**Abstract**

**Objective:** To investigate the changes in proteomics of peripheral polymorphonuclear neutrophils (PMNs) of rats with acute necrotizing pancreatitis (ANP).

**Methods:** 60 rats were randomly divided into 2 groups (30 per group): ANP group and sham operation (SO) group. At 3 h, 6 h and 12 h after ANP, animals were independently sacrificed and blood was obtained from the inferior vena cava for the detection of serum amylase. In addition, density gradient centrifugation was done for separation of PMNs. The apoptosis of PMNs was determined by flow cytometry. Pancreatic tissues were collected for hematoxylin-eosin staining (H&E) staining and pathological evaluation. In addition, at 12h after ANP, PMNs were lysed, and label-free technique was employed to determine the differentially expressed proteins. The apoptosis related proteins with differential expression were identified.

**Results:** In the ANP group, PMN apoptosis was delayed. The apoptosis rate was the lowest at 12 h. Significant difference was noted in the apoptosis rate between two groups at different time points (p<0.01). Four apoptosis related proteins were identified with differential expression: 78KDA glucose regulatory proteins, RhoGTPases, L-lactate dehydrogenase A chain and hemoglobin $\alpha_2$ chain.

**Conclusion:** In ANP, PMN apoptosis was delayed. The apoptosis-related proteins with differential expression may be involved in the delayed apoptosis of PMN in ANP rats.

**Key words:** Necrotizing pancreatitis; delayed apoptosis; neutrophils; proteomics.

**Received** April 20, 2013; **Accepted** May 08, 2013

**Introduction**

Numerous polymorphonuclear cells aggregate in the pancreas and play important roles in the development of acute necrotizing pancreatitis (ANP). Peripheral polymorphonuclear neutrophils (PMNs) are major cells inducing systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS)\(^1\). O’Neills et al\(^2\) found the apoptosis of peripheral PMN was inhibited significantly in ANP. Currently, the changes in proteins related to PMN apoptosis in ANP are not extensively studied. In the present study, an ANP animal model was prepared in rats, and peripheral PMN apoptosis was investigated to identify the changes in proteins related to PMN apoptosis with proteomics techniques.

**Materials and methods**

**Materials**

Health male Sprague-Dawley rats (n=60; specific pathogen free) weighing 250-300 g were purchased from the Experimental Animal Center of Affiliated Sixth People’s Hospital of Shanghai Jiaotong University. Sodium taurocholate (Sigma, USA), neutrophil separation solution for rat (LZS1091; Tianjin Haoyang Biotech Co., Ltd) and apoptosis detection kit (BD company, USA) were used in this study.

**Grouping**

These rats were randomly assigned into 2 groups: ANP group and sham operation (SO) group. In ANP group, animals were sacrificed at 3 h, 6 h and 12 h after introduction of ANP.
Establishment of ANP animal model

The ANP animal model was prepared according to previously described\(^3\) with modification. Food deprivation was done for 12 h before surgery but animals were given ad libitum access to water. Then, animals were intraperitoneally anesthetized with 3% pentobarbital sodium (1ml/kg), and put in a supine position. A midline incision was made in the abdomen, and the duodenum was exposed. A needle was inserted into the pancreatic duct (about 0.4 inward) through the intestinal wall contralateral to the duodenal mesentery. Then, the pancreatic duct was clamped at 2 sites, followed by injection of 5% sodium taurocholate (1 ml/kg) at a rate of 0.2 ml/min. Five minutes later, the needle was withdrawn, and the wound was closed. In the SO group, laparotomy was done, and the pancreas was exposed without other procedures. When animals recovered from anesthesia, they were given ad libitum access to water but received food deprivation.

Processing

At 3 h, 6 h and 12 h after ANP, 10 rats were sacrificed and blood was obtained from the inferior vena cava. Then, 1 ml of blood was used to detect serum amylase and 6 ml of blood for separation of PMN. The pancreatic tissues were collected and fixed in 10% formalin, embedded in paraffin and sectioned followed by HE staining. Schmids method\(^4\) and Rongione method\(^5\) were employed for pathological evaluation of pancreatic tissues by two experienced pathologists blind to this study (Table 1). Label free technique was used for proteomic analysis of PMN.

Detection of serum amylase

Serum amylase was detected by colorimetry with an automatic biochemical analyzer.

Separation of PMNs

Percoll density gradient centrifugation was done for separation of PMN. In brief, 6 ml of blood was obtained and added into 2 tubes (3 ml in each). The blood was mixed with PMN separation solution at a ratio of 1:1 followed by centrifugation at 400 xg for 15 min (15-cm rotor). The red blood cells were removed, and the supernatant and white layer were collected into 3 ml of separation solution, followed by centrifugation at 2000 xg for 15 min. The third PMN layer and cloudy solution (containing some PMN) were collected into a tube containing 4 ml of PBS followed by centrifugation at 2000 xg for 15 min. After washing in PBS twice, the PMNs were collected. These cells were suspended in PBS and counted. The cell density was adjusted to 1×106/ml. Cells collected at different time points were subjected to detection of apoptosis. In addition, these cells were lysed in Trizol (106 cells/ml Trizol) and stored in liquid nitrogen. Label free technique was employed for proteomic analysis of peripheral PMN.

