Original Article

Therapeutic effect of human umbilical cord-derived mesenchymal stem cells in rat severe acute pancreatitis

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Received September 29, 2013; Accepted October 28, 2013; Epub November 15, 2013; Published December 1, 2013

Abstract: Aim: To investigate the therapeutic effect of umbilical cord-derived mesenchymal stem cells (UC-MSCs) on rat severe acute pancreatitis (SAP). Methods: Rats were randomly divided into three groups (n = 15 per group): control group, SAP group, and SAP+MSCs group. SAP was established by retrograde pancreatic duct injection of 3% sodium taurocholate. In SAP+MSCs group, UC-MSCs at 1×10^7 cells/kg were injected via the tail vein 12 h after SAP. Rats (n = 5 per group) were sacrificed on days 1, 3 and 5, and the blood and pancreatic tissues were collected. The levels of serum amylase, lipase, inflammatory cytokines, and anti-inflammatory cytokines were determined. Pathological changes of the pancreas (HE staining) and apoptotic acinar cells (TUNEL staining) were observed under light microscope. Results: The levels of serum amylase and lipase in SAP group were significantly higher than those in control group (P<0.05). The pancreas in SAP group showed significantly massive edema, inflammation, hemorrhage and necrosis when compared with control group. There were numerous TUNEL-positive apoptotic acinar cells after SAP. However, in SAP+MSCs group, the levels of serum amylase were significantly reduced on days 1, 3, and 5 after MSC transplantation (P<0.01). The serum lipase level in SAP+MSCs group was significantly lower than that in SAP group on days 3 and 5 (P<0.01). The edema formation, inflammatory cell infiltration, hemorrhage, and necrosis were reduced significantly attenuated in SAP+MSCs group as compared to SAP group (P<0.05). MSCs significantly reduced the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), but increased the levels of anti-inflammatory cytokines (IL-4 and IL-10) in SAP rats. The number of TUNEL-positive acinar cells was significantly reduced on days 3 and 5 after MSCs transplantation (P<0.01). Conclusion: Transplantation of UC-MSCs significantly inhibits inflammation and decreases pancreatic injury secondary to SAP.

Keywords: Umbilical cord-derived mesenchymal stem cells, severe acute pancreatitis, pancreatic pathology, inflammation, apoptosis

Introduction

Acute pancreatitis (AP) is a clinically common disease seriously affecting human health and threatening life of patients. About 15-20% of patients with AP may develop severe acute pancreatitis (SAP) which is an acute abdominal disease with rapid progression, multiple complications and high mortality [1-3]. Among SAP patients with multiple organ dysfunction syndrome (MODS) and infectious pancreatic necrosis, the overall mortality is as high as 47%. With the improvement of pharmacotherapy and surgical interventions, the overall mortality reduces to about 17%. However, there are no effective strategies for the treatment of SAP currently. Thus, it is imperative to develop ideal therapeutic strategy to reduce the complications and mortality of SAP.

Mesenchymal stem cells (MSCs) belong to adult stem cells with self-renewal and multilineage differentiation potentials. It has been confirmed that MSCs may exert anti-inflammatory, immunoregulatory and repairing effects in animal models of lung injury, acute kidney failure, acute myocardial infarction, Crohn’s disease and diabetes [4-8]. MSCs were first identified in the bone marrow [9, 10]. However, collection of bone marrow is invasive and the amount of MSCs reduces over age, which significantly limits the clinical application of MSCs [11-13]. In
UC-MSCs for treatment of severe acute pancreatitis

Table 1. Criteria for pathological scoring of the pancreas

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<th>Pathological findings</th>
<th>Scoring</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>Edema</td>
<td>No</td>
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<tr>
<td>Infiltration of inflammatory cells</td>
<td>0-1 white blood cells/HPF</td>
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<tr>
<td>Bleeding</td>
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<td>Necrosis</td>
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2003, the umbilical cord blood was found to be rich in MSCs which have surface markers and induced differentiation as in MSCs from the bone marrow. Thus, umbilical cord blood derived MSCs have been widely applied in studies [14-16]. In the present study, SAP was established in rats by retrograde pancreatic duct injection of sodium taurocholate, which has similar pathogenesis of human SAP [17, 18]. Then, transplantation of umbilical cord blood derived MSCs was performed in these rats aiming to investigate the therapeutic efficacy of umbilical cord blood derived MSCs in SAP. Our findings may provide experimental evidence for the clinical application of stem cells.

