Influence of platelet activating factor on expression of adhesion molecules in experimental pancreatitis

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Abstract
AIM: To determine whether Platelet activating factor (PAF) has a regulatory role in the expression of adhesion molecules and accumulation of neutrophils in a murine model of acute pancreatitis.

METHODS: One hundred twenty-eight Kunming mice were divided into four groups. Group 1 received 0.1 ml saline s.c. every hour for three hours (sham). Group 2 received cerulein (50 µg/kg dose s.c.) every hour for three hours. Group 3 received AP and additional challenge of PAF (50 µg/kg in absolute ethanol) (AP/PAF). Group 4 received AP plus therapeutic treatment with GAB (25 mg dose i.p.) immediately after the first challenge of cerulein (AP/GAB). Animals were sacrificed at 12 h after the first challenge of saline or cerulein. Adhesion molecules of pancreas were semi-quantified by SP methods. Standard assays were performed for serum amylase and myeloperoxidase activity (MPO) of pancreas. Histology of pancreas was scored in a blind manner. Water content of pancreas was also measured at the same time.

RESULTS: Control pancreata showed negligible adhesion molecule expression and neutrophil accumulation. There were evident adhesion molecules expression and neutrophil accumulation in AP and AP/PAF compared with sham (P<0.05). AP/GAB had a lower level of adhesion molecules, neutrophils, and water content versus AP and AP/PAF (P<0.05). Histology showed a trend toward improvement in AP/GAB, but did not reach statistical significance.

CONCLUSION: PAF can induce the expression of adhesion molecules that mediate neutrophil accumulation. The PAF antagonist reduces the expression of adhesion molecules and the severity of inflammation when given immediately after the induction of mild AP in mice. These results suggest that PAF antagonism may be useful in the treatment of mild pancreatitis after its clinical onset.

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INTRODUCTION
Acute pancreatitis (AP) was characterized as local and systemic inflammatory reactions. Adhesion molecules play a pivotal role in neutrophil immigration and accumulation[1]. Platelet activating factor (PAF) is a biologically active phospholipid which is thought to function as one of the proximal mediators in the inflammatory cascade of AP. However, the relationship between PAF and the expression of adhesion molecules in AP remains largely unknown, although Blackstone MO has ever speculated[2].

The present study was conducted to determine whether PAF has a regulatory role in the expression of adhesion molecules and the accumulation of neutrophils. To examine this effect, a murine model of edematous pancreatitis was established by overdose administration of cerulein, as described by Tani et al[3]. PAF and its antagonist (GAB) were also used in this experiment.

MATERIALS AND METHODS

Materials
Cerulein and PAF were purchased from Sigma Chemical Co. (St.Louis, Missouri). GAB (Ginkgolide AB) was a generous gift from associate professor Di-Qing Zhang in Guizhou University. MPO assay kit was purchased from Nanjing Jiancheng Bioengineering Co. Ltd. China. Rabbit anti-rat E-selectin polyclonal antibody and Rabbit anti-rat ICAM-1 polyclonal antibody were purchased from Boster Bioengineering Co. Ltd. China. SP assay kit was purchased from Maixin Bioengineering Co. Ltd. China. Cerulein was dissolved in normal saline at the concentration of 10 µg/ml. PAF was first dissolved in absolute ethanol, and then diluted with normal saline when it was to be used. GAB was dissolved in 30 % Dimethyl Sulfoxide (DMSO).

128 six-week-old Kunming mice, weighing 34-36 g each, were purchased from the experimental animal center of Wuhan University. The animals were randomly divided into 4 groups (described in methods) and fed standard laboratory chow. All animals were allowed to acclimatize for a minimum of 1 week prior to experimentation. Before the day of the experiment, animals were fasted overnight, but allowed free access to water.

Animals model
Acute pancreatitis was established in 32 mice (AP group) by subcutaneous injection of cerulein (50 µg/kg dose) every hour for three hours. Sham group (32 mice) received subcutaneous injection of normal saline every hour for three hours. AP/PAF group (32 mice) received cerulein (50 µg/kg dose) every hour for three hours, plus PAF (50 µg/kg dose) injected peritoneally immediately after the first cerulein challenge. AP/GAB group (32 mice) received cerulein (50 µg/kg dose) every hour for three hours, plus therapeutic treatment with GAB (25 mg/kg i.p.) immediately after the first cerulein challenge.

