Therapeutic Efficacy of Spleen-Derived Mesenchymal Stem Cells in Mice with Acute Pancreatitis

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Abstract

**Background:** Acute pancreatitis (AP), a sudden inflammation of the pancreas, can cause severe complications and high mortality despite treatment. The use of mesenchymal stem cells (MSCs) for the treatment of AP has attracted significant attention in novel treatment strategy; however, the mode of action of spleen-derived MSCs (sp-MSCs) in AP remains unknown.

**Method:** MSCs isolated from mouse spleen (msp-MSCs) were used to investigate the effects in animal models of cerulein-induced acute pancreatitis (CAP) and pancreatic ischemia injury (PII).

**Results:** Msp-MSCs had multipotent differentiation capacities and immunoregulatory functions. A greater number of Qtracker-labeled msp-MSCs were detected in pancreas of mice with CAP than of control mice. Infused msp-MSCs reduced serum levels of amylase, lipase, and myeloperoxidase, and pancreatic edema, necrosis level, expression of inflammation cytokines, and CD3+ T cell infiltration. In PII model, infused msp-MSCs promoted cell growth and thus improved pancreatic dysfunction.

**Conclusion:** Msp-MSCs exert protective effects on CAP- and PII-induced pancreatic injury and might be developed as a potential therapeutic agent for pancreatitis treatment.

Keywords: Acute pancreatitis; Inflammation; Spleen-derived mesenchymal stem cells; Stem cell therapy

Abbreviations: AP: Acute Pancreatitis; hbm-MSCs: Human bone marrow-derived Mesenchymal Stem Cells; hsp-MSCs: human spleen-derived Mesenchymal Stem Cells; CAP: Cerulein-induced Acute Pancreatitis; PII: Pancreatic Ischemia Injury; MPO: Myeloperoxidase; IPGT: Intraperitoneal Glucose Tolerance Test

Introduction

Acute pancreatitis (AP) is one of the most common gastrointestinal disorders worldwide, with a reported annual incidence of 13 to 45 cases per 100000 persons [1]. About 20% of patients with AP have severe disease, and the mortality rate for those cases is approximately 30% when they progress to multisystem organ failure [2]. Moreover, an increase in the annual incidence for AP has been observed in most recent studies [3] and is associated with increased risk of pancreatic cancer [4]. Despite improvements in intensive care treatment during the past few decades, the rate of death from AP has not significantly declined [5].

AP, an inflammatory disorder of the pancreas, was characterized by activation of pancreatic digestive enzyme production, widespread inflammatory cell infiltration, leukocyte activation, and release of various pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin (IL) [6,7]. The progression of AP can be viewed as a three-phase continuum: local inflammation of the pancreas, a generalized inflammatory response, and the final stage of multi-organ dysfunction [8]. Experimental studies suggest that the prognosis for AP depends upon the degree of pancreatic necrosis and the intensity of multisystem organ failure generated by the systemic inflammatory response [2]. Therefore, the new therapeutic approach to arrest the disease process would be greatly valuable.

