Underexpression of Receptor for Activated C Kinase 1 (RACK1) in Leukocytes from Patients with Severe Acute Pancreatitis

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Receptor for activated C kinase 1 (RACK1) plays an important role in regulating the immune response and cytokine expression. However, little is known about its role in acute pancreatitis (AP). We therefore investigated the role of RACK1 in AP and explored its relationship with interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), both of which are related to AP severity. Two rat models of chemically induced AP with different severities were used: acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP). The expression levels of IL-6 and TNF-α mRNAs and proteins were significantly increased in leukocytes from AEP and ANP rats, compared with the levels in the control animals, while the expression levels of RACK1 mRNA and protein were significantly decreased in leukocytes from these AP rats. Moreover, the RACK1 levels in leukocytes were significantly lower in ANP rats than those in AEP rats. Consequently, AP patients and healthy volunteers (HVs) were enrolled in this study. Compared with the HVs (n = 5), the expression levels of IL-6 and TNF-α mRNAs and proteins were significantly higher in leukocytes from 15 AP patients, including patients with mild AP (n = 5). By contrast, the expression levels of RACK1 mRNA and protein in leukocytes were significantly lower among patients with severe AP (n = 5) and with moderately severe AP (n = 5), compared with the HVs. The expression levels of RACK1 mRNA were negatively correlated with the IL-6 and TNF-α mRNA levels. Thus, RACK1 may alleviate the severity of AP.

Keywords: acute pancreatitis; IL-6; leukocyte; receptor for activated C kinase 1; TNF-α

Introduction

Acute pancreatitis (AP), i.e., inflammation of the pancreas, is among the most common digestive diseases requiring hospitalization. The disease presents with variable degrees of severity, with an overall mortality rate of 2%, but severe AP patients have a high fatality rate of up to 30% (Forsmark et al. 2016). Despite significant advances in the past decades, the exact mechanisms regulating the severity of AP remain elusive. It is known that AP begins with the premature intra-acinar activation of digestive enzymes, involves the immune system in an inflammatory response and results in potential multiple organ failure (Shamoon et al. 2016). Despite significant advances in the past decades, the exact mechanisms regulating the severity of AP remain elusive. It is known that AP begins with the premature intra-acinar activation of digestive enzymes, involves the immune system in an inflammatory response and results in potential multiple organ failure (Shamoon et al. 2016). 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RACK1 binds to PKC, which in turn activates nuclear factor kappa B (NF-κB) and the transcription of its target genes (Chen et al. 1998; Leppanen et al. 2008). Fluoxetine, a selective serotonin reuptake inhibitor used for depression treatment, blocks the inflammatory response of LPS-stimulated peripheral blood mononuclear cells through the downregulation of RACK1-PKC/II-κB interactions (Waiskopf et al. 2014). Many other studies have also demonstrated the important role of RACK1 in regulating cytokine expression in cell lines and primary cells (Corsini et al. 1999; Racchi et al. 2006; Corsini et al. 2009; Corsini et al. 2014; Yao et al. 2014).

Based on the importance of cytokines in the progression of AP, together with the complicated role of RACK1 in immune cell activation, we investigated the expression level of RACK1 in the peripheral leukocytes of rat models with different severities of disease and its correlations with interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Moreover, we verified the potential role of RACK1 in the peripheral leukocytes of AP patients at the early stage of disease.

**Materials**

**Animals**

Adult male Sprague-Dawley rats (250-280 g) were purchased from the Experiment Animal Center of Sichuan University, and they were maintained at 22 ± 2°C under a 12-hour day-night cycle and fed standard mouse chow and tap water ad libitum for 1 week of acclimation before the experiment. The animal experiments performed in this study were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee for Animal Experiments of Sichuan University.

