Original Article
Human umbilical cord mesenchymal stem cells alleviate intestinal barrier injury in rats with severe acute pancreatitis

Dongye Wu, Guan Yang, Ying Gou, Heda Xiao, Weihui Liu, Hongyun Liang, Jing Zhou, Zhu Huang, Yaolei Zhang, Zhen Tan, Hongyu Sun, Lijun Tang

Abstract: Objective: To study the protective effects of human umbilical cord mesenchymal stem cells (ucMSCs) on intestinal barrier injury in rats with severe acute pancreatitis (SAP). Methods: Male Sprague-Dawley rats (n = 45) were randomly assigned to a SHAM group, SAP group and SAP+ucMSCs group (n = 15). The SAP group was subject to injection of 5% sodium taurocholate into the pancreatic duct. In the SAP+ucMSCs group, CM-Dil-labeled ucMSCs were intravenously injected into the rats with SAP. Twenty-four hours after the surgery, the samples of small intestine, blood, pancreas and mesenteric lymph nodes were collected for histological, bacteriological, serum and molecular analyses. Results: ucMSCs located in the injured region of the small intestine, and lowered the 24-h mortality rate of the SAP rats. ucMSC transplantation also significantly lowered the levels of the inflammatory factors tumor necrosis factor-α and interleukin-1β and of the pathological score of the small intestine. Importantly, ucMSC transplantation increased the expression of intestinal keratinocyte growth factor and improved the intestinal barrier function, as evidenced by the decrease in D-lactate, endotoxins and bacterial translocation and the increase in tight junction proteins zona occludens 1 and occludin expression. Conclusion: Human ucMSCs reduced intestinal barrier injury in rats with SAP, which may be developed as a therapeutic strategy for SAP.

Keywords: Human umbilical cord mesenchymal stem cells, severe acute pancreatitis, intestinal barrier injury, tight junction proteins

Introduction

Severe acute pancreatitis (SAP) is a sudden inflammation of the pancreas, which has severe complications and a high mortality rate [1, 2]. Being a natural barrier, the intestines can block intestinal bacteria and viruses, and are frequently destroyed during SAP. Once the intestinal barrier function (IBF) is interrupted, intestinal bacteria and endotoxin will migrate to the mesenteric lymph nodes (MLN) and/or distant organs, resulting in systemic inflammatory response syndrome and multiple organ dysfunction syndromes. This is the main reason that SAP patients have high mortality [3]. Currently, antibiotic application, early intra-intestinal nutrition and probiotic application are generally used to protect against the intestinal injury in SAP patients. However, their efficacy remains unsatisfactory. Therefore, there is an urgent need to develop effective strategies to prevent intestinal barrier injury caused by SAP.

Mesenchymal stem cells (MSCs) are adult multipotent stromal cells that can differentiate into specific cell types. Most importantly, increased evidences have shown that MSCs secrete cytokines that have immunomodulatory effects. Numerous studies have to date demonstrated the therapeutic potential of these cells in gastro-intestinal (GI) tract diseases. For example, bone marrow-derived MSCs have been applied...
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in radiation intestinal injury, ischemia/reperfusion injury, Crohn’s disease and ulcerative colitis, showing that MSCs have satisfactory protective effects [4-7]. However, bone marrow-derived MSCs have known disadvantages, including invasive sample collection, limited donor supply and reduced proliferation/differentiation capacity in aging donors [8, 9], which limit their clinical application. In contrast, human umbilical cord mesenchymal stem cells (ucMSCs) are considered an attractive alternative for clinical applications due to their ready collection, high proliferation and low immunogenicity. However, the therapeutic effects of ucMSCs on intestinal injury remain unclear.

Based on the above considerations, we systematically investigated the protective effects of ucMSCs on IBF in rats with SAP, which might provide an experimental basis for their clinical application.

