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Ulinastatin promotes T lymphocyte apoptosis in rats with severe acute pancreatitis via mitochondrial pathways

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ABSTRACT. We explored the influence of ulinastatin on apoptosis of T lymphocytes in rats with severe acute pancreatitis (SAP) and the effect of ulinastatin on mitochondrial apoptosis pathways in spleen lymphocytes. Thirty-six Wistar rats were randomly divided into three groups (N = 12): a sham operated group, a SAP group, and an ulinastatin-treated SAP group. The SAP model was established by injecting 5% sodium taurocholate into the intrapancreatobiliary duct. Study rats were sacrificed after 24 h, and splenic lymphocytes were then collected. CD_4^+ and CD_8^+ T lymphocytes were labeled by direct immune fluorescence assays; the percentage of apoptotic cells, mitochondrial membrane potential levels, and mitochondria permeability transition pore opening levels were measured by flow cytometry. In the ulinastatin-treated SAP group, the ratio of CD_4^+/CD_8^+ T lymphocytes was significantly higher than that in the SAP group, and the apoptosis percentage of CD_4^+ T lymphocytes was significantly decreased. The percentage of lymphocytes with an abnormal opening of the mitochondrial permeability transition pore and lymphocytes

with decreased mitochondrial membrane potential in the ulinastatin-treated SAP group were significantly lower than that in the SAP group. Ulinastatin can directly enhance immunological function and attenuate immune suppression in SAP rats through inhibiting the apoptosis of CD_4^+ T lymphocytes. These study findings demonstrate that therapeutic effects may occur through inhibiting the apoptosis induced by mitochondrial signaling pathways.

Key words: Ulinastatin; Severe acute pancreatitis; Immune suppression; Cell apoptosis; Mitochondrion

INTRODUCTION

Severe acute pancreatitis (SAP) is a systematic disease with ongoing immune disorders throughout the course of the disease. In the early stage, SAP is characterized by overactive inflammatory reactions, with clinical manifestation of systematic inflammatory response syndrome (SIRS) and functional damage of related organs including the heart, lungs, and kidneys (Martin et al., 2008). In the late stage, SAP is characterized by severe immune suppression and local complications, including sepsis, sepsis-related organic damage, secondary abdominal hemorrhage, and/or digestive tract fistula. Late in the disease course, infectious complications that are induced by immune suppression are difficult to control even with active anti-infectious treatment and surgical intervention. The mortality rate of necrotic infection is greater than 30%, and the mortality from multiple organ failure is between 20 and 30%; over 80% of SAP deaths are associated with infectious complications. The severe suppression of immune function is considered the fundamental cause of infection (Kylanpaa et al., 2010), which occurs as a result of the disproportion of CD_4^+ to CD_8^+ T lymphocytes, a Th1/Th2 shift, the immune energy of lymphocytes, and excessive apoptosis of lymphocytes. These immunosuppressive effects further inhibit the function of macrophage cells and/or dendritic cells, including co-stimulator expression and pro-inflammatory cytokine secretion, but increase anti-inflammatory cytokine secretion.

Ulinastatin (urine trypsin inhibitor, UTI) is a glycoprotein derived from human urine. UTI activity in urine increases when the body suffers from stresses such as infection, fever, shock, and surgery, and UTI levels return to normal after the body recovers. As an endogenous protection mechanism, the UTIs can counteract stress and attenuate the effects of stress-related injury. Kim et al. (2009) found that the UTI has strong enzyme inhibitory activity, which inactivate multiple enzymes, including leukocyte elastase, hyaluronidase, thiol-enzyme, fibrinolytic enzymes, and the serine protease-like trypsin, α-chymotrypsin. The UTI has anti-inflammatory effects and can directly affect the neutrophils and mononuclear macrophages; inhibit neutrophil infiltration; and the release of elastase, inflammatory mediators and mononuclear macrophage inflammatory cytokines like TNF-α, IL-1, and IL-8 (Yang et al., 2011). The UTI has been studied as a pancreatitis therapy (Uemura et al., 2008). Other studies indicated that the UTI can stabilize the lysosomal membrane and inhibit the release of lysosomal enzymes and inflammatory mediators as well as the production of the myocardial inhibitor. Based on these research results, the UTI may be curative in treating shock, diffuse intravascular coagulation, and may play a role in therapy during the perioperative period. The immune indices in patients, including T lymphocyte counts, T lymphocyte function, and cytokine pro-

duction, in the perioperative period were recovered to different extents after the application of UTIs. Consequently, it has been suggested that the UTI improves immune function (Sato et al., 2002; Ma et al., 2006).

Based on previous research, this study examined the effect of UTIs on a SAP animal model and explored the effects on CD_4^+/CD_8^+ T lymphocytes, lymphocyte apoptosis, and changes to mitochondrial function.

