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Research Article

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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.
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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).

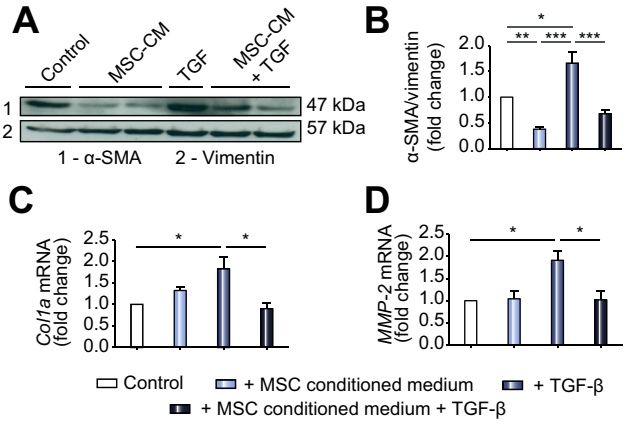

Fig. 1. Effect of mesenchymal stem cell conditioned medium (MSC-CM) on alpha smooth muscle actin in LX-2 cells, and collagen and MMP-2 expression in primary human HSC. (A) Total protein extracts from LX-2 cells, treated with MSC-CM (n = 4), TGF-β (n = 13), TGF-β and MSC-CM together for 5 days (n = 10), and untreated cells (control) (n = 4) were subjected to SDS-PAGE, transferred to nitrocellulose and blotted with anti-α-SMA and anti-vimentin antibodies. (B) Quantification of α-SMA signals in LX-2 cells by densitometry. The expression of α-SMA was normalized to vimentin. Data are mean values ± SEM and are expressed as fold change with respect to the control condition, which was set as 1. (C and D) Primary HSCs were isolated from human liver biopsies and cultured for 2 days under the following conditions: untreated (control) (n = 8), treated with MSC-CM (n = 7), treated with TGF-β (n = 10), and treated with TGF-β and MSC-CM together (n = 4). Total mRNA was extracted from cells, and mRNA levels of collagen type 1 alpha 1 (*Col1a*) (C) and *MMP-2* (D) were determined by real-time PCR (data are mean values obtained from 3 independent experiments). $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

Mesenchymal stem cell conditioned medium decreased HSC activation in vitro

The effect of MSC-CM on α-SMA expression, in immortalized (LX-2) and primary human HSCs, was studied. LX-2 cells were treated with control medium, MSC-CM, TGF-β (used as positive control), and MSC-CM together with TGF-β for 5 days, and analyzed by Western blotting (Fig. 1). LX-2 cells cultured with MSC-CM showed decreased levels of α-SMA compared to untreated cells (Fig. 1A). The treatment of LX-2 cells with MSC-CM together with TGF-β also resulted in decreased expression of α-SMA compared to cells treated with TGF-β alone (Fig. 1A), demonstrating neutralization of the activation induced by TGF-β. Quantification of α-SMA levels is shown in Fig. 1B. In primary HSCs, levels of collagen type I and *MMP-2* mRNA were also increased upon TGF-β treatment, while the increase was statistically significantly lower in the presence of MSC-CM (Fig. 1C and D). These results indicate that MSC-CM was able to reduce LX-2 and primary HSC activation when cells were cultured in profibrogenic conditions (i.e., upon treatment with TGF-β).

Survival, proliferation, and differentiation of encapsulated mesenchymal stem cells in vitro

First, the quality of MSCs after microencapsulation was analyzed in *in vitro* assays. MSCs were microencapsulated in recently developed alg-PEG hybrid microspheres [26,27,29] (Supplementary Fig. 2A). Cell viability was assessed at day 0 and at 5, 15, 45, and 218 days after microencapsulation (Supplementary Fig. 3A). Immediately after trypsinization and encapsulation, MSCs were

