Nuclear Import of Proinflammatory Transcription Factors Is Required for Massive Liver Apoptosis Induced by Bacterial Lipopolysaccharide*

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Stimulation of macrophages with lipopolysaccharide (LPS) leads to the production of cytokines that elicit massive liver apoptosis. We investigated the in vivo role of stress-responsive transcription factors (SRTFs) in this process focusing on the precipitating events that are sensitive to a cell-permeant peptide inhibitor of SRTF nuclear import (cSN50). In the absence of cSN50, mice challenged with LPS displayed very early bursts of inflammatory cytokines/chemokines, tumor necrosis factor α (1 h), interleukin 6 (2 h), interleukin 1 β (2 h), and monocyte chemoattractant protein 1 (2 h). Activation of both initiator caspases 8 and 9 and effector caspase 3 was noted 4 h later when full-blown DNA fragmentation and chromatin condensation were first observed (6 h). At this time an increase of pro-apoptotic Bax gene expression was observed. It was preceded by a decrease of anti-apoptotic Bcl2 and BclXl gene transcripts. Massive apoptosis was accompanied by microvascular injury manifested by hemorrhagic necrosis and a precipitous drop in blood platelets observed at 6 h. An increase in fibrinogen/fibrin degradation products and a rise in plasminogen activator inhibitor 1 occurred between 4 and 6 h. Inhibition of SRTFs nuclear import with the cSN50 peptide abrogated all these changes and increased survival from 7 to 71%. Thus, the nuclear import of SRTFs induced by LPS is a prerequisite for activation of the genetic program that governs cytokines/chemokines production, liver apoptosis, microvascular injury, and death. These results should facilitate the rational design of drugs that protect the liver from inflammation-driven apoptosis.

Programmed cell death (apoptosis) is the major mechanism of embryonic development and remodeling of tissues and organs, homeostatic control of immune cells that recognize self and non-self antigens, and removal of virally infected cells (1). Apoptosis of hepatocytes may occur in fulminant hepatitis, an inflammatory process that is caused by viral and non-viral agents (2). For example, recent gene therapy approaches to correct an inborn error of metabolism led to fulminant liver failure (3). This inflammation-related complication of gene therapy impedes broader application of viral vectors (4, 5). The sequence of intracellular signaling events that underlie inflammation-driven development of ultimately fatal liver apoptosis remains incompletely understood.

Fulminant liver apoptosis has been studied in several animal models. These studies indicate that activation of T cells with concanavalin A (6) or with agonists that interact with T cell receptor such as staphylococcal enterotoxin B can lead to massive apoptosis (7, 8). Staphylococcal enterotoxin B-induced apoptosis occurs under conditions of metabolic stress imposed by 2-amino-2-deoxy-D-galactosamine (D-Gal).1 Similarly, activation of macrophages with their Toll-like receptors (TLR) agonists, such as lipopolysaccharide (LPS, endotoxin), induces massive liver apoptosis when animals are treated with ethanol or D-Gal (9, 10). By reversibly depleting hepatic stores of uridine diphosphate (UDP), D-Gal sensitizes hepatocytes to the cytotoxic effects of tumor necrosis factor α (TNFα) (10, 11). Accordingly, massive liver apoptosis induced by a macrophage agonist, LPS, or a T cell agonist, staphylococcal enterotoxin B, in combination with a metabolic inhibitor, D-Gal, was abrogated in animals deficient in TNFα receptor 1 (TNFR-1) (12–14). These in vivo models of liver apoptosis offer an excellent way to study fulminant liver injury mediated by inflammatory cytokines because they provide a well defined and reliable end point, which is relevant to human disease states.

