Fibronectin Prevents Endotoxin Shock After Partial Hepatectomy in Rats *via* Inhibition of Nuclear Factor-κB and Apoptosis

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Fibronectins (Fns) are involved in a number of biologic processes, such as cellular adhesion, motility, differentiation, apoptosis, hemostasis, wound healing, and ischemic injury. We investigated the possible mechanism underlying the protective action of plasma Fn (pFn) on endotoxin shock following partial hepatectomy in rats. Lipopolysaccharide (LPS) was administered intravenously to male Sprague-Dawley rats within 48 hrs of 70% hepatectomy. Prior to LPS administration, pFn or human serum albumin was given intravenously. The survival rate of the pFn-treated group was improved markedly compared with that of the controls. The levels of inflammatory cytokines and nitric oxide (NO) in serum were significantly lower in the pFn-treated group than in the control group. Expression of inducible nitric oxide synthase (iNOS) in hepatocytes also was reduced following pFn treatment. The degree of apoptosis and necrosis in the remnant liver was significantly lower in the pFn-treated rats than the controls. Furthermore, pFn pretreatment greatly inhibited the activation of nuclear factor-κB (NF-κB), caspase 3 and 8 activities, and cytochrome *c* release, and caused a decrease in mitochondrial Bcl-x_L. Plasma Fn prevents endotoxin-induced liver injury at least in part through inhibition of NF-κB activation, which causes the reduction of iNOS expression and NO production by hepatocytes, and through the downregulation of inflammatory cytokines and promotion of Bcl-x_L expression.
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Key words: fibronectin; endotoxin; hepatectomy; inflammatory cytokines; apoptosis; nuclear factor-κB; nitric oxide

Introduction

Despite advances in surgical techniques and perioperative management, liver failure occasionally occurs after extended hepatectomy. Clinically, liver failure associated with postoperative infections sometimes leads to multiple organ failure (MOF) and death of the patient (1). Although a two-thirds resection of the liver is not fatal, there is increased sensitivity to endotoxin in the period following experimental hepatectomy. Hepatic upregulation of Toll-like receptor 4 (TLR4) after hepatectomy causes hypersensitivity to endotoxemia (2); therefore, intravenous injection of a sublethal dose of lipopolysaccharide (LPS) at 48 hrs after surgery results in a high mortality (3). LPS directly activates Kupffer cells—macrophages residing in the liver—to produce tumor necrosis factor-α (TNF-α) and other inflammatory cytokines (4). These inflammatory cytokines are induced through the activation of the transcription factor, nuclear factor-κB (NF-κB), and participate, either individually or by forming a network, in the process of endotoxemia and liver injury, leading to MOF (5, 6). Thus, the activation of NF-κB in Kupffer cells is a key event during endotoxemia (7).

Fibronectin (Fn) is a high–molecular weight glycoprotein found in plasma (plasma Fn) and the extracellular matrices of tissues (cellular Fn). Plasma fibronectin (pFn) is expressed by hepatocytes and secreted in a soluble form into the plasma**.** Cellular Fn is an insoluble form expressed locally by fibroblasts and other types of cells and deposited into and assembled within the extracellular matrix (8). Fn modulates cell proliferation, migration, adhesion, and survival, and it also plays an important role in wound healing, inflammation, and reticuloendothelial function (9). Fn exerts its multiple effects through binding to the integrin receptor on the cell surface or to many other biologic molecules, such as heparin, collagen, and fibrin (10). Plasma levels of Fn decrease in patients with fulminant hepatic

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failure (11), and the effects of endotoxin are enhanced in Fn-deficient rats (12). Moreover, pFn-deficient mice show increased neuronal apoptosis and larger infarction areas following transient focal cerebral ischemia (13). Although the opsonic activity of pFn has been documented, little is known about the effects of pFn on endotoxemia. We reported previously that exogenous pFn protected against endotoxin-induced liver injury after partial hepatectomy in rats (14); however, the mechanisms for this effect remain unclear. In this study, we investigated the possible mechanism underlying the protective action of pFn against endotoxin shock following partial hepatectomy *in vivo*.

Materials and Methods

Purified Human Plasma Fibronectin. Human pFn was purified from plasma cryoprecipitate. The purification process was performed by means of heat defibrinogenation at 50°C for 30 mins (with EDTA, citrate, and aprotinin), fractionation following 25% saturation with ammonium sulfate, and ion-exchange chromatography with DEAE-sephadex A50 (Pharmacia Biotech Inc., Uppsala, Sweden). Heat treatment of purified pFn was carried out at 60°C for 10 hrs in a solution containing sucrose and citrate. The purity of pFn was about 98% (high-performance liquid chromatography analysis). The purified pFn (44.4 mg/ml) was stored in a 0.15 *M* sodium chloride solution at –40°C.

Experimental Design. Male Sprague-Dawley strain rats (250–300 g) were obtained from Simizu Co. Ltd., Kyoto, Japan, and were kept at a controlled temperature under a 12:12-hr light:dark cycle, with food and water *ad libitum*. Rats were anesthetized with ether prior to undergoing 70% hepatectomy or sham operation (celiotomy). At 48 hrs after surgery, 1.5 mg/kg body wt LPS (Sigma Chemical Co., St. Louis, MO) was injected into the penile vein. Rats were administered intravenously with several doses (50, 100, or 200 mg/kg) of pFn or the same dose of human serum albumin (Benesis Co., Osaka, Japan) 30 mins before LPS injection, and then the animals were divided randomly: some animals were used for the survival experiment and some for tissue collection. The animals for survival were monitored over the following 14 days. In the case of the animals for tissue collection, animals were sacrificed using an overdose of sodium pentobarbital (300 mg/kg ip) at 1, 3, and 6 hrs after LPS injection, and then their blood and tissue samples were obtained immediately for biochemical analysis. All experimental animals used in this study were treated according to guidelines set down by the Animal Care and Use Committee of Kansai Medical University Animal Center.