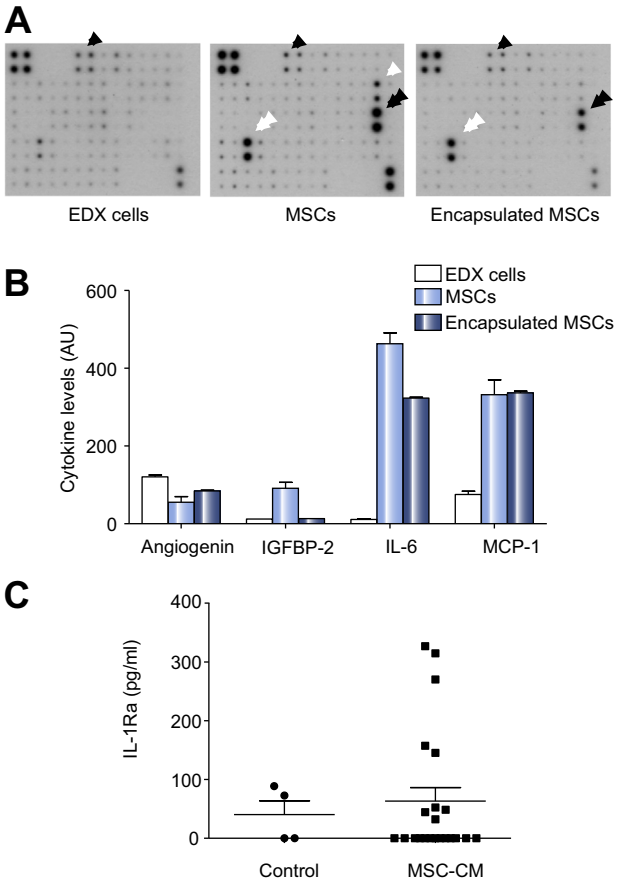

Fig. 2. Cytokine secretion of free and microencapsulated mesenchymal stem cells. (A) Human cytokine antibody array was used to analyze 48 h-conditioned medium from either free EDX cells, free MSCs and microencapsulated MSCs (single black arrow, angiogenin; single white arrow, IGFBP-2; double black arrow, IL-6; double white arrow, MCP-1). (B) Quantification of signals by densitometry reveals that cytokines like IGFBP-2, IL-6, and MCP-1 are present at similar levels in conditioned medium from encapsulated MSCs and free MSCs (performed in duplicate, data presented are in arbitrary units (AU), mean value ± SEM). (C) Quantification of human IL-1Ra by ELISA in 22 different MSC-conditioned media obtained from 11 human donors compared to four control media; data are presented for individual measurements and mean values ± SEM.

round-shaped (Supplementary Fig. 2B) and viability was 66.8 ± 3.8%, compared to 74.4 ± 2.9% among free MSCs (after trypsinization) (mean ± SEM, n = 4 independent experiments, difference not statistically significant (p = 0.418)) (Supplementary Fig. 3B). Five days after microencapsulation, MSCs recovered their typical spindle-shaped morphology (Supplementary Fig. 3C), almost without any cell death (viability close to 100%). Viability of MSCs and integrity of alg-PEG microspheres were maintained up to 6 months of culture (Supplementary Fig. 3A). Similar levels of proliferation were observed in encapsulated MSCs (2.7 ± 0.6%) and free MSCs (2.4 ± 0.2%) (mean ± SEM, n = 4 independent experiments, difference not statistically significant (p = 0.673)), as shown by analysis of 24 h-EdU incorporation (Supplementary Fig. 3C and D). When cultured in the respective differentiation media, free MSCs and microencapsulated MSCs were able to differentiate into adipocytes storing large lipid droplets (oil-red-O staining), osteoblasts producing calcific depositions (Alizarin red staining), and chondrocytes producing

collagen type I to IV (Goldner's trichrome), (Supplementary Fig. 3E). These results indicate that microencapsulated MSCs remain fully functional in terms of viability, proliferation, and differentiation potential.

Cytokine profile of free and microencapsulated mesenchymal stem cells

The cytokine profile of MSCs was analyzed using a human cytokine antibody array, with the aim of identifying molecules that might implicate an anti-fibrotic or anti-inflammatory effect (Fig. 2C). Medium conditioned by free EDX cells was used as control for media from free MSCs and microencapsulated MSCs (Fig. 2A). MSCs secreted several cytokines (Fig. 2A, Supplementary Fig. 4A), with the most elevated ones being angiogenin, IGFBP-2, IL-6, and MCP-1 (Fig. 2B). With the exception of angiogenin, EDX cells secreted modest levels of cytokines when compared to MSCs (Fig. 2B, Supplementary Fig. 4B). Conditioned medium from microencapsulated MSCs and medium from free MSCs showed a similar cytokine profile *in vitro* (i.e., angiogenin, IL-6, MCP-1) (Fig. 2A and B).