The genetic programs for inflammation and apoptosis are regulated by stress-responsive transcription factors (SRTFs) either alone or in various combinations (15). These SRTFs include nuclear factor kB (NFkB), nuclear factor yB; cSN50, cyclized form of SN50 peptide carrying an NLS derived from NFXB1 (p50); SM, control peptide carrying a non-functional NLS mutation; RAW, murine macrophage cell line RAW 264.7; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FDP, fibrin degradation products; PAI-1, plasminogen activator inhibitor-1; TUNEL, TdT-dependent dUTP-biotin nick end labeling; ANOVA, analysis of variance; NLS, nuclear localization sequence.

1 The abbreviations used are: n-Gal, 2-amino-2-deoxy-D-galactosamine; TLR, Toll-like receptors; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; TNFR-1, tumor necrosis factor α receptor 1; SRTF, stress-responsive transcription factors; NFkB, nuclear factor yB; cSN50, cyclized form of SN50 peptide carrying an NLS derived from NFXB1 (p50); SM, control peptide carrying a non-functional NLS mutation; RAW, murine macrophage cell line RAW 264.7; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FDP, fibrin degradation products; PAI-1, plasminogen activator inhibitor-1; TUNEL, TdT-dependent dUTP-biotin nick end labeling; ANOVA, analysis of variance; NLS, nuclear localization sequence.

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Importin/karyopherin α2 (Rch1, KPN2A) is the target for a cell-permeant peptide-cyclized form of SN50 (cSN50), which prevents the nuclear import of SRTFs (17, 18). Here we report in vivo studies with cSN50 showing that this cell permeant peptide prevents liver apoptosis and death in a murine model of LPS toxicity. These findings demonstrate a key role for SRTFs in the development of fulminant liver injury induced by LPS and mediated by inflammatory cytokines and chemokines.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Purification—**cSN50 and SM were synthesized, purified, filter-sterilized, and analyzed as described elsewhere (7, 18).

**Maintenance and Treatment of Cell Line—**Murine macrophage cell line RAW 264.7 (RAW) was obtained from the American Type Culture Collection (Manassas, VA; TIB-71). These cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro, VA) supplemented with 10% heat-inactivated fetal bovine serum containing no detectable LPS (<0.006 ng/ml as determined by the manufacturer, Atlas Biological, Notre Dame, IN). Cytosine and then incubated in a 37 °C water bath for 5 min. After were measured in serum according to the modified manufacturer’s instructions (7, 19).

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**Cytokine Assays of Plasma and Cultured Cell Supernatants—**RAW cells were treated in 96-well plates (200 μl/well at 2 × 10^5/ml) with LPS from Escherichia coli 0127:B8 (Sigma). Each experimental sample was run in duplicate or triplicate. Cells were incubated for 6 h at 37 °C in 5% CO2. Supernatant samples from the medium of RAW cells treated in 96-well plates were collected and frozen at −80 °C until assayed for cytokine levels.

**Animal Treatment Protocols—**Female C57BL/6 mice (8–12 weeks old, ~20 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were injected intraperitoneally with 1 μg of LPS (5 μg/ml, Sigma) and 20 mg of n-Gal (100 mg/ml, Sigma), both in pyrogen-free saline. Mice were randomly divided into two groups: dengue control, which was treated with dimethyl sulfoxide in sterile H2O as diluent. The treatment group received seven intra-peritoneal injections before (30 min) and after (30, 90, 150, and 210 min) and 6 and 12 h) LPS and d-Gal challenge. However, the control group usually received five intra-peritoneal injections of diluent because of the worsening condition of the animals and their rapid death. An additional group of 15 mice received the SM peptide (cell-permeant but functionally inactive analog of cSN50) in a dose of 2 mg given intraperitoneally before (30 min) and after (30, 90, 150, and 210 min). Due to the rapid demise of these mice, two additional injections at 6 and 12 h could not be administered. Animals were observed at hourly intervals for signs of acute toxicity (piloerection, ataxia, and the lack of reaction to an external stimulus). Cytosolic extracts from liver tissue were prepared by Dounce homogenization in hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM Pefabloc, and 1 μg/ml each peptatin, leupeptin, and aprotinin) and subsequently centrifuged (15 min, 13,000 rpm, 4 °C) (19). The protein concentration of supernatant was adjusted to 1 mg/ml with extraction buffer and stored at −80 °C. An equal volume of reagents and 10 μg/ml cytosolic protein were added to a white-walled 96-well plate and incubated at room temperature for 1 h. The luminescence of each sample was measured in a plate-reading luminometer.