**Experimental groups**

The rats were fasted for 12 hours and given free access to tap water until 2 hours before the experiments. Then, they were randomized into the AP and control groups. The AP rats were further grouped into the acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP) groups (n = 5 for each group). The AEP model was induced by bile duct ligation (BDL) and the ANP model was induced by two intraperitoneal injections of 50 μg/kg calcium chloride (Sigma, United States of America) at a 1-hour interval (Ishibashi et al. 2008). The ANP model was induced by two intraperitoneal injections of 50 μg/kg cerulein (Sigma, United States of America) at a 1-hour interval (Ishibashi et al. 2008). The ANP model was induced by a retrograde main pancreatic duct injection of 5% sodium taurocholate (1 ml/kg, Sigma, United States of America) using a micropump at a speed of 0.2 ml/min (Ye et al. 2013; Wu et al. 2015). All of the rats in both the control and ANP groups received a pancreatic duct injection of saline with the same volume of sodium taurocholate for those in the AP group. In addition, the animals in both the control and ANP groups received the intraperitoneal injection of saline twice. After the operation, the rats in each group were subsequently injected with normal saline (20 ml/kg) to compensate for fluid loss due to the surgery.

**Sample collection**

The rats were sacrificed at 6 hours after the establishment of each model. Blood samples were collected from the rats by intracardiac puncture. After the blood samples underwent centrifugation at 600 g for 20 minutes at 20°C, the leukocyte layer laid on the interface between the plasma and red cells. The plasma samples at the top were collected and stored at −80°C until the assay was performed. Then, the leukocyte layer was carefully collected into a 15 ml centrifuge tube, lysing red cell contamination with Red Blood Cell Lysis Buffer (Roche, Germany). Lastly, the leukocytes were stored at −80°C for Western blot analysis and quantitative polymerase chain reaction (qPCR) analysis.

**Inflammatory cytokine assay and histopathological examination of rat samples**

The IL-6 and TNF-α levels in the plasma of the rats were determined by commercially available ELISA kits (sensitivities: 30 pg/ml and 7 pg/ml, respectively, Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer’s instructions. The pancreatic tissue was placed in 10% buffered formalin solution for histopathological grading. A blinded histologic analysis was performed to evaluate for edema, inflammatory infiltration, hemorrhage and acinar cell necrosis under a × 200 microscope (Schmidt et al. 1992), and a final score was calculated based on the histological examination.

**Subjects**

The study was approved by the Clinical Trials and Biomedical Ethics Committee of West China Hospital, Sichuan University. Blood samples and clinical data were obtained from AP patients and healthy volunteers (HVs) enrolled in a clinical study (ChiCTR-COC-15007555). All procedures in study with human participants were performed in accordance with the ethical standards of the institutional or national research committee and with the Declaration of Helsinki. All subjects provided written informed consent before enrolling.

Patients with AP between 18 and 70 years of age who were admitted to West China Hospital, China, within 24 hours after the onset of disease were enrolled. These patients were classified into mild AP (MAP), moderately severe AP (MSAP) and severe AP (SAP) groups, according to the Revised Atlanta Classification (Banks et al. 2013). Patients were excluded if they (1) were pregnant, (2) had any malignancy, (3) had serious primary diseases of any other system or (4) were less than 18 years or more than 70 years. The healthy volunteers were enrolled by posting a poster in our hospital. They are families of inpatients or someone who had physical examination in our hospital.

**Blood collection**

Peripheral blood samples were collected from the patients with AP using BD Vacutainer EDTA tubes as soon as possible after they were admitted (within 24 hours after the onset of symptoms). The blood samples from the AP patients and healthy subjects were processed using the same methods as those used to process the blood samples from the rats.

**Inflammatory cytokine assay of human plasma**

The IL-6 and TNF-α levels in the human plasma were determined by ELISA kits (sensitivities: 2 pg/ml and 1.7 pg/ml, respectively, Siemens Healthcare Diagnostics Products Ltd., Llanberis, United Kingdom) following the manufacturer’s instructions.