Measurement of Biochemical Parameters. Serum aspartate transaminase (AST) and creatinine were measured at several dosages (50, 100, or 200 mg/kg) of pFn or the same dose of human serum albumin (control) 6 hrs after LPS injection. We reported previously that using the same experimental model, the AST levels in the control group began to increase obviously 6 hrs after LPS administration (15). Therefore, in this study we evaluated hepatic function at 6 hrs after LPS injection. Serum AST and creatinine levels were analyzed using an autoanalyzer (Model 7170; Hitachi High-Technologies Co., Tokyo, Japan).

Assay of Plasma Cytokines. The blood samples were centrifuged, and the serum was collected and stored at –80°C until use for cytokine determination. The levels of TNF-α, interleukin-1β (IL-1β), interferon-γ (IFN-γ; all from Biosource International, Camarillo, CA), and cytokine-induced neutrophil chemoattractant 1 (CINC-1; Immuno-Biological Laboratories, Gunma, Japan) were measured in serum and tissue using commercial enzyme-linked immunosorbent assays (ELISAs).

Assay of Serum Nitric Oxide (NO) Production. The sum of serum metabolites of NO, nitrite (NO_2^-) and nitrate (NO_3^-) was derived using the Griess reagent method (16) using a commercial kit (Roche, Mannheim, Germany).

Histopathology and TUNEL Staining. Excised liver specimens were fixed in buffered 10% formalin, embedded in paraffin, cut into 3-μm sections, and stained with hematoxylin and eosin. The extent of liver necrosis and hepatocyte damage was estimated from five independent sections. The degree of necrosis was evaluated using a 0–4 scoring system: 0, negative; 1, 1 focus or less per ×10 objective; 2, 2–4 foci per ×10 objective; 3, 5–10 foci per ×10 objective; and 4, more than 10 foci per ×10 objective (magnification ×100). Apoptotic bodies were detected by terminal deoxynucleotidy1-transferase (TdT)–mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL), which was performed using an *in situ* apoptosis detection kit (Takara Shuzo Co., Shiga, Japan). TUNEL-positive cells were counted by randomly selecting 15 high-power fields distributed over at least three independent sections. The mean number of TUNEL-positive cells per 1000 hepatocytes represented the TUNEL-positive index.

Measurement of Cytochrome *c* by ELISA. Cytochrome *c* released into the cytosolic fraction was measured ($n = 5$ in each group) using a commercial ELISA (MBL, Nagoya, Japan). Standards and 200 μg protein from each S100 fraction were assayed using a one-step sandwich ELISA with an affinity-purified anti–cytochrome *c* polyclonal antibody and a peroxidase-conjugated anti–cytochrome *c* polyclonal antibody. The optical densities (ODs) were measured at 450 nm.

Caspase 8 and 3 Activities. Caspase activity was determined ($n = 5$ in each group) using a colorimetric protease assay kit (Bio Vision, Mountain View, CA). The S100 fractions containing 200 μg protein were incubated with 200 μ*M* chromogenic substrates for caspase 8 (IETD-*p*NA) and caspase 3 (DEVD-*p*NA) at 37°C for 1 hr. Absorbances were measured at 405 nm. Caspase activity was normalized to the absorbance of the normal control.

Western Blotting Analysis of Bcl-x_L and Inducible Nitric Oxide Synthase (iNOS). Bcl-x_L was determined by Western blotting of the mitochondrial fractions. Frozen liver sections (–80°C, 0.2 g) were homogenized with a Dounce homogenizer (Fisher Scientific, Springfield, NJ) in 1.8 ml ice-cold tissue-homogenizing buffer (250 m*M* sucrose; 20 m*M* Tris-HCl, pH 7.4; 10 m*M* KCl; 1.5 m*M* $MgCl_2$; 1 m*M* EDTA; 1 m*M* EGTA; 1 m*M* dithiothreitol [DTT]; 1 m*M* phenylmethylsulfonyl fluoride [PMSF]; 500 U/ml Trasylol; 1 m*M* Na_3VO_4; and 1 m*M* β-glycerophosphate [βGP], pH 7.4). Unlysed cells and nuclei were pelleted *via* a 10-min, 750 *g* spin. The supernatant was spun at 10,000 *g* for 25 mins, and the resulting supernatant and pellet contained the S100 and mitochondrial fractions, respectively. The supernatant was then spun at 100,000 *g* for 1 hr, and final supernatant was the S100 fraction, which we used for assay of caspase activities and release of cytochrome *c*. The pellet containing the mitochondrial fraction then was resuspended in 100 μl lysis buffer (10 m*M* Tris-HCl, pH 7.4; 1% Triton X-100; 150 m*M* NaCl; 1 m*M* EDTA; 1 m*M* EGTA; 1 m*M* PMSF; 1 m*M* Na_3VO_4; 1 m*M* βGP; and 1× complete protease inhibitors) and spun at 15,000 *g* for 15 mins. The final supernatant comprised the mitochondrial fraction and was electrophoresed on a 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to a polyvinylidene-difluoride membrane (Bio-Rad, Hercules, CA). Immunostaining was performed using an ECL blotting detection agent (Amersham, Little Chalfont, UK) and antibodies against Bcl-x_L (monoclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA). A whole-cell lysate, Bcl-x_L (Santa Cruz Biotechnology), was used as the positive control.