MATERIAL AND METHODS

For *in vivo* experiments, 36 male 3-month-old clean grade Wistar rats that weighed between 250 g and 300 g were used. The rats were kept in cages and had free access to food and water, temperature of the room was controlled in the range of 20-29°C, and a diurnal cycle was maintained with 12 h of fluorescent illumination.

After adapting to the laboratory environment for 1 week, the 36 rats were randomly divided into 3 groups (N = 12): a sham-operated control group, a SAP group, and a UTI-treated SAP group. Animals were fasted for 12 h (with free access to water) before the operation. Each animal was weighed and given an intraperitoneal injection of 10% chloral hydrate (300 mg/kg). Aseptic operation included skin disinfection followed by central abdominal incision below the xiphoid process. The lower margin of the liver, duodenum, pancreatic duct, and partial pancreas were exposed. A disposable IV catheter was used to puncture the lateral wall of the duodenum where the two cholecystopancreatic ducts form an opening. Part of the needle core was withdrawn immediately after the insertion into the duodenal cavity. Then, the trocar entered approximately 0.5 cm along the opening of the cholecystopancreatic duct, and the needle core was completely pulled out. The cholecystopancreatic duct in the hilar region was clamped with a microclip to prevent the sodium taurocholate from entering the liver; finally, the end of the catheter tube was connected to the syringe pump.

In the SAP and SAP UTI-treated groups, rats were injected with 5% sodium taurocholate at a speed of 0.1 mL/min (1 mL/kg, based on weight). The catheter was aseptically removed after 5 min. The abdomen was closed layer by layer. In the sham group, the injection of the sodium taurocholate was excluded. After the surgery, each group was given subcutaneous injections of 5% glucose and sodium oxide (50 mL/kg, based on weight). The UTI-treated group received an injection of UTI through the tail vein (10,000 U/kg, based on weight).

Rats were anesthetized 24 h after the initial surgery. The spleen was quickly harvested under aseptic conditions and filled with serum-free Roswell Park Memorial Institute (RPMI)-1640 medium to remove the blood. The fat and fascia tissue were removed; the tissue was separated using a wire screen (200 mesh) and flushed with RPMI-1640 medium. The resulting cell suspension was washed twice with RPMI-1640 medium, suspended again to approximately 2.5 mL, and layered over 5 mL lymphocyte cell isolation in a 10 mL centrifuge tube. Cells were stratified by centrifuge (1500 rpm for 15 min at 20°C) and the monocytes were collected and separated by centrifuge (1500 rpm for 10 min at 20°C); the supernatant was removed, and the cells were suspended in RPMI-1640 medium and incubated for 60 min at 37°C to allow attachment of the monocytes. The remaining cell suspension was used as the spleen lymphocyte suspension.

Statistical analysis

The data were analyzed with the SPSS 18.0 software. The measurement data are reported as means ± SE, the multiple mean comparisons were evaluated by one-way analysis of variance (ANOVA), and the comparison between any two means was by least significant difference (LSD) test. Differences with $P < 0.05$ were considered to be statistically significant.

RESULTS

Compared with the sham group, the percentage of CD_4^+ T lymphocytes and ratio of CD_4^+/CD_8^+ T lymphocytes in the SAP group were significantly lower ($P < 0.01$). In the UTI-treated group, the percentage of CD_4^+ T lymphocytes and CD_4^+/CD_8^+ T lymphocytes was significantly higher than those in the SAP model group (Table 1).

Table 1. Percentage and ratio of CD_4^+ to CD_8^+ T cells for each group (N = 12).

Groups	CD_4^+ (%)	CD_8^+ (%)	CD_4^+/CD_8^+
Sham	45.22 ± 4.38	20.28 ± 2.11	2.23 ± 0.12
SAP	32.10 ± 2.87*	28.41 ± 2.31	1.15 ± 0.12*
Ulinastatin	37.63 ± 3.41#	24.31 ± 2.62	1.66 ± 0.12#

All the values are given in mean ± SE *P < 0.01 SAP *vs* sham group; #P < 0.01 ulinastatin *vs* SAP group.

Compared with the sham group, the percentage of apoptotic CD_4^+ and CD_8^+ T lymphocytes in SAP model group was significantly higher ($P < 0.01$). However, the percentage of the apoptotic CD_4^+ T lymphocytes in UTI-treated group was significantly lower than that in the SAP group (Table 2).

Table 2. Percentage of apoptotic CD_4^+ and CD_8^+ T cells for each group (N = 12).

Groups	CD_4^+ T cells, apoptosis (%)	CD_8^+ T cells, apoptosis (%)
Sham	3.82 ± 0.50	1.97 ± 0.36
SAP	17.70 ± 2.10*	2.78 ± 0.45*
Ulinastatin	8.58 ± 1.09#	2.46 ± 0.36▲

All the values are given in mean ± SE *P < 0.01 SAP *vs* sham group; #P < 0.01 ulinastatin *vs* SAP group; ▲P > 0.05 ulinastatin *vs* SAP group.