**Western blot analysis**

Leukocytes from the different groups were lysed using RIPA buffer (Beyotime, China) supplemented with 10 μg/mL phenylmethylsulfonyl fluoride (Beyotime, China) and protease inhibitor cocktail.
Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from leukocytes was extracted using TRIzol reagent. The integrity of the RNA was determined using an ultra violet spectrophotometer (Thermo Fisher, Waltham, United States of America). The total RNA was reverse-transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer’s instructions. Primers for the RACK1, IL-6, TNF-α and β-actin genes were synthesized by Shanghai Biotechnology Co. Ltd. (Shanghai, China; Table 1). The qRT-PCR analysis was conducted with 30 μl qRT-PCR reaction buffer: 5 μl cDNA, 3 μl 10 × buffer (Mg²⁺ free), 3 μl MgCl₂ (25 mM), 0.36 μl dNTP (25 mM), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1 μl probe (10 nM) and 15.34 μl deionized double-distilled water. An FTC-3000 RT-PCR detection system (Funglyn, Richmond Hill, Canada) was used with the following program: pre-denaturation at 94°C for 2 minutes followed by 45 cycles at 94°C for 20 seconds, at 50°C (RACK1), 50°C (IL-6), 54°C (human TNF-α), 52°C (β-actin) for 20 seconds and at 60°C for 30 seconds. All of the reactions were run in triplicate. The β-actin gene was used as an internal housekeeping gene. The threshold cycle (Ct) was defined as the cycle number of the fluorescence that passed through a given threshold. The target mRNA expression was analyzed by the 2-ΔΔCt method (ΔCt = Ct target mRNA − Ctβ-actin).

Statistical analysis

The statistical analysis was performed using SPSS 20.0. The data are expressed as the mean ± standard error (SE). A one-way ANOVA or Kruskal-Wallis test was used, when appropriate, to determine significant differences among the groups. The Pearson correlation coefficient was used to analyze correlations between the expression of RACK1 and cytokines. A two-sided P < 0.05 was considered statistically significant.

Results

Histopathological changes

The histopathological changes in the pancreatic tissues are shown in Fig. 1. No obvious changes that were indicative of AP were found in the control group (Fig. 1A). Interstitial edema, granulocyte infiltration and sporadic acinar cell necrosis were observed in the pancreatic tissues of the AEP rats, while more severe histopathological damage was detected in the ANP group rats (Fig. 1B, C). As shown in Fig. 1D, the total severity score was significantly higher in the AP groups than that in the control group (both P < 0.05). Additionally, the ANP group exhibited more severe pancreatic injury than did the AEP group (P < 0.05).

IL-6 and TNF-α levels in rat plasma

The plasma levels of TNF-α and IL-6 in the AEP and ANP groups were significantly higher than those in the control group (all P < 0.05, Fig. 2), while both of them in the ANP group were significantly higher than those in the AEP group (both P < 0.05).

<table>
<thead>
<tr>
<th>Gen target</th>
<th>Probe</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product (bp)</th>
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<td>CAAGAGGTAT CAGACTCAG CAGTCCAG AAACACCA</td>
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<td>148 148</td>
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<td>GAAGATCAAGA</td>
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The levels of IL-6 and TNF-α mRNAs were increased in the AP leukocytes compared with the control group (all \( P < 0.05 \), Fig. 3). Moreover, both levels were significantly higher in the ANP group than those in the AEP group (\( P < 0.05 \)). Western blot analysis showed that the levels of IL-6 and TNF-α proteins corresponded with their mRNA levels (Fig. 3). By contrast, RACK1 mRNA levels were significantly lower in leukocytes from the AEP rats than those from the control group, and the levels were much lower in leukocytes from the ANP group. Moreover, the RACK1 protein expression levels were lower in leukocytes from the
Fig. 3. Expression levels of IL-6, TNF-α and RACK1 in rat leukocytes.
A, IL-6 mRNA; B, IL-6 protein; C, TNF-α mRNA; D, TNF-α protein; E, RACK1 mRNA; F, RACK1 protein; G, Representative Western-blotting images of protein.
*P < 0.05 vs. control group, †P < 0.05 vs. AEP group (n = 5 in each group, mean ± SE).
AEP rats than those in the control rats, and that its expression level was further reduced in the ANP rats.