Since it has been suggested that MSCs secrete interleukin 1 receptor antagonist (IL-1Ra) [16], this was investigated for 22 MSC-CM preparations using a human-specific IL-1Ra ELISA (Fig. 2C). IL-1Ra was detected at levels above background (i.e., 100 pg/ml) in 5 out of 22 (23%) MSC-CM preparations and was not detectable in control media (n = 4) [16]. These results revealed that both free and microencapsulated MSCs secreted cytokines such as IGFBP-2, IL-6, MCP-1, and IL-1Ra.

Survival of microencapsulated mesenchymal stem cells after transplantation into mice

At one and six months after transplantation in mice, viable MSCs were found, as shown by the presence of nucleated, spindle-shaped, and human vimentin-positive cells in microspheres (Supplementary Fig. 5A and B, Supplementary Fig. 6A and B). The fibrotic reaction around the microspheres visualized by Masson's trichrome staining was moderate, did not increase from one to six months, and was not different between empty microspheres and microspheres containing MSCs (Supplementary Fig. 6A). A cellular infiltration was observed around the microspheres with MSCs at one month (Supplementary Fig. 6A). The infiltration included IBA-1-positive macrophages and CD4 and CD8-positive T cells. The relative presence of CD4 and CD8-positive T cells (normalized to the total number of cells) was significantly higher around microspheres with MSCs than around empty microspheres (n = 3 mice per group, $p = 0.042$ and $p = 0.011$, respectively). Similar levels of IBA-1-positive macrophages, expressed with respect to the total number of nuclei, were present around microspheres, in animals receiving empty microspheres or microspheres with MSCs (n = 3 mice per group, difference not statistically significant ($p = 0.176$)). Quantification of F4/80-positive cells on cryosections gave similar results (data not shown). Together, these results indicated that encapsulated MSCs are durably protected from rejection in mice and cause a modest fibrotic reaction, with emergence of an immune infiltrate around microspheres.

Reduced liver fibrosis and increased MMP-9 expression in the liver of mice after bile duct ligation and transplantation with microencapsulated mesenchymal stem cells

DBA-1 mice were treated with BDL or CCl_4 and underwent intraperitoneal transplantation with empty microspheres, encapsulated EDX cells or microencapsulated MSCs. The extent of liver fibrosis was analyzed by Masson's trichrome staining and by measuring the expression of fibrosis-related genes. Quantification of collagen revealed significantly less liver fibrosis in mice transplanted with microencapsulated MSCs than in mice transplanted with microencapsulated EDX cells or empty microspheres (8.2% *vs.* 12.0% and 11.0%, BDL model; 1.0% *vs.* 1.4% and 4.4%, CCl_4 model) (Fig. 3A–D). Histology revealed microencapsulated MSCs around the liver (Supplementary Fig. 5C). Liver collagen type I mRNA levels were increased compared to mice without BDL (>23-fold) or without CCl_4 (10-fold). Collagen type I expression was reduced in the liver of mice treated with microencapsulated MSCs compared to mice treated with microspheres containing EDX cells or empty microspheres: the respective differences did not reach statistical significance (Fig. 3E and F). Compared with control mice that were not treated with BDL or CCl_4, all three treatment groups showed a substantial increase in liver mRNA levels of *α-SMA* (Fig. 3G and H), *MMP-9* (Fig. 3I and J), and *MMP-13* (Fig. 3K and L). In the BDL model, *α-SMA* mRNA levels were significantly increased in the liver of mice treated with empty microspheres (8.1-fold) or microencapsulated EDX cells (9.1-fold), but not in mice treated with microencapsulated MSCs (1.3-fold): the difference reached statistical significance (Fig. 3G). No significant difference was observed in the CCl_4 model (Fig. 3H). The number of liver α-SMA and IBA-1 positive cells was reduced in mice treated with encapsulated MSCs compared to control (data not shown). Microencapsulated MSCs induced a substantial increase in MMP-9 expression in BDL and CCl_4 models (7.6-fold and 8.3-fold, respectively) whereas microencapsulated EDX cells or empty microspheres gave a lower increase (1.8 and 2.0-fold, respectively (BDL) and 2.9 and 5.7-fold, respectively (CCl_4)) (Fig. 3I and J). *MMP-13* mRNA levels increased compared to mice without BDL or CCl_4, and without significant differences between the three groups (Fig. 3K and L). We concluded that microencapsulated MSCs transplanted into bile duct-ligated mice have a protective effect during the development of liver fibrosis since collagen accumulation was reduced. The results further suggest that regulation of MMP-9 contributes to this effect.

