Mesenchymal stem cells improve intestinal integrity during severe acute pancreatitis

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Abstract. Severe acute pancreatitis (SAP) is an acute inflammatory disease of the pancreas that involves various distant tissues and organs. This study aimed to investigate post-tissue injury repair by mesenchymal stem cells (MSCs) in a rat model of SAP. A total of 54 pathogen-free adult male SD rats were randomly assigned to the groups SAP, SAP + MSCs and sham-operated (SO). SAP was induced by 4% sodium taurocholate, and MSCs were injected via the dorsal penile vein 1 h later. The amylase activity, and tumor necrosis factor (TNF)-α and diamine oxidase (DAO) levels were measured with an enzyme-linked immunosorbent assay (ELISA), while the expression of aquaporin (AQP)-1 was evaluated by immunohistochemical staining. The pathological score of intestinal tissues was also compared among groups. Marked improvement in intestinal necrosis, villi shedding and infiltration of inflammatory cells was observed in the SAP + MSCs group compared to the SAP and SO groups. Amylase, TNF-α, and DAO levels were significantly increased in the SAP + MSCs group. The intestinal expression of AQP-1 was increased at 12 and 24 h post-MSC transplantation compared to the SO group. Rats of the SAP + MSCs group displayed higher pathological scores compared to the SAP group at all time points. Overall, these data showed that MSCs can inhibit systemic inflammation and reduce TNF-α release in a rat model of SAP-induced intestinal injury, suggesting that MSCs exert protective effects on the intestinal barrier during SAP.

Introduction

Severe acute pancreatitis (SAP) is an acute abdominal disease with high prevalence, severe symptoms, complicated pathogenesis and mortality as high as 20-30% (1,2). Although the mechanisms underlying SAP have not been fully elucidated, changes in secretion patterns of pancreatic acinar cells, intracellular activation of proteases and generation of inflammatory mediators may be linked to SAP pathogenesis (1). Approximately 10% of patients with acute pancreatitis show necrosis of the pancreatic and peripancreatic tissues, leading to infection of the necrotic tissue, multiple organ failure, and mortality (2). A few studies emphasized that the major damage occurring in SAP patients is not necrosis of the pancreas, but intestinal bacterial translocation, enterogenic endotoxia and secondary pancreatic infection (3,4). The small intestine may become damaged during SAP due to alterations in microcirculation associated with fluid loss, hypovolemia, splanchic vasoconstriction and ischemia-reperfusion injury, and failure of the small intestine tends to aggravate the course of SAP (5). Intestinal permeability, which develops as a result of intestinal barrier damage early in acute pancreatitis, is directly associated with endotoxia (6). Furthermore, increased gut permeability has been suggested to be the initial event in the bacterial contamination of pancreatic necrosis in SAP (4). Samel et al (3) focused on bacterial translocation across the gut as a functional aspect of mucosal barrier function during SAP in rats. The investigators directly observed the translocation of fluorescent bacteria from the small bowel to the pancreas, providing evidence supporting the gut origin of microorganisms responsible for the infectious complications in SAP (3). Lu et al (7) reported an increase in small intestinal capillary leakage in a rat model of SAP; they concluded that various inflammatory mediators and cytokines released during SAP may directly attack capillary endothelial cells, resulting in cell apoptosis and necrosis, and increasing intestinal barrier permeability. Although disruption of the intestinal barrier function appears to be a key step and possibly a turning point in the progression of SAP, the patients with highest mortality risk are those whose inflammatory response to pancreatic injury leads to organ failure (5). Through the action of a number of cascades, systemic inflammatory response syndrome can eventually lead to multiple organ dysfunction syndrome (8).

