Correlation of fibrinogen-like protein 2 with progression of acute pancreatitis in rats

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Abstract

AIM: To examine fibrinogen-like protein 2 (fgl2) expression during taurocholate-induced acute pancreatitis progression in rats and its correlation with pancreatic injury severity.

METHODS: Forty-eight male Sprague-Dawley rats were randomly divided into the severe acute pancreatitis (SAP) group (n = 24) and the sham operation (SO) group (n = 24). Sodium taurocholate (4% at doses of 1 mL/kg body weight) was retrogradely injected into the biliopancreatic ducts of the rats to induce SAP. Pancreatic tissues were prepared immediately after sacrifice. At the time of sacrifice, blood was obtained for determination of serum amylase activity and isolation of peripheral blood mononuclear cells (PBMCs). Pancreatic tissue specimens were obtained for routine light microscopy including hematoxylin and eosin staining, and the severity of pancreatic injury was evaluated 1, 4 and 8 h after induction. Expression of fgl2 mRNA was measured in the pancreas and PBMCs using reverse transcription polymerase chain reaction. Expression of fgl2 protein was evaluated in pancreatic tissues using Western blotting and immunohistochemical staining. Masson staining was also performed to observe microthrombosis.

RESULTS: At each time point, levels of fgl2 mRNAs in pancreatic tissues and PBMCs were higher (P < 0.05) in the SAP group than in the SO group. For pancreatic tissue in SAP vs SO, the levels were: after 1 h, 3.911 ± 1.277 vs 1.000 ± 0.673; after 4 h, 9.850 ± 3.095 vs 1.136 ± 0.609; and after 8 h, 12.870 ± 3.046 vs 1.177 ± 0.458. For PBMCs in SAP vs SO, the levels were: after 1 h, 2.678 ± 1.509 vs 1.000 ± 0.965; after 4 h, 6.922 ± 1.984 vs 1.051 ± 0.781; and after 8 h, 13.533 ± 6.575 vs 1.306 ± 1.179. Levels of fgl2 protein expression as determined by Western blotting and immunohistochemical staining were markedly up-regulated (P < 0.001) in the SAP group compared with those in the SO group. For Western blotting in SAP vs SO, the results were: after 1 h, 2.183 ± 0.115 vs 1.110 ± 0.158; after 4 h, 2.697 ± 0.090 vs 0.947 ± 0.361; and after 8 h, 3.258 ± 0.094 vs 1.208 ± 0.082. For immunohistochemical staining in SAP vs SO, the results were: after 1 h, 1.793 ± 0.463 vs 0.808 ± 0.252; after 4 h, 4.535 ± 0.550 vs 0.871 ± 0.318; and after 8 h, 6.071 ± 0.941 vs 1.020 ± 0.406. Moreover, we observed a positive correlation in the pancreas (r = 0.852, P < 0.001) and PBMCs (r = 0.735, P < 0.001) between fgl2 expression and the severity of pancreatic injury. Masson staining showed that microthrombosis (%) in rats with SAP was increased (P < 0.001) compared with that in the SO group and it was closely correlated with fgl2 expression in the pancreas (r = 0.842, P < 0.001). For Masson staining in SAP vs SO, the results were: after 1 h, 26.880 ± 9.031 vs 8.630 ± 3.739; after 4 h, 53.750 ± 19.039 vs 8.500 ± 4.472; and after 8 h, 80.250 ± 12.915 vs 10.630 ± 7.003.
CONCLUSION: Microthrombosis due to fgl2 overexpression contributes to pancreatic impairment in rats with SAP, and fgl2 level may serve as a biomarker during early stages of disease.