**Caspase Assays—**Caspase 3, 8, and 9 activities in liver tissue were measured using a Caspase-Glo assay kit (Promega) and modified protocol. Briefly, the luminescent substrate containing the DEVD, AETD, or LEHD (sequences are in a single-letter amino acid code) is cleaved by caspase-3, caspase-8, and caspase-9, respectively. After caspase cleavage, a substrate for luciferase (aminoluciferin) is released; this results in the luciferase reaction and the production of luminescent signal. Cytosolic extracts from liver tissue were prepared by Dounce homogenization in hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM Pefabloc, and 1 μg/ml each peptatin, leupeptin, and aprotinin) and subsequently centrifuged (15 min, 13,000 rpm, 4 °C) (19). The protein concentration of supernatant was adjusted to 1 mg/ml with extraction buffer and stored at −80 °C. An equal volume of reagents and 10 μg/ml cytosolic protein were added to a white-walled 96-well plate and incubated at room temperature for 1 h. The luminescence of each sample was measured in a plate-reading luminometer.

**RNA Preparation and cDNA Synthesis—**Total RNA was extracted from frozen liver tissue with Versagene RNA tissue kit (Genta Systems, Minneapolis, MN) and treated with DNase (Versagene DNase treatment kit, Genta Systems, Inc.) according to the manufacturer’s instructions. The integrity of RNA preparations was assessed using a NanoDrop ND-1000 spectrophotometer and agarose gel electrophoresis. First-strand cDNA was synthesized with a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Briefly, 1 μg of total RNA was used as the template for synthesis of cDNA in a 50-μl reaction and incubated at 25 °C for 10 min followed by 37 °C for 120 min.

**RNA Quantification with Specific Probes by Real-time PCR—**Detection of mRNA expression levels by real-time PCR with a reporter probe has been established in a multiplexed kinetics studies using reverse-transcribed transcripts as template (20, 21). RNA quantification of specific genes was performed using a TaqMan assay (Applied Biosystems). A TaqMan probe for eukaryotic 18 S rRNA endogenous control (product number 4319413E) was VIC/minimal merge binder-labeled. The primers and FAM/minimal merge binder-labeled probes for the following genes were purchased from ABI Technologies (Applied Biosystems, Foster City, CA): Bcl2 (assay ID Mm00477631_m1), BclXl (assay ID Mm00477673_m1), and Bax (assay ID Mm00432050_m1). Eukaryotic 18 S RNA was used as an endogenous control in a multiplex PCR reaction with a primer/probe set for the gene of interest. For each experiment, 2 μg of cDNA and a TaqMan Universal PCR Master mix (Applied Biosystems), 900 nm primers, and 250 nm probes in 10 μl were added to 384-well plate. Real-time PCR and subsequent procedures were performed with the ABI Prism 7900HT sequence detection system (SDS v2.1) (Applied Biosystems) using the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of amplification (95 °C denaturation for 15 s, 60 °C annealing/extension for 1 min). All PCR reactions were performed in triplicate for each sample and were repeated three times.

**Platelet Count, Detection of Fibrin Degradation Products (FDPs), and Plasminogen Activator Inhibitor 1 (PAI-1) Total Antigen—**Heparinized fresh blood was diluted 1:100 in 1% ammonium oxalate (EM Science) and rocked for 20 min. The sample was added to hemacytometer, and after 20 min, platelets were counted. FDPs in serum were detected by staphylococcal clumping test as described elsewhere (22). Briefly, staphylococci (Staphylococcus aureus sp. aureus ATCC 25904) that express clumping factor were grown and processed to prepare a standardized smooth bacterial suspension for determining a clumping titer in serum samples. The clumping titer was expressed as a reciprocal of the highest dilution of tested serum giving a positive clumping reaction. PAI-1 total antigen in plasma was measured by an ELISA kit according to the manufacturer’s instructions (Molecular Innovations, Inc., Southfield, MI).