Materials and methods

Experimental animals and reagents

Healthy adult male Sprague-Dawley (SD) rats weighing 250-300 g (n = 45) were obtained from Chengdu Dasuo Experimental Animal Co., Ltd. (Chengdu, China). Human ucMSCs (5th generation, lot number B06160356) were obtained from Chengdu Kangjing Biotechnology Co., Ltd. (Chengdu, China). Sodium taurocholate was purchased from Sigma (St. Louis, MO, USA). CM-Dil was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). ELISA kits for tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), keratinocyte growth factor (KGF), D-lactate and endotoxins were purchased from Shanghai Meilian Biotechnology Co., Ltd. (Shanghai, China). Luria-Bertani (LB) agar was purchased from Shanghai Haoran Biotechnology Co., Ltd. (Shanghai, China). Rabbit anti-mouse tight junction protein 1 (ZO-1) antibody, occludin antibody and goat anti-rabbit fluorescent antibodies were purchased from Abcam (Cambridge, UK).

Preparation of CM-Dil-labeled ucMSCs

A cell suspension (1 x 10⁶ cells/ml) was prepared with serum-free medium, and CM-Dil labeling solution was added at 5 μl/ml of medium. The cells were suspended 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 twice with phosphate-buffered saline (PBS).

Transplantation of CM-Dil-labeled ucMSCs

SD rats, deprived of food for 12 h but given access to water ad libitum, were randomly assigned to three groups: SHAM group (n = 15), SAP group (n = 15) and SAP+ucMSCs group (n = 15). Following intraperitoneal anesthesia with 4% chloral hydrate at 1.0 ml/100 g, the SHAM group rats received a laparotomy, and the pancreas was turned several times, followed by wound closure, and a tail intravenous (i.v.) injection of 1 ml of 0.9% saline was performed. The SAP group experiments followed the Aho protocol [10]. A micro-infusion pump was used for the injection of 5% sodium taurocholate (0.1 ml/100 g) into the pancreatic duct at 12 ml/h. The pressure was maintained for 10 min before abdominal closure. For the SAP+ucMSCs group, the SAP model was first created, and then a tail i.v. injection of CM-Dil-labeled ucMSCs (1 x 10⁷ cells/kg) was performed. All of the procedures were performed under sterile conditions following the surgery guidelines. The rats were fasted after surgery, but were allowed free access to water.

Sample collection

All of the rats were subject to anesthesia 24 h after the surgery, followed by open abdominal surgery for blood sample collection. Blood samples were centrifuged and the serum was stored at -80°C until further use. MLN and pancreatic tissues were collected under asptic conditions, then they were homogenized in sterile saline (10 ml/g) for further use. The terminal ileum (approximately 5 cm) was collected and divided into three parts. One part was subject to Tissue-Tek OCT Compound and stored at -80°C until further use, one was fixed in 4% paraformaldehyde (PFA) for at least 24 h for further use, and one was stored at -80°C until further use.

ucMSC colonization in the small intestine

Frozen small-intestine tissues from the SAP+ucMSCs group that were subject to OCT embedding solution were sectioned into slices (10 mm thick). The colonization of the ucMSCs in the small intestines was observed under a fluorescent microscope.
Pathology

The small-intestine samples that were fixed in 4% PFM were dehydrated according to a routine procedure, followed by paraffin embedding, sectioning and H&E staining. The sealed sections were observed under a light microscope. One pathologist blindly examined the sections and scored them according to the Chiu guidelines [11]. The mean score across 10 fields was used as the final score.

Detection of tight junction proteins by Immunofluorescence

Intestinal tissue samples were lysed in buffer (50 mmol/l Tris-HCl, pH 8.0, 100 μg/ml phenylmethylsulfonyl fluoride, 1% Triton X-100, 150 mmol/l NaCl) for 30 min on ice. Then, 50 μg of protein samples were boiled for 5 min in sample buffer, separated by 10% or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Nonspecific reactivity was blocked by 5% non-fat dry milk in TBST (10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 0.05% Tween 20) for 1 h at room temperature. The membranes were then incubated with rabbit anti-rat polyclonal ZO-1 and occludin antibodies (1:500) at 4°C overnight. The membranes were washed three times in TBST and then incubated with the required secondary antibody HRP-conjugated goat anti-rabbit IgG (1:2000) for 30 min. The protein expression was detected by chemiluminescence system.