The correlation between RACK1 mRNA levels and IL-6 or TNF-α mRNA levels in rat leukocytes

A Pearson correlation analysis showed that leukocyte RACK1 mRNA levels were negatively correlated with IL-6 or TNF-α mRNA levels (r = -0.557, *P* = 0.031; and r = -0.583, *P* = 0.023, respectively).

**Subject demographics**

In all, 20 subjects, including 5 MAP patients, 5 MSAP patients, 5 SAP patients and 5 healthy volunteers, were enrolled. The demographic characteristics of the subjects are shown in Table 2. Details of the clinical data of the AP patients are found in Table 3.

**Plasma IL-6 and TNF-α levels in subjects**

All of the patient groups with AP had significantly higher levels of plasma IL-6 and TNF-α than did the HVs (Fig. 4). A greater amount of IL-6 and TNF-α were released into the plasma in the SAP patients than were for the MAP patients (both *P* < 0.05). No statistically significant difference was observed between the MSAP and SAP patients or between the MAP and MSAP patients (all *P* > 0.05).

**IL-6, TNF-α and RACK1 in leukocytes from human subjects**

In comparison with the HVs, both the IL-6 and TNF-α mRNA levels were higher in leukocytes from the AP patients (Fig. 5A, C). Furthermore, both of them were significantly higher in the leukocytes from the MSAP and SAP patients compared to the MAP patients (both *P* < 0.05). However, there was no statistically significant difference

<table>
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LC, local complications; OF, organ failure; HV, healthy volunteer; MAP, mild acute pancreatitis; MSAP, moderately severe acute pancreatitis; SAP, severe acute pancreatitis; HTG, hypertriglyceridemia; APFC, acute peripancreatic fluid collections; ANC, acute pancreatic and/or peripancreatic necrosis; IPN, infected (peri)pancreatic necrosis.
between the levels in the MSAP and SAP patients (P > 0.05). Western blot analysis showed that both IL-6 and TNF-α proteins were nearly undetectable in leukocytes from the HVs but were detected in leukocytes from all of the AP patients and that higher expression levels were found in the MSAP and SAP patients (Fig. 5B, D). In the SAP patients, the RACK1 mRNA level was significantly lower than those of the HVs and MAP patients (both P < 0.05, Fig. 5E). The RACK1 mRNA level was also significantly lower in the MSAP patients than in those of the HV group. No difference was detected between the MAP group and the HV group or MSAP group or between the MSAP and SAP patients. Moreover, the RACK1 protein levels were consistent with the corresponding mRNA expression levels (Fig. 5F).

**Discussion**

Complex immunological events underlie the pathogenesis and progression of AP. Leukocytes are the major cell population that participates in the propagation of the disease (Shamoon et al. 2016), which occurs through the release of proinflammatory mediators, including cytokines and chemokines. Regardless of the etiology of AP, it is widely accepted that inflammatory mediators, such as TNF-α, interleukin-1β and IL-6, play a key role in the development and spread of AP from a local inflammatory process to a systemic disease (de Beaux et al. 1996; McKay et al. 1996b; Frossard and Pastor 2002). Circulating leukocytes are a mixed cell population. As an integrated immune system in the peripheral circulating blood, these cells are interrelated and interact with each other. In this study, leukocyte from whole blood, rather than any one category of purified peripheral blood cells, were analyzed.