Mesenchymal stem cells (MSCs) have been shown to involve replacement, repair or enhancement of the biological function of a damaged organ or tissue and appeared as a potent therapeutic strategy for many diseases including AP [9,10]. MSCs from bone marrow, skin, vascular and adipose tissues are multipotent and differentiate into a range of cell types [11,12]. Recently, MSCs isolated from spleen were similar to those derived from bone marrow in that they comparable levels of the cell-surface markers CD90, CD105, CD166, and HLA class I, were negative for CD34, CD45, HLA class II, CD80, and CD86 expression, and were capable of adipogenic, osteogenic, chondrogenic, and neural-like differentiation [13,14]. T-cell anti-proliferative effects of bone marrow derived-(bm-MSCs) and spleen-derived MSCs were also comparable [13,15]. *In vitro*, both human (hsp-MSCs) and mouse spleen-derived MSCs (msp-MSCs) exhibited immunophenotypic characteristics and differentiation potential completely comparable to bm-MSCs [13,16]. Not only bm-MSCs but also msp-MSCs contributed efficiently to cellular treatment of a murine STZ-induced experimental diabetes model [15]. It has been suggested that bm-MSCs have therapeutic effects on AP; however, the effect of msp-MSCs on treatment of AP in mice is not investigated yet. Studies suggest the spleen of not only animals but also of normal
human adults to be a source of naturally-occurring multipotent stem cells with expressing the Hox11 [17,18]. Hox 11 is an embryonic transcription factor not found in bone marrow but persists throughout life in the adult human spleen [19]. In disease or injury, splenic stem cells are capable of self-renewal, differentiation, restoration and/or changes in function of a broad range of tissues, such as pancreatic islets, salivary epithelial cells, osteoblast-like cells, cranial neurons, inner ear structures, lymphocytes, and more differentiated immune cells that repair damaged heart cells after ischemic injury or extended lymphocyte lineages [18]. Additionally, the mesenchyme that accumulates around the dorsal pancreas promotes growth and differentiation of the pancreatic epithelium and also contributes to development of the spleen during early stages of development [20]. A evidence show that interactions between splenic mesenchyme and pancreas proceed via a highly orchestrated morphogenetic program [21]. Therefore, we hypothesized that msp-MSCs would be beneficial in mice with AP. In this study, we sought to explore whether msp-MSCs can migrate to the damaged tissues and play a role in improving pancreatic injury after onset of AP.

Materials and Methods

Cell preparation and characterization

MSCs isolated from mouse (msp-MSCs) and human spleen (hsp-MSCs) or human bone marrow (hbm-MSCs) was gifts from Dr. Shian-Min Hwang (Bioresources Collection and Research Center, Taiwan). Briefly, juvenile C57BL/6 mice were sacrificed by CO2 asphyxiation and the spleen were removed. Cells from the spleen were obtained by mechanical disruption as follows [15]: the dissected organs were washed with PBS, transferred into culture medium, cut into smaller fragments and subsequently minced with needles. The samples were then washed and filtered through a 60-μm nylon mesh filter to remove debris. Cells were then washed twice in Hanks’ balanced salt solution, plated in a 25cm² flask (BD Falcon, Bedford, MA, USA) and cultured in a humidified 5% CO2 incubator at 37°C for 72 h. Non-adherent cells were removed by sequential changes of the medium twice a week. Confluent primary cultures were washed with 1×PBS and lifted by incubation with 0.05% trypsin-EDTA (Life Technologies) at 37°C for 5 min. Subsequent passages were performed similarly. All cells were maintained at 37°C under 5% CO2 in alpha-MEM (Stem cell technologies) supplemented with 20% fetal bovine serum (Invitrogen). These cells were characterized for several stem cell markers by flow cytometry. The antibodies used for the analysis were anti-CD4, anti-CD29, anti-CD34, anti-CD44, anti-CD73, anti-CD90, anti-CD105 (BD Biosciences), anti-CD133 (Miltenyi Biotec), anti-Sca-1 antibodies (BD Biosciences Pharmingen, San Diego, CA). The cells were analyzed in a FACSCanto II flow cytometer (BD Biosciences).

Mesenchymal differentiation of msp-MSCs, hsp-MSCs, and hbm-MSCs

msp-MSCs, hsp-MSCs, and hbm-MSCs were seeded in a 6-well plate at a density of 1×10⁵ cells per well and incubated in adipogenic or osteogenic differentiation medium (Stem cell technologies). Cells maintained in expansion medium served as negative controls. The cells were differentiated into adipocytes or osteoblasts for 21 days. These cells were fixed with 4% formaldehyde and then stained with Oil Red O (Abcam) or subjected to Alizarin Red S (Sigma-Aldrich) staining. The cells were fixed with 4% formaldehyde and then stained with Oil Red O (Abcam) or subjected to Alizarin Red S (Sigma-Aldrich) staining. The cells were fixed with 4% formaldehyde and then stained with Oil Red O (Abcam) or subjected to Alizarin Red S (Sigma-Aldrich) staining.