Western blotting

Intestinal tissue samples were lysed in buffer (50 mmol/l Tris-HCl, pH 8.0, 100 μg/ml phenylmethylsulfonyl fluoride, 1% Triton X-100, 150 mmol/l NaCl) for 30 min on ice. Then, 50 μg of protein samples were boiled for 5 min in sample buffer, separated by 10% or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Nonspecific reactivity was blocked by 5% non-fat dry milk in TBST (10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 0.05% Tween 20) for 1 h at room temperature. The membranes were then incubated with rabbit anti-rat polyclonal ZO-1 and occludin antibodies (1:500) at 4°C overnight. The membranes were washed three times in TBST and then incubated with the required secondary antibody HRP-conjugated goat anti-rabbit IgG (1:2000) for 30 min. The protein expression was detected by chemiluminescence system.

Determination of TNF-α, IL-1β, KGF and D-lactate

An automatic ELISA machine was used and the procedures followed the enzyme linked immunosorbent assay (ELISA) kit instructions.

Endotoxin determination

Serum samples (100 μl) were mixed well with TAL solution (100 μl), followed by incubation at 37°C for 10 min. Development solution (100 μl) was added and mixed, followed by incubation at 37°C for 10 min. Finally, nitride solution (500 μl) was added and mixed. The samples were allowed to settle for 5 min and a spectrometer was used for detection.
Bacterial migration

The homogenate samples of MLN and pancreas (1 ml) were well distributed on LB agar plates. LB agar plates were incubated in the aerobic chamber at 37°C for 24 h. Finally, bacterial colony counts were counted. The bacterial translocation rate (BTR) was calculated as follows: \( \text{BTR} = \frac{\text{the number of bacterial positive sample}}{\text{total number of samples}} \) [12].

Statistical analysis

Statistical software SPSS 13.0 was used. Data are expressed as mean ± standard deviation (X ± s) and were compared using a one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison tests. Data with an asymmetrical distribution are presented as median values and the range, and Kruskal-Wallis H tests followed by Mann-Whitney U tests with Bonferroni corrections were performed. \( P < 0.05 \) was considered statistically significant.

Results

ucMSCs colonization in the small intestine

CM-Dil was used to label ucMSCs and an inverted phase contrast microscope was used to observe cell morphology and labeling. The CM-Dil-labeled ucMSCs were red with high fluorescence intensities; however, the nuclear region did not show any fluorescence (Figure 1A). CM-Dil-labeled ucMSCs were intravenously administrated to rats with SAP, and after 24 h, injected cells were observed to be distributed in the small intestine (Figure 1B).

ucMSCs decreased the mortality rate of SAP rats

The mortality rates at 24 h in the SHAM, SAP and SAP+ucMSCs groups were 0/15 (0%), 8/15 (53.3%) and 3/15 (20%), respectively. There was a significantly lower mortality rate in the SAP+ucMSCs group than in the SAP group (\( P < 0.05 \), Figure 2), indicating that ucMSC transplantation could lower the mortality rate of SAP rats.

ucMSCs decreased the pathological score of the small intestine

The pathological analysis showed that the SHAM group had normal ileum mucosa with an intact and continuous villi structure, and mesenchymal tissues did not reveal edema or inflammatory infiltration. In the SAP group, villi defects/reduced numbers, edema and inflammatory infiltration were observed in the intestinal tissues. However, these parameters were significantly improved in the SAP+ucMSCs group compared with the SAP group (Figure 3A). Additionally, the pathology score of the SAP+ucMSCs group were significantly lower compared with that of the SAP group (Figure 3B).

ucMSCs decreased the levels of TNF-α and IL-1β and increased the KGF content in the intestinal mucosa

As shown in Table 1, the SAP group had significantly higher levels of TNF-α and IL-1β compared with the SHAM group (\( P < 0.05 \)). Interestingly, compared with the SAP group, the SAP+ucMSCs group had significantly lower levels of TNF-α and IL-1β (\( P < 0.05 \)), indicating that...
ucMSCs alleviate intestinal barrier injury in rats with SAP