Decreased ALT and AST levels and increased levels of IL-10 and IL-1Ra in mice transplanted with microencapsulated mesenchymal stem cells

Mice with bile duct ligation- or CCl_4-induced liver fibrosis showed a substantial increase in ALT and AST after bile duct ligation, reflecting liver parenchymal damage (Fig. 4A–D). Liver enzymes were lower in mice receiving microencapsulated MSCs in both models, and the difference was statistically significant in the bile duct ligation model ($p < 0.05$). Mice treated with microencapsulated EDX cells showed a lower reduction, which did not reach statistical significance in either model. Levels of IL-10 were significantly higher in mice after transplantation with microencapsulated MSCs than in control mice in which no bile duct ligation was performed ($p < 0.05$); an increase in IL-10 was also seen in mice after bile duct ligation and transplantation with

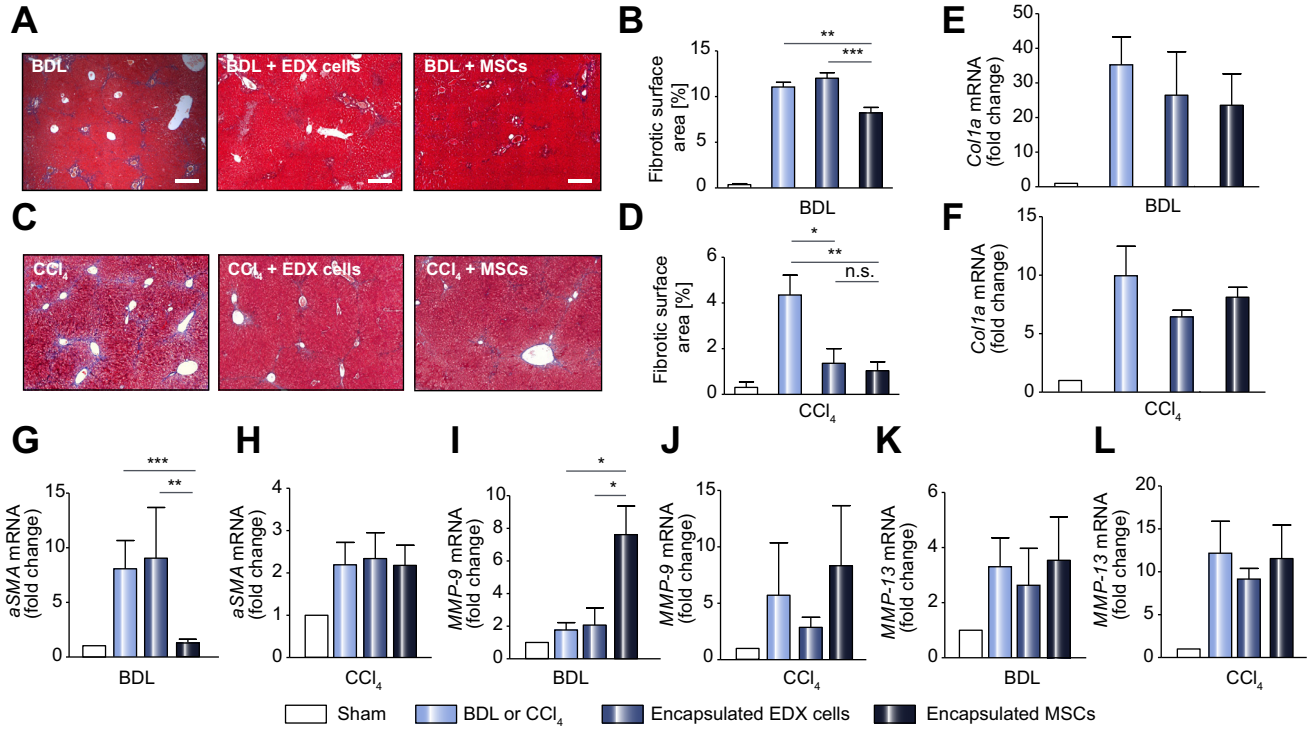

Fig. 3. Effect of microencapsulated mesenchymal stem cells on liver fibrosis in mice and correlation with MMP-9 expression in the liver. (A–D) Livers from mice were collected 15 days after BDL or 4 weeks of CCl_4 treatment and transplantation with either empty microspheres (n = 8 (BDL), n = 8 (CCl_4)), microencapsulated EDX cells (n = 5 (BDL), n = 8 (CCl_4)), or microencapsulated MSCs (n = 4 (BDL), n = 8 (CCl_4)). Mice without BDL (n = 6) or mice treated with corn oil (n = 3) were used as controls. Samples were fixed in formalin and embedded in paraffin. Liver sections were stained with Masson's trichrome. Liver parenchyma appears in red and fibrotic areas in blue. Scale bars 400 μm. (B and D) Morphometric quantification of fibrosis was performed on multiple liver sections and expressed as percentage fibrotic surface area. Data are presented as mean value ± SEM. (E–L) Liver collagen type I alpha 1 mRNA (E and F), *α-SMA* (G and H), *MMP-9* (I and J), and *MMP-13* (K and L) quantification by real time-PCR in liver samples of mice (n = 7 (without BDL), n = 3 (treated with corn oil)), and in mice with BDL and transplanted with empty microspheres (n = 11 (BDL), n = 8 (CCl_4)), microencapsulated EDX cells (n = 5 (BDL), n = 8 (CCl_4)), and microencapsulated MSCs (n = 12 (BDL), n = 8 (CCl_4)). Data presented are fold change with respect to housekeeping genes (see Materials and methods) and expressed as mean value ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

