Role of Kupffer cells in acute hemorrhagic necrotizing pancreatitis-associated lung injury of rats

Hong-Bin Liu, Nai-Qiang Cui, Dong-Hua Li, Chang Chen

INTRODUCTION
Acute lung injury is a severe complication of acute hemorrhagic necrotizing pancreatitis (AHNP)\(^{[1-3]}\). However, its pathogenic mechanism is not well understood and its treatment remains supportive. Recent researches suggest that inflammatory cytokines derived from the liver, especially hepatic cytokine released from Kupffer cells (KCs) may cause distant organ failure and death in severe pancreatitis and that KCs are an important source of inflammatory cytokines and may be the main factor causing lung damage in AHNP\(^{[4,5]}\). Here we studied the KC contribution to lung injury associated with AHNP.

MATERIALS AND METHODS
Experimental animals, agents, and ELISA kits
Male Wistar rats were provided by Experimental Animal Center of Capital Medical University (Beijing). Sodium taurocholate and gadolinium chloride (GdCl\(_3\)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TNF-\(\alpha\), IL-1 ELISA kits were purchased from TPI Ltd. (USA). TransAM\(^{TM}\) NF-\(\kappa\)B P\(^{\text{65}}\) chemi ELISA kit was provided by Active Motif (USA). Central LB 960 microplate luminometer was from Berthold Ltd.

Induction of AHNP model
Experimental AHNP was induced as previously described\(^{[6]}\). Briefly, a small median laparotomy was performed, then the pancreas was exteriorized and the hepatic duct was closed at the liver hilum with a soft microvascular clamp to prevent reflux of the infused...
material into the liver. The biliopancreatic duct was cannulated through the duodenum and 5% sodium taurocholate (1 mL/kg body weight, 0.1 mL/min) was retrogradely injected into the biliopancreatic duct. The clamp was removed 5 min after the injection. The abdominal wound was closed, and the animals were sent back to their cages with free access to water and food after surgery.

**Animals**

Forty-two male Wistar rats (weighing 250-280 g) were randomly divided into four groups: sham operation, AHNP group, GdCl₃ pretreatment, and GdCl₃ control. In the GdCl₃ pretreatment group, GdCl₃ solution (4%, 10 mg/kg) was administrated by caudal vein injection 24 h before the AHNP model was established. Blood from the iliac artery, alveolar macrophages, and tissues from the pancreas and lung, were collected in six animals per group 3 and 6 h after acute pancreatitis induction. Serum levels of TNF-α and IL-1 were determined by enzyme-linked immunosorbent assay. Myeloperoxidase (MPO) level in the lung and NF-κB activation of the alveolar macrophages were detected. Serum AST and ALT in sham operation group and GdCl₃ control group were tested by biochemical method and expressed as U/L.

**Alveolar macrophage isolation and nuclear protein extraction**

Bronchoalveolar lavage (BAL) was performed five times in the left lung, using 5 mL of sterile normal saline per lavage given through a tracheal cannula[1]. The whole BAL fluid (BALF) was centrifuged at 280 r/min for 10 min at 4 °C. Cell pellets were resuspended (1×10⁷ cells/mL) in RPMI 1640 medium. Cell suspension was then placed in plastic petri dishes (Nunc, Denmark) and incubated at 37 °C for 1 h in a CO₂ incubator (50 mL/L CO₂:95% air). Non-adherent cells were removed from adherent macrophages by washing with RPMI 1640 medium. Purified alveolar macrophages were recovered by gently rubbing the dishes with a rubber policeman. Nuclear protein was extracted from purified alveolar macrophages as previously described[8]. Protein content was determined by Bradford method, stored at -70 °C for subsequent examination of NF-κB activity.

**Amylase estimation**

Serum amylase activity was measured by iodoamylum method and expressed as U/L.

**Serum TNF-α, IL-1 estimation**

Serum TNF-α and IL-1 were measured by ELISA method according to the instructions of the kits.

**Lung tissue MPO estimation**

Lung tissue MPO activity was detected according to the instructions of commercial kit.