Inducible NOS was detected in a whole-cell lysate. Frozen liver was homogenized in five volumes of cell-solubilizing buffer (10 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.5% Nonidet P-40, 1 m*M* EGTA, 1 m*M* EDTA, 150 m*M* NaCl, and 1 m*M* PMSF) and centrifuged at 16,000 *g* for 15 mins; the resulting supernatant was subjected to 7.5% SDS-polyacrylamide gel electrophoresis. Rabbit polyclonal antibody against mouse iNOS (Affinity-Bio Reagent, Neshanic Station, NJ) was used as the primary antibody.

Electrophoretic Mobility Shift Assay for NF-κB. Livers were homogenized in homogenization buffer (10 m*M* Hepes-KOH, pH 7.9; 10 m*M* KCl; 1.5 m*M* $MgCl_2$; 1 m*M* DTT; 1 m*M* PMSF; and 500 U/ml Trasylol) and centrifuged at 1100 *g* for 5 mins at 4°C. The pellet was suspended in homogenization buffer supplemented with 0.1% Triton X-100 and was centrifuged (1100 *g*, 10 mins). The nuclear pellet was resuspended in extraction buffer (20 m*M* Hepes-KOH, pH 7.9; 1 m*M* DTT; 1 m*M* PMSF; 500 U/ml Trasylol; 0.2 m*M* EDTA; and 25% v/v glycerol), incubated for 30 mins, and centrifuged (15,000 *g*, 20 mins). The supernatant was taken as the nuclear fraction. An NF-κB oligonucleotide probe (Promega, Madison, WI) was labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. The nuclear extract (4 μg) was incubated with reaction buffer (20 m*M* Hepes-KOH, pH 7.9; 1 m*M* EDTA, 60 m*M* KCl; 10% glycerol; and 1 μg poly dI-dC) and the labeled probe for 20 mins. Then the products were electrophoresed on a 4.8% polyacrylamide gel in high–ionic strength buffer. Dried gels were analyzed by autoradiography.

Statistical Analyses. All data were expressed as means ± standard error of the mean (SEM). Differences between groups and in survival were identified by the one-way ANOVA and the log-rank tests, respectively. A P value less than 0.05 was considered statistically significant.

Results

Effect of Plasma Fibronectin on Animal Survival and Biochemical Parameters. Rats began to die at 12 hrs, and all of the rats died within 48 hrs after LPS injection in the pFn-untreated group. Survival was improved to approximately 70% by treatment with 100 mg/kg pFn, and these animals were still alive more than 14 days after the injection. However, there was no further increase in the survival rate at 200 mg/kg pFn (Fig. 1A). Administration of LPS increased serum levels of AST and creatinine in partially hepatectomized rats (Fig. 1B and C). The 100 and 200 mg/kg doses of pFn significantly inhibited increases of these markers of tissue injury, indicating that pFn attenuated the cellular damage that occurred following treatment with LPS. Biochemical parameters improved slightly with the 200 mg/kg dose compared with the 100 mg/kg dose of pFn; therefore, we used a 200 mg/kg dose of pFn in subsequent experiments.

Effect of Plasma Fibronectin on Serum Cytokines. In the sham group, LPS increased serum levels of TNF-α, IL-1β, and INF-γ significantly (Sham + LPS vs. Sham; Fig. 2A, B, and D, left), but the LPS-induced increases in cytokine levels in the sham group (LPS + Sham) were lower than in rats treated with LPS after hepatectomy (PH + LPS; Fig. 2). Administration of pFn inhibited LPS-induced increases of these cytokines significantly in the sham group (Sham + LPS + Fn vs. Sham + LPS; Fig. 2, left). In the partially hepatectomized group, pFn significantly prevented increases in serum TNF-α levels at 1 and 3 hrs after LPS injection compared with the PH + LPS rats (PH + LPS + Fn vs. PH + LPS; Fig. 2A, right). Serum IL-1β, CINC-1, and INF-γ levels reached a peak 3 hrs after LPS injection in the PH + LPS rats, and pFn also inhibited increases in serum levels of these cytokines significantly at 3 hrs compared with the PH + LPS rats (PH + LPS + Fn vs. PH + LPS; Figs. 2B, C, and D, right).

Effect of Plasma Fibronectin on Necrosis and Apoptosis in the Liver. Histopathologic analysis revealed that focal hepatocyte necrosis with associated severe neutrophil and lymphocyte infiltration was prominent at the midzone and periportal regions in LPS-treated rats after hepatectomy. Hepatic necrosis scores increased with time after LPS injection, and pFn inhibited the increases at 9 hrs (Fig. 3A). Moreover, pFn significantly inhibited increases in