**Histology Analyses—**Organ samples (liver, spleen, kidney, lung, and heart) were collected from mice showing typical signs of acute toxicity shortly before death or from surviving mice that were euthanized after recovery from the treatment. Tissues were fixed in 10% formalin for 24 h, cycloxed 24 h, and then dehydrated and embedded in paraffin. Sections were stained with hematoxylin and eosin or periodic acid-Schiff and hematoxylin to assess injury and hemorrhage. Apoptosis of the liver was evaluated by characteristic cytolysis changes and by TdT-dependent dUTP-biotin nick end-labeling (TUNEL) assay using the Apop Tag reagent (Intergen) according to the manufacturer’s instructions.

**Statistical Analysis—**All experimental data except survival were expressed as the mean ± S.E. A one-way analysis of variance, a two-way
they regulate a myriad of genes encoding mediators of inflammation and apoptosis (15). A cell-permeant nuclear import inhibitor, cSN50 peptide, was developed by us to suppress the deployment of SRTFs in the nucleus (18). This bipartite inhibitor contains a membrane-translocating motif, which allows rapid penetration of cell membrane, and a “cargo” comprised of a cyclized nuclear localization sequence (NLS) that enables this peptide to competitively inhibit cytoplasmic/nuclear import of NLS-containing SRTFs. To validate the dependence of LPS-induced inflammatory cytokines/chemokines production on nuclear import of SRTFs, we evaluated the potency of cSN50 peptide as compared with its mutated analog, SM peptide, in cultured murine macrophage RAW cells stimulated with LPS. As shown in Fig. 2, the cSN50 peptide in a range of concentrations from 5 to 50 μM significantly inhibited LPS-induced expression of inflammatory cytokines TNFα (p < 0.0001), IL-6 (p < 0.0001), IL-1β (p < 0.0001), and chemokine MCP-1 (p < 0.0001). In contrast, the cell-permeant SM peptide that contains mutated NLS as cargo was without effect on LPS-induced inflammatory cytokine/chemokine expression, attesting to the specificity of a nuclear import inhibitory sequence. Importantly, these two peptides, cSN50 and SM, did not affect the viability of LPS-stimulated RAW macrophages (>80% under these experimental conditions). These results extend our previous findings of inhibition of SRTF nuclear import in LPS-stimulated macrophages (18) to the concentration-dependent inhibition of inflammatory cytokine/chemokine expression.

**Time Course of Inflammatory Cytokines and Chemokine Expression**—We serially monitored the levels of inflammatory cytokines/chemokines in blood to investigate the sequence of events preceding massive apoptosis of the liver, which was not fully apparent until 6 h after administration of LPS and d-Gal (Fig. 1). As shown in Fig. 3, TNFα levels rose very rapidly in the circulation, reaching a peak in plasma at 1 h. Bursts of IL-6 and chemokine MCP-1 at 2 h followed a very early rise in TNFα. On the other hand IL-1β showed a more progressive rise in systemic levels. Administration of LPS alone induced similar response of inflammatory cytokines and chemokine, but d-Gal alone did not have a detectable effect on inflammatory cytokines and chemokine production in vivo (data not shown), thereby confirming the requirement for LPS to induce an inflammatory cytokine/chemokine response. This response was suppressed significantly by the cSN50 peptide, affirming the dependence of the in vivo production of inflammatory mediators on the nuclear import of SRTFs.