Mesenchymal stem cells (MSCs) isolated from various tissues, including the stroma of bone marrow and adipose tissue, have been demonstrated to exert therapeutic effects on intestinal injury as well as inflammatory, cardiovascular, degenerative and skeletal diseases (9-14). MSCs have the
potential for proliferation and multipotent differentiation into cells of mesodermal, ectodermal and endodermal lineages (15-17). Viable strategies to foster lineage-specific differentiation of MSCs have been proposed, rendering feasible novel applications (10). MSCs possess immunoregulatory properties and have great potential for the treatment of inflammatory response; they respond to inflammation by homing to the inflamed tissues, which provides local control of inflammation and facilitates tissue repair (14). During SAP, where translocation of bacteria and toxins promotes the development of inflammation, human bone marrow-derived MSCs were shown to reduce inflammation and pancreatic tissue damage in a rat model of SAP, reducing the levels of cytokines and suppressing rat T-cell proliferation (18). Considering the unpredictable course of SAP and the absence of effective therapies, a cell-based therapeutic strategy may be promising for SAP treatment. Additional studies are needed to better understand the potential of MSCs to limit pancreatic damage in SAP, which may possibly rely on restoring the structure and function of pancreatic acinar cells (18).

In the present study, MSCs obtained by multiple digestions and passages of cells isolated from rat bone marrow were injected into male Sprague Dawley (SD) rats with taurocholate-induced SAP, in order to investigate the effects of MSC transplantation on intestinal barrier function and bacterial translocation. Our study aimed to investigate the mechanism underlying MSC-induced repair of tissue injury. Results of this study provide evidence for effective treatment of SAP with stem cell transplantation.

Materials and methods

Materials. A total of 54 specific-pathogen-free adult male SD rats, weighing 300±30 g, were purchased from the Shanghai Laboratory Animal Co. (SLAC), Ltd. (animal license no. SCXK [Hu] 2007-0005; Shanghai, China). Animals were grown at 20-28˚C in an environment with 40-70% humidity. The animals were allowed to accommodate to the environment for one week prior to the experiments. This study used sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA), CM-DiI (Thermo Fisher Scientific Inc., Waltham, MA, USA), enzyme-linked immunosorbent assay (ELISA) kits, and diamine oxidase (DAO) (Robert & Co., Tokyo, Japan) at a 1:2,000 dilution and biotin-conjugated rabbit anti-rat secondary polyclonal antibody targeting aquaporin  (AQP)-1 (Cell Signaling Technology, Danvers, MA, USA) at a 1:2,000 dilution and biotin-conjugated rabbit anti-rat secondary polyclonal antibody at a 1:100 dilution (Cell Signaling Technology, Danvers, MA, USA). The amylase detection kit was purchased from Meikang Biotechnology (Ningbo, Jiangsu, China).

Preparation of CM-DiI-conjugated MSCs. MSCs were isolated from aseptically collected and cultured bone marrow from sacrificed male rats by multiple digestions and passages as follows: First, MSCs were digested with 0.25% trypsin (Hyclone, Logan, UT, USA) in 0.1% EDTA (Gibco-BRL, Hercules, CA, USA), and then serum containing medium was used to terminate the digestion reaction. Cells were harvested by centrifugation at 139.875 x g for 10 min. The supernatant was removed, and cells were washed in phosphate-buffered saline (PBS) once. The cell suspension (10^6 cells/ml) was prepared with serum-free medium, and then CM-DiI labeling solution, a fluorescent dye that covalently conjugates to the thiol group in the cells, was added at 5 µl/ml of medium. The cells were resuspended and incubated at 37˚C for 20 min. After centrifugation at 139.875 x g for 5 min, the supernatant was removed and cells were washed in PBS twice. Following trypsin blue staining, viable cells were counted. CM-DiI-conjugated MSCs were injected into the rats via the dorsal penile vein. The rat intestine was collected, freeze-sectioned (5-µm sections) and observed by fluorescence microscopy (IX51 Biological Inverted Microscope, Olympus, Tokyo, Japan).

