CD14-Dependent Alterations in c-Jun Expression in the Liver after Burn Injury

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Background. Burn injury-triggered activation of lipopolysaccharide signaling via the CD14 pathway alters the expression of a variety of downstream genes contributing to pathogenic changes in distant organs. The regulation of CD14 and its role in the immediate-early response of c-Jun in the liver after burn injury were investigated in this study.

Materials and methods. An incidental identification of the differential induction of CD14 mRNA after an approximately 18% TBSA burn injury in mice was confirmed by RT–PCR and immunohistochemical analyses of CD14 expression. Subsequently, CD14’s role in the immediate-early regulation of c-Jun expression in the liver after injury was examined by Western blot analysis using CD14 knockout (KO) mice.

Results. RT–PCR analysis demonstrated a rapid and transient induction of CD14 mRNA in the liver and lungs of mice after injury. Immunohistochemical analysis revealed a peak induction of CD14 reactivity in cells appearing to be Kupffer cells at day 1 after injury. Furthermore, an augmented and delayed induction of c-Jun mRNA was observed in the liver of CD14 KO mice after injury compared to wild-type controls. The induction of phosphorylated (serine 63 or serine 73) forms of c-Jun after injury was lower in the livers of CD14 KO mice than that in WT controls.

Conclusions. This study provides evidence that injury elicits CD14 induction as well as hyperphosphorylation of the c-Jun N-terminus activation domain and that CD14 is involved in the modulation of c-Jun’s transactivation potential via phosphorylation, which may be associated with hepatic pathogenesis after injury. © 2004 Elsevier Inc. All rights reserved.

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INTRODUCTION

Burn injury can initiate proinflammatory responses leading to the immune disorder and often sepsis [1–3]. Lipopolysaccharide (LPS), an important membrane component of gram-negative bacteria, is directly associated with most episodes of bacterial sepsis [4]. CD14 has been identified as a receptor for the endotoxin LPS. Binding of LPS to LPS binding protein (LBP) facilitates the interactions between LPS and membrane-bound CD14. Subsequently, the signal is transmitted to toll-like receptor 4 (TLR4), leading to the initiation of a cascade of intracellular signaling events [5].

CD14 is a 55-kDa glycoprotein and its membrane-bound form is expressed in mature myeloid cells as well as most tissue macrophages [6–8]. CD14, lacking a transmembrane domain, is attached to the plasma membrane via a glycosyl-phosphatidyl inositol anchor. Proteolytic cleavage of membrane-bound CD14 by a serine protease generates the 49-kDa soluble form of CD14 found free in plasma [9]. The expression of membrane-bound and soluble forms of CD14 is mainly regulated by cytokine profiles in the environment.

The exposure of monocytes and macrophages to LPS can initiate the IκB kinase–NF-κB and three mitogen-activated protein kinase (MAPK) signaling cascades: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 [4, 10]. Activation of these signaling pathways contributes to the induction of genes, such as c-Jun, c-Fos, and NF-κB transcription factors, leading to the transactivation of a.
variety of cytokines (both inflammatory and anti-inflammatory); cis-acting LPS responsive elements have been identified in the 5' flanking promoter region of many genes encoding cytokines [11]. The rapid phosphorylation of transcription factors following LPS stimulation is one of the key molecular events facilitating the activation of these genes [12].

c-Jun forms dimers with other inducible as well as constitutive transcription factors and it is a potent component within the activator protein-1 (AP-1) complex [13,14]. Our laboratory has reported an immediate-early induction of c-Jun in the liver of mice after burn injury [15]. Stimulation of stress-activated protein kinases (SAPKs) in response to extracellular stress signals (e.g., oncoproteins, ultraviolet light, growth factors) may lead to the hyperphosphorylation of the c-Jun N-terminal transactivation domain at serine 63 and serine 73 [16–18]. Alterations in phosphorylation profiles at these serine sites are directly associated with c-Jun’s activity as a transcription factor and the transactivation potential of the AP-1 transcription complex.

In this study, we investigated the involvement of CD14 and c-Jun, including its phosphorylation, as well as their interactions in signaling events, which may be associated with pathologic alterations in the liver after burn injury.