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Key words: Fibrinogen-like protein 2; Microthrombosis; Fibrin; Severe acute pancreatitis; Peripheral blood mononuclear cell

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INTRODUCTION

Severe acute pancreatitis (SAP) is a pathogenic condition that progresses rapidly and has a high mortality,[1-3] but the underlying pathophysiological mechanisms remain incompletely defined. SAP is currently considered to be complicated by microcirculatory disturbances and coagulation abnormalities.[4,8]. Inflammatory mediators such as interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α) released during acute inflammatory reactions are not just involved in the inflammatory process but may also be responsible for the systemic activation of hemostasis in patients with SAP[6,7]. Intravascular coagulation and thromboembolism are believed to play an important role in the pathogenesis of SAP and are related to its severity.[6,9]. Acute inflammatory events during disease progression can lead to dysregulation of the coagulation cascade.[10]. In SAP patients, thrombin and platelets are deposited not only in the local pancreatic blood vessels but also in the connective tissue and intercellular spaces[8]. Studies suggest that biochemical variables such as prothrombin time, D-dimer, and clotting time may have prognostic value, and direct anticoagulant therapy has been shown to be helpful in the treatment of SAP.[8,9,10]. These facts suggest that coagulation and inflammation in SAP are correlated, thus microthrombosis plays a crucial role in SAP[10]. However, the exact pathophysiological mechanism remains unknown.

Fibrinogen-like protein 2 (fgl2)/fibroleukin (also termed fgl2 prothrombinase) was determined to be a new member of the fibrinogen-related protein superfamily (fibrinogen-related domain), which includes fibrinogen, tenascin, ficolin, and angiopoietin[13-15]. fgl2 is a direct prothrombinase with serine protease activity. fgl2 can cleave prothrombin to thrombin via a noncanonical pathway, resulting in fibrin deposition[16,17]. fgl2 leads to histopathological lesions and ischemic injury by mediating “immune coagulation”, fibrin deposition, and microthrombosis.[18-21]. Microvascular disturbances are caused by microthrombi that are activated and produced as a consequence of fgl2 action[19,21-23]. Nevertheless, whether fgl2 contributes to the pathogenesis of SAP is unclear.

In the present study, we used 4% sodium taurocholate to induce SAP in rats. We then investigated the expression and localization of fgl2 in pancreatic tissues. We also assessed fgl2 expression and its correlation with severity of pancreatic injury and microthrombi in rats with SAP to provide new insight into the pathogenesis of this disease.

MATERIALS AND METHODS

Animals

Forty-eight male Sprague-Dawley rats, weighing 200-250 g, were obtained from the Experimental Animal Center of Wenzhou Medical College, Wenzhou, China. All animals were fed standard rat chow, had free access to water, and were housed at a constant room temperature of 25 °C and a 12-h day/night cycle. All animals were acclimated for at least one week before the experiments were initiated. All procedures were performed in accordance with the Guidelines for Animal Experiments of Wenzhou Medical College.

Induction of SAP

All rats received intraperitoneal injection of 10% chloralhydrate (2 mL/kg body weight; Solarbio, Beijing, China) for anesthesia. The rats were divided into the SAP group (n = 24) and the sham operation (SO) group (n = 24). In the SAP group, a laparotomy was performed through a midline incision. Sodium taurocholate (4%; 1 mL/kg body weight; Sigma, St. Louis, MO, United States) was retrogradely injected into the biliopancreatic duct through the papilla using a segmental epidural catheter via a microinjection pump at a speed of 0.2 mL/min. A microclip was placed in the hepatic portion of the biliopancreatic duct to avoid reflux before the injection. SO rats underwent surgery but without infusion. After each operation, the abdomen was closed in two layers. All procedures were carried out using sterile techniques.

Sample collection and determination of serum amylase

At defined time points (1, 4 and 8 h; n = 8 per time point) after SAP induction, rats were anesthetized with 10% chloralhydrate (2 mL/kg body weight) and euthanized by exsanguination. Pancreatic tissues were harvested immediately and divided into two pieces. Portions of the tissues were fixed in 4% paraformaldehyde for immunohistochemical staining and microscopic observation, and other portions were removed and stored in liquid nitrogen until use. Blood samples (5 mL) were obtained via postcava puncture, and 4 mL of each sample was collected and stored in 5-mL tubes without anticoagulants (Generay, Shanghai, China). The blood samples were centrifuged at 1200 × g for 20 min, and the serum was collected for determination of amylase activity (U/L) with a fully automatic biochemical analyzer (Hitachi, Tokyo, Japan). The remaining 1 mL blood was stored in
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ethylenediaminetetraacetic acid-containing tubes (Gen
eray) and used to isolate peripheral blood mononuclear cells (PBMCs).