Preparation and treatment of infusion cells

msp-MSCs were labeled with Qtracker (Life Technologies, Qtracker® 655 Cell Labeling Kit) according to the manufacturer’s instructions. 1×10⁶ Qtracker-labeled msp-MSCs suspension in 100 μl 1×PBS were intravenously administered to each animal only once at 1 hour after induction of CAP or PII.

Animal experiments

C57BL/6 mice were purchased from the Laboratory Animal Center of National Cheng Kung University (Tainan, Taiwan). All animal experiments were approved by the Institutional Animal Care and Use Committee. The mice were used for experiments at 6-8 weeks of age. For induction of CAP study, mice were induced by 5 intraperitoneal injections of cerulein (Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% saline to a total dose of 50 μg/kg/body weight at 1 hour intervals [22]. Control group mice were injected intraperitoneally with 100 μl 0.9% saline. After induction of CAP, 40 mice were divided randomly into 4 groups of 10 mice; saline+PBS group (n=10), Saline+MSCs group (n=10), CAP+PBS (n=10), and CAP+MSCs (n=10). PIH model was established using a modified method of ischaemia/reperfusion model [23]. For induction of pancreatic ischemia injury study, mice were anesthetized using intraperitoneal injection of zoletil (50 mg/ml; Virbac group, Taiwan). The abdomen was opened by a midline laparotomy. The splenic pancreas portion was carefully separated from the stomach, and a vascular clamp was applied to the pancreatic vessels. After the clamp obstructed blood flow in a part of pancreas, the clamp was not taken out after ischemia induction. The flank was then closed, and the mice were placed under a heat lamp. The mice were divided into four groups: Sham group (submitted to operative procedure without PII; n=4), Sham+MSCs group (submitted to operative procedure without PIH and received msp-MSCs treatment; n=4), PIH group (submitted to PIH procedure without treatment; n=6), PIH+MSCs group (submitted to PIH and received msp-MSCs treatment; n=6). Blood and pancreas tissues were collected after CAP or PIH induction.

Histopathological examination

Tissue specimens from pancreas were fixed in 4% paraformaldehyde and embedded in paraffin according to standard histological procedures. Sections were subjected to conventional hematoxylin and eosin staining (H&E) and examined under light microscopy. To quantify acinar cell injury, 20 randomly chosen microscopic fields were scored as previously described [24]. Briefly, edema was graded from 0 to 3 (0 = absent, 1 = focally increased between lobules, 2 = diffusely increased between lobules, and 3 = acini disrupted and separated), and acinar necrosis was graded as 0–3 (0 = absent; 1 = periductal necrosis, 5%; 2 = focal necrosis, 5%–20%, and 3 = diffuse parenchymal necrosis, 20%–50%).

Pancreatic function parameters

Pancreatic function was assessed by measuring serum amylase and lipase. The levels of amylase and lipase were analyzed at 6 h using activity assay kit (BioVision) according to the manufacturer’s instructions. All samples were analyzed in duplicate.

Determination of MPO activity

Neutrophil infiltration in the serum was monitored by measuring myeloperoxidase (MPO) activity. Briefly, serum was added to 50 mg/ml in phosphate buffer (50 mM, pH 6.0) with 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich). The samples were diluted 1:100 with assay buffer consisting in 50 mM phosphate buffer pH 6.0 with 0.167 mg/ml o-dianisidine (Sigma-Aldrich) and 0.0005% H₂O₂, and the colorimetric reaction was measured at 450 nm between 1 and 3 min in a spectrophotometer. MPO activity per gram of wet tissue was calculated as: MPO activity (U) = (A₄₅₀) × (13.5), where
$A_{\text{os}}$ is the change in the absorbance of 450nm light from 1 to 3 min after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1U MPO activity represents the amount of enzyme that will reduce 1 µmol peroxide/min.