The animals were sacrificed at 12 h after the first cerulein challenge or saline injection. Some mice (eight in every group) were decapitated and the blood was collected in vials for the analysis of serum amylase. Pancreata were dissected. Some were weighted and grounded for the analysis of MPO, and the others were fixed with formalin for histological scoring and
immunohistochemical staining. Some left pancreatic tissues were dried and weighted.

MPO measurement
MPO assay of pancreas was performed according to the instructions of commercial kit.

Amylase
Serum amylase levels were measured using starch-iodine method.

Water content
Pancreas was dissected from its attachment and the fluid on its surface was dried with filter paper. Then the pancreas was placed in oven at a temperature of 90-92 °C for 10 hours. Water content = \( \frac{\text{Weight}_{\text{wet}} - \text{Weight}_{\text{dry}}}{\text{Weight}_{\text{wet}}} \times 100 \% \).

Histological grading
Pancreatic sections were stained with hematoxilin and eosin and graded in a blind manner by two investigators. Severity of pancreatitis was scored on a scale of 0 to 4 (normal to severity) in the categories (vacuolization, inflammation, edema) for a total score of 0 to 12, as described by J.Schmidt[4].

Immunohistochemical staining
Pancreata were fixed in formalin for 12 h and then embedded in paraffin wax. Sections were cut at 4 µm in thickness and mounted on slides. They were deparaffinized by passing them through two changes of xylene and graded series of ethanol, followed by rinses in tap water and 0.01 mmol·l\(^{-1}\) phosphorate buffered saline (PBS),respectively. Endogenous peroxidase activity was quenched by treating the section with 3 % hydrogen peroxide for 10 minutes. Nonspecific binding was blocked by incubating sections in 1 % bovine albumin in PBS for 10 minutes, and then incubated for an hour in primary antibody (rabbit anti-E-selectin or rabbit anti-ICAM-1 polyclonal antibody). After rinsing in PBS, the sections were treated sequentially with biotinconjugated second antibody for 10 minutes and then with streptavidin-peroxidase for another 10 minutes with PBS rinsing after each step. Sections were stained subsequently with freshly prepared DAB reagent for 3 minutes, and terminated by rinsing in water. then the sections were immerged in hematoxylin for 3-5 minutes and 0.5 mmol·l\(^{-1}\) HCl for 10 seconds. Finally, when passed through two changes of xylene and graded series of alcohol, the sections were covered with coverslip for light analysis. Every slice was counted on ten different fields by two investigators independently. Vessel stained brown was considered positive vessel. (-), score 0, means no positive vessel was observed. (+), score 1, means 1-2 positive vessels were observed. (++) , score 2,means 3-4 positive vessels were observed. (+++), score 3, means more than 5 positive vessels were observed.

Statistical analysis
Numerical variables are reported as means ± SEM and compared between groups using Newman-Keuls method. Rank data are reported as means and analyzed with Nemenyi method. Difference with \( P<0.05 \) are considered significant.

RESULTS
Expression of adhesion molecules
In control group there was no staining of E-selectin and ICAM-1 in pancreatic venules. Expression of the two adhesion molecules was eminent in AP and AP/PAF. But there was no difference between the two groups. AP/GAB has a lower level of E-selectin and ICAM-1 expression compared with AP (Table 1, Figures 1-4).
**Amylase and water content**

Serum amylase levels and water content was used to evaluate the severity of pancreatic injury. Amylase rose at 12 h in AP and AP/PAF group against control group, but there was no difference between the two groups. GAB treatment significantly reduced the amylase level at 12 h (P<0.05). Water content reflects the extent of pancreatic edema. At 12 h AP and AP/PAF showed a higher level of water content against sham and AP/GAB. AP/GAB still has a higher level of water content than sham (Table 2).

**MPO activity**

Measurement of neutrophil accumulation by MPO activity revealed that AP and AP/PAF had an increase of MPO activity almost 10-fold greater than sham. AP/GAB showed significantly lower level of MPO Activity than AP (P<0.05). And this level did not return to the values seen in sham treated animals (Table 2).

**Pancreatic histological analysis**

Sham group was microscopically intact, with normal histological structure. Pancreatic lesions, such as edema, vacuation and infiltration of PMN were found in AP, AP/PAF and AP/GAB. There was a trend toward improvement in histological parameters in AP/GAB, but it failed to reach statistical significance. (Data not shown).