**Serum AST and ALT estimation**

Serum levels of AST and ALT were determined using Toshiba VF-A5/ASP Bio-Chemical analyzer.

| Group              | Serum amylase (U/L, mean±SD) |
|--------------------|------------------------------|
|                   | 3 h                          | 6 h                          |
| Sham               | 499.4±86.3                   | 503.8±91.2                   |
| AHNP               | 10 444.5±1 863.2             | 13 316.4±1 374.1             |
| GdCl₃-pretreatment | 9 107.1±569.9               | 12 420.4±1 779.2             |

[^1]: P<0.001 vs sham group.

| Group              | Serum AST (U/L, mean±SD) | Serum ALT (U/L, mean±SD) |
|--------------------|--------------------------|--------------------------|
| Sham 3 h           | 74.2±13.6                | 31.6±9.3                 |
| Sham 6 h           | 71.3±15.0                | 32.7±11.3                |
| GdCl₃-control      | 82.5±14.6                | 28.9±8.7                 |

**NF-κB of alveolar macrophage estimation**

NF-κB activation of alveolar macrophages was determined using TransAM[9] NF-κB kit with chemi ELISA kit by Central LB 960 microplate luminometer and expressed as relative light units (RLU).

**Histology**

Pancreas and lung tissues were collected and evaluated by a pathologist blinded to the experimental assignment of the animals. After embedded with paraffin, the tissue was stained with hematoxylin-eosin (H&E). Pulmonary lesion was scored following Lei’s criteria[10].

**Statistical analysis**

All data were expressed as mean±SD. Comparisons among multiple experimental groups and between each time point were made using ANOVA. P<0.05 was considered statistically significant.

**RESULTS**

Table 1 illustrates the levels of serum amylase in all the three groups. Three and six hours after AHNP induction, serum amylase increased significantly in AHNP group compared to that in sham operation group. But there was no significant difference between GdCl₃-pretreatment group and AHNP group 3 and 6 h after AHNP induction.

Table 2 shows the influence of GdCl₃ on hepatic functions of experimental animals. No changes were found in GdCl₃-treated animals and the values of ALT and AST in GdCl₃ control group were similar to those in sham group measured at all time points.

Serum TNF-α and IL-1 in AHNP group were significantly increased compared to those measured in sham group at all time points (P<0.01). In GdCl₃ pretreatment group, 3 and 6 h after AHNP induction, serum TNF-α and IL-1 were significantly decreased (P<0.01, Table 3).

Table 4 shows the MPO activity of lung injury in
The MPO levels in AHNP group were significantly higher than those in sham operation group ($P < 0.01$). In GdCl$_3$ pretreatment group, 3 and 6 h after AHNP induction, the lung MPO levels were significantly decreased ($P < 0.01$).

Figure 1 shows that the NF-κB activity of alveolar macrophages in AHNP group was significantly higher than that in sham operation group ($P < 0.01$) and was significantly decreased in GdCl$_3$ pretreatment group ($P < 0.01$).

Histopathological study of the pancreas, 3 and 6 h after AHNP induction (Figure 2A) revealed extensive necrosis of pancreatic tissue, intense edema, and inflammatory infiltrate. The necrosis of pancreatic tissue and edema in GdCl$_3$ pretreatment group were similar to those in AHNP group (Figure 2B). The sham operation group was normal.

Diffuse alveolar blood stasis, intense alveolar septum swelling and heavy infiltration of inflammatory cells mostly neutrophils were found in the lung tissue of AHNP group with slight infiltration of neutrophils in GdCl$_3$ pretreatment group (Figure 2C). The mean histopathologic scores of AHNP group were significantly higher than those in sham operation group ($P < 0.05$, Table 5). In GdCl$_3$ pretreatment group, the major histopathological findings were mild edema of the alveolar walls and mild alveolar blood stasis with slight infiltration of neutrophils (Figure 2D). The mean histopathologic scores of GdCl$_3$ pretreatment group decreased significantly compared to those of AHNP group ($P < 0.05$).

**DISCUSSION**

Acute hemorrhagic necrotizing pancreatitis (AHNP) is a potentially fatal disease with a morbidity and mortality rate of approximately 30%. Acute lung injury (ALI)
is a common complication of AHNP, but the events that link AHNP and pulmonary damage are not fully understood. Many factors, such as oxygen free radicals, platelet activating factor, phospholipase A₂ (PLA₂), cyclooxygenase-2 (COX-2), cytokines and arachidonic acid metabolites are related to AHNP and ALI[10-13].

