Original Paper

Depletion of Neutrophils Protects Against L-Arginine-Induced Acute Pancreatitis in Mice

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Key Words
Acute pancreatitis • Neutrophils • NF-κB

Abstract

Background/Aims: Acute pancreatitis (AP) is an inflammatory disease characterized by acinar cell damage and inflammation of the pancreas with infiltration of leukocytes, predominantly neutrophils. We investigated whether neutrophil depletion protects against experimental AP induced by L-arginine. Methods: AP was induced in C57BL/6 mice via two intraperitoneal L-arginine (4 g/kg) injections. Mice were pretreated with 250 and 100 µg anti-Gr-1 antibody via intraperitoneal injection at 24 and 4 h, respectively, before L-arginine challenge for neutrophil depletion. At 48 and 72 h after injection, the severity of AP was determined with the aid of biochemical and histological analyses. Amylase and MPO activity was detected using specific assay kits. The plasma cytokines levels were detected using ELISA. Results: Neutrophil depletion resulted in significantly reduced plasma amylase levels in pancreas, myeloperoxidase (MPO) activity in pancreas and lung, reactive oxygen species (ROS) generation and cell apoptosis, and decreased circulating neutrophil, tissue damage as well as expression levels of nuclear factor NF-κB. Conclusion: Neutrophil depletion is capable of reducing tissue damage of pancreas and lung in mice with acute pancreatitis.

Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas. Diagnosis of AP is based on increased concentrations of amylase and lipase in serum, along with acute abdominal pain [1, 2]. Clinically, most patients with AP display mild pancreatic injury without complications after therapy, while about 20% have a life-threatening disease with
local and systemic complications and experience multiple organ dysfunction [3, 4]. To date, intense efforts have been made to uncover the exact mechanisms regulating the initiation and severity of AP. Earlier studies showed that the severity of AP can be determined based on pathophysiological components within pancreatic acinar cells, such as activation of trypsinogen and nuclear factor (NF)-κB, release of pro-inflammatory cytokines, recruitment of neutrophils and apoptosis of acinar cells [5-8]. In this light, the inflammatory response plays crucial roles in AP, and effective therapy should focus on key components of the immune system, such as neutrophils.

Several studies have reported that upregulated activity of myeloperoxidase (MPO), a biochemical marker of neutrophil infiltration, is associated with disease development in sodium taurocholate-induced, cerulein-induced, and L-arginine-induced AP in both mice and rats [4, 5, 9-12]. Moreover, neutrophil depletion induced by pretreatment of mice with anti-neutrophil serum reduces the severity of pancreatitis and prevents pancreatitis-associated lung injury [13, 14]. An earlier study by Pandol and colleagues demonstrated that the activity of neutrophils infiltrating the pancreas in cerulean-induced AP is mediated through NADPH oxidase products [15].

In the present study, L-arginine-induced AP in mice was characterized by increased pancreatic injury, plasma amylase and MPO activities, and higher frequency of neutrophils in the pancreas. Depletion of neutrophils via intraperitoneal injection with an anti-Gr-1 antibody (RB6-8C5) [16] resulted in significant protection against L-arginine-induced pancreatic inflammation, as evident from the marked reduction in plasma amylase in pancreas and MPO activity in pancreas and lung, suppression of the reactive oxygen species (ROS) content, and decreased apoptosis of pancreatic cells. These effects of neutrophil depletion on AP were associated with decreased NF-κB expression. Our findings collectively indicate a critical role of neutrophils in the development of AP.

Materials and Methods

Experimental procedures

All animal related procedures were approved by the Animal Research Committee at Soochow University. Animal experiments were performed in accordance with the established International Guiding Principles for Animal Research. C57BL/6 mice (male, 20–25 g) were maintained under a 12 h light/dark cycle at 21–24°C in the Animal Housing Unit. Animals were allowed to acclimatize for at least 1 week before experimental procedures were undertaken, and randomly assigned to control or experimental groups.