The percentage of lymphocytes with decreased mitochondrial membrane permeability (MMP) and lymphocytes with an abnormal opening of the mitochondrial permeability transition pore (MPTP) in the SAP group was significantly higher than those in the sham group ($P < 0.01$), while the percentage of lymphocytes with decreased MMP and lymphocytes with an abnormal opening of the MPTP in the UTI-treated group was significantly lower than those in the SAP group ($P < 0.01$; Tables 3, 4).

Table 3. Percentage of spleen lymphocytes with decreased MMP for each group (N = 12).

Groups	Percentage of lymphocytes with decreased MMP
Sham	3.69 ± 0.45
SAP	46.94 ± 3.49*
Ulinastatin	17.30 ± 1.60#

All the values are given in mean ± SE *P < 0.01 SAP *vs* sham group; #P < 0.01 ulinastatin *vs* SAP group.

Table 4. Percentage of spleen lymphocytes with abnormal openings of MPTP for each group (N = 12).

Groups	Percentage of lymphocytes with abnormal opening of MPTP
Sham	7.69 ± 0.77
SAP	51.31 ± 3.23*
Ulinastatin	30.14 ± 2.46#

All the values are given in mean ± SE *P < 0.01 SAP *vs* sham group; #P < 0.01 ulinastatin *vs* SAP group.

DISCUSSION

SAP, which has acute onset and rapid progression, is characterized by pancreatic diffuse hemorrhage and tissue necrosis. SAP is one of the most complicated acute abdominal diseases associated with surgery and often has complications such as SIRS and multiple organs dysfunction syndrome (MODS). Past experiments showed that, in the early stage of SAP, the body triggers a cascade of inflammatory reactions as well as anti-inflammatory reactions followed by excessive anti-inflammatory reactions and apoptosis of lymphocytes, which leads to immune suppression and immunologic paralysis. Immunologic paralysis, also called immunologic unresponsiveness, refers to the condition in which the immune cells neither proliferate nor secrete cytokines under stimulation of the specific antigen. Excessive lymphocyte apoptosis is closely related to immune suppression (Feng et al., 2010). Previous research found that immune suppression may be caused by acceleration of lymphocyte apoptosis (especially B lymphocytes and T helper cells) and dendritic cell apoptosis (Kylanpaa et al., 2010). Inflammation of the pancreas and surrounding tissue, which is caused by decreased immunity, is a common and severe complication and the main cause of death in late stage SAP, with mortality rates reaching 80% (Cicalese et al., 2001).

Our research found that the percentage of apoptotic CD_4^+ T lymphocytes in the SAP group was higher than the percentage of apoptotic CD_8^+ T lymphocytes, which resulted in a lower ratio of CD_4^+/CD_8^+ T lymphocytes in the SAP groups than in the sham-operation group. This indicates that, when SAP occurs, the body stimulates CD_4^+ T lymphocyte apoptosis and downregulates the inflammatory reaction. This reaction can reduce the injury from excessive inflammatory reactions, but also result in low cellular immune function, which causes immune suppression.

UTI, a glycoprotein secreted by the liver (Watanabe et al., 2003), is involved in several key steps of pancreatic disease, such as activation of pancreatic enzymes and cytokines and release of inflammatory mediators; consequently, it has been used in pancreatitis treatment (Uemura et al., 2008). Clinical and basic research studies have shown the value of UTI. A clinical study by Sato et al. (2002) found that UTI has a corrective effect on immune deficiency-caused surgical stress (Sato et al., 2002); UTI can enhance immune function and improve the immune imbalance in sepsis cases through increasing the ratio of inducer T lymphocytes. In 2009, Huang et al. (2009) initiated UTI immunotherapy and revealed that UTI therapy can noticeably improve the quality of life of sepsis patients. There is increasing interest in the role that UTI plays in the immune system, including its role in anti-inflammation.

In this study, the percentage of CD_4^+ T lymphocytes and ratio of CD_4^+/CD_8^+ T lymphocytes in the UTI-treatment group were higher than those in the SAP groups. This is attributed to the high percentage of apoptotic CD_4^+ cells. Through reducing abnormal apoptosis of CD_4^+ T lymphocytes in the SAP rats, UTI has a direct effect on improving the state of immune depression and thus reduces the susceptibility to secondary infection, prevents occurrence of

septicopyemia and MODS, and decreases the SAP mortality rates.

