Original article

Investigation for role of tissue factor and blood coagulation system in severe acute pancreatitis and associated liver injury

Zhi-Bing Ou\textsuperscript{a}, Chun-Mu Miao\textsuperscript{a}, Ming-Xin Ye\textsuperscript{a}, Ding- Pei Xing\textsuperscript{a}, Kun He\textsuperscript{a}, Pei-Zhi Li\textsuperscript{a}, Rong-Tao Zhu\textsuperscript{b}, Jian-Ping Gong\textsuperscript{a,\textast}}

\textsuperscript{a} Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, People’s Republic of China

\textsuperscript{b} Department of Hepatobiliary and Pancreatic Surgery, Institute of Hepatobiliary and Pancreatic Diseases, School of Medicine, First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, People’s Republic of China

\textbf{A R T I C L E  I N F O}

Article history:
Received 12 September 2016
Received in revised form 9 November 2016
Accepted 9 November 2016

Keywords:
Severe acute pancreatitis
Tissue factor
Blood coagulation system
Kupffer cells
Liver injury

\textbf{A B S T R A C T}

This study aims to investigate the molecular mechanisms underlying the pathogenesis of severe acute pancreatitis (SAP) and SAP-associated liver injury, we performed an association analysis of the functions of tissue factor (TF) and blood coagulation system in both SAP patients and mouse SAP model. Our results showed that serum TF and tissue factor-microparticle (TF-MP) levels were highly up-regulated in both SAP patients and mouse SAP model, which was accompanied by the dysfunction of blood coagulation system. Besides, TF expression was also highly up-regulated in the Kupffer cells (KCs) of SAP mouse model. After inhibiting KCs in SAP mouse model, the amelioration of blood coagulation system functions was associated with the decrease in serum TF and TF-MPs levels, and the reduction of SAP-associated liver injury was associated with the decrease of TF expression in KCs. In conclusion, the dis-regulated TF expression and associated dysfunction of blood coagulation system are critical factors for the pathogenesis of SAP and SAP-associated liver injury. TF may serve as a potential and effective target for treating SAP and SAP-associated liver injury.

© 2016 Published by Elsevier Masson SAS.

\section*{1. Introduction}

Acute pancreatitis (AP) is a severe abdomen disease characterized by the activation of Trypsin in pancreas, which will cause pancreatic tissue self-digestion, edema, hemorrhage and even necrotic inflammation [1,2]. The patients with severe acute pancreatitis (SAP) suffer from severe pancreatic hemorrhage and necrosis which are usually accompanied by liver injury, infection, peritonitis and shock [3,4]. Therefore, the mortality of SAP is high.

The blood leaving pancreas is processed by the liver before returning to the heart, and the liver is frequently injured extrapancreatic organ in AP patients. As early as 1984, Blamey et al., report that 80% of the AP patients suffer from liver injury, and its severity is positively correlated with the progression of AP [5].

The pathological changes in liver cells can be observed in AP patients. Conversely, liver injury can contribute to the progression of AP [6,7]. Clinically, liver injury is an important indicator of AP severity and has significant predictive value for AP prognosis. Kupffer cells (KCs) account for 80–90% of the total cell number in the entire monocyte-macrophage system. They are the largest fixed macrophage population in human body. Previous studies indicate that KCs may contribute to AP-associated liver injury [8–10].

Currently, the pathogeneses of SAP and SAP-associated liver injury are not well-understood. Recent studies suggest that microcirculation has critical roles in AP occurrence and development [11,12]. The disturbance of pancreatic microcirculation is an important factor for the transformation from moderate acute pancreatitis (MAP) to SAP and the main cause of multiple organ failures and even death. Actively correcting or ameliorating the dysfunction of microcirculation can significantly reduce the severity of AP and improve AP prognosis. The imbalance between blood coagulation and fibrinolysis systems can largely contribute to the disturbance of pancreatic microcirculation. Abnormal blood
coagulation system can be observed during early AP and is obvious at SAP [13,14]. Although both blood coagulation and fibrinolysis systems can be activated during AP, the functions of fibrinolysis system are relatively insufficient. The exact mechanism underlying the dysfunction of coagulation and fibrinolysis during AP remains unclear.

