stellate cell activation.

Key words: lactoferrin, liver fibrosis, dimethylnitrosamine, hepatic stellate cells

INTRODUCTION

According to statistics tabulated by the Department of Health in Taiwan, chronic liver disease and cirrhosis constituted the ninth leading cause of death in 2010. Recent studies show that alcohol abuse, chemical intoxication, viral hepatitis infection, and autoimmune disorders contribute to chronic liver fibrosis, which often advances to liver cirrhosis (Friedman, 2003; Kisselева and Brenner, 2006). Liver cirrhosis is generally irreversible, and treatment usually focuses on preventing progression and complications. In advanced stages of cirrhosis, the only option is a liver transplant.

Dimethylnitrosamine (DMN), a family of N-nitrosoamine compounds, is a potent hepatotoxin, carcinogen, and mutagen. Dimethylnitrosamine causes liver necrosis, fibrosis, and cirrhosis through the metabolic activation of cytochrome P450 2E1 (Guengerich et al., 1991). Activation of liver cytochrome P450 2E1 stimulates Kupffer cells to generate reactive oxygen species, thus leading to liver cell damage (Teufelhofer et al., 2005). The injured liver cells release several cytokines that cause further liver damage. Therefore, DMN-induced liver fibrosis presents a valuable animal model for studying the mechanisms of hepatic fibrosis that may facilitate the rapid screening of antifibrotic agents.

The DMN-induced liver fibrosis model closely resembles the development of liver damage in humans, which includes ascites, nodular regeneration, overproduction of the extracellular matrix (including collagen), histopathological manifestations, and biochemical alterations (George and Chandrakasan, 2000; Bataller and Brenner, 2005). Lee et al. (2013) also noted that DMN intoxication inhibited the growth of rats; damaged liver function; activated transforming growth factor beta 1
or Smad signaling; increased the expression of α-smooth muscle actin (α-SMA), matrix metalloproteinase-2, and collagen; and relieved hepatic fibrogenesis. Thus, the rat model of DMN-induced liver injury, which displays similar characteristics to human patients based on clinical observations, biochemical alterations, and histopathological determinations, has been studied to gain insight into the underlying mechanisms and to discover potential therapeutic interventions.

Lactoferrin (LF), an siderophilic protein with 2 iron-binding sites, is mainly found in exocrine secretions, such as breast milk, tears, saliva, and biliary tracts, with the highest concentration (5–7 mg/mL) being found in colostrum (Levay and Viljoen, 1995). Lactoferrin has been reported to have a wide range of biological activities, including antiinflammatory, anticancer, antimicrobial, antioxidant, and immunomodulatory-enhancing effects (Yamauchi et al., 1998; Hayashida et al., 2004; Ishikado et al., 2005). Lactoferrin has been demonstrated to have protective effects against cutaneous inflammation, colitis, and rheumatoid arthritis by inhibiting the levels of the proinflammatory cytokines tumor necrosis factor-α and IL-1β and stimulating the expression of the antiinflammatory cytokine IL-10 (Cumberbatch et al., 2000; Togawa et al., 2002). Lactoferrin has also been demonstrated to possess anti-inflammatory activity, as it can directly modulate cytokine production by immune cells, such as macrophages and lymphocytes, through receptor-mediated signaling pathways (Van Snick and Masson, 1976; Mazurier et al., 1989). Lactoferrin can also downregulate inflammatory responses by preventing iron-catalyzed free radical damage at inflammation sites (Chodaczek et al., 2007). In the current study, we induced hepatotoxicity in rats with DMN to establish a situation that would enable us to evaluate the hepatoprotective effects of LF against hepatic injury induced by DMN in rats.