The initiation and process of cell apoptosis are both related to and controlled by unique and complex signal systems. Mitochondria are not only the location of the tricarboxylic acid cycle, oxidative phosphorylation, and fatty acid oxidation, but also a crucial organelle for apoptosis (Zamzami and Kroemer, 2001). According to some studies, the key factors that could open up the MPTP have excessive Ca^{2+} in the mitochondria, oxidative stress effects in response to a large number of oxygen radicals, exhaustion of adenine nucleotides, and mitochondrial membrane depolarization (Javadov et al., 2009).

Normal mitochondrial membrane potential is a prerequisite to maintain mitochondrial oxidative phosphorylation and generate adenosine triphosphate (ATP). It is also necessary to retain mitochondrial function. Researchers have shown that, when the potential difference between the inside and outside of the mitochondria reduces, the mitochondrial membrane potential is decreased, which leads to a series of biochemical changes on both sides of the mitochondrial membrane. For example, when cytochrome C, the caspase activator that can regulate energy mechanism and cell apoptosis, is released, mitochondrial membrane permeability changes and both the Bcl-2 family and caspase are activated, which then leads to the cascade of cell apoptosis and finally stimulates cell apoptosis. Compared with morphological changes in apoptotic cells, there were changes in the amount of chromatic nuclear pycnosis, fragmentized DNA, sheared PARP, activated apoptotic protease (CPP32, caspase-3) in the cytoplasm, cell shrinkage, the calcium entry, potassium outflow, and exposure of phosphatidylserine of the cell membrane. Additionally, permeability was strengthened, and the mitochondrial membrane potential disappeared earlier. These findings demonstrate that the reduction of mitochondrial trans-membrane potential is an irreversible course to cell apoptosis (Green and Reed, 1998). Another study indicated that inhibiting mitochondrial membrane potential reduction can inhibit cell apoptosis; thus, this reduction is a specific change during apoptosis.

Rhodamine123 is a positive lipophilic fluorochrome and is permeable to the cell membrane. It can be selectively absorbed by mitochondria and stored in the mitochondrial matrix, which owns potential. It is very sensitive to mitochondrial membrane potential changes, which can affect rhodamine123's absorbing, and different absorbed doses could linearly change fluorescence intensity (Huang et al., 2007). When apoptosis occurs, there are decreases in the turnover capacity of the mitochondrial membrane, electronegativity, mitochondrial capacity to collect rhodamine123, and fluorescence intensity. Mitochondrial membrane potential changes can be measured based on the aforementioned reductions.

The MPTP, which is a polyprotein composite structure (Bernardi and Forte, 2007), called the cell's fortune switch, is composed of an voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane and an adenine nucleotide translocator (ANT) in the mitochondrial inner membrane (Halestrap, 2009). Simultaneously, the pathway also includes hexokinase interacting with VDAC and cyclophilin D (CyP D) interacting with ANT. The present consensus is that the functional pathway model should be VDAC-ANT-CyP D, while hexokinase is only involved in pathway regulation by glucose. Under physiological conditions, the MPTP is closed or in a low permeable state and only opens to small molecules; under oxygen-poor pathological conditions, the state is entirely opposite (Xue et al., 2002). The opening of MPTP makes the mitochondria swell, and the ruptured outer membrane directly leads to the exhaustion of ATP, rising of cytoplasm Ca^{2+} density, releasing of cytochrome C and apoptosis-inducing factor (AIF), and dropping of mitochondrial membrane potential. Finally, through the mitochondrial apoptosis pathway, cells undergo apoptosis and necrosis

(Weiss et al., 2003; Baines et al., 2003; Korsnes et al., 2006). Thus, the state of MPTP directly controls the cell's destiny (Kim et al., 2003; Gomez et al., 2009). A previous study (Hotchkiss and Nicholson, 2006) found that pyemic splenic lymphocytic mitochondrial membrane potential dropped, and the authors suspected that this decrease is the result of MPTP's abnormal opening.

Compared with the control group, lymphocytes with lower mitochondrial membrane potential or an abnormally opened MPTP remarkably accelerates, which indicates that, during SAP, splenic lymphocytic mitochondrial membrane permeability is enhanced and the potential decreases; therefore, the mitochondrial apoptosis pathway plays an important role in regulating apoptosis. UTI can effectively protect mitochondria by stabilizing the mitochondrial membrane structure and enhancing the activity of mitochondrial Na^+-K^+-ATP enzyme. Other studies found that UTI could minimize mitochondrial damages and inhibit apoptosis induced by the mitochondrial pathway by limiting the expression of ischemia-reperfusion organic cytochrome C and AIF. Our experiment revealed that UTI can dramatically decrease the ratio of lymphocytes to opened MPTPs in SAP rats and the ratio with dropped MMP. Our study demonstrated that UTI attenuated lymphocyte apoptosis, improved immunological function in SAP rats, and exerted therapeutic effects by inhibiting apoptosis induced by mitochondrial signaling pathways.

Conflicts of interest

The authors declare no conflict of interest.

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