**Time-dependent Induction of Enzyme Markers for Hepatocyte Injury**—ALT and AST measured in serum provide an index of hepatocyte integrity. Leakage of ALT/AST into the extracellular compartment and a subsequent rise in serum reflect hepatocyte damage. These enzymes are significantly elevated in a number of conditions that cause liver injury including viral and bacterial infections, alcohol, and drug toxicity (27). As shown in Fig. 4, the serum ALT and AST activity increased rapidly during the first 4 h after administration of LPS and d-Gal and then dropped precipitously at 6 h. This drop most likely reflects liver failure (see Fig. 1). Significantly, the cSN50 peptide prevented the rise in liver enzymes ALT and AST. Thus, by suppressing expression of inflammatory mediators, a nuclear import inhibitor exerts a cytoprotective effect on liver cells in this model. Solo administration of LPS or d-Gal to the control groups of mice produced a moderate increase in serum ALT and AST levels with delayed peaks of activity at 8 and 24 h, respectively, and without massive apoptosis or reduced survival (data not shown).

**Activation Kinetics of Initiator and Effector Caspases**—Although the peak of TNFα required for activation of its cognate
death receptor TNFR-1 occurs at 1 h, activation of initiator and effector caspases is observed much later. This family of intracellular aspartate-specific cysteine proteases exists as inactive proenzymes ("zymogens"). Caspase activation can be measured using specific substrates. Caspase 8 mediates TNFR-1-proximal events in cell death signaling. Caspase 9 is activated by cytochrome c released from mitochondria. Caspase 3 is dubbed DEVDase because it cleaves a DXXD motif, a substrate shared with caspase 7; it is an "executioner caspase," which can be activated directly by caspase 8 or by caspase 9 (28–32). Thus, a cascade of proteolytic events initiated by TNFα and mediated by caspases leads to nucleosomal DNA fragmentation and chromatin condensation as documented in Fig. 1. Despite a very early burst in TNFα production (see Fig. 3), the caspase cascade was considerably delayed. As shown in Fig. 5, the initiator caspases 8 and 9 were activated between 4 to 6 h in mice given LPS and d-Gal. Consistent with these findings, "effector" caspase 3 was not activated during the first 4 h. Caspase 3 (and caspase 7) showed a burst of proteolytic activity at 6 h. Thus, anti-apoptotic mechanisms significantly slowed death receptor signaling initiated by TNFα. Moreover, caspase activation was almost totally suppressed in the livers of mice treated with the cSN50 peptide. Thus, nuclear import of SIRTs is a rate-limiting step for initiation of pro-apoptotic signaling by TNFα and
and cSN50 peptide-treated animals (triangles) over the 6-h time period after LPS/D-Gal challenge. Error bars indicate the ± S.E. of the mean value in five mice that are represented by each data point. p values represent the significance of the difference between the control and the cSN50 peptide-treated groups (two-way ANOVA). U/L, unit/liter.

FIG. 5. Time-dependent activation of initiator and effector caspases in control and cSN50 peptide-treated mice. Wild-type C57BL/6 mice were treated with cSN50 peptide (0.7 mg in 200 μl of 5% dimethyl sulfoxide) or diluent before and after intraperitoneal administration of LPS with D-Gal according to the protocol described under “Experimental Procedures.” Caspase activities in liver were measured in diluent controls (open bar) and cSN50 peptide-treated animals (solid bar). Error bars indicate the ± S.E. of the mean value in four mice that are represented by each data point. p values represent the significance of the difference between the control and the cSN50 peptide-treated groups (two-way ANOVA). RLU, relative light units.

other inflammatory cytokines in the LPS-induced model of liver apoptosis (10, 11, 24).

Alteration in the Balance between Gene Expression of Anti-apoptotic and Pro-apoptotic Proteins Induced by LPS and D-Gal—The observed delay in caspase activation could be due to the initial balance between anti-apoptotic Bcl2 family proteins e.g. Bcl2, BclXl, and pro-apoptotic proteins, e.g. Bax, Bid. Such a balance is important for maintaining cell homeostasis (33, 34). Quantitative analysis of the liver transcripts of the pro-apoptotic gene bax indicated that its expression was significantly increased at 6 h after challenge with LPS and D-Gal (Fig. 6A). Conversely, expression of Bcl2 and BclXl was significantly decreased at 2 h (Fig. 6, B and C). Treatment with the cSN50 peptide suppressed the transcriptional activation of Bax gene and prevented the subsequent shift in balance of these transcripts that favors apoptosis.