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley rats (aged 8 wk and weighing 271 ± 12 g) were purchased from the Livestock Research Institute (Taipei, Taiwan), given a standard laboratory diet (Altromin, Lage, Germany) and distilled water ad libitum, and kept on a 12 h light-dark cycle at 21 to 27°C (Liu et al., 2013). This animal study was approved by the Institutional Animal Care and Utilization Committee of National Chung Hsing University (IACUC Approval No: 100-104). In the current study, we induced hepatotoxicity in rats with DMN (Sigma, St. Louis, MO) to evaluate the hepatoprotective effects of LF from bovine milk, the purity approximately 90% (Sigma; L9507). For the examination of both aspartate aminotransferase and alanine aminotransferase activities in the serum, 24 rats were randomly assigned to 4 groups (n = 6). The normal control group received 0.90% wt/vol NaCl by gastric gavage plus a 0.90% wt/vol NaCl injection. The DMN group received 0.90% wt/vol NaCl by gastric gavage plus a DMN injection. The low-dose LF (LF-L) group received oral administration of LF at 100 mg/kg of BW by gastric gavage (concentration = 100 mg of LF/1 mL of 0.90% wt/vol NaCl; volume = 1 mL of LF/1 kg of BW) plus a DMN injection. Finally, the high-dose LF (LF-H) group received oral administration of LF at 300 mg/kg of BW by gastric gavage (concentration = 300 mg of LF/1 mL of 0.90% wt/vol NaCl; volume = 1 mL of LF/1 kg of BW) plus a DMN injection. The DMN-induced hepatotoxic rats were injected i.p. with 10 mg/kg of DMN (3 consecutive days each week) for 4 wk, whereas rats in the normal control group were injected with 0.90% wt/vol NaCl only. Ochoa et al. (2008) observed no adverse effects of children that received 1 g of LF for 6 d/wk for 9 mo. Thus, in the current study, we simulate the diet of LF to treat the mice (300 mg/kg of BW of mice = 33.294 mg/kg of human = 33.294 × 30 mg/30 kg of children = 998.82 mg/30 kg of children). At the end of the experiment, BW, liver index (liver-to-BW ratio), and spleen index (spleen-to-BW ratio) were recorded. Accordingly, each rat was anesthetized, and the liver was immediately perfused with ice-cold 0.90% wt/vol NaCl, then carefully removed, rinsed in ice-cold 0.90% wt/vol NaCl, blotted dry, and weighed. All samples were stored at −80°C for further assays.

**High-Frequency Ultrasound Examination**

The animals fasted for 3 h before high-frequency ultrasound (HFU) scanning. During the surgical procedures, animals were lightly anesthetized with gas consisting of 0.5 to 1.0 L/min of oxygen-enriched air mixed with 2.0 to 2.5% isoflurane vapor. The animals were placed in supine positions and were able to breathe freely. After being anesthetized, each rat abdomen was shaved and further cleaned with a chemical hair remover to minimize ultrasound attenuation. For ultrasound imaging, the Vevo 770 micro-imaging system (VisualSonics Inc., Toronto, Canada) with a single element probe at a center frequency of 40 MHz was used for small animal examinations. The Vevo 770 ultrasound probe has a 40-MHz center frequency with a 6-mm focal depth, providing an axial resolution of 40 μm with a 14.6-mm field of view. Ultrasound gel was placed on the skin as a coupling fluid before using the transducer (Chen et al., 2012b).
Tissue Hydroxyproline

The hydroxyproline content in the liver tissue was measured as described by the method of Jamall et al. (1981). Briefly, the liver tissue was dehydrated in 95% alcohol for 5 to 6 h and defatted with acetone for 2 d. The defatted tissue was dried in an oven at 65°C for 2 d. The tissue was then added to a tube with HCl (6 M; according to the ratio 1 mL of HCl:100 mg of dried liver) and hydrolyzed at 121°C for 1 h. Sodium hydroxide (6 N) was added to hydrolysates that were neutralized and filtered to pH of 6 to 6.5. Then, 450 μL of 0.056 M chloramine T was placed in a new tube and left for 25 min at room temperature, followed by the addition of 500 μL of 1 M Ehrlich’s reagent and a reaction for 20 min at 60°C. Finally, the test tube was placed on ice for 5 min to stop the reaction. The absorbance was measured at 550 nm against a reagent blank using a spectrophotometer (DU650, Beckman Coulter Inc., Indianapolis, IN).