microencapsulated EDX cells, but this difference did not reach statistical significance (Fig. 4E). In the bile duct ligation model, human IL-1Ra was detected in the serum of 4 out of 11 mice (36%) transplanted with microencapsulated MSCs, and in none of the mice transplanted with empty microspheres (n = 7) (Fig. 4F). These results showed that encapsulated MSCs reduced liver damage induced by bile duct ligation or CCl_4, promoted endogenous anti-inflammatory IL-10 secretion, and secreted anti-inflammatory cytokine IL-1Ra *in vivo*.

Discussion

MSC transplantation is currently investigated in 22 clinical trials as potential treatment for chronic liver diseases (www.clinicaltrial.gov) and several phase 1-2 studies are published [5,30,31]. These trials are based on pre-clinical studies where MSCs were shown to reduce liver fibrosis upon systemic injection [10,11,14]. The mechanisms underlying this beneficial effect are not well understood and may include MSC ability to differentiate into hepatocyte-like cells [32], to reduce inflammation [33] and to enhance tissue repair at the site of injury [34]. The aim of the present study was to analyze the potential anti-fibrotic effect of molecules secreted by MSCs. We used cell microencapsulation to avoid cellular interactions between recipient's cells and transplanted MSCs, and to analyze solely the effect of released cytokines on liver fibrosis.

We first showed that conditioned medium from human bone marrow-derived MSCs impeded the activation of HSC *in vitro*. To analyze whether this effect persisted *in vivo*, MSCs were microencapsulated to avoid intercellular contact after transplantation into mice. We used newly developed alg-PEG microspheres [27] allowing reduced inflammation and better mechanical resistance, when compared to conventional calcium-alginate microspheres [29]. *In vitro*, microencapsulated MSCs continued to proliferate and kept their capacity to differentiate into adipocytes, osteocytes and chondrocytes, demonstrating that microencapsulated MSCs were fully functional after encapsulation.

To identify molecules implicated in the anti-fibrotic effects of MSCs, we performed a cytokine antibody array using MSC-CM. We compared medium from free and microencapsulated MSCs to verify that cytokine release from MSC was not inhibited by the microencapsulation procedure. The results confirmed that both free and microencapsulated MSCs secrete cytokines at similar levels. IL-6, IGFBP-2, and MCP-1 were among the cytokines showing the highest level of secretion. Of note, IL-6 has known anti-apoptotic effects and may contribute to the protective effect of MSCs [35,36]. IGFBP-2 regulates insulin-like growth factor-I, which is a potent cytoprotective and anabolic hormone produced by the liver; upregulated insulin-like growth factor-I was shown

