Research Article

JOURNAL OF HEPATOLOGY

Microencapsulated human mesenchymal stem cells decrease liver fibrosis in mice

Raphael P.H. Meier[1,*], Redouan Mahou[2], Philippe Morel[1], Jeremy Meyer[1], Elisa Montanari[1], Yannick D. Muller[1], Panayiotis Christofilopoulos[3], Christine Wandrey[2], Carmen Gonelle-Gispert[1,†], Leo H. Bühler[1,†]

[1]*Cell Isolation and Transplantation Center, Department of Surgery, Geneva University Hospitals and Medical School, Geneva, Switzerland;* [2]*Institut d'Ingénierie Biologique et Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland;* [3]*Orthopedic Surgery, Department of Surgery, Geneva University Hospitals and Medical School, Geneva, Switzerland*

Background & Aims: Mesenchymal stem cell (MSC) transplantation was shown to be effective for the treatment of liver fibrosis, but the mechanisms of action are not yet fully understood. We transplanted encapsulated human MSCs in two mouse models of liver fibrosis to determine the mechanisms behind the protective effect.

Methods: Human bone marrow-derived MSCs were microencapsulated in novel alginate-polyethylene glycol microspheres. *In vitro*, we analyzed the effect of MSC-conditioned medium on the activation of hepatic stellate cells and the viability, proliferation, cytokine secretion, and differentiation capacity of encapsulated MSCs. The level of fibrosis induced by bile duct ligation (BDL) or carbon tetrachloride (CCl_4) was assessed after intraperitoneal transplantation of encapsulated MSCs, encapsulated human fibroblasts, and empty microspheres.

Results: MSC-conditioned medium inhibited hepatic stellate cell activation and release of MSC secreted anti-apoptotic (IL-6, IGFBP-2) and anti-inflammatory (IL-1Ra) cytokines. Viability, proliferation, and cytokine secretion of microencapsulated MSCs were similar to those of non-encapsulated MSCs. Within the microspheres, MSCs maintained their capacity to differentiate into adipocytes, chondrocytes, and osteocytes. 23% (5/22) of the MSC clones were able to produce anti-inflammatory IL-1Ra *in vitro*. Microencapsulated MSCs significantly delayed the development of BDL- and CCl_4-induced liver fibrosis. Fibroblasts had an intermediate effect against CCl_4-induced fibrosis. Mice transplanted with encapsulated MSCs showed lower mRNA levels of collagen type I, whereas levels of matrix metalloproteinase 9 were significantly higher. Human IL-1Ra was detected in the serum of 36% (4/11) of the mice transplanted with microencapsulated MSCs.

Conclusions: MSC-derived soluble molecules are responsible for an anti-fibrotic effect in experimental liver fibrosis.

Keywords: Mesenchymal stem cells; Liver fibrosis; Inflammation; Interleukin 1 receptor antagonist; Mice; Cell transplantation; Microencapsulation; Alginate.
Received 20 January 2014; received in revised form 15 October 2014; accepted 20 October 2014; available online 30 October 2014

* Corresponding author. Address: Surgical Investigation Unit, Transplantation and Visceral Surgery, University Hospital and Medical Faculty, Geneva, Switzerland.
E-mail address: Raphael.meier@hcuge.ch (R.P.H. Meier).
† These authors contributed equally to this work.

Abbreviations: ALT, alanine aminotransferase; α-SMA, alpha smooth muscle actin; AST, aspartate aminotransaminase; BDL, bile duct ligation; BSA, bovine serum albumin; CCl_4, carbon tetrachloride; DTT, dithiothreitol; EdU, 5-ethynyl-2′-deoxyuridine; EDTA, ethylene diamine tetraacetic acid; EDX, foreskin fibroblasts; EEF1A1, eukaryotic translation elongation factor 1 alpha; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FDA, fluorescein diacetate; GAK, cyclin G-associated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSC, hepatic stellate cells; IBMX, 3-isobutyl-1-methylxanthine; IL-1Ra, interleukin 1 receptor antagonist; IMDM, Iscove's modified Dulbecco's medium; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; MSC-CM, MSC-conditioned medium; Na-alg, sodium-alginate; PDGF-BB, platelet derived growth factor BB; PBS, phosphate buffered saline; PE, phycoerythrin; PEG, polyethylene glycol; PI, propidium iodide; PS, penicillin-streptomycin; RT, room temperature; RT-PCR, real-time polymerase chain reaction; SRP72, signal recognition particle 72 kDa; TGF-β, transforming growth factor-beta 1.