Tissue factor (TF) is a transmembrane protein mainly expressed in extravascular tissues [15,16]. Recent studies suggest that TF is involved in both extrinsic coagulation pathway and intrinsic coagulation pathway [16,17]. During microcirculation, the major vector of TF is TF-microparticles (TF-MPs) which are released from activated mononuclear cells, endothelial cells and platelets, etc. by exocytosis. After the stimulation by lipopolysaccharide (LPS), tumor necrosis factor α (TNF-α) or interleukin-1 (IL-1), etc., mononuclear cells can have high TF expression level. TF can then be transferred to platelet and participate in pathological blood coagulation process.

Therefore, to understand the pathogenesis of SAP and SAP-associated liver injury, we simultaneously analyzed serum TF/TF-MP levels, KC/TF/TF-MP levels and the functional changes of blood coagulation system in both SAP patients and SAP mouse model. In addition, we also examined the effects of KC inhibition on KC TF/TF-MP level and the associated changes of blood coagulation system and liver injury in SAP mouse model. This study may further reveal the relationship among TF level, blood coagulation system and the pathogenesis of SAP and SAP-associated liver injury. Besides, it may provide valuable clues on how to treat SAP and SAP-associated liver injury.

2. Materials and methods

2.1. Subjects

A total of 40 SAP patients in the Second Affiliated Hospital of Chongqing Medical University from May 2013 to May 2014 were enrolled in this study. There were 16 male cases, and 24 female cases. The mean age of the patients was 64.2 ± 6.8 years. In addition, 40 health old people (20 male cases and 20 female cases. The mean age was 63 ± 6.5 years) receiving physical examination in the same hospital during the same period were served as the control group. There was no significant difference in gender and age between SAP and control group. All procedures performed in the experiments involving human participants were in accordance with the ethical standards of Chongqing Medical University Research Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consents were obtained from all individual participants included in this study. AP diagnosis and classification were performed according to a new international classification system for acute pancreatitis severity [4]. SAP was diagnosed if patients had clinical and biochemical changes of AP and met at least one of the following conditions: local complications (pancreatic necrosis, pseudocyst or pancreatic abscess), organ failure, Ranson score ≥3, APACHEII score ≥8, Balthazar CT classification D or E.

Exclusion criteria: 1) occurred less than 72 h after admission; 2) with malignant tumor; 3) oral administration of the drugs influencing coagulation system, such as anticoagulant drugs; 4) children and pregnant women; 5) with hematological system

Fig. 1. Serum TF and TF-MP levels are highly up-regulated in both SAP patients and SAP mouse model. A ELISA showed that the TF levels in SAP patients were much higher than those in healthy control subjects at 1d, 2d and 7d after admission (3–5 folds, p < 0.01). B FCM showed that the TF-MP levels in SAP patients were also much higher than those in healthy control subjects at the above timepoints (10–25 folds, p < 0.05). At 6 h, 12 h and 24 h after the establishment of SAP mouse model, ELISA and FCM showed that TF (C) and TF-MP (D) levels were both significantly higher than those in normal control mice and SO mice (p < 0.01). n.s., no significant difference. *, P < 0.05. **, P < 0.01. ***, P < 0.001. P < 0.001.
disease; 6) with hepatocirrhosis; 7) with diabetes; 8) with renal insufficiency; 9) without complete 24 h APACHE II score or 72 h enhanced CT scan data.

2.2. Establishment of SAP mouse model

The Six-week-old and healthy adult male C57BL/6 mice (20–30 g, clean grade) were used to establish SAP mouse model. They were purchased from the Experimental Animal Center of the Chongqing Medical University and raised according to animal ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. For each experimental group, 18 mice were randomly selected for the following experiments. Before the operation, all mice were fasted for 12 h and free to drink water. The normal healthy control mice (control group) received no operation. Mice were anesthetized by intraperitoneal injection of 4% chloral hydrate and received routine skin preparation and disinfection. A middle epigastric incision was made to get into the abdominal cavity. Found the opening of bile papilla along bile duct and choledoch (about 0.5 cm below). A venous infusion needle was used to retro-gradely puncture the anterior wall of duodenum at the opening of biliopancreatic duct until 0.5 cm to the distal biliopancreatic duct. Iarcerated the both ends of bile duct near porta hepatis and at the opening of biliopancreatic duct. Injected 5% sodium taurocholate (0.1 mL/100 g) at the flow rate of 0.2 mL/min to establish a SAP mouse model. After injection, kept the needle for 5 min and then withdrew the needle. Removed the artery clamp and oppressed the puncture site, no bile leakage should be observed. If hyperemia was observed at the inferior bile duct and pancreatic duct, SAP model was successfully established. Sutured the abdominal incisions. For the sham operation (SO) mice (SO group), just turned the duodenum and touched the pancreas for several times.