Materials and methods

Materials

The umbilical cord was collected from the Department of Obstetrics of Shanghai 10th People’s Hospital (2012-07/2012-11) and MSCs were separated. DMEM-LG, fetal bovine serum, penicillin, streptomycin, trypsin (Gibco, USA), antibodies for flow cytometry (BD Biosciences, USA), medium for differentiation induction (Cyagen Biosciences, USA), SPF male SD rats (180-220 g; 6-8 weeks) (Shanghai SLAC Animal Co., Ltd), sodium pentobarbital (Beijing Dingguochangsheng Biotech Co., Ltd), instruments for establishment of animal model (Shanghai Yuanchang Medical Devices Co., Ltd.), serum amylase and lipase detection kits (BioVision, USA), ELISA kit for serum inflammatory cytokines (IL-1β, IL-6, TNF-α) and pro-inflammatory cytokines (IL-4, IL-10) (R&D, USA) and TUNEL detection kit (Roche, Swiss) were used in the present study.

Cell culture

Umbilical cord (1 mm³) was cut into pieces which were maintained in medium followed by collection of primary MSCs. These MSCs were maintained in DMEM-LG containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humified environment with 5% CO₂ for 3 weeks. When the cell confluence reached about 80%, cells were digested with 0.25% trypsin and passaging was done at a ratio of 1:3. Cells of the third generation were subjected to flow cytometry for identification and used for differentiation induction and transplantation.

Preparation of rat SAP model and cell transplantation

Before surgery, rats were fasted for 12 h but given ad libitum access to water. Rats were randomly assigned into 3 groups (n = 15 per group) and anesthesia was done by intraperitoneal injection of 3% sodium pentobarbital. Normal control group: animals were anesthetized for 2 h and laparotomy was not performed. SAP group: A midline incision was made at the abdomen (2 cm in length) and laparotomy was performed. The duodenum was identified along the pylorus and then taken out of the abdominal cavity. The pancreas was identified and the duodenal papilla, common bile duct and pancreatic duct were located. A 9/0 nylon thread was placed at the shared outlet of common bile duct and pancreatic duct at the head of pancreas. A small clamp was used to clamp the common bile duct. Then, a needle was inserted via the duodenal papilla into the middle common bile duct. Once the head of the needle was located, ligation was done with the pre-existing thread. Then, 3% sodium taurocholate (1 ml/kg) was slowly injected at a speed of 5 min/ml. Focal bleeding and diffuse edema were observed in the pancreas. The needle was withdrawn, and the thread and the clamp were removed. When there was no active bleeding in
Figure 1. Identification of umbilical cord derived MSCs. A: Morphology of MSCs (×40); B: Proliferation of MSCs; C: Surface markers of MSCs; D: Osteogenesis (×100), adipogenesis (×200) and chondrogenesis (×40) of MSCs.
the pancreas and duodenum, the viscera were placed back into the abdominal cavity followed by wound closure. In SAP+MSC group, MSCs ($1 \times 10^7$/ml) were injected via the tail vein at $1 \times 10^7$/kg at 12 h after surgery.

**Morphological examination of the pancreas**

At 1, 3 and 5 days after surgery, rats (n = 5 per group) were anesthetized, and 5 ml of blood was collected from the heart and anti-coagulated. The pancreas was harvested, fixed in 4% paraformaldehyde over night and embedded in paraffin. The paraffin-embedded tissues were cut into sections (4 µm) followed by HE staining and observation under a light microscope. Ten fields were randomly selected, and pathological scoring was done according to the pathological criteria for pancreatitis (Table 1).