Introduction

Mesenchymal stem cells (MSCs) are adult progenitor cells that contribute to stromal tissue renewal [1]. Originally found in the bone marrow [2], MSCs are present in all types of tissues [3] and were recently recognized as closely related to blood vessel pericytes [4]. MSC-based cell therapy is currently investigated with the aim to treat acute and chronic liver injury [5]. Indeed, it was suggested that MSCs might be able to transdifferentiate into hepatocytes [6]. Several other studies demonstrated that MSCs have immunosuppressive and anti-inflammatory properties [7–9], which could represent another mechanism by which MSCs improve chronic liver injury.

In experimental models, MSCs reduce liver fibrosis in rodents [10–14]. The mechanisms of action remain largely unknown but may involve the secretion of anti-inflammatory cytokines, such as IL-10 [15] or IL-1 receptor antagonist (IL-1Ra) [16], or the secretion of growth factors such as hepatocyte growth factor [7,17], vascular endothelial growth factor [18] or insulin-like growth factor-binding proteins [17]. Further, MSCs secrete matrix metalloproteinases that could mediate a fibrolytic effect [12].

Other studies showed that *in vitro* hepatocyte-like pre-differentiation of MSCs has a therapeutic effect in experimental liver fibrosis [19,20]. Consequently, it is currently not clear whether molecules secreted by MSCs are sufficient to mediate the anti-fibrotic effect or whether cell-cell interactions and/or the presence of hepatocyte-differentiated MSCs are necessary.

A further issue is phenotype stability: even if MSCs engraft in the injured liver and differentiate into hepatocyte-like cells, it is likely that induction of chronic injury (e.g., high levels of transforming growth factor-beta 1 (TGF-β)) precludes those cells from maintaining epithelial-like characteristics. It was shown that cells recruited from the bone marrow to an experimentally-induced fibrotic liver finally became collagen-producing fibrocytes [21].

In the present study, we investigated whether immunoprotection by microencapsulation prevents MSCs from participating to scar formation and allows MSCs to mediate an anti-fibrotic effect by releasing soluble molecules *in vitro* and *in vivo*. We found that MSC-conditioned medium (MSC-CM) reduced alpha smooth muscle actin (α-SMA) expression, a marker of hepatic stellate cell (HSC) activation (the key event in liver fibrosis).

We used recently developed alginate-polyethylene glycol (alg-PEG) hybrid hydrogel to encapsulate MSCs. This hydrogel is permissive to soluble factors (e.g., O_2, glucose, cytokines) but not to immune cells or antibodies, thus protecting MSCs from immune rejection upon *in vivo* administration. We first verified that cells maintained normal viability, proliferation, differentiation, and cytokine secretion. We further observed that microencapsulated MSCs decreased liver fibrosis and inflammation in mouse models of chronic liver injury induced by bile duct ligation (BDL) or carbon tetrachloride (CCl_4), suggesting that these effects can be attributed solely to factors secreted by MSCs.

Materials and methods

Cell culture

This research project was accepted by the local ethical committee of the University Hospitals of Geneva (protocols NAC 01-015).

Human adult bone marrow MSCs were isolated from femoral heads of 11 adult orthopedic patients undergoing total hip replacement. Written informed consent was obtained from each patient.

Cells were isolated and cultured as previously described [22]. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Cambrex, Verviers, Belgium) with 10% fetal calf serum (FCS) (Invitrogen, Basel, Switzerland), 100 IU/ml penicillin, 100 mg/ml streptomycin (P-S) (Gibco-Invitrogen), dithiothreitol (DTT, Sigma, St-Louis, USA) and 10 ng/ml platelet derived growth factor BB (PDGF-BB, PeproTech EC Ltd, London, UK). Cells were expanded as previously described [23,24], produced and used for experiments between passages 3 to 6. MSC-CM was obtained after incubation of 5×10^6 cells in 10 ml IMDM with 5% FCS for 48 h. We used MSC-CM issuing from 22 different MSC clones (i.e., two per donor). Primary human foreskin fibroblasts (designated in this study as EDX cells, a gift from DFB Bioscience, Fort Worth, TX) were maintained in expansion medium consisting of IMDM supplemented with 10% FCS and P-S. The medium was changed every 3 days. EDX-conditioned medium was obtained after incubation of 5×10^6 - cells in 10 ml IMDM with 5% FCS for 48 h. Human HSCs were obtained from biopsies of healthy liver parenchyma from 3 patients undergoing partial hepatectomy. The protocol was approved by the University Hospitals of Geneva ethics committee and informed consent was obtained from all patients (protocols 01-172/chir01-015). HSCs were isolated as previously described [25]. Cells were cultured in 24-well plates (100,000 cells/dish) with IMDM medium containing 10% FCS and P-S (Invitrogen) at 37 °C with 5% CO_2. HSC cells were treated with either control condition, MSC-CM, TGF-β (PreproTech, USA) or TGF-β and MSC-CM together, during 48 h. α-SMA and vimentin protein detection was performed in LX-2 cells (a human HSC line provided by Prof. Scott Friedman, Mount Sinai School of Medicine, New York, NY) after 5 days of culture.