2.3. Measurement of serum TF level using enzyme-linked immuno sorbent assay (ELISA)

Human and mouse TF levels were measured using ELISA kit (USCNK, USA) according to manufacturer’s instructions. A standard curve was plotted by the data obtained from gradient dilution. On reaction plate, 100 μL first antibody solution was added to each sample. After washing, 100 μL substrate solution was added, and the reaction solution was incubated at 22–25 °C for 15 min. At last, 100 μL stop solution was used to terminate the reaction. OD492 nm was measured within 5 min. TF levels were calculated according to the standard curve.

2.4. Measurement of serum TF-MP level

A total of 2 mL blood plasma samples were collected from each human or mouse subject. Sodium citrate was added for anti-coagulation. Under room temperature, centrifuged the samples at 1900 × g for 15 min. The supernatant was centrifuged at 1900 × g for another 15 min, and the final supernatant was used for detection. Serum TF-MP levels were detected using flow cytometry (FCM; Becton Dickinson, San Jose, CA, USA). PE-IgG1, PE-CD142 and FITC-Annexin V antibodies (EBioscience, USA) were used. FITC

---

**Fig. 2.** SAP patients have dysfunctional blood coagulation system. A–D In SAP patients, all the four blood coagulation system indexes showed significantly higher scores than those in healthy control subjects at all three examined time points (P < 0.05). Serum WBC (E) and amylase (F) levels were both significantly higher in SAP patients (P < 0.05). Meanwhile, the correlation between the FIB levels (G) or DD levels (H) and the APACHE II scores have also been analyzed by using the Pearson correlation test. n.s., no significant difference. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
positive events were defined as the detection of MPs. If PE events also occurred, then they were defined as the detection of TF-MPs.

2.5. Examination of blood coagulation and biochemical indexes

For human subjects with an empty stomach, venous blood was collected on day 1, 3 and 7 after admission, respectively. For mouse subjects, blood was collected from abdominal aorta at 6 h, 12 h and 24 h after surgery, respectively, after the establishing of SAP model. The prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), D-dimer and fibrinogen (FIB) levels were measured by the CA7000 Automatic Blood Coagulation Analyzer (SYSMEX Co., LTD). The blood samples were centrifuged at 3000 r/min for 8 min, and the supernatant was analyzed by the
Fig. 4. TF expression is highly up-regulated in the KCs of SAP mouse model. At 6 h, 12 h and 24 h after the establishment of SAP mouse model, RT-PCR showed that TF mRNA levels were significantly higher in the KCs of SAP mouse model compared with those in healthy control mice and SO mice (11–22 folds, *P < 0.01). B. Western blot demonstrated that TF protein levels were also significantly up-regulated (*P < 0.05, **P < 0.01, ***P < 0.001).

2.6. Mouse KC isolation, culture and identification

With reference to Li PZ’s method [18], Hepatic portal vein was perfused with PBS buffer. Mouse liver tissues were collected and transferred to culture dish. Removed fat and fibrous tissues, and washed liver tissues with PBS for 3 times. Liver tissues were then cut into tiny pieces, and digested with 30 mL type IV collagenase (1 mg/mL) (Sigma-Aldrich, USA) in 37°C for 30 min. During digestion, gently pipetted the tissues for 3 times. After the filtration using 200-mesh, the filtered fluid was centrifuged four times to isolate KCs. Cultured the isolated KCs with DMEM culture medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Finally, F4/80 and CD68 immuno-fluorescence and flow cytometry were used for KCs cell identification. The method resulted in a satisfactorily high yield of 5 to 6 × 10^6 KCs per liver [18]. We calculated the proportion of the positively stained cells compared to the total cells (positively stained cells plus the unstained cells) in a least six selected fields as the percentage of staining cells.

2.7. HE staining for neutrophil

Nasal mucosa samples were fixed with 4% polyoxymethylene, then paraffin embedding within 24 h. HE staining was employed to observe morphological changes of nasal mucosa tissue under optical microscope, and 5 high power fields for counting of neutrophil.