Establishment of the SAP rat model. Animals were deprived of food for 12 h, but given access to water ad libitum. Following intraperitoneal anesthesia with 10% chloral hydrate at 3.0 ml/kg, the rat abdomen was sterilized and a midline incision was performed at 3 cm from the lower part of the xiphoid. The pancreas was exposed, and the bile duct was clamped with a non-invasive clamp at the porta hepatic. Then, a 24-G trocar was inserted via the contralateral intestine, and the stylet was withdrawn. The outer cannula was inserted forward to the main pancreatic duct (0.5-cm) and fixed. Next, 4% sodium taurocholate (0.1 ml/100 g) was injected by a micropump at a rate of 0.1 ml/min. The cannula was left in the pancreatic duct for 5 min and the injection pressure was maintained. Pancreatic edema and congestion were observed, and then the clamps and cannula were removed. The intestine was sutured and returned to the abdominal cavity, followed by wound closure. After surgery, 2 ml of normal saline were subcutaneously injected for fluid supplementation.

Study design and procedures

Animal groups. SD rats were randomly assigned to 3 groups (n=24 each): SAP, SAP + MSCs and sham-operated (SO). Rats in the SAP and SAP + MSCs group received retrograde injection of 4% sodium taurocholate via the biliopancreatic duct to induce SAP, as described above. MSCs (2x10^6 cells/100 g) were injected into half (n=24) of the SAP-induced rats via the dorsal penile vein 1 h later (SAP + MSCs group). Rats in the SO group received laparotomy, and the pancreas and intestine were massaged, followed by wound closure. The SAP and SAP + MSCs group animals were sacrificed at 6, 12, 24 and 48 h after SAP induction. The SO group animals were sacrificed 6, 12, 24 and 48 h after surgery.

Detection of α-amylase. The temporal changes in the level of amylase were measured in the 3 groups at 6, 12, 24 and 48 h after SAP induction. The enzymatic activity of serum amylase was detected using ethylidene-4-nitrophenyl-α-D-maltotetraoside as a substrate, and were quantified using a spectrophotometer (7150; Hitachi Ltd., Tokyo, Japan). The amylase activity was calculated using the equation: Amylase activity (U/L) = (DA_sample/min-DA_control/min) * F, where F = V/ν/V_t, total volume; V_s, sample volume; P=1 cm, mm (molar extinction coefficient) for p-nitrophenylcarbinole (pNP) = 10.567, and F=4826 in the test.

Detection of TNF-α and DAO. Blood was collected from the rat abdominal aorta and the plasma was stored at -70˚C. The TNF-α level and the DAP activity were detected by ELISA kits, following the manufacturer's recommendations.
Intestinal histopathology and immunohistochemical detection of AQP-1. The terminal ileum (~5 cm) was collected and processed in paraffin-embedded sections (4 µm). Then, hematoxylin and eosin (H&E) staining was performed, and sections were observed under a light microscope (Leica DM 2000, Leica Microsystems, Wetzlar, Germany). The intestine was collected and fixed in 4% paraformaldehyde. Following dehydration, tissues were embedded in paraffin, followed by sectioning (4 µm). After deparaffinization and hydration, antigen retrieval was performed in citrate acid (pH 6.0). Endogenous peroxidase was inactivated with 3% H$_2$O$_2$. Following incubation with the primary anti-AQP-1 antibody at room temperature, sections were treated with biotin-conjugated rabbit anti-rat secondary antibody, followed by visualization with 3,3'-diaminobenzidine (DAB). Counterstaining was done with H&E, followed by dehydration, transparentization and drying. After mounting in neutral gum, the sections were observed under a light microscope.

Pathological scoring of intestinal tissue. Intestinal tissue samples were fixed in 10% formaldehyde and processed in paraffin-embedded sections. Two sections were selected from each rat. H&E staining was performed, and the sections were observed under a light microscope. For each section, 50 fields (x200) were randomly selected and scored using the scored by Chilu et al (19).