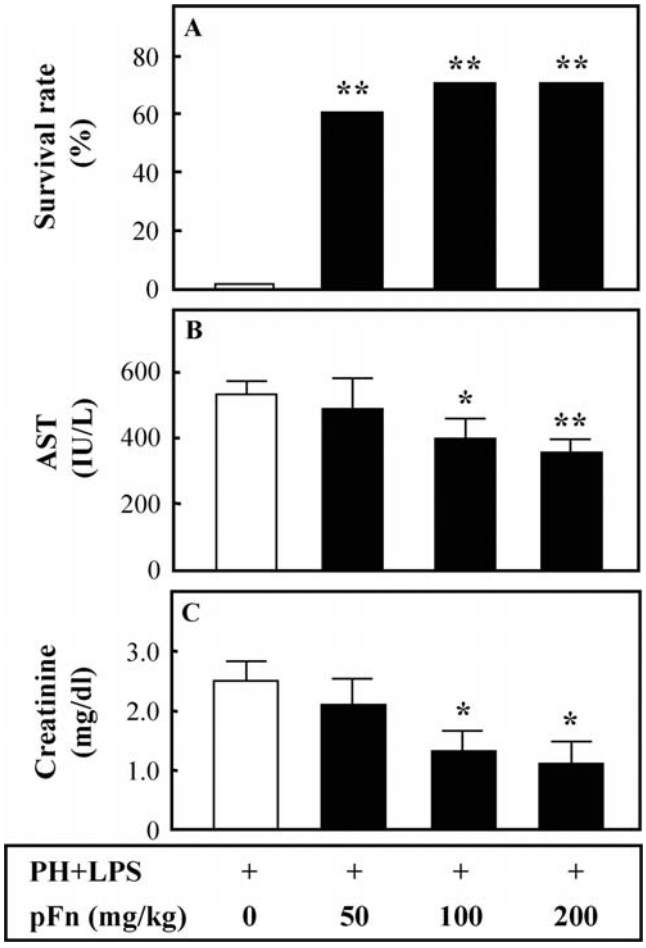

Figure 1. Effects of pFn pretreatment on animal survival and biochemical parameters. Forty-eight hours after partial hepatectomy (PH), rats were administered various doses (50, 100, or 200 mg/kg) of pFn or 200 mg/kg human serum albumin at 30 mins prior to LPS injection. (A) Survival rate. (B) AST. (C) Creatinine. All data represent the mean ± SEM ($n = 10$ and $n = 5$ to 7 rats for survival rate and biochemical parameters, respectively). *$P < 0.05$ and **$P < 0.01$ vs. pFn-untreated.

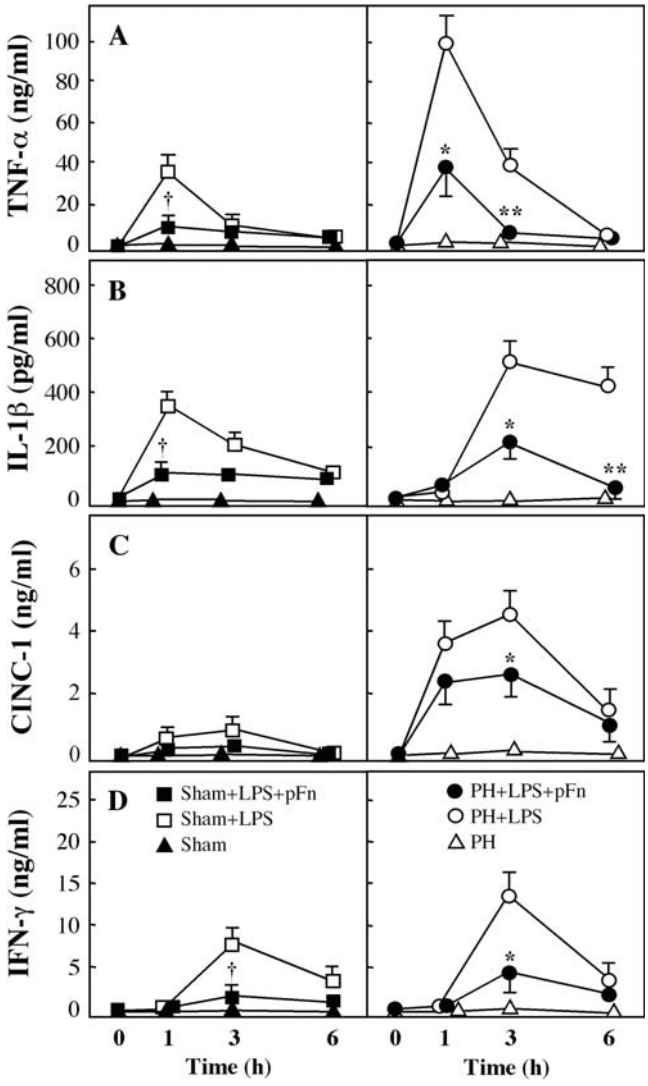

Figure 2. Effect of pFn on serum levels of TNF-α, IL-1β, CINC-1, and IFN-γ. Forty-eight hours after partial hepatectomy (PH) or sham hepatectomy, rats with LPS were treated with 200 mg/kg pFn (Sham + LPS + pFn; PH + LPS + pFn) or human serum albumin at the same dose as pFN (Sham + LPS; PH + LPS) Controls were partially hepatectomized and sham rats without either LPS or pFn and human serum albumin treatment (Sham; PH). Serum samples were obtained at the times indicated. (A) TNF-α. (B) IL-1β. (C) CINC-1. (D) IFN-γ. Data represent mean ± SEM ($n = 5$ to 7 and $n =$ five rats for PH and sham groups, respectively). †$P < 0.05$ vs. Sham + LPS; *$P < 0.05$ and **$P < 0.01$ vs. PH + LPS. The time indicated is the time after LPS injection.

apoptosis detected by TUNEL staining in the liver at 6 and 9 hrs after LPS injection compared with the PH + LPS rats (Fig. 3B). LPS had no significant effects on necrosis, neutrophil infiltration, and apoptosis in the sham group.

Effect of Plasma Fibronectin on Plasma NO Production and Expression of iNOS in the Liver. Because NO derived from iNOS in hepatocytes plays a crucial role in hepatic failure, we determined whether pFN influences the induction of iNOS and its product NO. Plasma NO levels were markedly inhibited by pFn administration at 6 and 9 hrs after LPS injection compared to the PH + LPS rats (Fig. 4). In addition, Western blot analysis of iNOS protein expression in liver showed that pFn significantly decreased iNOS (130 kDa) expression in liver at 6 and 9 hrs after LPS injection compared with the pFn-untreated rats (Fig. 5).