Time-dependent Changes in Markers for Microvascular Injury—in this model of LPS-induced liver apoptosis the DNA fragmentation is demonstrated by 6 h along with extensive hemorrhagic necrosis of the liver (see Fig. 1A). Hemorrhage reflects a break in the integrity of microvascular endothelium associated with the formation of intravascular platelet thrombi (Fig. 1B). The mechanism of microvascular injury remains unexplained.

To sequentially analyze this process, we monitored circulating platelets. The platelet count demonstrated that its normal range is maintained during the first 4 h after administration of LPS and D-Gal (Fig. 7). However, a precipitous drop in circulating platelets occurred between 4 and 6 h. In tandem with platelet count, we measured FDP in murine serum by the staphylococcal clumping test that detects this marker of intravascular coagulation (22). FDP level was significantly increased at 4 and 6 h. For comparison, PAI-1, which promotes vascular thrombosis in mice (35), was significantly increased at 6 h. The mice injected with LPS alone (n = 4) or D-Gal alone (n = 4) did not show alterations in platelet count. In contrast, PAI-1 levels were elevated in LPS-challenged mice but not in those that received D-Gal alone (data not shown). Thus, these markers of microvascular injury peak at 6 h when there is histologic evidence of massive apoptosis of the liver and widespread hemorrhagic necrosis in response to LPS and D-Gal (Fig. 1, A and B). More importantly, these markers of microvascular injury were significantly suppressed when mice were treated with the cSN50 peptide, further indicating the overall dependence of this process on the nuclear import of proinflammatory SRTFs.

Massive Apoptosis of the Liver and Survival of the Mice Are Dependent on Nuclear Import of SRTFs—A combination of LPS and D-Gal in this model leads to death with massive apoptosis and hemorrhagic necrosis of the liver. As documented in Fig. 8, control mice treated with diluent showed characteristic progressive signs of sickness resulting in the early death of 26 of the 28 mice within 6–12 h. In contrast, the administration of the cSN50 peptide produced a dramatically protective effect. Twenty of 28 mice recovered fully from LPS/D-Gal challenge and survived at least 72 h. Thus, the cSN50 peptide increased survival from 7 to 71%. Based on the log rank test, the difference in the survival rate between cSN50 peptide-treated mice and the control mice was statistically significant (p < 0.0001). Another group of 15 mice, which were treated with the SM peptide (twice the cumulative dose level of cSN50), showed rapid signs of LPS/D-Gal toxicity and died within 6–12 h (results not shown). These control experiments with the SM peptide containing a mutated NLS confirm the essential role of
this sequence in nuclear import blockade achieved with cSN50. Mice that received either LPS (1 μg) alone (n = 5) or d-Gal (20 mg) alone (n = 10) did not show signs of sickness and survived (data not shown). These survival data correlated with suppression of apoptotic injury and hemorrhagic necrosis of the liver (Fig. 8B). Non-survivors exhibited severe liver injury characterized by extensive apoptosis and hemorrhagic necrosis. In contrast, the mice that were treated with the cSN50 peptide and survived showed normal tissue architecture with normal contrast, the mice that were treated with the cSN50 peptide restored the liver from these devastating complications. This study demonstrated that blocking nuclear import of proinflammatory SRTFs counteracts a full-blown apoptotic and necrosis of the liver and has a death-sparing effect in this model prevents the entire process of massive liver apoptosis and microvascular injury of the liver. (i) TNF-α-mediated apoptotic liver injury. These highly reproducible models allow experimental study of an important biologic process. inflammation-driven and TNF-α-mediated apoptotic liver injury (36–38). The need for new therapeutic approaches to proinflammatory cytokine responsible for development of liver apoptosis world-wide, ~20 million will develop fulminant liver failure associated with apoptosis (6). Similarly, scores of alcoholic liver disease cases can be complicated by concomitant infection/inflammation-driven and TNFα-mediated apoptotic liver injury (36–38). The need for new therapeutic approaches to protect the liver from these devastating complications is apparent. Targeting nuclear import of proinflammatory SRTFs comprises one of the potential approaches to the control of inflammation-driven liver apoptosis.