Induction of AP and neutrophil depletion

L-Arginine hydrochloride was purchased from Sigma Chemical (St. Louis, MO). A sterile solution of L-arginine hydrochloride (8%) was prepared in normal saline (pH 7.0). Mice were randomly assigned to four groups. In the control group, animals were injected twice intraperitoneally using normal saline 1 h apart. In the AP group, animals were injected intraperitoneally with L-arginine solution at two doses of 4 g/kg body weight each 1 h apart [17]. After treatment, animals were returned to their cages and allowed free access to food and water. In the AP+aGr-1 group, to deplete neutrophils, animals were injected intraperitoneally with 250 and 100 µg anti-Gr-1 monoclonal antibody (mAb) per mouse (RB6-8C5, BD Biosciences, San Diego, CA) at 24 and 4 h before the L-arginine challenge, respectively [16]. In the AP+IgG2b Group, control rat IgG2b (BD Biosciences) for anti-Gr-1 treatment was injected at equivalent doses. Animals were killed at specific time-points via CO₂ asphyxia and blood samples collected to determine serum amylase levels. Pancreas samples from mice were rapidly collected and fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining. Portions of organs were stored at −80°C for further investigation.

Histological scores

Pancreatic damage was evaluated using a scoring system ranging from 0 (absent) to 4 (extensive) as previously described [18]. Total histological scores represent the sum of scores for inflammatory cell infiltration, edema, acinar cell necrosis and hemorrhage. Lung injury was assessed according to pulmonary
edema, inflammatory changes, alveoli and interstitial tissue hemorrhage. Pathological features were scored on a scale from 0 (normal) to 3 (severe) in terms of changes.

Amylase estimation
A kinetic spectrophotometric assay was used to measure plasma amylase activity, as described previously [19]. Plasma samples were incubated with amylase reagent for 2 min at 37°C, and absorbance measured at 405 nm according to the manufacturer’s protocol. The changes in absorbance were used to estimate amylase activity.

MPO estimation
Fresh tissue samples were homogenized immediately on ice in 5 volumes of normal saline. The MPO assay kit (Nanjing Jiancheng Corp., Nanjing, China) was used to measure MPO activity according the manufacturer’s recommendations. One unit of MPO activity was defined as that required to degrade 1 mmol hydrogen peroxide at 37°C. MPO activity was expressed as units per milligram (U/mg).

Reverse transcription and quantitative real-time PCR
Total RNA was extracted from tissue using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) was used to synthesize complementary DNA from 500 ng total RNA. Quantitative real-time PCR was performed with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). NF-κB p65 expression was determined using the primers 5′-ATACCACCAAGACCCACCCC-3′ (forward), 5′-TGAGGAGGGTCCTTGGTGAC-3′ (reverse). The relative expression of p65 was measured with the 2−ΔΔCT method using β-actin mRNA for normalization.

Western blot analysis
Pancreatic tissues were homogenized in RIPA lysis buffer, along with protease inhibitor (Sigma-Aldrich), and electrophoresed on a 10% SDS gel. Proteins were transferred to polyvinylidene fluoride membrane, followed by blocking with 5% skimmed milk. Blots were subsequently incubated with primary antibodies against cleaved caspase 3, p65 and GAPDH (Abcam, Cambridge, UK) at 4°C overnight. After further incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA), proteins were detected using enhanced chemiluminescence (Pierce, Rockford, IL).

Measurement of ROS
ROS production was detected using CM-H2DCFDA (Molecular Probes, Carlsbad, CA), a cell-permeable dye that remains non-fluorescent until acetate groups are removed [20]. In brief, 200 µl cell suspensions (10^5 cells/ml) were seeded in a 96-well plate in the presence of CM-H2DCFDA for 45 min at 37°C. Analysis of signal intensity was carried out using a fluorescent plate reader and ROS production calculated based on a H_2O_2 standard curve.

TUNEL assay
Apoptosis was determined using TUNEL, a method for detecting DNA fragmentation. Pancreatic tissues were fixed by immersion in 10% buffered formaldehyde phosphate for at least one day, dehydrated and embedded in paraffin wax. The TUNEL assay was performed on 5-μm thick sections using a cell apoptosis detection kit (Promega, Madison, WI), according to the manufacturer’s protocol.