2.8. Measurement of TF mRNA and protein levels in mouse KCs

Mouse total RNA was extracted according to the protocol provided by manufacturer. cDNA was synthesized using a reverse transcription kit (Takara Biotechnology Co., LTD). TF mRNA level was determined by Real-time PCR which was performed according to manufacturer’s protocol (TF primer was synthesized by Sangon Biotech, China).

Mouse total protein was extracted according to the protocol provided by manufacturer and quantified by the BCA method. Protein samples were separated by SDS-PAGE and detected by Western blot. The rabbit anti-mouse tissue factor monoclonal antibody (1:1000; Catalogue No: EPR88986; Abcam, UK), and the goat anti-rabbit HRP labeled IgG (1:3000; Catalogue No. ab97051; Abcam, UK) were used in this study. Chemiluminescence was used recorded by photographic film, and the gray levels of target protein bands were analyzed by the UVP BioSpectrum Imaging System (Bio-Rad Co., LTD, USA).

2.9. GdCl3 treatment in SAP mouse model

About 24 h before the procedure to establish SAP mouse model, half of the mice in each group were treated by 1 mL 0.1 mg/kg GdCl3(Gadolinium chloride) (Sigma, U.S.) using intravenous injection. After the establishment of SAP mouse model, the KCs of GdCl3-treated mice were isolated and analyzed by the methods described above.

2.10. Detection of SAP-associated liver injury in SAP mouse model

Liver injury was evaluated by immunohistochemistry. Mouse liver tissues were isolated, fixed in 4% polyformaldehyde for 24 h, and then dissected. After gradient dehydrating, hydration and drying,
tissue slices were stained hematoxylin-eosin (HE). Light microscopy was used to observe liver injury at the cellular level.

2.11. Statistical analysis

Data were expressed as means and standard deviations (SDs). Differences between the two groups were analyzed by using the Student’s t-test. When comparing three or more groups and the data are normal distribution, the one-way ANOVA method was used. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Serum TF and TF-MP levels are highly up-regulated in both SAP patients and SAP mouse model

To investigate the role of TF in SAP, we firstly examined the serum TF and TF-MPs levels in SAP patients. ELISA showed that the TF levels in SAP patients were much higher compared to those in healthy control subjects at 1 d, 2 d and 7 d after admission (Fig. 1A, 3–5 folds, P < 0.01). In addition, FCM showed that the TF-MPs levels in SAP patients were also much higher compared to those in healthy control subjects at the above timepoints (Fig. 1B, 10–25 folds, P < 0.01).

To further investigate the role of TF in SAP, we established a SAP mouse model. At 6 h, 12 h and 24 h after the establishment of SAP mouse model, ELISA and FCM showed that TF and TF-MPs levels were both significantly higher than those in normal control mice and SO mice (Fig. 1C, D, P < 0.01). The representative flow cytometry images in both of SAP patients and SAP mouse model were illustrated as the Supplementary Fig. 1.

3.2. Both SAP patients and SAP mouse model have dysfunctional blood coagulation system

Since TF has critical roles in blood coagulation, we also examined four typical blood coagulation indexes, PT, APTT, FIB and D-dimer, in both SAP patients at 1d, 2d and 7d and mouse model at 6 h, 12 h and 24 h after admission. In SAP patients, all these four indexes showed significantly higher scores than those in healthy control subjects at all three examined time points.
3.3. TF expression is highly up-regulated in the KCs of SAP mouse model

Liver injury is a common and severe complication of SAP. KCs are the critical mediator of SAP-associated liver injury. To investigate the molecular mechanisms underlying SAP-associated liver injury, we examined TF expression in KCs. At 6 h, 12 h and 24 h after the establishment of SAP mouse model, RT-PCR showed that TF mRNA levels were significantly higher in the KCs of SAP mouse model compared with those in healthy control mice and SO mice (Fig. 4A, 11~22 folds, P < 0.01). In addition, Western blot demonstrated that TF protein levels were also significantly up-regulated (Fig. 4B, C, P < 0.01).