**Detection of serum amylase and lipase**

The anti-coagulated blood was centrifuged at 250 g for 25 min at 4 °C, and the serum was collected. Then, 5 µl of serum was added into 96-well plates and distilled water was added with the final volume of 50 µl in each well. Subsequently, detection buffer (50 µl) and substrate solution (50 µl) were independently added to each well, and the absorbance (OD) was measured at 405 nm immediately ($T_0$). Then, OD was also measured at different time points ($T_1$). The serum amylase and lipase levels were determined according to the standard curves. Serum amylase/lipase level = $B / [V \times (T_1 - T_0)]$ mU/ml, where $B$ is the nitrophenol concentration (nmol) in the standard curve when the $OD_{405}$ was measured at $T_0$; $V$ is the volume of samples (5 µl); $T_1 - T_0$ refers to the time from immediately after addition of substrate to a specific time point.

**Detection of serum pro-inflammatory cytokines and anti-inflammatory cytokines**

The ELISA kits (R&D, USA) were used to detect the content of IL-1β, IL-6, IL-4, IL-10 and TNF-α.
Detection of acinar cell apoptosis

The paraffin embedded sections were deparaffinized and dehydrated, and then treated with protease K at 20 µg/ml for 20 min to increase the tissue permeability. After washing in PBS twice, 100 µL of balance buffer was added to each section followed by incubated at room temperature for 5-10 min. Then, the biotin conjugated nucleoside mixture buffer (100 µl) and Terminal Deoxynucleotidy1 Transferase (100 µl) were added to each section followed by incubation at 37 °C for 60 min. The sections were subsequently immersed in SSC solution for 15 min to stop reaction. After washing in PBS thrice, sections were washed in 0.3% hydrogen peroxide solution for 5 min to inhibit the endogenous catalase. Following washing in PBS thrice, 100 µl of horseradish peroxidase solution was added, followed incubation for 30 min. After washing in PBS thrice, visualization was done with DAB followed by mounting. These sections were observed done under light microscope. In detection of acinar cell apoptosis, 5 fields were randomly selected.

in the serum according to manufacturer's instruction.
from each section at a high magnification (×400), and the apoptotic cells were counted.

Statistical analysis

Statistical analysis was done with SPSS version 14.0. Data were expressed as mean ± standard deviation (SD). Comparisons were done with one way analysis of variance and a value of P<0.05 was considered statistically significant.

Results

Culture and identification of MSCs

MSCs were long spindle-shaped (Figure 1A) and had potent proliferation activity. Flow cytometry showed umbilical cord derived MSCs had no expressions of CD45, CD34, CD11b, CD19 and HLA-DR (0.61%) but high expressions of CD44 (99.99%), CD73 (99.98%), CD90 (99.99%) and CD105 (99.97%) (Figure 1C). Experiment of induction differentiation showed the MSCs had the capabilities of osteogenesis, adipogenesis, chondrogenesis (Figure 1D). These findings suggest these cells are MSCs.

Pathological examination of pancreas after MSC transplantation

At different time points, there were no edema, infiltration of inflammatory cells, bleeding and necrosis in the pancreatic lobules in control group. In SAP group, the pancreatic edema was observed immediately after surgery. One day after surgery, evident expansion of alveolar septum, parenchymal bleeding and infiltration of inflammatory cells were noted. Three days after surgery, the pancreatic parenchymal necrosis (focal patchy necrosis) deteriorated. Five days after surgery, the necrotic area merged, and tubular complexes were observed. In SAP+MSCs group, pancreatic edema, bleeding, necrosis and infiltration were also noted, but these pathological changes were milder than in SAP group. Moreover, the pathological changes in SAP+MSCs group improved over time, and a small amount of fibrous tissues were observed (Figure 2). On the basis of pancreatic parenchymal necrosis, pathological scoring was performed (Figure 3). Results showed the pathological scores of pancreatic edema, pancreatic parenchymal bleeding and necrosis and infiltration of inflammatory cells in SAP group were markedly higher than those in control group (P<0.01). In SAP+MSCs group, the pancreatic scores were significantly reduced when compared with SAP group (scores of edema and bleeding on days 1, 3 and 5: P<0.01; scores of inflammatory cell infiltration and acinar cell necrosis on day 1: P<0.05; scores of inflammatory cell infiltration and acinar cell necrosis on days 3 and 5: P<0.01).