Detection of PMN activity

In brief, 0.9 ml of PMN suspension was mixed with 0.1 ml of 0.4% trypan blue. Viable cells were not stained, and dead cells were blue. A total of 200 cells were counted and the proportion of viable cells was calculated. Generally, the proportion of viable cells was >95%. PMN viability = non-stained cells/200 ×100%.

Detection of PMN apoptosis

Cells were washed in PBS at 4oC twice. Then, the cell suspension was mixed with 60 µl of binding buffer and 5 µl of Annexin V-FITC solution. Incubation was done in dark at room temperature for 15 min. Five minutes before detection, 5 µl of Propidium Iodide and 150 µl of binding buffer were added. Apoptosis was measured by flow cytometry. Annexin V cells were apoptotic cells, PI positive cells were necrotic cells and cells negative for both Annexin V and PI were viable.

<table>
<thead>
<tr>
<th>Pathological findings</th>
<th>Pathological score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema</td>
<td>Interlobular edema</td>
<td>Diffused interlobular edema</td>
<td>Enlargement of pancreas and interlobular septa</td>
<td>Obvious lobular separation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>&lt;20 cells</td>
<td>20–50 cells</td>
<td>50–100 cells</td>
<td>&gt;100 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>&lt;25% pancreatic duct parenchyma</td>
<td>25%–50% parenchyma</td>
<td>50%–75% parenchyma</td>
<td>&gt;75% parenchyma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td>Destruction of parenchyma around pancreatic duct</td>
<td>&lt;20% spotty destruction of parenchyma</td>
<td>20%–50% of loss of lobule</td>
<td>&gt;50% of loss of lobule</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Criteria for pathological evaluation of pancreatic tissues.
**Changes in proteomics of PMN with non-labeled quantitative proteomic technique**

LC-MS/MS: In brief, 10 µg of products were subjected for MS for 2 h. Capillary High Performance Liquid Chromatography: The Capillary solution was 200°C. Solution A was 0.1% formic acid. Solution B was 0.1% formic acid in acetonitrile solution (84%). The column was balanced with 95% solution A, and samples were loaded onto Trap column via an automatic sampler. About 105 min later, the concentrations of solution B were increased linearly from 4% to 50% within 9 min and then increased linearly from 50% to 100% within 6 min. Then, the concentration of solution B was maintained at 100%.

Analysis of data from MS: The mass-to-charge ratio of polypeptides and fragments of polypeptides was determined as follow: After each full scan, spectrum of 20 fragments was collected by MS1 scan with profile mode and MS2 scan with centroid mode. Data analysis: the raw file was qualitatively analyzed with DeCyder MST2.0. MASCOT algorithm was employed to identify and quantify the polypeptides.

**Qualitative analysis:** MS was done three times for each sample. The list of identified proteins was output via the Buildsummary 4.8.0. Non-labeled quantitative method was used with DeCyder MST2.0. MASCOT algorithm was employed to identify and quantify the polypeptides.

**Pathological examination**

Sections were routinely prepared for H&E staining. The pathological features of pancreatic tissues were evaluated.

**Statistical analysis**

Data were expressed as mean ± standard deviation (±s) . Statistical analysis was done with SPSS version 17.0. Comparisons between two groups were done with t test, and those of intragroup qualitative data performed with one way analysis of variance. A value of P<0.05 was considered statistically significant.

**Results**

**Changes in serum amylase in two groups**

In ANP group, the serum amylase level increased after introduction of ANP and reached a maximal level at 12 h (9888.00±1498.06). The serum amylase level in ANP group was markedly higher than that in SO group at different time points. In SO group, the serum amylase level remained unchanged (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP</td>
<td>2577.00±890.22</td>
<td>5521.30±1177.08</td>
<td>9888.00±1498.06</td>
</tr>
<tr>
<td>SO</td>
<td>770.60±161.25</td>
<td>788.10±192.01</td>
<td>713.80±199.97</td>
</tr>
</tbody>
</table>

Table 2: Serum amylase level at different time points after introduction of ANP (n=10, ±s, IU/L). Note: a, p<0.05 vs SO group; b, p<0.05 between two time points in ANP group.

**Pathological findings of pancreatic tissues**

**Gross findings:** In SO group, there were no marked changes in the pathology of pancreatic tissues. In ANP group, bloody ascite was observed. Necrotic foci were found in the pancreatic tissues. Adhesion of intraabdominal organs and massive saponification spots in the mesentery and omentum were also noted.