3.4. The amelioration of blood coagulation system is associated with the decrease of serum TF and TF-MP levels in SAP mouse model

GdCl3 is an effective inhibitor of KCs. After GdCl3 treatment, ELISA and FCM showed that both TF and TF-MP levels were significantly down-regulated compared with the SAP mice without GdCl3 treatment (Fig. 5A, B, P < 0.05). In addition, it was accompanied by the amelioration of blood coagulation system
in SAP mice. The scores of PT, APTT, FIB and D-dimer levels were significantly lowered in the SAP mice treated by GdCl3 (Fig. 5C–F, P < 0.05). As predicted, serum WBC and Amylase levels were also significantly down-regulated (Fig. 5G, H, P < 0.05).

3.5. The reduction of SAP-associated liver injury is associated with the decrease of KC TF level in SAP mouse model

After GdCl3 treatment, RT-PCR and Western blot showed that both KC TF mRNA and TF protein levels were significantly down-regulated in SAP mice (Fig. 6A, B, P < 0.01). In addition, HE staining demonstrated that it was accompanied by the significant reduction of SAP-associated liver injury (Fig. 6C–F).

3.6. GdCl3 triggers the KCs depletion in SAP mouse model

In order to investigate the effects of GdCl3 on the KCs depletion, the KCs specific biomarker, CD68 and F4/80, were examined by using the immunofluorescence tests. The results indicated that the CD68 (Supplementary Fig. 2A) and F4/80 (Supplementary Fig. 2B) staining positive cells in GdCl3 treatment cells were significantly more compared to the no GdCl3 treatment (SO and SAP group) (P < 0.01).

4. Discussion

Although AP is severe acute disease, its pathogenesis is still not well understood. In this study, by simultaneously measuring serum/KC TF/TF-MP levels, the functional changes of blood coagulation system and SAP-associated liver injury, we revealed a significantly positive association among the up-regulation of TF/TF-MP levels, the dysfunction of blood coagulation system and the severity of SAP-associated liver injury. In addition, we also demonstrated that the down-regulation KC TF/TF-MP levels was accompanied by the amelioration of blood coagulation system and the reduction of liver injury.

Our study indicates that abnormal TF level and the dysfunction of blood coagulation system may be important factors contributing to the development and pathogenesis of SAP. In addition, our study also indicates that TF may serve as a new target for treating SAP. If TF level is down-regulated in KCs, the functions of blood coagulation system in SAP patients may be significantly ameliorated. Besides, the SAP-associated liver injury may be also significantly reduced.

However, we have to notice that our experiments were mainly based on association study. It will be very helpful to further confirm our discoveries by more subsequent studies. For example, if the up-regulation of TF level in SAP model is inhibited, the severity of SAP and SAP-associated liver injury may be largely reduced. If TF level is artificially up-regulated in healthy control or moderate acute pancreatitis (MAP) animals, the severity of SAP and SAP-associated liver injury may be increased, and more MAP cases may develop to SAP cases. TF is also involved in other human diseases [15–17]. Actually, TF has been selected as a potential target for treating some of these diseases [19–22].

In this study, the results also indicated that GdCl3 is an effective inhibitor of KCs. After GdCl3 treatment, both TF and TF-MP levels were significantly down-regulated compared with the SAP mice without GdCl3 treatment. Meanwhile, the GdCl3 could also decrease the expression of CD68 and F4/80 staining, which are the specific biomarker for the KCs. Therefore, the GdCl3 can inhibit the KCs amounts and the related biomarkers. However, whether the GdCl3 could inhibit the KCs in the other organs, such as spleen and pancreas, have not been investigated in the present study. Liu et al. [23] reported that the GdCl3 inhibits the macrophages in the spleen and the pancreas. Therefore, we would also investigate the effects of GdCl3 on macrophages in spleen and pancreas, which could provide more evidence that the protective effect of GdCl3 on macrophages.

Although some of the better findings have been observed, there were also a few limitations. Firstly, the staining patterns of the two antibodies of anti-C600 and anti-F4/80 did not show membranous structures that typically observed due to our limited instruments. Secondary, the nuclei has not been stained also because the lower-resolution instruments. In the following study, we would observe the membranous structures by using a higher-resolution instruments.

5. Conclusions

Our study suggests that TF and blood coagulation system may not only serve as import factor for SAP pathogenesis but also be selected as a candidate target for treating SAP. The studies of TF and blood coagulation system in different diseases may assist or have synergic effects on the progress of each other.

Conflict of interest

Authors declare that they have no competing interests.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81301618).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biопhа.2016.11.039.

References