Serum amylase and lipase levels after MSCs transplantation

On day 1, the serum amylase level increased markedly (2100±245 mU/ml), and further elevated on days 3 and 5 (2380±194 mU/ml and 2920±231.5 mU/ml) when compared with control group (P<0.01). This, together with pathological findings, suggests the SAP animal model
was stable and reliable. After MSC transplantation, the serum amylase level reduced to 1080±172 mU/ml, 1020±283.5 mU/ml and 1180±278.6 mU/ml on days 1, 3 and 5, respectively, which were significantly lower than those in SAP group (P<0.01; Figure 4A). In SAP group, the serum lipase level increased slightly on day 1 and was 3 folds higher than that in control group since day 3 (day 3: 4010±357.7 mU/ml; day 5: 4560±441 mU/ml; P<0.01). One day after transplantation, the serum lipase level remained unchanged (P>0.05), but reduced markedly since day 3 (day 3: 1880±204 mU/ml; day 5: 2160±387.8 mU/ml) when compared with SAP group (P<0.01; Figure 4B).

**Serum levels of inflammatory cytokines after MSC transplantation**

When compared with SAP group, the serum TNF-α content reduced in SAP+MSCs group, and significant difference was noted since day 3 day 3: P<0.05; day 5: P<0.01). In SAP+MSCs group, the serum IL-1β and IL-6 contents were markedly lower than those in SAP group on day 1 (P<0.05), and they further reduced over time (day 5: P<0.01). In addition, MSC transplantation significantly increased the serum IL-4 and IL-10 contents which were 0.0812±0.0153 ng/
ml and 0.306±0.052 ng/ml on day 3 and 5, respectively and significantly higher than those in SAP group (P<0.01) (Figure 5).

Apoptosis of pancreatic acinar cells after MSCs transplantation

TUNEL staining showed there was almost no apoptotic cells in control group (Figure 6G-I). In SAP group, massive apoptotic cells were observed in the pancreas, and the number of apoptotic cells increased over time (Figure 6A-C). After MSCs transplantation, the number of apoptotic acinar cells reduced since day 3, and these apoptotic cells were scattered (Figure 6D-F). In SAP+MSCs group, the number of apoptotic cells was markedly lower than that in SAP group on days 3 and 5 (P<0.01, Figure 7).

Discussion

AP is one of common acute abdominal diseases and characterized the self-digestion, edema, bleeding and necrosis (features of inflammation) due to the activation of trypsin in the pancreas. The incidence of AP increases with the elevation of living standard [19]. Less than 20% of patients with AP may develop SAP which has a lot of complications and high mortality. Thus, SAP has been a dangerous and refractory acute abdominal disease [1]. Currently, SAP is managed with traditional supportive therapy or surgical interventions, and no effective strategy has been developed for the treatment of SAP to date. Cell therapy with MSCs has been found to promote the repairing of acute and chronic injury, regulate immune function and attenuate inflammatory response [8]. It has been confirmed that MSCs can improve the inflammatory response in the liver, kidney and lung, and have favorable promise in clinical application in myocardial infarction, Crohn’s disease and organ transplantation. Studies have shown that multiple inflammatory cytokines are involved in the pathogenesis of SAP including pro-inflammatory cytokines (IL-1, IL-6, TNF-α) and anti-inflammatory cytokines (IL-4, IL-10) [20, 21]. Thus, MSCs have the potential for the treatment of pancreatitis due to the anti-inflammatory effect of MSCs. In addition, MSCs can be induced to differentiate into different cell types including insulin secreting cells and endothelial cells [22, 23]. Thus, it is feasible to promote pancreatic regeneration and repairing of microvascular endothelium of the pancreas with MSCs.