Both caerulein- and sodium taurocholate-induced AP models are widely used by investigators of pancreatic diseases. Different degrees of severity can be achieved using these two AP models. The sodium taurocholate-induced ANP rats demonstrated more significant histopathological changes and higher plasma TNF-α and IL-6 levels than AEP rats and had successful induction of more severe AP. It would be better to represent pancreatic injury conditions from mild edematous pancreatitis to severe necrotizing pancreatitis by combining these two models, which might be used to mimic the complicated processes of AP.

Cytokine secretion during AP has been related to the severity of systemic manifestations and eventual death. In this study, both the IL-6 and TNF-α mRNA levels were significantly increased in the leukocytes of the AP rats compared with the control rats. Consistent with the results that were obtained with the AP rats, higher expression levels of IL-6 and TNF-α were detected in the leukocytes from the AP patients. This result is consistent with the results of previous studies; namely, Ramudo et al. (2010) and de Dios et al. (2002) reported that the production of TNF-α was significantly increased in the peripheral blood monocytes from rats with AP induced by bile-pancreatic duct obstruction. Another study found that peripheral blood monocytes could produce TNF-α in rats with AP induced with 3.5% sodium taurocholate (Yubero et al. 2009). In the patients with AP, the complex upregulation of proinflammatory cytokines released from peripheral blood leukocytes has been demonstrated to be related to the clinical progress of the disease (de Beaux et al. 1996; Dambrauskas et al. 2010). Upon comparing the IL-6 or TNF-α levels between the MSAP and SAP patients, no significant difference was detected. The plasma levels of IL-6 and TNF-α were not significantly different between the MAP and MSAP patients. However, western blot or qPCR analysis showed that both the IL-6 and TNF-α expression levels were significantly higher in the MSAP patients than in the MAP patients in this study. The poor performance may be attributed to the complex
Fig. 5. Expression levels of IL-6, TNF-α and RACK1 in human leukocytes.
A, IL-6 mRNA; B, IL-6 protein; C, TNF-α mRNA; D, TNF-α protein; E, RACK1 mRNA; F, RACK1 protein; G, Representative Western-blotting images of protein.
*P < 0.05 vs. HVs, #P < 0.05 vs. MAP group (n = 5 in each group, mean ± SE).
sources of cytokines released into the plasma or to the small sample size used in this study. Alternatively, these results might simply support the limited ability of IL-6 and TNF-α as biomarkers for predicting AP severity in the early stage of the disease (Nieminen et al. 2014).

Accumulating evidence supports the important role of RACK1 in cytokine expression. With LPS treatment, TNF-α production was reduced in macrophages treated with RACK1 antisense oligonucleotides (Corsini et al. 1999). Moreover, a good positive correlation was observed between RACK-1 mRNA and protein levels and TNF-α or interleukin-8 release in THP-1 cells (Corsini et al. 2014). Furthermore, a decreased expression level of RACK1 underlies immune deficits in aged people and Alzheimer’s patients (Racchi et al. 2006; Corsini et al. 2015). RACK1 also plays an important role in the anti-inflammatory effects of glucocorticoids (Corsini et al. 2014). However, Yao et al. (2014) identified RACK1 as a novel negative regulator of NF-κB signaling, NF-κB-mediated cytokine induction in inflammatory reactions in RAW264.7 cells after TNF-α stimulation. It seems that RACK1 regulates the immune response in a stimulus- and cell-dependent manner.

Corsini et al. (2014) found that all corticosteroids, including cortisol, prednisone, prednisolone, budesonide, betamethasone and methylprednisolone, reduced RACK-1 expression in LPS-treated THP-1 cells. Serum corticosterone levels in severe AP rats were elevated and peaked from 1 h until 3 h and then declined to the control level at 12 h (Deng et al. 2014). Muller et al. (2006) showed that throughout the study period (from day 1 to day 6), the daily serum concentrations of total and calculated free cortisol in 109 consecutive patients with AP were significantly increased in comparison with those of the healthy controls. They were higher in severe pancreatitis patients than they were in mild pancreatitis patients and were also higher in necrotizing pancreatitis patients than they were in edematous pancreatitis patients. Similarly, patients with moderate to severe AP showed significantly higher plasma concentrations of cortisol than did those with mild AP in the study reported by Nebiker et al. (2018). In this study, we first observed the reduction of RACK1 in the leukocytes of experimental AP rats and AP patients. The observed reduction in RACK1 levels may reflect an increase in the plasma levels of corticosterone in AP. However, this requires further investigation.