Flow cytometric analysis
Pancreatic cell suspension was prepared as described previously [21]. Briefly, pancreatic tissues were sheared gently and digested with collagenase V (1 mg/ml) for 15 min, followed by passage through a 40 μm filter. The cell suspension was centrifuged at 1500 rpm for 10 min, and subsequently washed in phosphate buffered saline. Cells were incubated for 10 min with a Fcy receptor blocker, CD16/32 (BD Biosciences). Aliquots of cells (10^6) were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD11b, anti-F4/80, and phycoerythrin (PE)-conjugated anti-NK1.1 and anti-Gr-1 antibodies (BD Biosciences). Peripheral blood mononuclear cells harvested from eyeballs of mice after density gradient centrifugation were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b and phycoerythrin (PE)-conjugated anti-Gr-1 antibodies to detect circulating neutrophils. Flow cytometric analysis was carried out...
on a FACScalibur (BD Biosciences) to calculate the quantity of each cell type (T cells, NK cells, NKT cells, neutrophils and macrophages).

Statistical analysis
All data were calculated as means ± SD from at least three separate experiments. Statistical analysis was performed by applying the unpaired two-tailed Student’s t-test, with significance assigned to \( P < 0.05 \) in all cases.

Results

Increased infiltration of neutrophils in L-arginine-induced pancreatitis of mice
The successful development of a novel mouse model of acute pancreatitis induced by L-arginine has been previously reported [17]. Evaluation of histology of the pancreas in the L-arginine-administered group using hematoxylin and eosin-stain (HE) revealed disruption of normal histoarchitecture, distended interlobular spaces, acinar cell vacuolization and neutrophil infiltration over time after induction of acute pancreatitis (Fig. 1A and B). Plasma amylase levels were significantly increased in the AP group at 48 and 72 h after L-arginine administration, compared with the control group (Fig. 1C). Furthermore, mice of AP group
displayed markedly increased pancreatic MPO activity at 48 h and 72 h after administration of L-arginine compared with the control group (Fig. 1D), consistent with the patterns of plasma amylase.

As neutrophil infiltration to the pancreas was observed in L-arginine-induced AP, we determined the number of neutrophils (Gr-1−Mac-1+) and other leukocytes, such as T cells (CD3−NK1.1−), NK cells (NK1.1−CD3+), NKT cells (NK1.1+CD3+) and macrophages (F4/80+), based on two-parameter flow cytometric analysis. As shown in Fig. 1E, the quantity of neutrophil significantly increased in L-arginine-induced AP mice, compared with the control group, while the number of CD3 T cells, NK cells, NKT cells and macrophages was increased to a moderate extent after L-arginine administration (Fig. 1F), indicating a crucial role of neutrophil in the development of AP.

Fig. 2. Neutrophil depletion inhibits tissue damage and innate immune cell accumulation. Normal mice were injected with L-arginine (4 g/kg) intraperitoneally on day 0. 250 and 100 µg anti-Gr-1 (RB6-8C5) mAb was injected intraperitoneally at 24 and 4 h, respectively, prior to the L-arginine challenge. IgG2b was injected at equivalent doses in the AP+IgG2b group. (A) Plasma amylase activity. (B) MPO activity. (C) Histological analysis of pancreatic sections with hematoxylin-eosin stain and histological score. (D) Representative flow cytometric analysis and quantity of neutrophils 72 h after IgG2b (AP+IgG2b) and anti-Gr-1 (AP+aGr-1) treatment. (E) Absolute numbers of CD3 T cells, NK cells, NKT cells and macrophages per pancreas in AP+IgG2b and AP+aGr-1 group. (F) Absolute number of neutrophil in peripheral blood mononuclear cells. Data are presented as means ± SD (n = 5 per group). *P < 0.05 versus AP+IgG2b group.
Depletion of neutrophils decreases pancreatic injury and innate immune cell accumulation in L-arginine-induced mice

To determine whether infiltrated neutrophils play a role in the development of L-arginine-induced AP, neutrophils were depleted via intraperitoneal injection of anti-Gr-1 mAb at 24 h (250 µg per mouse) and 4 h (100 µg per mouse) before L-arginine administration (4 g/kg intraperitoneally). As shown in Fig. 2A and B, anti-Gr-1 treatment led to a significant decrease in plasma amylase and MPO activities, compared with those of IgG2b-treated control mice. Furthermore, histological analysis of pancreas sections revealed that the area of tissue damage is considerably reduced in neutrophil-depleted AP mice (AP+aGr-1), compared with IgG2b mAb-treated mice (AP+IgG2b) in which L-arginine caused severe tissue damage (Fig. 2C). Our findings demonstrate that depletion of neutrophils is an effective means to decrease pancreatic injury in an L-arginine-induced AP mouse model.