**Immunohistochemistry staining and measurement**

Paraffin-embedded sections were stained with anti-CD3 (abcam), anti-Ki67 (Santa cruz), and anti-PCNA (DAKO) antibodies at 4°C overnight followed by incubation with biotinylated secondary antibody (DAKO). Development was carried out with a high-sensitivity substrate-chromogen system (DAB and DAB plus Chromogen Solution, DAKO). At the end of the procedure, sections were counterstained with hematoxylin for 5 min. The number of CD3+ cells was counted in 20 high-power fields per section. The percentage of Ki-67- or PCNA-positive cells in each sample was further quantified by TissueQuest software (Tissue Gnostics, Vienna, Austria).

**RNA extraction and quantitative real-time PCR**

Total RNA from pancreas tissues were extracted using RNeasy Mini kit (Qiagen. Cat. No. 74106) and was then reverse transcribed (ImPromIITM Reverse Transcription System, Promega. Cat. No. A3800) according to the manufacturer's instructions. The OD260/OD280 ratios of total RNA were between 1.8 and 2.0. Samples were assayed in duplicate in 1 µl of template DNA, 5 ul of GoTaq real-time PCR systems with SYBR Green dye (Promega), and 12.5 pmol of forward primer and reverse primer. The cycling conditions used were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min each. During the annealing-extension step, the LC480 (Roche) monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. To normalize the amount of RNA, amplification of the GAPDH gene would be used as a control.

**Cell lysis and western blot analysis**

The harvested MSCs were washed twice with ice-cold PBS and lysed in ice for 30 minutes with lysis buffer (Cell Signaling Technology, 9803). Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C, and protein concentration was measured by the Bradford assay (Bio-Rad Laboratories, 50-0006). Equal amounts of protein (30 µg) were separated on 10% polyacrylamide gels by SDS-gel electrophoresis and transferred to PVDF membranes (ImmobilonTM, Millipore; Bedford, MA). The membrane was blocked with 5% skim milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for one hour and then stained with anti-Hox11 (abcam) and anti-β-actin antibody (Genetex) at 4°C overnight. Subsequently, the membrane was washed with TBST buffer and incubated with anti-rabbit HRP conjugates (Sigma). The binding of each antibody was detected using an enhanced Immobilon Western Chemiluminescent HRP Substrate (Millipore). The signals were captured by Biospectrum Imaging System (UVP Ltd., BioSpectrum™ 500 Imaging System).

**Intraperitoneal glucose tolerance test**

Glucose tolerance was analyzed in overnight fasted mice as described by Garcia-Ocana et al. [25]. Briefly, the mice were subjected to intraperitoneal injection of glucose at 2 g/kg of body weight. Blood samples were obtained from the snipped tail at 0, 15, 30, 60, and 120 min after injection and analyzed for glucose levels using a glucometer (Roche).

**Statistical analysis**

Data are expressed as means±SE from three independent experiments. All calculations in this article were performed by T-test, one- or two-way ANOVA using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Significance was set at P < 0.05.

**Results**

**msp-MSCs express functional characterization of MSCs**

To determine the immunophenotype of MSCs isolated from mouse spleen (msp-MSCs), the cell population was compared by means of surface antigen characterization using flow cytometry. Similar to human spleen-derived MSCs (hsp-MSCs) and bone marrow-derived MSCs (hbm-MSCs), msp-MSCs expressed comparable levels of the cell-surface marker CD44. In addition, msp-MSCs also expressed high levels of CD29, CD73, CD90, and Sca-1, but were negative for CD4 and CD34 expression (Figure 1A). Given that splenic stem cells have been reported to express Hox11 [18], we therefore examined Hox11 expression in msp-MSCs and hsp-MSCs. The Western blotting results showed that both msp-MSCs and hsp-MSCs expressed Hox11 (Figure 1B). Like hsp-MSCs and hbm-MSCs, msp-MSCs exhibited typical spindle fibroblast-like morphology at the fourth passage (Figure 1C). To explore the ability to undergo differentiation into multilineage, msp-MSCs were cultured in differentiation condition medium for three weeks. Cell type-specific staining revealed that the msp-MSCs can be successfully differentiated into adipocytes and osteoblasts (Figure 1D).