MATERIALS AND METHODS

Burn Model

Female C57BLKSJ, CD14 knockout (KO), and their wild-type (WT) control (C57BL/6J) mice from The Jackson Laboratory (Bar Harbor, ME) were housed in the Animal Resources Service at the University of California at Davis. The experimental protocol was approved by the Animal Use and Care Administrative Advisory Committee of the University of California at Davis. The burn protocol was described previously [15]. Briefly, under methoxyflurane anesthesia, a Teflon template measuring 2.1 x 4.0 cm was placed over the back of each mouse. Within the cut portion of the template, 0.5 ml of 70% ethanol was ignited and measuring 2.1

Total RNA was extracted using a RNEasy kit (Qiagen Inc., Valencia, CA); 100 ng of total RNA from each sample was subjected to reverse transcription using Sensiscript reverse transcriptase (Qiagen Inc.) and accompanying reagents based on the protocol provided except for the oligo(dT) primer and RNase inhibitor (Promega, Madison, WI). The sequence of the oligo(dT) primer was as follows: 5'-GCC CAC GCG TCG ACT AGT TTG TTT TTT TTT TTT TTT T-3'. The primer sequences for histone H4, which differentially amplified CD14, were as follows: 5'-CTA TGT CTG GTC GCG GCA AG-3' (H41D) and 5'-CAC GTC CAT GGC GGT GAC-3' (H42C). A primer set of CD1A/CD2A was used to amplify the mouse CD14 sequence: 5'-GAC CAT GGA GCG TGT GCT TG-3' (CD1A) and 5'-GCC ACT GCT GCA GTT CTG CGA G-3' (CD2A).

Western Blot Analysis

Tissues were homogenized for extract preparations in ice-cold mild lysis buffer, containing 1% Nonidet P40, 0.15 M sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 1 µg/ml of aprotinin. The tissue homogenates were centrifuged at ~20,000g for 15 min and supernatants were collected. SDS–PAGE was performed on equivalent amounts of protein samples using precast 10% resolving/4% stacking Tris–HCl gels (Bio-Rad, Hercules, CA). Separated proteins were then transferred to PVDF membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were blocked in 5% nonfat dry milk in TBS buffer containing 0.1% Tween 20 for 1 h at room temperature. Blocked membranes were incubated in one of three rabbit polyclonal antibodies specific for mouse c-Jun, phospho-c-Jun (serine 63), and phospho-c-Jun (serine 73) (Cell Signaling Technology, Beverly, MA) at a concentration of 1:1000 in 1 x TBS–0.1% Tween and 5% BSA overnight at 4°C. Anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Inc.), at a concentration of 1:2000 in 1 x TBS–0.1% Tween, and 5% nonfat dry milk was added to membranes and incubated for 1 h at room temperature. The protein signal was amplified and visualized via chemiluminescence using the ECL Plus Western Blotting Detection System and Hyperfilm ECL autoradiography film (Amersham Pharmacia Biotech, Inc.).
FIG. 1. (A) Differential display of CD14 in the lungs of mice (C57BLKS/J) after burn injury. Lung tissues collected at multiple time points (no-burn control, 3 h, 6 h, and 7 days) after an 18% TBSA burn injury were subjected to RT–PCR analysis of the mouse histone H4 subtype. A nonspecific amplification, which was transiently induced at 3 and 6 h, was identified as mouse CD14 cDNA by cloning and sequencing analysis. (B) RT–PCR screening of CD14 regulation in the liver and lungs of mice after burn injury. Liver and lung tissues harvested at multiple time points (no-burn control, 3 h to 29 days) after an 18% TBSA burn injury were subjected to RT–PCR screening for CD14 expression. A rapid and transient induction of CD14 mRNA was observed in both tissues. Note that there is an extended induction of CD14 in the liver until day 1 after injury compared to lungs. Comparability between samples was provided by the β-actin amplification of each sample. The relative intensity of each CD14 band in comparison to the respective amplified β-actin is summarized in a graph. Each bar represents the average value of normalized band intensities and variability among samples within each group is indicated by an error bar.
RESULTS