We additionally examined the effects of neutrophil depletion on leukocyte accumulation in pancreas. Leukocytes were isolated from mouse pancreas 72 h after L-arginine treatment. The frequency and number of neutrophils were significantly decreased in mice treated with anti-Gr-1 mAb (AP+aGr-1), compared to those treated with IgG2b mAb (AP+IgG2b) (Fig. 2D). However, anti-aGr-1 had a weak inhibitory effect on infiltration of other innate immune cells in the pancreas (Fig. 2D). Furthermore, circulating neutrophils from peripheral blood mononuclear cells were reduced by about 90% in the AP+aGr-1 group, compared with the AP+IgG2b group (Fig. 2F).

Effect of neutrophil depletion on lung MPO and histology in L-Arginine-induced AP

The effects of neutrophil depletion on lung injury were additionally analyzed. As shown in Fig. 3A, MPO activity in lung was significantly increased in AP group at 48 and 72 h after L-arginine administration, compared with control group, which was markedly decreased upon neutrophil depletion. Hematoxylin and eosin staining (HE) was performed for detection of lung injury in mice with L-Arginine-induced AP. As shown in Fig. 3B and C, pulmonary edema, alveolar thickening and neutrophils infiltration were more severe in the AP group, compared with control group. Lung tissue damage was significantly attenuated upon neutrophil depletion at the 72 h time-point.

Effect of neutrophil depletion on plasma cytokines level in L-Arginine-induced AP

Inflammatory reaction is reported to be associated with AP and a number of cytokines, such as tumor necrosis factor α (TNF-α), IL-6 and IL-10 play regulatory roles in AP [22].
Accordingly, plasma levels of these cytokines were determined using ELISA. As shown in Fig. 4, levels of all the cytokines examined were significantly higher in the AP group than the control group. Notably, neutrophil depletion led to decreased plasma levels of TNF-α and IL-6, but increased plasma IL-10, compared with the AP group.

Fig. 5. Neutrophil depletion decreases ROS generation and apoptosis. (A) ROS production by pancreatic acinar cells of control, AP and AP+aGr-1 mice. (B) Western blot detection of cleaved caspase 3 protein expression. (C) TUNEL detection of cellular apoptosis. Data are presented as means ± SD. *P < 0.05 versus control group, **P < 0.05 versus AP group.

Effects of neutrophil depletion on L-arginine-induced apoptosis in pancreas

Previous studies have reported the ability of neutrophils to produce and release a variety of cytotoxic agents, such as reactive oxygen and nitrogen species, which cause apoptosis [23]. As shown in Fig. 5A, reactive oxygen species (ROS) generation was markedly increased in leukocytes isolated from L-arginine-induced AP mice, compared with control mice. However, upon depletion of neutrophils with anti-Gr-1 treatment (AP+aGr-1), we observed significant reduction of ROS generated from leukocytes, clearly indicating an important role of neutrophils in ROS generation. Expression of cleaved caspase-3, a commonly used marker...
of cell apoptosis, was detected using western blot analysis. Our results disclosed markedly higher caspase-3 expression in AP mice, compared with that in control mice, which was significantly reduced under conditions of neutrophil depletion (Fig. 5B). Apoptosis levels were additionally determined using transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL). As shown in Fig. 5C, cellular apoptosis in AP mice was significantly increased, compared with that in control mice, but decreased markedly upon anti-aGr-1 treatment. These results collectively suggest that neutrophil depletion results in reduction of ROS generation and cell apoptosis and exerts a protective role in pancreatic acinar cells during AP.

**Effects of neutrophil depletion on NF-κB expression in pancreas**

Given that the NF-κB pathway participates in the pathogenesis and development of AP [6], we examined the mRNA and protein expression levels of NF-κB p65 via real-time PCR and western blot analysis to determine the effects of neutrophil depletion on NF-κB in pancreas. As shown in Fig. 6A, p65 mRNA expression was significantly downregulated upon neutrophil depletion (AP+aGr-1), compared with that in non-treated AP mice. The patterns of NF-κB p65 protein expression were consistent with those of mRNA expression (Fig. 6B).