**Isolation of PBMCs**

PBMC isolation was performed with density gradient centrifugation. The blood sample (1 mL) was diluted with 1 mL 0.9% saline. Subsequently, the diluted cell suspension was carefully layered over 2 mL Bandicoot per
coll (Solarbio) and centrifuged at 2000 × g for 20 min at 20 °C. The PBMC layer was carefully transferred into a new tube, and the volume was brought to 5 mL with 0.9% saline and centrifuged (2000 × g, 5 min, 20 °C). This step was repeated. Finally, the supernatant was carefully re
moved, reserving the PBMCs at the bottom of the tube. PBMCs were immediately stored in liquid nitrogen until use.

**Histological analysis and assessment of pancreatic tissue injury**

Pancreatic tissue samples were fixed in 4% paraformal
dehyde for histological analysis. The samples were de
hydrated and embedded in paraffin. Pancreas sections were stained with hematoxylin and eosin (HE) for rou
tine light microscopy. Slides were examined in a blinded fashion by two pathologists who were unaware of the treatment protocol according to the modified method of Schmidt et al[24] and Eşrefoğlu et al.[25]. Variables of edema, hemorrhage, acinar cell degeneration, and interstitial inflammation were scored in 10 random fields of each slide to assess the severity of pancreatic injury under a light microscope (CX31, Tokyo, Japan; HE staining, × 200). Each variable was scored as follows: (1) edema: 0 = absent, 1 = focially in the interlobular space, 2 = in
creased in the intralobular space, 3 = isolated-island ap
pearance of pancreatic acinus; (2) hemorrhage: 0 = ab
sent, 1 = slight, 2 = moderate, 3 = severe; (3) acinar cell degeneration: 0 = absent, 1 = focal (< 5%); 2 = and/or sublobular (< 20%); 3 = and/or lobular (> 20%); and (4) inflammation: 0 = absent, 1 = slight, 2 = moderate, 3 = severe. The sum of these four variables for each pan
creas section could have a maximum score of 12.

Pancreatic tissue sections were also stained with Mass
son stain to observe microthrombi in microvessels. One hundred microvessels on each slide were randomly se
lected, and the percent of microthrombus-positive ves
sels was calculated.

**Real-time fluorescence-based quantitative polymerase chain reaction**

Total RNA was extracted from pancreatic tissues and PBMCs using Trizol reagent (Invitrogen, Carlsbad, CA, United States), and cDNA was synthesized with the First Strand cDNA synthesis kit (MBI Fermentas, Burlington, Canada) according to the manufacturer’s protocols. The samples were subsequently amplified using Moloney mu
rine leukemia virus reverse transcriptase and Taq DNA polymerase (Invitrogen) using an ABI 7500 Sequence De
tection System (Applied Biosystems Inc., Carlsbad, CA, United States). The sequences of the primers (Generay) were as follows: fgl2 (153 bp): 5'-CCTGGAGATTGG-GTTTCGT-3' (forward) and 5'-TACCATGCCCTTCTC
CAAGG-3' (reverse), β-actin (153 bp): 5'-TGTCAC
CAACTGGGACGATA-3' (forward) and 5'-GGGGT
GTTGAAGGTCTCAA-3' (reverse). The cDNA was denatured at 95 °C for 5 min and amplified for 40 cycles of 95 °C (15 s), 60 °C (45 s), and 72 °C (60 s), followed by a final extension at 72 °C (5 min). The samples were tested in triplicate, and the results were calculated using the 2−ΔΔCT method. The expression of fgl2 mRNA was shown as relative to β-actin.

**Western blotting**

Pancreatic proteins were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Bil
lerica, MA, United States). The membrane was blocked with 5% skim milk in Tris-buffered saline and then incubated with a polyclonal antibody against fgl2 (Biosynthesis Biotechnology, Beijing, China; 1:200) at 4 °C overnight. The membrane was washed three times with Tris-buff
ered saline and incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) conjugated to horseradish peroxidase for 2 h at room temperature. The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Pierce, Rockford, IL, United States). Protein expression levels were normalized to β-actin.