Rapid and Transient Induction of CD14 mRNA Expression in the Liver and Lungs Following Burn Injury

During the course of histone H4 subtype RT–PCR analysis in the lungs of mice after burn injury, an unexpected and differentially expressed band was observed at the 3- and 6-h time points (Fig. 1A). Cloning and sequencing analyses revealed that the band was mouse CD14 cDNA. Subsequently, the regulation of CD14 expression in the liver and lungs after burn injury was examined by RT–PCR. Liver had a rapid and significant induction of CD14 mRNA starting at 3 h \( (Z > 1.964; P < 0.05) \) and it lasted at least until 1 day \( (Z > 1.964; P < 0.05) \) after injury compared to that in no-burn controls (Fig. 1B). In addition, CD14 was also significantly up-regulated in the lungs at both 3 and 6 h \( (Z > 1.964; P < 0.05) \) after injury in comparison to no-burn controls and it returned to the basal level at day 1 (Fig. 1B). Since we were interested in investigating the role of CD14 in liver pathogenesis after injury, the following studies focused only on the liver.

Induction of CD14 Protein in Kupffer Cells of the Liver after Burn Injury

To determine whether the induction of CD14 mRNA levels correlates with protein expression, CD14 expression in the liver was examined by immunohistochem-
istry. Cells appearing to be Kupffer cells had elevated levels of reactivity against CD14 antibody at 3 h which peaked at day 1 after injury (Fig. 2). CD14 staining returned to the no-burn control levels by day 8. Also, a moderate and diffuse induction of CD14 reactivity was observed throughout the liver section at day 1.

CD14-Dependent Regulation of c-Jun mRNA Expression in the Liver after Burn Injury

Our laboratory previously reported the transient induction of c-Jun in the liver after burn injury [15]. It also has been demonstrated that burn injury can activate the LPS signaling pathway involving CD14 leading to sepsis [1–3]. Following the identification of the rapid and transient induction of CD14 in the liver after injury, the potential interactions between CD14 and c-Jun in the liver were investigated using CD14 KO mice. There was a transient induction of c-Jun mRNA at 3 h in WT mice after injury, which returned to the basal level at day 1 (Fig. 3). These findings were consistent with our previous report [15]. In contrast, no significant change in the level of c-Jun mRNA expression was observed at 3 h in CD14 KO mice; instead, a delayed and marked induction of c-Jun was present at day 1 after injury. These data suggest that the absence of CD14 expression resulted in a delayed but augmented c-Jun induction after burn injury.

Induction of c-Jun Phosphorylation and CD14-Dependent Regulation of c-Jun Phosphorylation in the Liver after Burn Injury

Western blot analysis of c-Jun expression demonstrated a transient induction of c-Jun protein at day 1 after injury in the liver of both WT and CD14 KO mice, which correlates with our previous findings (Fig. 4) [15]. Interestingly, there was a substantial induction of phospho-c-Jun (serine 63) at day 1 in WT mice, while no significant change was noted in CD14 KO mice (Fig. 4). Also, both WT and CD14 KO mice had an elevation of phospho-c-Jun (serine 73) at day 1; however, the induction was less in CD14 KO mice. It appears that the presence of basal level CD14 expression, and probably its induction, is associated with the phosphorylation of both serine sites in the c-Jun activation domain in the liver after injury.

DISCUSSION

One potential pathogenic mechanism for the liver damage after burn injury is that alterations in the gut and its flora lead to the release of endotoxin (LPS) into the portal circulation [19, 20]. Influx of LPS into the liver after burn injury may elicit a CD14-mediated proinflammatory response predominantly through the activation of Kupffer cells. Activated Kupffer cells produce CD14 as well as various other inflammatory mediators (e.g., macrophage inflammatory protein-2, intercellular adhesion molecule-1, prostaglandin E2, tumor necrosis factor (TNF)-α, reactive oxygen species) which might be detrimental to the liver [21–23]. The rapid and transient induction of CD14 in the liver in this study suggests that stress signals originating from burn injury trigger a cascade of signaling events via CD14, which probably contributes to phenotypic alterations in the liver.