Table 1. TNF-α, IL-1β and KGF levels in the small intestine determined by ELISA

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/g)</th>
<th>IL-1β (pg/g)</th>
<th>KGF (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>63.25±14.61</td>
<td>57.38±12.37</td>
<td>89.71±13.06</td>
</tr>
<tr>
<td>SAP</td>
<td>350.27±19.47</td>
<td>248.72±36.19</td>
<td>143.63±23.71</td>
</tr>
<tr>
<td>SAP+ucMSCs</td>
<td>225.71±23.19*</td>
<td>160.53±25.83*</td>
<td>175.92±34.19*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. SHAM group; *P < 0.05 vs. SAP group.

Figure 4. ucMSCs decreased the levels of D-lactate and endotoxins. A. D-lactate levels in the peripheral blood. B. Endotoxin levels in the peripheral blood. *P < 0.05 vs. SHAM group, *P < 0.05 vs. SAP group.

Table 2. Bacterial translocation of MLN and pancreas

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>MLN n</th>
<th>%</th>
<th>Pancreas n</th>
<th>%</th>
<th>BTR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SAP</td>
<td>7</td>
<td>5</td>
<td>71.4</td>
<td>4</td>
<td>57.1</td>
<td>64.3*</td>
</tr>
<tr>
<td>SAP+ucMSCs</td>
<td>12</td>
<td>5</td>
<td>41.7</td>
<td>3</td>
<td>25.0</td>
<td>33.3*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. SHAM group; *P < 0.05 vs. SAP group. BTR: bacterial translocation rate.

ucMSC transplantation decreased the inflammatory response to SAP.

As shown in Table 1, the SAP group had a significantly higher level of KGF compared with the SHAM group (P < 0.05). Furthermore, the SAP+ucMSCs group had a significantly higher level of KGF compared with the SAP group (P < 0.05), indicating that ucMSC transplantation upregulated KGF expression of intestinal tissue in rats with SAP.

ucMSCs decreased the levels of D-lactate, endotoxins and bacterial migration

The serum D-lactate and endotoxin levels in the rat pancreatitis model 24 h after its creation were significantly higher compared with those in the control group (Figure 4A and 4B). Twenty-four hours after ucMSC transplantation, the serum D-lactate and endotoxin levels were significantly lower compared with those in the SAP group.

Immunofluorescent staining was used to observe the distribution of ZO-1 and occludin in the intestinal tissue. We found that in the SHAM group, the ileum end of the intestine had a high level of the junction protein, occludin, which was ubiquitously distributed (Figure 5A). In contrast, in the SAP group, the level of occludin protein was low and the distribution was non-ubiquitous. In some regions, the protein was absent. In the SAP+ucMSCs group, the distribution of occludin was ubiquitous with strong signals, and there was a greater increase when compared to that in the SAP group (Figure 5A).

Western blotting was further used to examine the expression of ZO-1 and occludin at the end of the ileum. Compared with the SHAM group, the SAP and SAP+ucMSCs groups had significantly lower expression of ZO-1 and occludin (Figure 5B, P < 0.05). Injection of ucMSCs increased the expression of ZO-1 and occludin compared with the SAP group (P < 0.05, Figure 5B).

Discussion

Currently, there is no ideal treatment strategy for SAP-related intestinal barrier dysfunction. In this study, we systematically evaluated the effect of ucMSCs on intestinal injury in rats with SAP. ucMSCs significantly reduced intestinal injury and the levels of inflammatory factors. Importantly, we found that ucMSCs could reduce the permeability of the intestinal mucosa.
ucMSCs alleviate intestinal barrier injury in rats with SAP

Bone marrow-derived MSCs have been widely used in various GI tract diseases, including radiation intestinal injury, ischemia/reperfusion injury, Crohn's disease and ulcerative colitis. These studies have shown that MSCs have protective effects. However, the limited donor supply restricts the further clinical application of bone marrow-derived MSCs. In the present study, we selected human ucMSCs, which have a wide range of sources, are easy to obtain and do not have ethical restrictions, as seed cells to evaluate their effects on intestinal injury in SAP rats. The results showed that ucMSCs significantly reduced the secretion of intestinal tissue injury and inflammatory factors, decreased the levels of D-lactate and endotoxin and the bacterial migration, and upregulated the expression of the tight junction proteins ZO-1 and occludin. These results showed that ucMSCs alleviated intestinal injury in SAP and protected the IBF.