Pathological Histology Examination

Liver tissue was fixed in 10% buffered formaldehyde (pH 7.0), embedded in paraffin, sectioned into 3-μm sections, and processed for histological examination according to the following 3 types of histopathological stains: hematoxylin and eosin, Masson, and Sirius red (Chen et al., 2013; Shen et al., 2013).

Reverse Transcription PCR

Total RNA from liver tissue was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) as specified by the manufacturer. Total RNA (2 μg) was resuspended in 9 μL of diethylpyrocarbonate-treated water, and the first strand of cDNA was synthesized with random primers and the ImProm-II reverse transcriptase (Promega, Madison, WI) in a total volume of 20 μL. The reaction was carried out at 42°C for 1 h (Tung et al., 2013a). For further PCR amplification, an aliquot (1:10) of the reverse transcription (RT) product was adjusted to contain 0.1 μg of each primer, and additional buffer was added to a total volume of 20 μL. Aliquots of the reverse transcriptase mix were used for the PCR amplification of collagen α-1(I) (ColIα-1), α-SMA, TGF-β, TIMP-1, TNF-α, IL-6, and GAPDH. The amplified RT-PCR products were subjected to electrophoresis in a 1.5% agarose gel for 22 min. The cDNA of GAPDH was used as an internal control. The RT-PCR method was analyzed as described previously (Chen et al., 2012a; Wen et al., 2013).

Western Blot Analysis

The protein expression of liver tissue was measured by Western blot. Liver tissues were homogenized in 300 μL of radio immunoprecipitation assay (RIPA) buffer [5 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP40, 0.25% sodium deoxycholate, 5 mM EDTA, and 1 mM ethylene glycol-bis(2-aminoethyl-ether)-N, N, N, N-tetraacetic acid]. The homogenates were centrifuged at 12,000 × g for 30 min at 4°C. Protein (30 μg) was then separated by SDS-PAGE in 10% polyacrylamide and electrotransferred to polyvinylidene difluoride membranes. The membranes were incubated in blocking solution (5% BSA) at room temperature for 1 h. The membranes were then incubated with the primary antibody [ColIα-1, α-SMA, low-density lipoprotein receptor-related -protein-1 (LRP-1), LF, and β-actin] for 2 h. After washing, the membranes were incubated with a goat antirabbit or a goat antimouse IgG peroxidase-conjugated secondary antibody directed against the primary antibody for 1 h. The membranes were developed by an enhanced chemiluminescence Western blot detection system as described previously (Tung et al., 2011, 2013b).

Statistical Analysis

All results are expressed as mean ± SE (n = 6). The significance of difference was calculated by Duncan’s test, and results with P < 0.05 were considered to be statistically significant.

RESULTS

Effects of LF on Rats with DMN-Induced Liver Fibrosis

After receiving DMN injections for 4 wk, rats were evaluated for liver fibrosis based on several parameters, including BW, liver index, spleen index, and hydroxyproline, as shown in Figure 1A. The decrease of liver index (liver-to-BW ratio) revealed liver cirrhosis induced by DMN. The DMN treatment caused a reduction in BW (264.1 g) when compared with the normal control group (383.7 g). The BW of the DMN-treated group significantly decreased by approximately 31% compared with the normal control group, and the BW of the LF-H treated group increased by 7% compared with the DMN-treated group. The liver index of the DMN-treated group significantly decreased by 38% compared with the normal control group. Furthermore, the normal rat liver was soft and pinkish-brown,