Polyethylene glycol-alginate microencapsulation of cells

Reagents

Sodium alginate (Na-alg) (PRONOVA UP LVM) was obtained from FMC BioPolymer (Novamatrix, Norway, batch no: FP-506-01). 8-arm polyethylene glycol (PEG), molar mass 20 kg/mol (PEG-8-20), was purchased from Shearwater Polymers (Huntsville, AL, USA). This PEG consists of a poly(glycerol) backbone with multiple PEG arms attached through an ether bond (PEG-OH). Divinyl sulfone, DTT, calcium chloride dihydrate, and sodium chloride were obtained from Sigma. All chemicals were of analytical grade and were used as supplied, unless indicated otherwise.

Formation of microspheres

Microspheres were prepared under sterile conditions. We used a co-axial air-flow droplet generator as previously described [26]. Briefly, MSCs or EDX cells were detached using 0.25% trypsin-EDTA (Sigma) for about 30 sec and washed twice. The cell suspension was centrifuged (1200 rpm, 5 min, RT) and the supernatant discarded. The pellet was resuspended in Na-alg/PEG-8-20 solution (1.5% (w/v) Na-alg + 10% (w/v) PEG-8-20 in DMEM (special formulation without NaCl and KCl, Culture Technologies, Gravessano, Switzerland)) to a final concentration of 500,000 cells/ml. The mixture was extruded through a 400 μm needle into the sterile gelation bath prepared by dissolving $CaCl_2$ and DTT, in DMEM (special formulation as indicated above) with osmolality adjusted to 300 mOs/kg (80 ± 5 mM $CaCl_2$). The receiving bath was incubated in a shaker (80 rpm) at 37 °C for 3 h to achieve optimal cross-coupling [27]. Microspheres were collected by filtration and cultured in IMDM 10% FCS. Microspheres without cells were prepared using the same protocol.

Fibrosis induction in mice

All animal studies were approved by the animal ethics committee of the Geneva Veterinarian Office and the University of Geneva, Geneva, Switzerland (protocol number 1043/3603/2). Eight to 10 week-old male DBA-1 mice were purchased from Janvier (Le Genest-St-Isles, France). All mice were maintained under standard conditions at the animal facility of the Geneva University. Water and food were provided *ad libitum*. Liver fibrosis was induced by BDL as previously described [28]. Briefly, mice were anesthetized with isoflurane and a midline laparotomy was performed in order to expose the hepatic hilum and to identify the common bile duct. We used a dissecting microscope to cut the common bile duct in between three ligatures. To obtain CCl_4-induced liver fibrosis, 2 ml/kilogram of CCl_4 50% (v/v) solution in corn oil (Sigma Co., Milan, Italy), containing 1.0 ml/kg of CCl_4, was administered by subcutaneous injections, twice a week for 4 weeks (to avoid intra-peritoneal damage of the encapsulated cells). Animals received intraperitoneally 1.5 million encapsulated MSCs in 1 ml alg-PEG microspheres, or 1.5 million encapsulated EDX cells in 1 ml alg-PEG, or 1 ml alg-PEG microspheres without cells. Sham operated mice were used as controls. The animals were sacrificed 15 days after BDL, and blood (transaminases, IL-10, and IL-1Ra measurements) and liver samples (histology and RT-PCR) were collected.

Statistical analysis

Results were expressed as mean values ± SEM. Differences between groups were analyzed using the Student *t*-test or Mann-Whitney U test (2 groups) and one-way analysis of variance with Bonferroni multiple testing correction (>2 groups). $p < 0.05$ was considered statistically significant.

Results

Isolation and characterization of mesenchymal stem cells

MSCs were isolated from 11 adult donors. MSCs showed typical spindle-shape morphology and were expanded during 3-6 passages to reach about 15 population doublings (Supplementary Fig. 1A). Analysis of the surface antigens on MSCs by flow cytometry showed patterns that are typical of MSCs. Cells were negative for HLA class1, CD34, CD36, and CD45, and positive for CD44, CD54, CD90, CD105, and CD106 (Supplementary Fig. 1B).