Statistical analysis. Data were expressed as mean with standard deviation (SD), and presented in bar charts. Comparisons were performed with analysis of variance (ANOVA), and resulting p-values were adjusted for multiple testing with the Bonferroni method (20). Data were analyzed using the SPSS 15.0 statistical software (IBM, Armonk, NY, USA). P<0.05 were considered to indicate statistically significant differences.

Results

In vitro tracing and location of CM-DiI-labeled MSCs in the intestinal tissue. Following CM-DiI labeling, cells were observed under a fluorescence microscope. CM-DiI-labeled MSCs displayed red fluorescence. The adherent cells were...
spindle-like. Passage 0 MSCs were circular, with numerous granules. The fluorescence intensity was high, but not detected in the nucleus (Fig. 1A and B). Fig. 1C shows the fluorescence of frozen sections at the blue channel, revealing CM-DiI-positive cells in the intestine. Fig. 1D shows a frozen observed under a phase contrast light microscope.

**Detection of markers for decreased intestinal barrier integrity.** The temporal changes in the levels of intestinal barrier integrity biomarkers amylase, TNF-α and DAO were measured in the SAP, SAP + MSCs and SO groups at 6, 12, 24, and 48 h after SAP induction. Amylase (Fig. 2A), TNF-α (Fig. 2B) and DAO values (Fig. 2C) were significantly increased in the SAP group compared to the SO group, and were decreased in the SAP + MSCs group compared to the SAP group (all P<0.05).

**Pathological analysis of pancreatic tissues.** The structure of the pancreatic lobules as observed under the light microscope was normal. Interstitial edema and infiltration were detected in a few inflammatory cells, and the alveoli were normal at 6 h after SAP induction in the SAP group (Fig. 3A). At 12 h post-SAP, infiltration of inflammatory cells was detected in the pancreatic interstitial tissue, accompanied by hemorrhage, which was more severe compared to that observed 6 h post-SAP induction. Vacuolar degeneration was observed in the pancreatic lobules, the alveoli were largely disrupted, and hemorrhage and necrosis were detected in the adipose tissues (Fig. 3C). At 24 h post-SAP, massive necrosis was observed in the pancreas, blood vessels in the interstitium were markedly enlarged and hemorrhage occurred, infiltration of inflammatory cells was obvious, and alveoli were severely disrupted (Fig. 3E). At 48 h post-SAP, necrosis of the pancreas was aggravated, and vacuolar degeneration, congestion of blood vessels in the interstitium, infiltration of numerous inflammatory cells, patchy necrosis of adipose tissues, hemorrhage and saponification were observed (Fig. 3G). Injection of MSCs had protective effects on rats with taurocholate-induced SAP, overall reducing the damage in the pancreas (Fig. 3B, D, F and H).

**Figure 3. Temporal changes in histology of the affected pancreas.** Pancreatic sections stained with hematoxylin and eosin (H&E) are from either SAP- (A, C, E and G) or SAP + MSCs-treated (B, D, F and H) rats at different time points (6 h, A and B; 12 h, C and D; 24 h, E and F; 48 h, G and H). Scale bar, 100 µm. SAP, severe acute pancreatitis; MSCs, mesenchymal stem cells.