To repair damaged tissues, ucMSCs need to reach the sites of tissue injury. Jiang H et al. [13] have created an ischemia-reperfusion injury model and found that MSCs were able to colonize in the damaged intestine. Consistent with this, Yabana et al. [14] have established a colitis model and found that MSCs were able to colonize in the damaged intestine. In the present study, we applied CM-Dil-labeled ucMSCs to SAP rats and detected cells with red fluorescence 24 h later, confirming the intestinal colonization of ucMSCs, which is consistent with previous reports.

Figure 5. ucMSCs could upregulate the expression of ZO-1 and occludin. (A) Immunofluorescence of ZO-1 and occludin in intestinal epithelium. In the SAP+ucMSCs group, the distribution of occludin was ubiquitous with strong signals, and there was a greater increase when compared to that in the SAP group. (B) Western blotting (B1) showing the expression of ZO-1 and occludin in the different groups; (B2) the normalized expression of ZO-1 and occludin (*P < 0.05 vs. SHAM group, and #P < 0.05 vs. SAP group).
ological conditions. During intestinal barrier dysfunction, large amounts of D-lactate, endotoxins and intestinal bacteria will enter the blood via the intestinal mucosa and cause a 'second attack', resulting in a secondary pancreatic infection and a cascade of inflammatory responses [3, 15]. In the present study, we showed that ucMSCs effectively decreased the levels of D-lactate and endotoxins as well as the bacterial migration. Increased permeability of intestinal mucosa is attributed to the increased D-lactate, endotoxins and bacterial translocation. The integrity of the tight junctions among intestinal epithelial cells has direct effects on intestinal mucosal permeability. Tight junctions are located at the apical end of the cells, consisting of the transmembrane proteins, occludin, claudins and junctional adhesion molecule (JAM), and cytoplasmic proteins, including ZO-1, ZO-2, ZO-3 and cingulin. During SAP, many factors contribute to the malfunction of intestinal epithelial tight junctions and decrease protein expression, which causes increased epithelial permeability and further intestinal barrier dysfunction (IBD). Among these proteins, ZO-1 and occludin have been extensively studied due to their apparent function. Therefore, we determined the expression of ZO-1 and occludin and found this to be upregulated by ucMSCs, which alleviated the intestinal damage in the SAP rats. In addition, our results were also in agreement with the findings of Zhang et al. [16] and Yabana et al. [14]. Zhang et al. used a rat model of heterotopic intestinal transplantation and confirmed that MSCs increased the expression of ZO-1 and occludin. Yabana et al. used a colitis model and found that MSCs increased the expression of ZO-1 and claudin-2, -7, -8, -12, -13 and -15.

Several studies have reported that TNF-α and IL-1β play important roles in SAP-related IBD via various direct/indirect pathways, disrupting the normal barrier functions [17, 18]. Many studies have confirmed that TNF-α and IL-1β lower the expression of ZO-1 and occludin [19-21]. Our experimental results also showed that ucMSCs significantly decreased the levels of intestinal TNF-α and IL-1β in the SAP rats.

KGF, a paracrine factor produced by the digestive tract, has been shown to promote the proliferation and differentiation of the epithelial cells in intestinal mucosa, thus promoting the repair of intestinal injury [22, 23]. MSCs can also secrete KGF [24, 25]. In our study, we found that the expression of intestinal KGF was significantly higher in the ucMSCs+SAP group than in the SAP group. Whether the increased level of KGF comes from intestinal cells/or ucMSCs needs further investigation.

In summary, we demonstrated that ucMSCs could protect rats against intestinal mucosal barrier dysfunction during SAP. The specific mechanism may be attributed to the immunosuppressive regulation of ucMSCs. Further studies are required to determine the exact mechanism underlying the therapeutic efficacy of stem cells.

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Disclosure of conflict of interest

None.

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