Pancreatic proteolytic enzymes or activated PLA₂ released into the circulatory system determines the development of lung injury[14]. Furthermore, other mediators in lung tissue such as platelet activating factor, arachidonic acid metabolites can stimulate inflammatory cell activation[15,16]. Also, interaction of polymorphonuclear granulocytes, endothelium, and endothelium-derived mediators seems to be important to amplify lung damage[17]. Recently, Cheng et al[18,19] noted that activation of alveolar macrophages may play an important role in lung injury associated with AHNP, and that TNF-α and nitric oxide (NO) secreted by alveolar macrophages are increased significantly in rats with AHNP. Bhatia et al[20] reported that inhibition of the production of hydrogen sulfide (H₂S) can significantly reduce the severity of cerulein-induced pancreatitis and associated-lung injury, suggesting an important proinflammatory role in regulating the severity of pancreatitis and associated-lung injury.

In recent years, some researchers found that the liver, especially KCs, might play a vital role in ALI caused by different factors. Okutan et al[21] reported that KCs blocked by GdCl₃ can attenuate lung damage caused by aortic ischemia reperfusion and malondialdehyde (MDA) level, an indicator of free radical generation and MPO activity, an indirect evidence of neutrophil infiltration in lung injury are decreased significantly[21]. Although there is no evidence that GdCl₃ can suppress the function of neutrophils, it was reported that GdCl₃ can suppress the accumulation of neutrophils and alveolar macrophages[22]. Feng et al[23] investigated the role of KCs in the pathogenesis of ALI during acute obstructive cholangitis (AOC) and found that the phagocytic function of KCs is damaged in AOC induced by AOC.

KCs, the resident macrophages in the liver, are the major component of mononuclear phagocytic system (MPS). These macrophages make up 90% of the MPS and have abundant cytoplasm where abundant ribosome and phagosomes are located. These typical structures are associated with their functions. It was reported that KCs are responsible for the increased levels of TNF, IL-1, IL-6 in trauma, hemorrhagic shock and resuscitation. Decreasing the number or functional ability of KCs can lead to decreased levels of inflammatory cytokines in serum in the models of liver resection and sepsis[24,25].

Also, KCs are regarded as the predominant source of inflammatory cytokines in AHNP at present[26,27]. Closa et al[28] performed an end-to-side portacaval shunt before AHNP induction in rats and found that portacaval shunting appears to exert a profound effect on ameliorating the inflammatory infiltrate. It is suggested that almost all the pancreatic enzymes and mediators released from the pancreas into the plasma during AHNP pass through the liver before their dilution in the systemic circulation, indicating that this step is a determinant in the development of the lung injury response. These observations point to the key role of liver as a triggering mechanism for inflammatory processes in the lung as a consequence of AHNP. Moreover, activation of hepatic inflammatory cells, especially KCs, plays a key role in the development of lung injury[29].

To improve our understanding of the role of KCs in AHNP-ALI, GdCl₃ was given 24 h prior to AHNP model induction to eliminate KCs in the present study. Moreover, the dose of GdCl₃ used in our study was 10 mg/Kg because administration of GdCl₃ at a dose 10 mg/kg can block the phagocytic activity of KCs completely[28]. In GdCl₃ pretreatment group, the levels of MPO in lung tissue and the levels of TNF-α, IL-1 in serum all decreased significantly compared to those in AHNP group. NF-κB activation of alveolar macrophages was also attenuated significantly in GdCl₃ pretreatment group compared to that in AHNP group. All these data suggest that GdCl₃ can ameliorate AHNP-ALI significantly and these results are in accordance with those reported by Folch et al[20]. It was reported that lung injury associated with acute necrotic pancreatitis (ANP) is ameliorated by GdCl₃ through inducing apoptosis of alveolar macrophages of ANP[30].

In our study, the serum amylase level did not decrease and the injury of pancreas was not prevented in GdCl₃ pretreatment group, suggesting that GdCl₃ cannot prevent lung injury by ameliorating pancreatic injury.

Using GdCl₃ as a tool to investigate the role of KCs is more reasonable than portacaval shunting operation which appears to exert a profound effect on animal's systemic circulation. Serum AST and ALT estimation suggests that GdCl₃ has no harmful effects on hepatic functions.

In conclusion, KCs play a vital role in AHNP and ALI.

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