It is likely that an increase in CD14 production may provide beneficial effects to the liver after injury [24]. In particular, CD14 induction may play a crucial role in the swift phagocytosis of cells undergoing apoptosis following burn injury. The enhanced phagocytic activity associated with the induction of CD14 expression...
may provide protection against the toxic effects of phlogistic macromolecules leaked from damaged or apoptotic cells after burn injury. In addition, since the circulating LPS is primarily cleared by the liver, it is probable that the induction of CD14 in the liver may participate in the LPS clearance mechanism [25].

While it has been known that monocytes, macrophages, and granulocytes are the major source of CD14, evidence suggests that CD14 mRNA and protein are expressed in human hepatocytes [25–28]. It is reasonable to interpret that not only Kupffer cells but also hepatocytes contributed to and/or participated in CD14 regulation after burn injury.

The delayed and augmented induction of c-Jun mRNA in the liver of CD14 KO mice provides evidence that CD14 is involved in the immediate-early regulation of c-Jun transcription. Instead of activating the signaling events associated with a rapid and transient (within 3 h) induction of c-Jun mRNA observed in WT mice, an alternative pathway may be used in the absence of CD14 which, in turn, leads to the unique pattern of delayed and augmented c-Jun mRNA induction.

One of the most crucial molecular events associated with AP-1 transcriptional activity is the phosphorylation of the c-Jun N-terminal activation domain at serine 63 and serine 73 [18, 29]. A substantial induction of phospho-c-Jun (serine 63) and phospho-c-Jun (serine 73) in the liver of WT mice after burn injury implies that a cascade of signaling events (e.g., SAPKs, ERKs) enhanced phosphorylation of c-Jun at these serine sites [17, 30–33]. One potential molecular mechanism could be that TNF-α, a key proinflammatory cytokine produced through the LPS/CD14/TLR4/NF-κB pathway following injury could be responsible for the activation of these c-Jun N-terminal kinases.

No induction of phospho-c-Jun (serine 63) and the lesser induction of phospho-c-Jun (serine 73) in the liver of CD14 KO mice compared to WT mice suggest a potential involvement of CD14 in the regulation of c-Jun activity through the transactivation of relevant protein kinases. The absence of CD14 expression in the liver of CD14 KO mice may alter a portion of or the entire stress-activated protein kinase pathways leading to the amelioration of c-Jun transactivation. It is possible that the malfunction of the CD14-mediated NF-κB pathway in CD14 KO mice may lead to failure of the activation of c-Jun N-terminal kinases. However, the underlying mechanism responsible for the differential regulation of phospho-c-Jun (serine 63) and phospho-c-Jun (serine 73) in the liver after injury is not clear. It is of interest to note that the regulation patterns of c-Jun mRNA, protein, and phospho-c-Jun in CD14 KO mice are different, suggesting the presence of multiple layers of signaling events in the liver after burn injury.

These data provide evidence supporting the following hypothesis in regard to the involvement of CD14 and c-Jun in the pathogenesis of the liver after injury: burn injury elicits the CD14-mediated signaling pathways, such as stimulation of SAPKs and NF-κB, leading to the transient enhancement of c-Jun transactivation potential through phosphorylation at its N-terminus activation domain.

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REFERENCES

2. Schwacha, M. G. Macrophages and post-burn immune dysfunc-

polybacterial sepsis in a regional burn unit. *Burns* **19**: 434,
1993.


5. Pugin, J., Heumann, I. D., Tomasz, A., Kravchenko, V. V.,
Akamatsu, Y., Nishijima, M., Glauser, M. P., Tobias, P. S., and
Ulevitch, R. J. CD14 is a pattern recognition receptor. *Immu-

6. Landmann, R., Muller, B., and Zimmerli, W. CD14, new aspects

7. Bazil, V., Baudys, M., Hilgert, I., Stefanova, I., Low, M. G.,
Zbrozek, J., and Horejsi, V. Structural relationship between the
soluble and membrane-bound forms of human monocyte surface

8. Ziegler-Heitbrock, H. W., and Ulevitch, R. J. CD14: Cell surface
receptor and differentiation marker. *Immunol. Today* **14**: 121,
1993.