**Immunohistochemical staining**

Immunohistochemical staining was performed to assess fgl2 protein expression in the pancreas using EnVision reagents (Dako, Glostrup, Denmark). Four-μm-thick par
affin sections were routinely cut. Microwave antigen retrieval was conducted for 20 min in citrate buffer (pH 6.0) to activate antigens before quenching endogenous peroxidase activity in 0.3% H2O2 for 10 min. After three times of washing with phosphate-buffered saline (Generay) and then the primary reaction solution, the sections were incubated with rabbit polyclonal anti-rat fgl2 (Bio
synthesis Biotechnology; 1:100) for 2 h at 37 °C. Follow
ing the same washing procedure, EnVision reagents were applied and incubated for 30 min at 37 °C. Finally, the reaction was developed with 0.05% dianinobenzidine and counterstained with hematoxylin for microscopy. Phosphate-buffered saline was used as a negative control instead of the primary antibody. To measure fgl2 protein expression, 10 randomly selected fields across each section were evaluated at × 200 magnification.

**Statistical analysis**

All data represent the mean ± SD. SPSS 15.0 software (SPSS, Chicago, IL, United States) was used for statisti
cal analysis. Differences between the SAP and SO groups were analyzed with the Student’s t test. One-way analysis of variance was used to check for statistical significance
Levels of serum amylase are elevated in rats with SAP

Serum amylase is the most commonly used biochemical indicator of acute pancreatitis. Levels of serum amylase were markedly elevated \((P < 0.01)\) in the SAP group compared with the SO group at each time point. There was no significant difference in the levels of serum amylase among the three time points in the same group. Pearson’s correlation coefficient was calculated to determine the strength of the association between two continuous variables. \(P < 0.05\) was considered statistically significant.

RESULTS

Levels of serum amylase are elevated in rats with SAP

Serum amylase was the most commonly used biochemical indicator of acute pancreatitis. Levels of serum amylase were markedly elevated \((P < 0.01)\) in the SAP group compared with the SO group at each time point. There was no significant difference in the levels of serum amylase among the three time points in the SO group (Figure 1).

Histopathology and pathological scoring of pancreatic tissues

Compared with the SO group, the pancreatic tissues in the SAP group (Figure 2A-C) at 1 h (A), 4 h (B), and 8 h (C) appeared much more severely damaged. The pancreatic tissue of the SO group (Figure 2D) appeared morphologically normal at 8 h. Microscopic examination of the pancreas in the SAP group showed edema, hemorrhage complicated by microthrombosis, acinar cell degeneration, and inflammation (Figure 2A-C). The mean pathological score of each rat with SAP was higher \((P < 0.01)\) than that of control rats at each time point. Pancreatitis worsened over time, as demonstrated by the increasing pathological score \((P < 0.01)\), Figure 2E).

Masson staining was used to observe microthrombosis, which was seen as very bright red regions under light microscope. Microthrombi were localized and tightly combined with the microvascular endothelium of the pancreatic tissues, which suggested that the microthrombi formed in situ with the formation of fibrin. The percent of Masson staining-positive microvessels in the pancreas of the SAP group was higher \((P < 0.01)\) than that in control rats at all time points and tended to increase \((P < 0.01)\), Figure 3).

DISCUSSION

SAP is an inflammatory disorder mediated by up-regulated expression of proinflammatory cytokines such as TNF-\(\alpha\). Inflammation and coagulation are interactive events during SAP. Microthrombosis is found in the early stages of the SAP rat model. The “immune coagulation” hypothesized by Levy means that fgl2 could be transcribed and the mRNA translated following the induction of cytokines such as IL-2 and TNF-\(\alpha\), resulting in immediate activation of coagulation. \(^{[17,23,26,27]}\) fgl2 functions as a bridge molecule between immune and coagulation reactions. fgl2 is highly expressed in endothelial cells due to the action of TNF-\(\alpha\). Otherwise, interferon-\(\gamma\) is necessary for macrophage induction of fgl2. \(^{[27,28]}\) In the present study, fgl2 was clearly up-regulated and localized in inflammatory regions of the pancreas sections, suggesting that fgl2 as an effector molecule may contribute to SAP pathogenesis by initiating and promoting coagulation through the induction of proinflammatory cytokines such as TNF-\(\alpha\).
fgl2/fibroleukin is a new procoagulant that belongs to the fibrinogen-related protein superfamily, which has a potent capability of inducing microthrombosis. fgl2 is expressed in activated macrophages, T cells, and endothelial cells. fgl2 expression and the subsequent fibrin deposition account for microthrombus formation in situ, which occurs via a novel way by directly producing thrombin in addition to the classic extrinsic and intrinsic coagulant pathway. Researches suggest that microcirculatory disturbance is an important aspect of the mechanism of SAP. Our data show that microthrombosis generated during pancreatitis (due to increased fgl2 expression) led to ischemia/hemorrhage injury and consequently resulted in necrosis and dysfunction of the pancreas. Moreover, we found that fgl2 plays a contributing role in pancreatic microthrombus formation in rats with SAP. Our study also shows that fgl2 has procoagulant activity in the pancreatic endothelial cells of microvessels in rats with SAP, and fgl2 expression correlates strongly with the severity of pancreatic injury.