The following lines of evidence establish the essential role of nuclear import of SRTFs in development of massive apoptosis and microvascular injury of the liver. (i) TNFα, a key inflammatory cytokine responsible for development of liver apoptosis (11, 24) was suppressed by our inhibitor of nuclear import of...
SRTFs, (ii) other inflammatory cytokines (IL-6 and IL-1β) and the chemokine MCP-1 were also suppressed, indicating a broad spectrum of inhibition of these inflammatory mediators by cSN50 peptide in contrast to the inactive SM peptide containing mutated NLS, (iii) suppression of inflammatory mediators was accompanied by a cytoprotective effect on hepatocytes reflected by normal level of ALT and AST in serum of animals treated with cSN50, (iv) initiator and effector caspases were suppressed, and a balance between anti-apoptotic and pro-apoptotic gene transcripts was maintained, (v) DNA fragmentation in the liver cells was arrested, (vi) microvascular injury was prevented, and (vii) survival of mice that were treated with cSN50, an inhibitor of SRTFs nuclear import, was significantly improved. In contrast to non-survivors that usually died within the first 12 h after administration of LPS and n-Gal, the surviving animals lived at least 3 days and did not display histologic evidence of liver injury. The lack of signs of liver and other organ injury in mice that received a nuclear import inhibitor persisted for at least a week when observation was extended. Thus, inhibition of nuclear import of SRTFs affords a lasting protection from highly deleterious effects of LPS and n-Gal that induce fulminant liver injury. The cSN50 peptide is rapidly (−20 min) distributed within mouse blood cells and organs after an intraperitoneal injection (18). However, further studies will be required to determine the pharmacokinetics, long-term toxicity, and therapeutic efficacy of this new class of nuclear import peptide inhibitors.

As depicted in Fig. 9, sequential analysis of the events leading to death due to LPS-induced fulminant liver injury indicates a lag phase of at least 4 h before activation of initiator and effector caspasases was detected in the liver. During this lag phase the production and action of TNFα and other mediators of inflammation depend on signaling to the nucleus in LPS-responsive cells that encompass liver macrophages (Kupffer cells) (25). This LPS-induced signaling depends on expression of TLR4 because TLR4-deficient C3H/HeJ mice escape massive apoptosis (24). Moreover, there is a requirement for metabolic changes; without the depleting action of n-Gal on UTP in hepatocytes, LPS is unable to induce massive apoptosis despite a robust burst in TNFα (18). When administered alone, LPS is responsible for a rise in TNFα and other cytokines. Neither LPS nor n-Gal administered alone induces massive apoptosis of the liver and death (10). Thus, development of fulminant apoptosis requires a combination of transient hepatocyte metabolic dysfunction and the burst of inflammatory cytokines to overcome anti-apoptotic defenses of the liver.