**Figure 4. Temporal changes in histology of the affected intestine.** Intestinal sections stained with hematoxylin and eosin (H&E) are from either SAP- (A, C, E and G) or SAP + MSCs-treated (B, D, F and H) rats at different time points (6 h, A and B; 12 h, C and D; 24 h, E and F; 48 h, G and H). Scale bar, 100 µm for panels A, B, E, F, G and H, and 50 µm for panels C and D. SAP, severe acute pancreatitis; MSCs, mesenchymal stem cells.
Pathological analysis of intestinal tissues. Under a light microscope, mild edema was observed in the ileal interstitium, accompanied by spotty hemorrhage. In addition, the epithelium and lamina propria were mildly separated at 6 h post-SAP induction in the SAP group (Fig. 4A). At 12 h, the mucosal injury of the ileum deteriorated as compared to that observed at 6 h post-SAP induction, and interstitial edema and infiltration of a few inflammatory cells were detected (Fig. 4C). At 24 h post-SAP, the villous edema of the ileum was obvious, and erosion, necrosis and shedding of intestinal villi were observed, accompanied by infiltration of numerous inflammatory cells (Fig. 4E). At 48 h post-SAP, the mucosal injury of the intestine was alleviated, and some repair of the intestinal mucosa was noted (Fig. 4G). At 6 h after treatment with MSCs, disruption at the top of the intestinal villi, interstitial edema and infiltration of neutrophils were attenuated when compared to those observed in the SAP group at corresponding time points (Fig. 4B). At 12 h after MSC transplantation, necrosis and shedding of the intestinal villi, and infiltration of inflammatory cells were improved compared to the SAP group at corresponding time points (Fig. 4D). At 24 h, lodging, shortening and loosening of intestinal villi were also observed, but the extent of the damage and the infiltration of inflammation were alleviated when compared to that observed in the SAP group at corresponding time points (Fig. 4F). At 48 h post-MSC transplantation, pathological changes were improved in the SAP + MSCs compared to the SAP group at corresponding time points (Fig. 4H).

Immunohistochemical detection of AQP-1. Immunohistochemistry analysis showed that AQP-1 is highly expressed in the capillaries, small blood vessels and endothelial cells of the central chyle duct of the intestine (brown cells; Fig. 5A). At 6 h after SAP, AQP-1 expression
AQP-1 expression was lower in the SAP compared to the SO group starting from 6 h after SAP, reaching the lowest expression level at 24 h post-SAP induction. However, following MSC treatment, AQP-1 expression was significantly increased in the intestine as compared to the SO group from the 12-h time point onwards. Thus, we hypothesize that MSCs may inhibit systemic inflammation and reduce TNF-α release, which enhances the activity of the AQP-1 promoter, and upregulates mRNA and protein expression of AQP-1, leading to the attenuation of the intestinal barrier dysfunction.

Under normal conditions, the intestinal barrier is composed of mechanical, immune, biological and chemical barriers, which can potently inhibit the translocation of intestinal bacteria and toxins into extraintestinal tissues and organs, and protect from injury induced by endogenous microorganisms and toxins. However, at the early stage of SAP, the white blood cells in the pancreas and intestinal mucosa are overactivated and produce numerous inflammatory cytokines [e.g., interleukin (IL)-1, IL-6, IL-8, TNF-α and endotoxin (ET)] that mediate mucosal inflammation, resulting in damage of the intestinal barrier. A functional intestinal barrier does not allow endotoxins and DAO from the mucosal epithelial cells to enter the circulation. However, when the integrity of intestinal epithelial cells is altered and gut permeability increases, DAO may be released into the circulation. The translocation of intestinal bacteria (21) and endotoxemia induced by intestinal endotoxins may cause a ‘second strike’ (8,22), resulting in secondary pancreatic infection and a cascade of inflammatory responses. Two studies by Towne et al (23,24) confirmed that TNF-α can activate the nuclear factor (NF)-κB pathway via the TNF-α receptor, which then downregulates AQP-5 expression. AQP-1 and AQP-5 are homologous. Thus, we hypothesize that TNF-α can regulate AQP-1 via a similar mechanism in SAP-induced intestinal barrier dysfunction. It has been shown that AQP-1 is widely expressed in the capillaries, small blood vessels and endothelial cells of the central chyle duct in the gastrointestinal system, and that it mediates the transmembrane transport of water in the gastrointestinal tract (25). Intestinal edema manifests at the early stage of SAP. It may affect cellular viability, aggravate ischemia/hypoxia-induced injury and result in diffusion of intestinal bacteria and endotoxins into other organs and tissues, which may finally lead to systemic infection and multiple organ dysfunction (8).