Effect of Plasma Fibronectin on Activation of Transcription Factor NF-κB. Recent evidence indicates that NF-κB is involved in the transcriptional activation of a variety of inflammatory genes, including TNF-α, IL-1β, CINC-1, and iNOS. The maximum activation of NF-κB was detected 1 hr after LPS administration. Pretreatment with pFn greatly attenuated the activation of NF-κB. The maximum inhibitory effect of pFn on NF-κB activation was observed at 3 hrs after LPS administration (Fig. 6).

Effect of Plasma Fibronectin on Caspase 3 and 8 Activities and Cytochrome *c* Release. To confirm the apoptosis of hepatocytes and antiapoptotic action of pFn in LPS-induced liver failure after partial hepatectomy, caspase 3

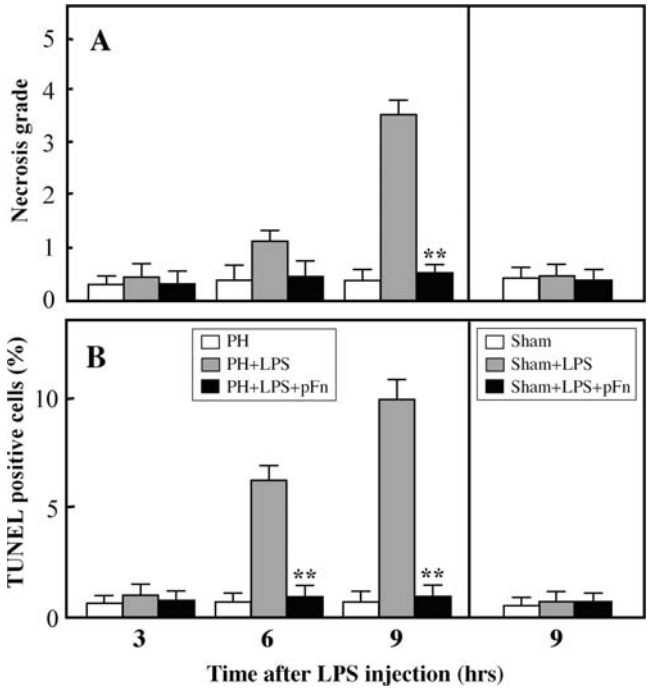

Figure 3. Effect of pFn on necrotic and apoptotic damages in the liver. Forty-eight hours after partial hepatectomy (PH) or sham hepatectomy, rats with LPS were treated with 200 mg/kg pFn (Sham + LPS + pFn; PH + LPS + pFn, solid column) or human serum albumin at the same dose as pFN (Sham + LPS; PH + LPS, hatched column). Controls were partially hepatectomized and sham rats without either LPS or pFn and albumin treatment (Sham; PH, open column). (A) Hepatic necrosis indicates the percentage of the total field at the times indicated. (B) Liver samples were stained with TUNEL and an apoptosis index was calculated for each sample as the proportion of positive hepatocytes out of the total number of hepatocytes evaluated, multiplied by 100. Data represent the mean ± SEM ($n = 5$ to 7 and $n = 5$ rats for PH and sham groups, respectively). $^{**}P < 0.01$ vs. PH+LPS.

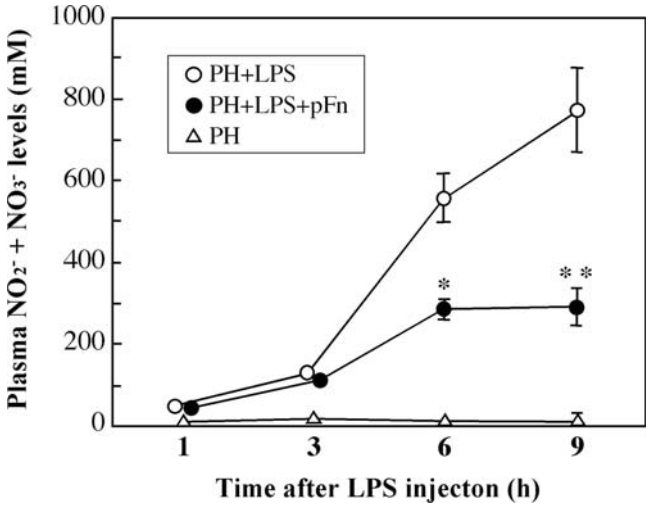

Figure 4. Effect of pFn on production of NO in blood. Blood samples were obtained at the times indicated from LPS-treated rats 48 hrs after partial hepatectomy (PH, open triangle), rats with LPS treated with 200 mg/kg pFn (PH + LPS + pFn, solid circle), and rats receiving the same dose of human serum albumin (PH + LPS, open circle). Metabolites of NO (NO_2^- and NO_3^-) were measured. Data are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. PH + LPS.