**msp-MSCs traffic to the injured pancreas in CAP mice**

To track the migration of msp-MSCs in vivo, Qtracker 655 nontargeted QDots was used for cell labeling. Using fluorescence microscopy, a higher number of Qtracker-labeled msp-MSCs was observed in the CAP with MSCs group than in the MSCs alone group (Figure 2), indicating the migration of intravenous injected msp-MSCs to the injured pancreas.

**Treatment with msp-MSCs ameliorates AP**

Cerulein, a cholecytokinin analog, has been frequently used to cause AP in rats, mice, and dogs. Using this hormone-induced model, structural changes of acinar cells are similar to human AP [26]. Mice were challenged with intraperitoneal injections of cerulein at a supramaximal dose (50 µg/kg) to explore the therapeutic effects of msp-MSCs on acute pancreatitis. H&E staining of CAP pancreatic sections showed significant mass edema and inflammation with necrosis compared with the control group (saline with PBS) at 24 h following cerulein injection. The extent of acinar cell necrosis, vacuolisation, and inflammatory cell infiltration progressed with time. The histological sections were scored for edema and necrosis. msp-MSCs treatment attenuated the severity of pancreatitis, as characterized by decreased edema and necrosis (Figure 3A). We next examined the influence of msp-MSCs on pancreatic injury by assessing pancreatic edema and levels of both amylase and lipase. CAP mice treated with a single dose of msp-MSCs resulted in reduced serum levels of amylase and lipase (Figures 3B and 3C). The pancreas-to-bodyweight ratio, which reflects pancreatic edema, was also significantly decreased in the CAP with MSCs groups compared with the CAP group (Figure 3D). MPO, which is a lysosomal protein stored in neutrophil azurophilic granules and in monocyte lysosomes, has been used to measure the extent of inflammatory infiltration. When msp-MSCs were infused to CAP mice, MPO activities was decreased significantly (Figure 3E).

**msp-MSCs regulate inflammatory response**

Because msp-MSCs display immunosuppressive capacity, we next...
investigated the effects of msp-MSCs on expression of inflammatory mediators that are involved in AP pathogenesis. The mRNA expression of pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, and IL-6, and anti-inflammatory cytokines, such as IL-4 and IL-10, in pancreas tissue were analyzed by qRT-PCR. Of these mentioned cytokines, IL-10 expression was increased in msp-MSCs-treated CAP mice compared with CAP mice. By contrast, TNF-α and IFN-γ (Figures 4A and 4B) were downregulated, and IL-4, IL-1β, and IL-6 were undetectable (data not shown). To further decipher the mechanism underlying the immunomodulation of msp-MSCs in AP, we explored the interplay between msp-MSCs and T cells in CAP mice. Immunostaining showed a high level of infiltrating CD3+ cells in CAP pancreatic tissues, indicating the participation of T cells in AP. Treatment with msp-MSCs decreased the level of infiltrating CD3+ cells in CAP tissues (Figures 4C and 4D). These results demonstrate that msp-MSCs can regulate inflammation reaction in AP mice, most likely because of enhanced IL-10 expression, decreased expression of TNF-α and IFN-γ, and lower T-cell infiltration.
msp-MSCs support blood sugar regulation and pancreatic cell proliferation