There was inconsistency in the measurements of RACK1 expression in the AP rats and in the AP patients. The AEP rats had significantly lower RACK1 expression levels than did the control rats, while no detectable difference was found between those of the MAP patients and HVs. This discrepancy may be attributed to species differences. In addition, the time point for blood collection may be a variable. However, no research can be referenced for the dynamic process of RACK1 expression in AP. As shown in Fig. 5E and F, RACK1 expression levels were significantly lower in the SAP patients compared to the MAP patients. Although no significant difference was detected between the MSAP patients and the MAP or SAP patients, the MSAP patients had medium expression levels of RACK1, compared to those of the MAP and SAP patients. It seems that the RACK1 expression level negatively correlates with the AP severity. However, extensive exploration should be conducted to uncover the potential functions of RACK1 in AP. Nevertheless, in this study, the RACK1 expression level was significantly lower in the MSAP and SAP patients compared with the HVs, which is consistent with the results in the more severe AP rat model, the sodium taurocholate-induced ANP. At the same time, this study showed significant negative correlations between the RACK1 mRNA expression level and the IL-6 and TNF-α mRNA expression levels. Based on the dual role of RACK1 in cytokine expression, the results mentioned above indicate that RACK1 might be a negative inflammatory factor in the peripheral leukocytes of AP. The study conducted by Yao et al. (2014) found that RACK1 regulated the activation levels of NF-κB, which had been widely demonstrated to be activated in AP, and that RACK1-knockdown cells transcribed more NF-κB target genes, including proinflammatory cytokines. Therefore, the reduction of RACK1 in AP leukocytes made sense of the increases in the IL-6 and TNF-α expression levels.

Despite the existing potential interest in our findings, there were several limitations in this study. The direct influences of RACK1 on IL-6 and TNF-α were not explored; thus, only a potential relationship between them could be concluded. We will next focus on uncovering the potential exact roles of RACK1 in the release of proinflammatory cytokines in AP. Furthermore, in the study conducted by Racchi et al. (2006), leukocytes from elderly subjects (age range: 65-89 years) showed a significant reduction in the expression of RACK-1 compared to young subjects (age range: 23-64 years). Our study included a patient aged 67 years and a patient aged 62 years in the SAP group, and the remaining three patients in this group were younger than 55 years. No definite age number to eliminate the influence of age on RACK1 expression can be noted for now. Considering the lack of difference based on the demographic data of the subjects enrolled in this study, we believe that there may be little influence on the average expression level of RACK1 in SAP patients. However, age is still a variable that should be considered in future research. In addition, only 5 subjects were included in each subject group, and no differences were found in the leukocyte RACK1 levels between the MAP patients and HVs or MSAP patients and between the MSAP and SAP patients. It is possible that the small sample size resulted in the inability to identify differences among these subjects.

In summary, our results show that the expression levels of the leukocyte IL-6 and TNF-α were increased, while those of RACK1 were decreased, in experimentally induced mild and severe AP rats, as well as in patients with AP. The reduction of the expression level of RACK1 mRNA was
significantly correlated with increases in those of IL-6 and TNF-α mRNAs. RACK1 may alleviate the degree of inflammation in AP. Further studies are needed to explore the true functions of RACK1 in AP.

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Conflict of Interest

The authors declare no conflict of interest.

References


Underexpressed RACK1 in Leukocytes of Acute Pancreatitis