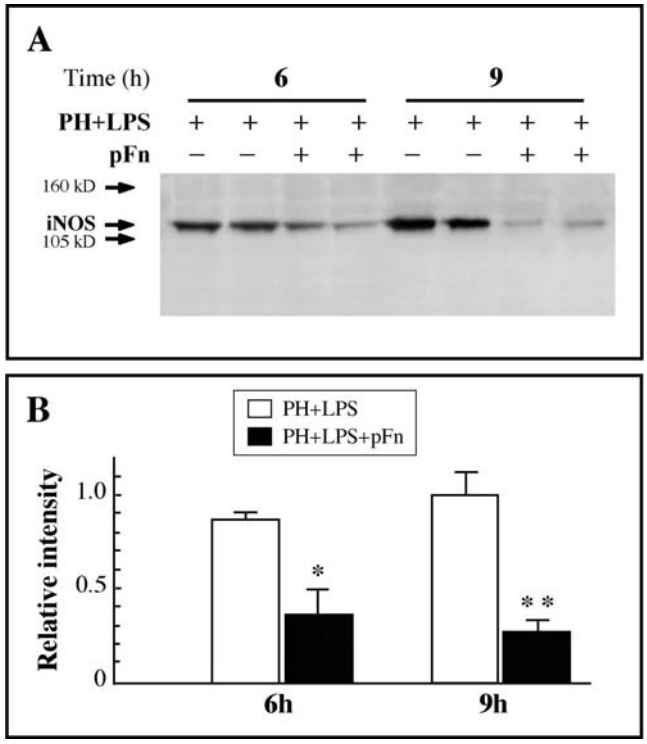

Figure 5. Effect of pFn on induction of iNOS. Liver samples were obtained at the times indicated from LPS-treated rats 48 hrs after partial hepatectomy (PH) with pFn (200 mg/kg) or human serum albumin (200 mg/kg) pretreatment. (A) Western blot analysis of iNOS protein expression in the liver extracts. (B) Summarized iNOS protein expressions from five independent observations. Expression of iNOS protein was quantified by a densitometric scan and was normalized to the iNOS protein expression in the extract from an LPS-treated control rat at 9 hrs detected on the same blot. Data are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. PH + LPS. The time indicated is the time after LPS injection.

and 8 activities were measured 1, 3, and 6 hrs after LPS injection with or without pFn pretreatment. Caspase 3 and 8 activities began to increase at 3 hrs and were markedly increased in the livers of LPS-treated rats at 6 hrs after LPS injection; however, those increases were significantly inhibited in rats with pFn pretreatment 6 hrs after LPS injection (Fig. 7A and B). Moreover, cytochrome *c* release was increased markedly at 6 hrs after LPS injection in pFn-untreated rats; pFn significantly attenuated the cytochrome *c* release (Fig. 7C).

Effect of Plasma Fibronectin on Expression of Bcl-X_L. To clarify further the mechanism of signal transduction, we examined expression of Bcl-x_L in mitochondrial fractions of liver extracts. Mitochondrial Bcl-x_L content decreased at 6 hrs after LPS injection in control rats; however, the decrease in Bcl-x_L contents was markedly enhanced in pFn-treated rats compared with pFn-untreated rats (Fig. 8).

Discussion

Plasma Fn is synthesized and secreted predominantly by hepatocytes and is present in human plasma at 300 μg/

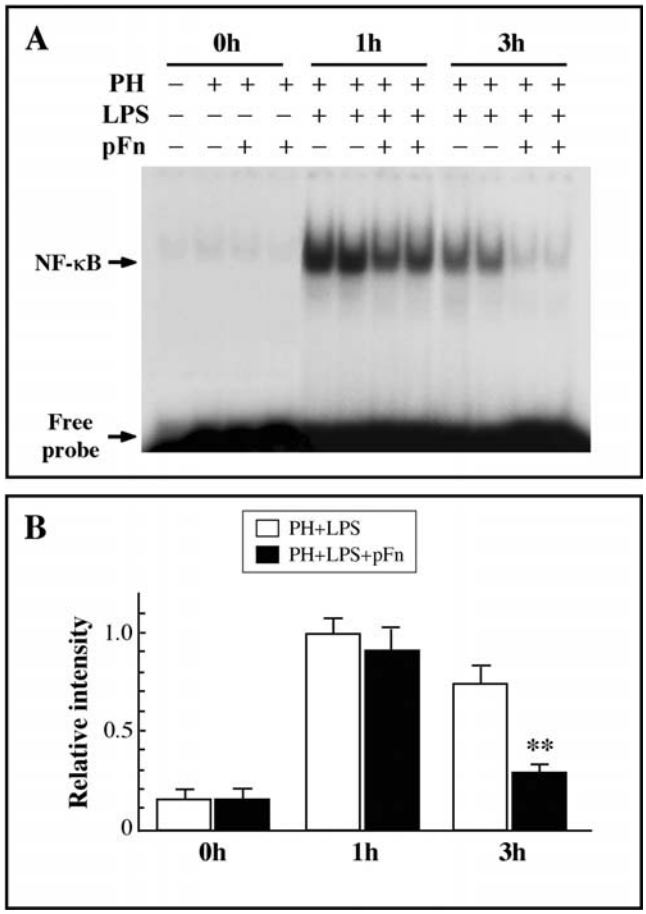

Figure 6. Effect of pFn on activation of NF-κB in the livers. Rats given a single intravenous injection of 200 mg/kg pFn or the same dose of human serum albumin were subjected to LPS injection 48 hrs after partial hepatectomy (PH). (A) Nuclear extracts from the livers were used for electrophoretic mobility shift assay. The specificity of the band was assessed by the competitor (250-fold), and time indicates the time after LPS injection. (B) Summarized NF-κB activity from five independent observations. NF-κB activity was quantified by a densitometric scan and was normalized to the NF-κB activation in the extract from an LPS-treated control rat at 1 hr detected on the same blot. Data are expressed as mean ± SEM ($n = 5$ per group per time point). $^{**}P < 0.01$ vs. PH+LPS. The time indicated is the time after LPS injection.