Eckerskorn, C., Schutt, C., and Engelmann, H. Soluble
lipopolysaccharide receptor (CD14) is released via two
different mechanisms from human monocytes and CD14 trans-

10. Downey, J. S., and Han, J. Cellular activation mechanisms in

11. Sweet, M. J., and Hume, D. A. Endotoxin signal transduction in
macrophages. *J. Leukocyte Biol.* **60**: 8, 1996.

12. Muller, J. M., Ziegler-Heitbrock, H. W., and Baueurle, P. A.
Nuclear factor kappa B, a mediator of lipopolysaccharide effec-

M. Oncogenic and transcriptional cooperation with Ha-Ras re-
quires phosphorylation of c-Jun on serines 63 and 73. *Nature*

14. Herdegen, T., and Leah, J. D. Inducible and constitutive tran-
scription factors in the mammalian nervous system: control of
gene expression by Jun, Fos and Krox, and CREB/ATF pro-

15. Cho, K., Zipkin, R. I., Adamson, L. K., McMurtry, A. L., Griffe,
S. M., and Greenhalgh, D. G. Differential regulation of c-Jun
expression in liver and lung of mice after thermal injury. *Shock*
**14**: 182, 2000.

16. Benetruy, B., Smeal, T., and Karin, M. Ha-Ras augments c-Jun
activity and stimulates phosphorylation of its activation do-

17. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie,
E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. The
stress-activated protein kinase subfamily of c-Jun kinases. *Na-

18. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T.,
Karin, M., and Davis, R. J. JNK1: A protein kinase stimulated
by UV light and Ha-Ras that binds and phosphorylates the c-Jun

19. Wilson, M. D., and Dziuwulski, P. Severe gastrointestinal
haemorrhage and ischaemic necrosis of the small bowel in a
child with 70% full-thickness burns: A case report. *Burns* **27**:

20. Varedi, M., Lee, H. M., Greeley, G. H. Jr., Herndon, D. N., and
Englander, E. W. Gene expression in intestinal epithelial cells,
IEC-6, is altered by burn injury-induced circulating factors.

2002.

22. Forman, H. J., and Torres, M. Reactive oxygen species and cell
signaling: Respiratory burst in macrophage signaling. *Am. J.

23. Gregory, C. D. CD14-dependent clearance of apoptotic cells:
Relevance to the immune system. *Curr. Opin. Immunol.* **12**: 27,
2000.

24. Woltmann, A., Hamann, L., Ulmer, A. J., Gerdes, J., Bruch,
H. P., and Retschel, E. T. Molecular mechanisms of sepsis.

S. C. CD14 expression and production by human hepatocytes.

26. Wheeler, M. D., and Thurman, R. G. Up-regulation of CD14 in
liver caused by acute ethanol involves oxidant-dependent AP-1

27. Pan, Z., Zhou, L., Hetherington, C. J., and Zhang, D. E. Hapa-
tocytes contribute to soluble CD14 production, and CD14 ex-
pression is differentially regulated in hepatocytes and mono-

Lipopolysaccharide induced synthesis of CD14 proteins and its
gene expression in hepatocytes during endotoxemia. *World J.
Gastroenterol.* **8**: 124, 2002.

29. Johnson, G. L., and Lapadat, R. Mitogen-activated protein ki-
nase pathways mediated by ERK, JNK, and p38 protein ki-

30. Dorion, S., and Landry, J. Activation of the mitogen-activated
protein kinase pathways by heat shock. *Cell Stress Chaperones*
**7**: 200, 2002.

31. Mielke, K., and Herdegen, T. 2000. JNK and p38 stresskinases—Degenerative effectors of signal-transduction-casuases in

32. Barr, R. K., and Bogoyevitch, M. A. The c-Jun N-terminal

33. Benhar, M., Engelberg, D., and Levitzki, A. ROS, stress-
avtivated kinases and stress signaling in cancer. *EMBO Rep.* **3**:
420, 2002.