We observed that both fgl2 mRNA and protein levels were higher in rats with SAP and that the levels gradually increased in parallel with the progression of SAP. We also observed that fgl2 expression was associated with microthrombus formation and that microthrombus formation in situ may be caused by fgl2, leading to partial impairment of the pancreatic tissues and the functions involved. We propose that fgl2 functions similarly as in other diseases: microthrombi form as a consequence of fgl2 expression in the pancreas, leading to microcirculatory disturbance and consequent hemorrhage/ischemia injury in rats with SAP, thus aggravating the pancreatic injury.
Figure 3  Masson staining of microthrombosis in pancreatic microvessels from severe acute pancreatitis and sham-operated rats (× 200). A-C: Microthrombosis in situ in pancreatic microvessels of rats with severe acute pancreatitis (SAP) (arrows) at 1 h (A), 4 h (B), and 8 h (C) in the SAP group; D: No microthrombi were detected in the sham operation (SO) group (arrow); E: The percent of Masson staining-positive microvessels. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean ± SD. "P < 0.01 vs SO group.
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Figure 4  Levels of fibrinogen-like protein 2 mRNA and protein in severe acute pancreatitis and sham-operated rats. A, B: The calculated levels of fibrinogen-like protein 2 (fgl2) mRNA in the pancreas and peripheral blood mononuclear cells (PBMCs). The expression of fgl2 mRNA is relative to β-actin; C, D: fgl2 protein expression revealed by Western blotting. Protein levels were normalized to β-actin. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ vs sham operation (SO) group. SAP: Severe acute pancreatitis.

Figure 5  Fibrinogen-like protein 2 expression in pancreatic tissues of severe acute pancreatitis and sham-operated rats with immunohistochemical staining (× 200). A-D: Fibrinogen-like protein 2 (fgl2) proteins were expressed in microvascular endothelial cells of rats with severe acute pancreatitis (SAP) (arrows in A-C) and control rats (arrow in D) at 1 h (A), 4 h (B), and 8 h (C, D) after initiation of SAP; E: fgl2 protein expression is indicated by the mean absorbance value. The data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ vs sham operation (SO) group.
To evaluate the relevance of fgf2 expression and the severity of pancreatic disease, a Pearson's correlation coefficient was calculated. Evaluation of fgf2 expression levels in both the pancreas and PBMCs (containing lymphocytes, monocytes, dendritic cells, and other cell types) revealed a strong correlation with the severity of pancreatic disease as illustrated by the pathological score. Thus, fgf2 expression may serve as a promising marker for predicting the occurrence of SAP in early stages.

Injection of a neutralizing antibody or genetic therapy against fgf2 in diseases involving fgf2, such as murine hepatitis virus 3-induced hepatitis and graft rejection, has been beneficial in terms of attenuating fibrin deposition and pathology and preventing death in mice11,13,14. Thus, we will perform an in-depth investigation to see whether inhibiting fgf2 activity or applying fgf2 antibodies will delay or ameliorate the disease course of SAP.

In summary, fgf2 functions as a novel prothrombinase and may initiate the coagulation reaction, finally leading to microthrombosis in microvessels of pancreatic tissues in an experimental model of rats with SAP, and resulting in ischemia/hemorrhage injury as well as necrosis and dysfunction of the pancreas. fgf2 expression is closely correlated with the severity of pancreatic disease, and thus fgf2 may serve as a useful biomarker for predicting SAP at the onset of disease. Whether inhibiting fgf2 or using antibodies against fgf2 will delay or ameliorate SAP requires further exploration.

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