Journal of Dairy Science Vol. 97 No. 6, 2014
whereas the DMN-treated rat liver was shrunken, relatively dark-brown, and coated with some yellowish material. The abnormal liver morphology induced by DMN treatment was effectively modulated by LF-L or LF-H administration (Figure 1B). The DMN treatment significantly increased the spleen index (spleen-to-BW ratio) compared with that of the normal control group. The increase of spleen index revealed that DMN stimulates inflammatory cell proliferation. However, the spleen index of the LF-L or LF-H treatment was not recovered. Hydroxyproline, a major component of the protein collagen, is produced by hydroxylation of the AA proline by the enzyme prolyl hydroxylase following protein synthesis. To evaluate extracellular matrix production, the hydroxyproline content was measured. The hepatic hydroxyproline content in the DMN-treated group significantly increased 8-fold over the normal control group. However, the hydroxyproline concentration significantly decreased in the DMN-induced group after LF-L or LF-H was administered.

**Effects of LF on HFU Imaging of Livers**

High-frequency ultrasound (40 MHz) has been used to monitor changes in the rat liver and other hollow organs longitudinally (Chen et al., 2012a). In ultrasonographic presentations, the normal liver parenchyma had a uniform, sponge-like texture of low-level echogenicity.
Passing through the liver were the blood vessels, which were observed as branching tubular structures that could be traced toward either the portal or hepatic veins. In the DMN-treated rats, the hepatic surface appeared as a dotted or irregular line, and the liver parenchyma was a coarse echotexture (Figure 2A, part b). However, livers from rats treated with LF-L or LF-H displayed a relatively homogeneous echotexture compared with the DMN-treated group (Figure 2A, parts c and d).

**Effect of LF on the Histological Changes of the Liver**

Hematoxylin and eosin staining shows that DMN-induced liver injury causes fatty degeneration and necrosis in the central vein (Figure 2B, parts e–h). The levels of fatty degeneration and necrosis were significantly reduced after either LF-L or LF-H treatment. Histological examination of hematoxylin and eosin staining revealed more remarkable preventive effects on DMN-induced hepatotoxicity from LF-H treatment than from LF-L treatment. Masson staining and Sirius staining (Figures 2C, parts i–l, and 3) showed that the livers of the normal control group had a normal lobular architecture with central veins and radiating hepatic cords. The DMN treatment caused severe hepatic pathological damages, such as inflammation, hepatic cell necrosis, and excessive collagen deposition. The semiquantitative hepatic fibrosis staging score for Sirius staining was significantly elevated to 18.0 ± 0.2 in the DMN-treated group. The livers of rats in both the LF-L and LF-H groups showed less collagen deposition and significantly decreased staging scores of 9.0 ± 0.4 and 5.8 ± 0.1, respectively. According to semiquantitative hepatic fibrosis staging score for Sirius staining, the collagen deposition of the DMN-treated group significantly increased by 1700% compared with that of the normal control group. However, the collagen deposition of the
LF-H and LF-H treated groups significantly decreased by 44 and 68%, respectively, compared with that of the DMN-treated group.

**LF Treatment and the mRNA and Protein Levels of ColIα-1 and α-SMA in DMN-Treated Rats**

The extent of liver fibrosis depends on the rates of hepatic collagen synthesis and degradation. The gene expression levels of ColIα-1 and α-SMA, which represent hepatic fibrosis factors, were analyzed using RT-PCR (Figure 4). The mRNA expression levels of both ColIα-1 and α-SMA were markedly increased in the DMN-treated group by 5.6- and 1.9-fold, respectively, compared with those of the normal control group. The LF-L or LF-H treatments significantly reduced the mRNA levels of ColIα-1 and α-SMA by 85 or 80% and 37 or 15%, respectively. Furthermore, Western blotting showed that the expression levels of the ColIα-1 and α-SMA proteins significantly increased in the DMN-treated group by 185 and 148%, respectively, compared with those of the normal control group (Figure 5). The DMN-treated groups that underwent oral administration of either LF-L or LF-H showed a significant reduction in the protein expression levels of both ColIα-1 and α-SMA when compared with DMN-treated group. These results demonstrate that oral administration of LF at either 100 or 300 mg/kg of BW is able to mark-
edly decrease both the mRNA and protein levels of ColIα-1 and α-SMA, which may in turn reduce DMN-induced hepatic fibrosis.