**Discussion**

Acute pancreatitis (AP) is a life-threatening pancreatic disease with significant morbidity and high mortality, but no specific therapies are currently available [24, 25]. The development of AP is a multistep process characterized by inflammation, edema and vacuolization [24]. Clarification of the exact mechanism regulating the pathogenesis and development of AP is essential for effective therapy, which remains an unmet medical need. Infiltration of neutrophils, an essential component of the host innate immunity system, is a well-known hallmark of tissue injury in AP and contributes to the pathogenesis and development of the disease [26]. In the present study, we showed that depletion of neutrophils in an L-arginine-induced AP mouse model protects against AP, as evident from downregulation of plasma amylase and MPO activity and reduction in pancreatic tissue damage. Lung tissue damage and plasma levels of inflammatory cytokines were decreased following neutrophil depletion. ROS generation and cleaved caspase 3 protein expression in pancreas were decreased significantly upon complete neutrophil depletion via anti-aGr-1 treatment. Apoptosis levels detected using TUNEL were markedly reduced under conditions of neutrophil depletion, consistent with the changes in caspase 3 expression, indicating that the protective role of neutrophil depletion against AP is achieved through suppression of cellular apoptosis in the pancreas. Furthermore, the NF-κB pathway is suggested to participate in the pathogenesis and development of AP, in view of the reduced NF-κB p65 protein levels under conditions of neutrophil depletion.

The AP model was successfully established in C57BL/6 mice with the administration of two doses of L-arginine (4 g/kg) 1 h apart. Plasma amylase and MPO activities in pancreas were increased significantly at 48 h and peaked at 72 h, compared with those in control mice.
mice. Histopathological examination revealed tissue damage in pancreas and lung over time after induction of AP, consistent with the time-points at which plasma amylase in pancreas and MPO activities in pancreas and lung increased. Induction of AP additionally resulted in infiltration of leukocytes into pancreas, particularly neutrophils, similarly increasing at 48 h and peaking at 72 h.

Accumulating evidence supports a crucial role of neutrophils in the progress of AP. Steer and colleagues found that in a choline-deficient ethionine (CDE) diet-induced mouse AP model, neutrophil depletion by pretreatment with antineutrophil serum reduces the severity of AP and completely prevents lung injury [13]. Another study by Regnér and co-workers showed that neutrophil depletion with the anti-Gr-1 antibody reduces the pancreatic trypsinogen activation peptide (TAP) level, plasma amylase and MPO activities in taurocholate-induced severe AP, confirming that neutrophils are critical in mediating pancreatic and lung tissue damage under these conditions [27]. The effects of neutrophil depletion on the L-arginine-induced mouse AP model presented in this study are consistent with previous findings.

Inflammatory factors, such as TNF-α and IL-6, are known to play critical roles in the development of AP [28]. In the present study, TNF-α, IL-6, and IL-10 plasma levels increased significantly in AP group. Notably, neutrophil depletion led to reduced plasma TNF-α and IL-6, but increased IL-10 levels. Our results suggest a crucial role of neutrophil in the regulation of inflammatory cytokines.

The signaling mechanism by which neutrophil depletion protects against AP is of significant interest. To this end, the pleiotropic transcription factor, NF-κB, is known to have a key function in the pathogenesis of AP. Activation of NF-κB is an early event in AP and promotes inflammatory reactions associated with severe pancreatitis [29], suggesting a pro-inflammatory role of NF-κB in development of the disease [30]. Expression of NF-κB is correlated with the severity of pancreatitis in a cerulein-induced AP mouse model and its inhibition has a positive role in improving survival of rats in a taurocholate-induced AP model [31, 32]. Among the NF-κB family members, NF-κB p65 plays crucial roles in the progression of AP [33]. In view of these findings, we examined the NF-κB p65 expression patterns. Our experiments showed that NF-κB p65 is upregulated during L-arginine-induced AP and downregulated upon depletion of neutrophils.

In conclusion, data from our present study clearly demonstrate that neutrophil depletion provides a novel effective therapeutic strategy for the management of L-arginine-induced AP, as evident from the resulting improvement in pancreatic injury, decreased ROS generation and cell apoptosis, and downregulation of NF-κB in an L-arginine-induced AP mouse model.

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