Thus, maintaining the intestinal barrier and preventing the translocation of bacteria and endotoxins may protect from the above-described ‘second strike’ following SAP.

MSCs are a group of non-hematopoietic stem cells having the potential for self-renewal and multilineage differentiation. MSCs have been used in autologous transplantation, transfection of exogenous genes and regulation of gene expression (9,11,12,13). In addition, MSCs have the unique property of specifically homing to damaged tissues. This is especially valuable in SAP, since the MSCs respond to inflammation by homing to the inflamed tissues, providing local control of the inflammation and facilitating tissue repair (11,14). MSCs may exert a protective effect on the intestinal barrier of animals with SAP via the following mechanisms: Numerous injury-induced cytokines in the microenvironment promote the differentiation of MSCs into specific tissues. MSCs also secrete numerous cytokines and chemokines at the injured site (25). MSCs enter the circulation at the injured sites, which improves the focal circulation and blood supply (26). This also improves the nutritional
status, which is beneficial for the recovery of the cells and subsequently, of the tissues. We suggest that this may be one of the mechanisms underlying MSC-induced repair of injured tissues. In addition, MSCs have potent ability to regulate the immune system. Previous studies showed that MSCs can downregulate the expression of pro-inflammatory cytokines such as IL-1 and TNF-α, and that of inducible nitric oxide synthase (iNOS), but upregulate the expression of anti-inflammatory cytokines such as IL-1 and transforming growth factor (TGF), which attenuate inflammation and improve tissue injury (14,27). Jung et al (18) showed that the migration of MSCs into the inflammatory site induces the expression of forkhead box P3 (Foxp3), inhibits T cell proliferation, attenuates inflammation and facilitates tissue repair. Finally, the transplanted MSCs localize in the submucosal layer of the injured intestine and then differentiate into epithelial cells, which promotes mucosal recovery (28,29). These cells can also differentiate into intestinal subepithelial myofibroblasts (ISEMFs), which may improve the microenvironment for stem cells and indirectly promote intestinal epithelial cell repair and angiogenesis in the intestine following injury (30).

Although previous studies have reported the effectiveness and safety of using bone marrow stem cells in the treatment of intestinal ischemia/reperfusion, the use of MSCs still has limitations: i) in vitro-transplanted MSCs tend to rapidly proliferate. Thus, following transplantation into animals, they may transform into malignant cells (potential tumorigenesis) (31); ii) MSCs have a large volume and potent adherent ability. The transplantation of MSCs via the tail vein usually has poor efficacy, since the majority of MSCs stay in the lung and only a few MSCs reach the injured site to exert therapeutic effects. Thus, it is difficult to optimize the number of MSCs and the frequency of transplantation; iii) although a few MSCs locate in the intestine, whether or not these cells can differentiate into intestinal epithelial cells for further repair of tissue is still unclear. These limitations in MSC transplantation may further limit the interpretation of our results.

Additional studies are needed to confirm our findings on the anti-inflammatory and immunomodulatory properties of MSCs. Our previous study (32) reported that MSCs can relieve injury of pancreatic acinar cells in a rat model of SAP, attenuate inflammation and injury in the epithelium of the small intestine, promote proliferation of the enteric epithelium and mucosal repair, thereby helping to maintain the integrity of the intestinal barrier function.

In conclusion, this study showed that SAP induces systemic inflammation in rats. Transplanted MSCs may migrate into the injured intestine, inhibiting the release of inflammatory mediators, increasing AQP-1 expression, reducing mucosal permeability of the intestine, promoting the recovery of intestinal epithelial cells and maintaining the integrity of the intestinal mucosal barrier. In the present study, transplanted MSCs inhibited systemic inflammation, reduced necrosis of intestinal epithelial cells and reduced TNF-α release in a rat model of SAP-induced intestinal injury, suggesting that MSCs exert a protective effect on the intestinal barrier during SAP. Our findings may provide evidence for the prevention of SAP-induced intestinal barrier dysfunction.

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References