**LF Treatment and the Protein Expression Levels of LF and LRP-1 in DMN-Treated Rats**

The protein expression level of the LRP-1, an LF receptor for signaling, of DMN treatment was significantly decreased by 55% compared with that of the normal control group (Figure 6). Both the LF-L and LF-H treatment increased the protein expression level of LRP-1 by 39 and 83%, respectively, compared with that of the DMN-treated group. Furthermore, LF-H treatment significantly increased the protein level of LF compared with that of the DMN-treated group, but no significant difference was observed between the normal control group and the DMN-treated group. These results demonstrate that oral administration of 100 or 300 mg/kg of BW of LF can increase the level of LRP-1, a signaling receptor for LF that mediates the liver response to LF, thus reducing the mRNA and protein levels of both ColIα-1 and α-SMA.

**DISCUSSION**

Liver diseases, which can be caused by viral infection, autoimmune conditions, alcohol abuse, or chemi-
cal intoxication, often progress into liver fibrosis and cirrhosis, which are a major health issue. Lactoferrin, an iron-binding glycoprotein, has multiple biological functions, including anti-inflammatory, anticancer, and antimicrobial effects, as well as immunomodulatory-enhancing functions (de Mejia and Dia, 2010). To exert multiple biological functions, LF or active LF fragments must survive passage through the stomach. Troost et al. (2001) demonstrated that a major proportion of orally administered bovine LF survives passage through the stomach in adults and intragastric degradation. Accordingly, previous studies showed a minor degradation of LF both in vivo in the entire digestive tract in newborns (Spik et al., 1982) as well as ex vivo in gastric juice of preterm infants (Britton and Koldovsky, 1989). Many studies have demonstrated the potent anti-inflammatory effect of LF in both in vitro and in vivo assays (Van Snick and Masson, 1976; Mazurier et al., 1989; Cumberbatch et al., 2000; Togawa et al., 2002). However, our understanding of the hepatic protection of LF and its mechanisms remains quite limited. In the current study, a rat model of DMN-induced hepatitis was used to demonstrate the hepatoprotective effects of LF caused by suppressing both the mRNA and protein expression of ColIα-1 and α-SMA.

Dimethylnitrosamine is a hepatotoxin and a well-known cause of acute liver injury in rats (Chen et al., 2012a). The DMN-induced liver fibrosis in rats is a reproducible model for studying the pathogenesis of liver fibrosis and cirrhosis (Jézéquel et al., 1987; Nakamuta et al., 2001). In the current study, HFU was used to quickly diagnose hepatic fibrosis or cirrhosis in the DMN-induced liver fibrosis in rats (Matsuhashi et al., 1996; Guimond et al., 2007; Yan et al., 2007; de Lima et