**Microscopic findings:** In the SO group, there was no significant change in pathology of pancreatic tissues. In ANP group, swelling of pancreatic acini and tissue necrosis, infiltration of inflammatory cells and necrotic foci were found to different extents at different time points. Moreover, these pathological features deteriorated over time after introduction of ANP (Figure 1). In ANP group, the pathological score was significantly higher than that in SO group (P<0.05) (Table 3).

**Changes in apoptosis of peripheral PMNs in two groups**

The apoptosis rate reduced over time in ANP group and reached a minimal level at 12 h (2.15±0.45). Significant difference was found in the
apoptosis rate between the two groups at different time points (P<0.01) (Figure 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>3h</th>
<th>6h</th>
<th>12h</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP</td>
<td>6.4±1.35a,b</td>
<td>8.3±1.34a,b</td>
<td>10.7±1.95a,b</td>
</tr>
<tr>
<td>SO</td>
<td>0.1±0.32</td>
<td>0.2±0.42</td>
<td>0.1±0.32</td>
</tr>
</tbody>
</table>

Table 3: Pathological score of pancreatic tissues in two groups (n=10, ±s).
Note: a, P<0.05 vs SO group; b, P<0.05 between two time points in ANP group

Proteomic analysis

The expression of a total of 513 proteins was quantified. Randomly paired t-test was used to screen these proteins, and significant difference in expression was noted in 320 proteins (P<0.05). In the ANP group, 219 proteins presented with up-regulation and 101 with down-regulation. In addition, 4 proteins closely related to apoptosis were identified: 78KDa glucose regulatory proteins, RhoGTPases, L-lactate dehydrogenase A chain and hemoglobin α2 chain, and the ratios of these proteins in ANP group to those in SO group were 1.953614, 3.526625, 1.766764 and 0.609825, respectively.

Discussion

Our results showed the peripheral PMN in ANP group presented with delayed apoptosis and obvious changes were found in the proteins of neutrophils. Among these differentially expressed proteins, 4 proteins related to apoptosis were identified; GRP78, RhoGTPases, L-lactate dehydrogenase A chain and hemoglobin α2 chain.

GRP78 is a marker of endoplasmic reticulum stress. In endoplasmic reticulum, GRP78 can block aggregation of nascent polypeptides, regulate calcium homeostasis, combat endoplasmic reticulum associated apoptosis and initiate response to unfolded proteins.

GRP78 may exert anti-apoptotic effect which is attributed to following functions of GRP78:
- GRP78 may combat the endoplasmic reticulum associated apoptosis;
- GRP78 may inhibit the mitochondrion dependent apoptosis. Lin et al found that GRP78 could inhibit the release of cytochrome c and apoptosis-inducing factor (AIF) from the mitochondria to exert anti-apoptotic effect under the endoplasmic reticulum stress condition;
- GRP-78 may up-regulate the expression of Bcl-2, an anti-apoptotic protein, and down-regulate the expressions of BAD, BAX and BAK, three pro-apoptotic proteins to inhibit apoptosis. Under the endoplasmic reticulum stress condition, the GRP78 expression increases, which may attenuate the endoplasmic reticulum stress and inhibit apoptosis.

RhoGTPases are a group of small GTPases and belong to Ras superfamily. These proteins can
bind to GDP in an inactive form or bind to GTP in an active form, which may confer a switch like effect\(^{(1)}\). Barberan et al found\(^{(12)}\) Rho could reduce the activities of caspase-3 and -9 to delay cell apoptosis. In addition, studies also revealed that Rac1 could activate reduced nicotine adenine dinucleotide phosphate oxidase, which increases the production of ROS and inhibit cell apoptosis\(^{(13)}\). It may inhibit cell apoptosis via a phosphatidylinositol 3 - kinase /Akt dependent manner\(^{(14)}\). There is evidence showing that, in ANP, the number of apoptotic cells was proportional to the NF-κB expression\(^{(15)}\). The activation of NF-κB in peripheral neutrophils might attribute to the delayed apoptosis of these cells in ANP\(^{(16)}\). RTKN, an effector of Rho, may activate Rho/RTKN/NF-kb pathway to induce the expressions of anti-apoptotic proteins (cIAP-2, BCL-XL, A1 and A20) resulting in inhibition of apoptosis\(^{(17,18)}\).

Our results showed delayed apoptosis predicted the deterioration of ANP. We speculated that proteins such as GRP78 and RhoGTPases in ANP rat were involved in the pathogenesis of delayed apoptosis of PMN. However, the exact mechanism is largely unclear and more studies are required. Once the mechanism is elucidated, measures can be taken largely unclear and more studies are required. Once the mechanism is elucidated, measures can be taken to induce or promote apoptosis of neutrophils, which may attenuate the inflammation in ANP and prevent the progression into SIRS and MODS. Our findings provide evidence for the treatment of ANP.

References


Request reprints from:
XIAN-HUA ZANG
Graduate Departamnt of Soochow University
Suzhou 215006
(China)