The experimental model employed in this study depends upon cross-talk between macrophages and hepatocytes as schematically depicted in Fig. 10. Macrophages respond to LPS via TLR-4 and produce TNFα along with other mediators of inflammation in a SRTFs nuclear import-dependent manner. Apparently, TNFα via its “death” receptor (TNFR-1) evokes a different pro-apoptotic signaling in a hepatocyte that is metabolically altered by n-Gal. The primary effect of n-Gal is its capacity to lower the level of UTP in hepatocytes (10, 24). A cascade of initiator and effector caspasases is activated in hepatocytes and ultimately leads to the execution of a program of DNA fragmentation and chromatin condensation. Sequential analysis of pro-apoptotic and anti-apoptotic genes expression in the liver indicates that in this model of fulminant liver injury there is an early block in transcription of anti-apoptotic genes, Bcl2 and BclXL, before pro-apoptotic gene Bax is transcriptionally activated (Fig. 6). The Bax expression between 4 and 6 h coincided with activation of initiator caspasases 8 and 9 (Fig. 7). Activation of caspase 8 reflects signaling by death receptors represented by TNFR-1. Activation of caspase 9 indicates that changes in mitochondrial integrity have occurred. Such changes are usually due to a rise in intracellular Ca2⁺, generation of reactive oxygen species, ceramide, and pro-apoptotic protein Bax (39, 40). These changes destabilize mitochondria and lead to the release of cytochrome c. Although we detected occasional DNA fragmentation in the liver using a TUNEL assay at 4 h, the most dramatic changes were observed at 6 h...
Thus, pro-apoptotic signaling induced by TNFα in D-Gal-sensitized liver cells requires at least 4 h to overcome anti-apoptotic mechanisms as documented in Fig. 1. Subsequently, sometime between the fourth and sixth hour, the consequences of the “life or death” decision made by hepatocytes become apparent. Thus, this 2-h time span is decisive for development of a full-blown apoptosis. Importantly, overexpression of Bcl2 prevents cells from undergoing apoptosis by blocking cytochrome c release from mitochondria induced by a variety of stimuli (41). Moreover, inhibition of caspase-3 activity with YVAD-chloromethyl ketone protected mice from liver apoptosis and death caused by LPS and D-Gal (42).

The association of massive apoptosis of the liver with hemorrhagic necrosis reflects a concomitant microvascular injury due to a loss of endothelial integrity with attendant extravasation of erythrocytes and intravascular formation of platelet aggregates (Fig. 1B). This is accompanied by a precipitous decrease in circulating platelets and generation of FDP. In view of the fulminant nature of liver failure in this model, our inability to detect fibrin in histologic sections is not surprising. Nevertheless, combination of acute platelet consumption and generation of FDP strongly suggests a process of microvascular injury with thrombosis (43). Consistent with this process, increased expression of PAI-1 was detected. The cSN50 peptide prevented all of these abnormal changes. Thus, three interwoven mechanisms, inflammation, apoptosis, and microvascular dysfunction, depend on induction of a genetic program regulated by SRTFs and controlled by their nuclear import. Broad inhibition of inducible SRTFs nuclear import prevents massive apoptosis of the adult liver, whereas disruption of physiologic signaling mediated by NFκB led to TNFα-dependent apoptosis of fetal liver (44–46). Although the cSN50 peptide inhibits nuclear import of NFκB, it also blocks nuclear translocation of activator protein 1, nuclear factor of activated T cells, and signal transducer and activator of transcription 1 (17, 18). Apparently, the coordinated regulation of genes that encode mediators of inflammation and apoptosis by multiple SRTFs exceeds the unique role of NFκB in protecting fetal liver from TNFα-mediated developmental injury.

Taken together, our experiments identify a key rate-limiting step in the development of LPS-induced apoptosis of the liver that may be amenable to therapeutic intervention with nuclear import inhibitors. TNFα production and subsequent hepatocyte apoptosis may contribute to the development of a number of inflammatory liver diseases, including viral hepatitis, alcoholic liver disease, Wilson disease, drug-induced liver failure, and...
ischemia/reperfusion liver damage (34, 47, 48). Moreover, our results may have therapeutic applications for other disease conditions, such as secondary organ injury after ischemia/reperfusion, due to the excessive production of inflammatory cytokines and subsequent neutrophil involvement (49). Thus, targeting nuclear import of proinflammatory SRTFs offers a new approach to suppress expression of inflammatory and apoptotic mediators in the liver and interrupt the underlying disease mechanisms.

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