**Figure 5.** Changes in the protein expression levels of collagen α-1 (ColIα-1) and α-smooth muscle actin (α-SMA) in the dimethylnitrosamine (DMN)-induced fibrosis group and the DMN-induced fibrosis and lactoferrin (LF) group (LF-L = low-dose LF group; LF-H = high-dose LF group). The ColIα-1 and α-SMA proteins were normalized with an internal control of β-actin. Presented as means ± SE (n = 6); different letters (a–c) are significantly different at the level of P < 0.05 according to the Duncan's test.
Recent studies showed a positive correlation between ultrasound images and the anatomical forms of chronic liver injury (Chen et al., 2012a,b). Ultrasound images revealed that the hepatic surface of the DMN-treated rats appeared heterogeneous, but the livers of LF-treated rats displayed a significantly homogeneous echotexture compared with those of the DMN-treated group (Figure 2A, parts a–d). These results demonstrate that LF treatment has a fast hepatoprotective effect on DMN-induced liver fibrosis, as shown by HFU imaging. In addition, Figure 1 shows that the DMN-damaged livers were reduced in size and contained dark discolorations, and DMN treatment significantly decreased both BW and liver index. The abnormal liver morphology induced by DMN treatment was effectively modulated by either LF-L or LF-H administration. Furthermore, treatment with LF significantly increased the BW and liver index (Figure 1A, part b), suggesting that such improvements may result from the attenuation of hepatocyte necrosis. In addition, DMN treatment damaged endothelial cells and caused cell morphological changes in adjacent hepatocytes (Figure 2B and C, parts e–l). The livers of rats treated with LF showed less hepatic pathological damages, such as inflammation, hepatic cell necrosis, and excessive collagen deposition. Thus, oral administration of 100 or 300 mg/kg of BW of LF significantly relieved the DMN-induced histopathological deterioration.

Liver fibrosis is traditionally viewed as a progressive pathological process involving multiple cellular and molecular events that ultimately lead to the deposition of excess matrix proteins in the extracellular space (Bataller and Brenner, 2005). Reeves et al. (1996) demonstrated a positive correlation between the degree of fibrosis and hepatic stellate cells (HSC) activation in the damaged liver both animals and humans.
Elsharkawy et al. (2005) also demonstrated an association between the apoptosis of HSC and a regression of fibrosis. Activated HSC are characterized by a high rate of proliferation, the expression of fibrotic cell markers, such as α-SMA, and the production of extracellular matrix. In the present study, RT-PCR and Western blotting of α-SMA (Figures 4 and 5) showed that HSC were activated in the DMN-induced liver fibrosis and that LF treatment significantly suppressed this HSC activation. In addition, LF treatment prevented the accumulation of collagen proteins, presumably by inhibiting the activation of HSC, leading to reduced mRNA and protein expression of collagen-1 (Figures 4 and 5) in proportion to the reduced production of collagens. Oxidative stress levels that override antioxidant defenses often trigger liver damage (Parola and Robino, 2001). Excess reactive oxygen species activate HSC, recruit proinflammatory cytokines, and initiate downstream responses. Increased expression of α-SMA and ColIα-1, as well as the secretion of inflammatory mediators, facilitate fibrotic processes (Gressner et al., 2007; Friedman, 2008). Oral administration of LF significantly relieved liver fibrosis, suggesting that such improvement may result from a reduction in the mRNA and protein expression of α-SMA and ColIα-1. Therefore, LF prevented the accumulation of collagen proteins, presumably by inhibiting the activation of stellate cells, leading to a reduction in the mRNA and protein expression of α-SMA and ColIα-1 in proportion to the reduced production of collagens and stellate cell activity. The result is in agreement with those obtained by Chen et al. (2012a). Chen et al. (2012a) demonstrated that Yi Guan Jian herbal reduced collagen deposition in the DMN model of hepatic fibrosis, and this was associated with reduced α-SMA and ColIα-1 mRNA expression in the liver. In addition, clinical studies and experimental models have demonstrated an association between the apoptosis of stellate cell and a regression of fibrosis (Elsharkawy et al., 2010).

In conclusion, DMN-induced acute hepatitis in rats has been widely used as a model system that closely mimics the cascade of events leading to clinical hepatitis caused by endotoxemia and sepsis. Our results demonstrate that treatment with LF attenuated DMN-induced liver injury, including hepatic inflammation and necrotic and apoptotic tissue injury. Lactoferrin may therefore represent a new type of hepatoprotective agent and may provide potent hepatoprotective effects for clinical use.

ACKNOWLEDGMENTS

This research was supported in part by grants NSC-97-2313-B-005-004-MY3 and NSC-100-2313-B-005-028-MY3 from the National Science Council and the Ministry of Education (Taipei, Taiwan, Republic of China), under the Aiming for the Top University plan.


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