**Table 1 Expression of adhesion molecules in pancreas. (x̅±s)**

| Group     | E-selectin | ICAM-1 |
|-----------|------------|--------|
| Sham      | 0          | 0      |
| AP        | 2.1±       | 2.3±   |
| AP/PAF    | 2.3±       | 2.4±   |
| AP/GAB    | 1.1±       | 1.1±   |

*P<0.05 vs sham, bP<0.05 vs AP.*

**Table 2 Measurement of amylase, MPO activity, water content. (x̅±s)**

| Group      | Amylase (×10^5 U/L) | MPO activity (AU/g) | Water content (%) |
|------------|---------------------|---------------------|-------------------|
| Sham       | 4.2±0.2             | 2.14±0.011          | 71.0±0.1          |
| AP         | 22.1±2.7±           | 2.213±0.039±        | 81.0±0.2±         |
| AP/PAF     | 22.9±0.9±           | 2.325±0.067±        | 79.8±0.2±         |
| AP/GAB     | 10.9±0.9±           | 0.795±0.004±        | 75.3±0.1±         |

*P<0.05, bP<0.05 vs sham, bP<0.05 vs AP.*

**DISCUSSION**

Platelet activating factor, 1-O-octadecyl-2-acetyl-Sn-glycero-3-phosphocholine, is a potent inflammatory mediator produced by endothelial cells, platelets, monocytes, neutrophils, and basophils. It has been widely studied that PAF played an important role in the pathogenesis of AP, and its local and systemic effects on AP have also been revealed. The systemic effects cause circulatory disturbances and multiple organ failure. Local effects include platelet aggregation, neutrophil accumulation, microvascular ischemia and increasing of capillary permeability[5-7]. Moreover, PAF itself also can induce pancreatitis when injected in peritoneal or superior pancreatoduodenal artery[8-10].

Accordingly, treatment of experimental pancreatitis with PAF antagonist has consistently shown significant local and systemic protection to reduce inflammatory changes[11-13]. But the mechanism of its protection has not been fully classified. One kind of PAF antagonist, lexipafant, has been studied in clinical trials in acute pancreatitis. In phase II and phase III clinical studies lexipafant showed better protection against AP, although a phase III clinical trial in UK showed little protection in the treatment of severe acute pancreatitis[16-18].

**In vitro** PAF can induce the expression of adhesion molecules[19], there was little information whether PAF and its antagonist could regulate the expression of adhesion molecules in AP. So, we conducted this experiment to testify the hypothesis.

Leukocyte accumulation from circulating blood to the site of inflammation includes multiple steps involving different kind of adhesion molecules on the endothelial cell. There are four steps in the adhesion cascade: (1) tethering, (2) triggering, (3) firm adhesion and (4) diapedesis- and these steps must occur in ordered sequence. Tethering interactions are the first step in the adhesion cascade. The molecules involved in this reaction come from the selectin family, such as E-selectin and P-selectin. The firm-binding phase involves superimunoglobulin family, such as ICAM-1 and VCAM-1. Both E-selectin and ICAM-1 have shown to be necessary for transendothelial migration of polymorphonuclear leukocytes[20]. In AP, neutrophils and adhesion molecules elevate and play an important role[21]. Treatment of severe acute pancreatitis with ICAM-1 monoclonal antibody reduces the severity of pancreatic and lung injury. The severity of AP is partially decreased in mice deficient in ICAM-1[22].

In our study adhesion molecules were elevated in AP. But additional PAF challenge couldn’t increase the expression of adhesion molecules. PAF antagonist (GAB) can reduce the expression of adhesion molecules and reduce the accumulation of neutrophils in pancreas in AP. The mechanism may be that internal PAF has enough capability to induce the expression of adhesion molecule, and exogenous PAF do not have accelerating ability to induce more expression of adhesion molecules. PAF antagonist may have different ways in AP to reduce the expression of adhesion molecules: (1) reduce the concentration of cytokines, such as TNF-α and IL-1[23,24], (2) reduce oxidative stress in pancreas[24,25], (3) repress the activation of NF-κB directly.

In conclusion, PAF plays an important role in the expression of adhesion molecules and accumulation of neutrophils in AP. PAF antagonist can be used early in mild AP. Further studies are necessary to determine whether PAF antagonist can prevent mild AP to transform into severe AP.

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