UC-MSCs for treatment of severe acute pancreatitis

Retrograde cholangiopancreatic injection of sodium taurocholate was employed to induce acute obstructive pancreatitis which has similarity in the pathogenesis of clinical pancreatitis. This animal model is easy to establish and feasible. The pathological examination and pancreatic enzyme detection showed this animal model was stable. The main pathological findings were pancreatic edema, infiltration of massive inflammatory cells, pancreatic parenchymal bleeding and acinar cell necrosis. The umbilical cord derived MSCs were injected into SAP rats via the tail vein, and results showed the serum levels of amylase and lipase reduced, pancreatic pathology and acinar cell apoptosis improved, serum levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) reduced, and those of anti-inflammatory cytokines increased (IL-4, IL-10). These findings suggest that MSCs have favorable therapeutic effects on SAP. When compared with SAP group, MSCs transplantation significantly reduced serum levels of amylase and lipase (amylase: 48% on day 1; 55% on day 3; 60% on day 5; lipase: 20% on day 1, 50% on day 3, 53% on day 5), suggesting that MSCs transplantation may protect the structural integrity of acinar cells. MSCs transplantation facilitates the recovery of pancreatic pathology which was characterized by improved pancreatic edema, reduction of inflammatory cells and attenuation of pancreatic parenchymal bleeding and necrosis. However, the specific mechanisms are still poorly understood. Previous studies have shown that bone marrow derived MSCs can migrate into the injured pancreas and differentiate into pancreatic cells to repair the injured pancreas. In the SAP, more MSCs migrate into the pancreas. This suggests that the homing ability of MSCs is related to the severity of injury [24]. However, in recent years, MSCs are found to secret some cytokines (IL-10, TGF-β, IL-1Reg and HGF) in a paracrine dependent manner to exert the anti-inflammatory effect [25]. More recent studies reveal that the TNF-α and IL-1 may activate MSCs which then secret anti-inflammatory protein TSG-6, which is found to be involved in the anti-inflammatory effect of MSCs [26-28]. Our results showed MSCs transplantation reduced the serum levels of pro-inflammatory cytokines and increased those of anti-inflammatory cytokines, which was consistent with the anti-inflammatory effect of TSG-6. Thus, the therapeutic effect of MSCs transplantation might be associated with the TSG-6 secreted by MSCs.
Pancreatic acinar cell apoptosis play an important role in the pathogenesis of SAP. The initiation of pancreatic acinar cell apoptosis is regarded as a protective response to inflammation related stimulation in cells [29]. The apoptosis and necrosis of pancreatic acinar cells have reciprocal transformation. Thus, to reduce the apoptotic acinar cells may also attenuate the necrosis of acinar cells to a certain extent [30]. In the present study, a lot of apoptotic acinar cells were observed in the pancreas of SAP rats, and the number of apoptotic acinar cells increased over time. However, in SAP+MSCs group, the apoptotic acinar cells reduced significantly when compared with SAP. This suggests that MSCs transplantation attenuate the pancreatitis and inhibit the apoptotic initiation, which prevent the further attack on acinar cells and improve the acinar cell necrosis.

In the present study, umbilical cord derived MSCs are transplanted into rats with SAP. Results demonstrate that MSCs transplantation may protect the structural integration of acinar cells, improve the pancreatic pathology, regulate inflammatory response and attenuate acinar cell apoptosis. The specific mechanism underlying the therapeutic effect of MSCs transplantation may be attributed to the homing and transdifferentiation of MSCs or the paracrine effect of MSCs. However, more studies are required to investigate the exact mechanism underlying the therapeutic efficacy of stem cells, which may provide evidence for a novel strategy for the treatment of SAP.

Acknowledgements

The study was supported by National Natural Science Foundation of China (No. 81170436); and Foundation of Shanghai Municipal Health Bureau (No. 2010015). We thank Fei Yin and Qianglin Duan for their kind help.

Disclosure of conflict of interest

None.

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