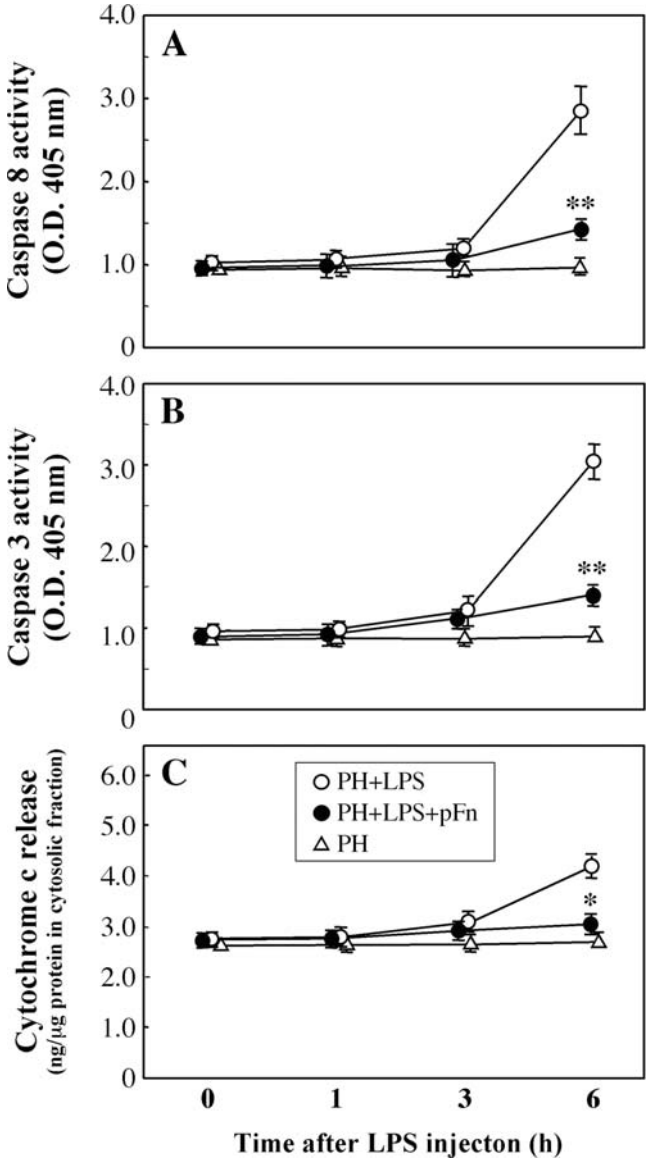

Figure 7. Effect of pFn on caspase 3 and 8 activities and cytochrome *c* release. Forty-eight hours after partial hepatectomy (PH, open triangle), rats with LPS were treated with 200 mg/kg pFn (PH + LPS + pFn, solid circles) or the same dose of human serum albumin (PH + LPS, open circles). The activities of caspases were measured using a colorimetric assay in the cytosolic fraction from liver extracts. (A) Caspase 8. (B) Caspase 3. The quantities of cytochrome *c* released were measured by ELISA in the cytosolic fractions of the liver extracts (C). Data are expressed as mean ± SEM ($n = 5$ for each group per time point). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. PH + LPS.

ml. Because pFn has specific binding sites for actin and collagen (10), blood-borne materials, such as actin and gelatinlike ligand, may bind to Fn and thereby deplete pFn during tissue injury. Plasma Fn opsonizes and promotes phagocytosis and may be depleted in the opsonization and clearance of bacterial endotoxins, cell debris, and other circulating particulate material released during tissue damage (11). The pFn levels decreased in patients with fulminant hepatic failure (11) and decreased immediately after hepatectomy (15), septic trauma (17), and burns (18). This acute depletion is due to its consumption after opsonic utilization, as well as its rapid binding to the injured tissues (19–21). Clinical observations revealed that the pFn levels increased in parallel with clinical improvement and that low pFn levels indicated a poor prognosis (22). In the present study, administration of pFn improved the survival rates and reduced the degrees of apoptosis and necrosis in the remnant liver in LPS-treated partial hepatectomized rats, demonstrating the protective effect of pFn on endotoxin-induced liver injury.

High levels of NO production generated by iNOS in hepatocytes are an important mediator associated with the liver damage during sepsis (23, 24). NO causes apoptosis through reduction of mitochondrial membrane potential,

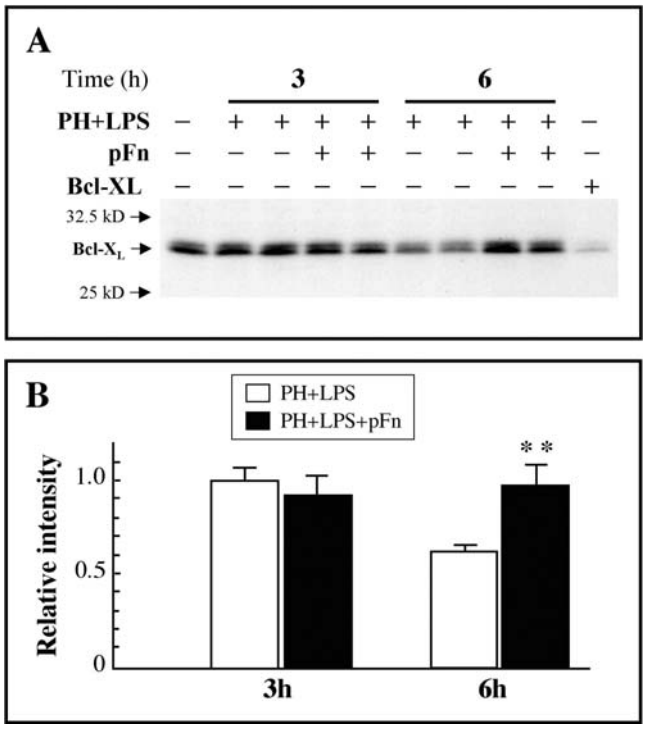

Figure 8. Effect of plasma fibronectin (pFn) on Bcl-x_L protein expressions. Liver samples were obtained at the times indicated from LPS-treated rats 48 hrs after partial hepatectomy (PH) with pFn (200 mg/kg) or human serum albumin (200 mg/kg) pretreatment. (A) Western blot analysis of Bcl-x_L protein expression in liver extracts. (B) Summarized Bcl-x_L protein expressions from five independent observations. Bcl-x_L expression was quantified by a densitometric scan and was normalized to the Bcl-x_L expression in the extract from an LPS-treated control rat at 3 hrs detected on the same blot. Data are expressed as mean ± SEM. $^{**}P < 0.01$ vs. PH + LPS. The time indicated is the time after LPS injection.