To examine the effect of msp-MSCs on pancreatic function in AP mice, we carried out the intraperitoneal glucose tolerance test (IPGTT) by administration of 2g glucose per kg body weight in mice. Unexpectedly, there were no significant differences in blood glucose levels between mice with and without CAP (Figure 5A). We next established a pancreatic ischemia injury (PII) mouse model by using the vessel champ to obstruct blood flow in a part of pancreas (Figure 5B). IPGTT results showed that, although there was a significant difference in blood glucose level at 30 min after glucose loading between the PII group and the sham group (Figure 5C), but no significant differences in serum amylase or lipase levels between the sham and PII groups were observed (Figure 5D). Moreover, IPGTT revealed that glucose tolerance in the msp-MSCs-treated PII group was increased as compared with that in the PII group. Blood glucose levels were decreased significantly at 60 and 120 min in PII mice after 3 weeks of msp-MSCs treatment (Figure 5E). Further tests of these mice after 5 weeks period exposed reinstating IPGTT responses compared with PII group (Figure 5F). To determine whether the msp-MSCs-mediated restoration of pancreatic function is due to increased cell proliferation, IHC was performed using antibodies against Ki67 and PCNA in pancreas tissues of PII mice. IHC quantification results displayed that msp-MSCs treatment increased the number of Ki67- and PCNA-expressing cells in pancreas section of PII (Figures 5G and 5H).

Discussion

The pathogenesis of AP involves the interplay of local and systemic immune responses, and uncontrolled local inflammation leads to organ failure, prolonged hospitalization, and death. Thus, understanding and targeting the inflammatory response is critical to improving therapeutic outcomes. Bone marrow- and umbilical cord-derived MSCs have been reported to have a potential therapeutic role on AP, but the studies about the spleen-derived MSCs are limited. In this study, we found that msp-MSCs possess MSC phenotypes. msp-MSCs exhibited immunoregulatory effects and reduced inflammation by trafficking to injured pancreas. msp-MSCs also supported pancreatic cell proliferation and thus improved blood sugar metabolism. These results show for the first time that msp-MSCs ameliorated AP and promoted recovery of pancreatic function.

MSCs are potentially capable of limiting pancreatic damage. Several studies observed that MSCs reduced the increase of amylase, lipase, and MPO levels in different rodent models of AP [10,27-29]. In vivo tracking techniques revealed active homing of hbm-MSC to the injured pancreas, whereas the severity of the pancreatitis was correlated...
directly with the number of hbm-MSC recruited to the pancreas [10]. Infused hbm-MSC ameliorated acinar cell necrosis, pancreatic edema, and inflammatory cell infiltration [10,30]. Transfusion of MSCs from bone marrow and umbilical cord significantly reduced the expression levels of pro-inflammatory mediators and cytokines, like TNF-α, IFN-γ, IL-1β, or IL-6, and increased the production of anti-inflammatory cytokines, like IL-4 or IL-10 [10,29]. Consistent with these reports, we found that msp-MSCs trafficked to the injured pancreas and exerted immunomodulatory ability to diminish pancreatic tissue damage by enhancing IL-10 expression, decreasing expression of TNF-α and IFN-γ, reducing serum levels of amylose, lipase, and MPO, and suppression T-cell infiltration. These observations argued that MSCs of different origin have similar properties that control inflammation, immune response, and tissue repair during AP.

Here we found Hox11-expressing msp-MSCs could mediate restoration of pancreatic function by enhancing the number of Ki67- and PCNA-expressing cells in pancreas section of PII. Hox11/11x1 is part of a homeodomain gene family essential for organogenesis of the spleen and for contributions to development of hindbrain, cochlea, pancreas, and salivary gland [31]. Recent research has found that Hox11 expressing stem cells can differentiate into fully functional insulin-producing islet cells of the pancreas [32,33] or partially functional osteoblast-like cells [34-36]. Accordingly we propose that msp-MSCs would be a potential therapeutic strategy for the repair and regeneration of tissues and organs.

In conclusion, we suggest that msp-MSCs are capable of improving pancreatic damage, exerting anti-inflammatory effects, and revolving pancreatic dysfunction. Our study has an implication for further exploration of msp-MSCs as a promising tool for stem cell-based therapies, including inflammatory diseases.

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References