releasing cytochrome *c* from mitochondria, and activation of caspases 9, 3, and 8 (25, 26). In the present study, the depression of iNOS expression in liver and the marked decrease in plasma NO production following pFn treatment in LPS-treated partially hepatectomized rats may be one of the major factors contributing to the protective action of pFn. Expression of iNOS is regulated by transcription factor NF-κB. This study shows that pFn treatment inhibited activation of NF-κB in the entire liver. Because the liver contains a large number of hepatocytes, in which NF-κB is activated in response to LPS stimulation (27), activation of NF-κB in the entire liver occurs predominantly in the hepatocytes. Therefore, it is possible that pFn exerts its protective action on the liver through the inhibition of NF-κB activation in hepatocytes, causing the suppression of iNOS in liver

In septic conditions, TNF-α is the initial and most important cytokine, because it not only causes the production of IFN-γ, IL-6, and IL-12, but it also directly activates caspase 8–dependent apoptotic signals by binding to the TNF receptor on the surface of hepatocytes, after which caspase 8 triggers the activation of caspase 3, a downstream cysteine proteinase, in multiple apoptosis signal pathways, and is critical for the programming of cells for apoptosis (28). Neutralization of TNF activity attenuated the release of other mediators during endotoxemia as well as an antibody against TNF-α decreased liver injury in LPS-treated rats (6, 29). In addition to TNF-α, inflammatory cytokines, such as IL-1β, IFN-γ, and CINC-1, also are considered to play an important role in liver injury during endotoxemia. IL-1β induces the release of CINC-1, which acts as a neutrophil chemoattractant and causes infiltration of neutrophils into the liver in sepsis (4, 30). In our study, serum levels of TNF-α and IL-1β increased at 1 hr after the administration of LPS in partial hepatectomized rats, followed by increases in IFN-γ and CINC-1. These cytokines also increased in the remnant liver (14) and are produced by Kupffer cells. Thus, the increased cytokine levels in serum were partly derived from Kupffer cells. Therefore, the protective action of pFn on liver seems to involve in the inhibition of TNF-α and other cytokines. The inhibition of TNF-α might be attributable to pFn directly blocking TNF-α *via* an extracellular mechanism (i.e., by binding directly to TNF-α; Refs. 31, 32).

It is known that LPS activates Kupffer cells by binding to CD14/TLR4, leading to activation of NF-κB (7), which induces production of TNF-α, IL-1β, IFN-γ, and CINC-1 (33, 34). Thus, we cannot exclude the possibility that the inhibition of TNF-α occurs through the suppression of NF-κB activation in Kupffer cells. Even though Kupffer cells account for only a small percentage of the cellular content of the liver, activated Kupffer cells have a considerable potential for production of TNF-α, IL-1β, and CINC-1. If NF-κB from Kupffer cells was not activated, production of these cytokines would not increase in liver in response to LPS stimulation (14). Therefore, the inhibition of these cytokines may result from the suppression of NF-κB activation in Kupffer cells.

Bcl-x_L is a member of the Bcl-2 family of proteins and is located in the outer membranes of the mitochondria. It prevents the release of cytochrome *c* from mitochondria, thereby blocking apoptosis signal transduction downstream of caspase 8 and upstream of caspase 3 (35, 36). In our model, treatment with pFn significantly inhibited the release of cytochrome *c*, the activation of caspases 8 and 3, and the decrease of mitochondrial Bcl-x_L content after the administration of LPS. Bcl-x_L has been documented as preventing hepatic apoptosis in TNF-α/GalN-treated mice (37). Moreover, Takehara *et al.* (38) suggested that hepatocytes frequently encounter various apoptotic insults that must be antagonized by Bcl-x_L to maintain cell integrity; therefore, Bcl-x_L is a crucial apoptosis antagonist in hepatocytes and plays an important role in contributing to the protective effect of pFn on LPS-induced hepatic injury.

Fns may be survival factors inhibiting the apoptosis of a variety of cell types (39, 40). This indicates that the pFn is interacting with cells to inhibit apoptosis. The most likely cell receptors for pFn are integrins. In some cell types, the interaction of pFn through $\alpha_5\beta_1$ and $\alpha_5\beta_3$ integrins leads to

increased levels of the antiapoptotic protein Bcl-2 and Bcl-2–mediated protection from apoptosis induced by serum deprivation (41, 42). In addition, pFn protects neuronal and nonneuronal cells from death by interacting with integrins to increase Bcl-2 expression (13). Furthermore, adhesion to Fns increases survivin expression levels in an AKT-dependent manner, and these increased survivin levels counteract the processing/function of caspase 9 and decrease apoptosis (43). Although intact pFn protects cells from apoptosis, it has been reported to induce apoptosis when Fn is fragmented in some specific domains (44). The exogenous pFn used in the present study has been shown to be pure and intact; hence, we eliminated the possibility of a fragment of pFn inducing apoptosis of hepatocytes.

In conclusion, pFn prevents endotoxin-induced liver injury after partial hepatectomy in rats. The possible mechanisms might be: first, to inhibit the activation of NF-κB in hepatocytes, which in turn causes a reduction of iNOS expression and NO production; second, to suppress inflammatory cytokines, such as TNF-α and IL-β1; and third, to promote Bcl-x_L expression by hepatocytes, directly blocking downstream intracellular apoptotic signals.

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