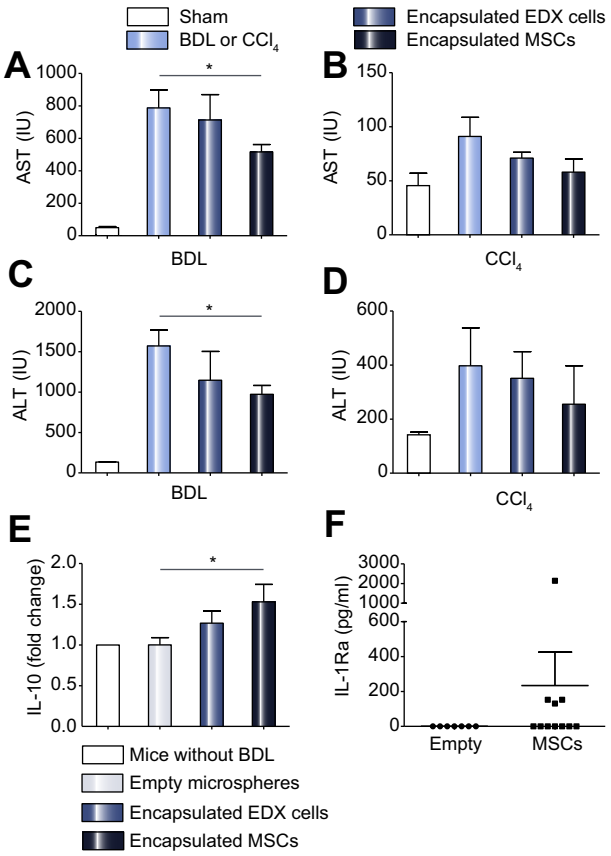

Fig. 4. Liver enzymes and IL-10 and IL-1Ra in serum of mice treated with microencapsulated mesenchymal stem cells or EDX cells, or with empty microspheres. (A–D) Serum levels of AST and ALT (expressed in international unit (IU)) in mice without BDL (n = 8) or treated with corn oil (n = 3), and in mice 15 days after BDL or 4 weeks after CCl_4 treatment and transplantation of empty microspheres (n = 4 (BDL), n = 8 (CCl_4)), microencapsulated EDX cells (n = 7 (BDL), n = 8 (CCl_4)), and microencapsulated MSCs (n = 8 (BDL), n = 8 (CCl_4)). (E) Serum levels of IL-10 in mice with BDL and after transplantation of empty microspheres (n = 6), microencapsulated EDX cells (n = 4), and microencapsulated MSCs (n = 5), expressed in fold change with respect to control mice without BDL (n = 5). (F) Human IL-1Ra in the serum of mice transplanted with empty microspheres (n = 7) and microencapsulated MSCs (n = 11). (A–C) Data are presented as mean value ± SEM, $^*p$ <0.05.

to have fibrinolytic effects during experimental liver fibrosis [37]. It is thus possible that IGFBP-2 plays a role in the decreased liver injury observed upon treatment with microencapsulated MSCs.

We further showed that MSCs secrete IL-1Ra *in vitro* and *in vivo*. This natural inhibitor of the pro-inflammatory effect of IL-1 could be responsible for the anti-inflammatory effects of MSCs, as suggested by others [16,38,39]. In contrast, increased levels of pro-inflammatory MCP-1 [40] in MSC-CM seem to be in contradiction with the anti-inflammatory effect of MSCs. However, macrophages are known to play a dual role in liver fibrosis [41]: in the present model, macrophages possibly contribute to liver fibrosis during the initial phase of tissue injury, and contribute to enhanced matrix degradation once liver fibrosis is established.

In vivo, we first demonstrated that microencapsulated MSCs survived well upon transplantation underneath the kidney capsule of mice, and triggered a modest reaction, visualized by the presence of CD4 and CD8-positive T cells around microspheres, one month after transplantation. A fibrotic reaction was observed that was similar for empty microspheres and microspheres with MSCs.

Second, we showed the effectiveness of microencapsulated MSCs in reducing BDL- and CCl_4-induced liver fibrosis in mice. Fifteen days after BDL and transplantation of microencapsulated MSCs, a significant reduction of α-SMA expression was observed, compared to mice treated with encapsulated EDX cells or with empty microspheres. This confirmed the *in vitro* results where MSC-CM reduced α-SMA expression in HSCs. After 4 weeks of CCl_4 treatment, this effect was not observed, possibly because the peak of α-SMA expression had passed, making potential differences difficult to detect.

We found that microencapsulated MSCs increased endogenous IL-10 secretion, confirming that MSCs promoted anti-inflammatory/anti-fibrotic signals in the mouse liver fibrosis model. Our results are in line with another study on liver fibrosis in mice, where mouse bone marrow MSCs reduced α-SMA expression in the fibrotic liver [10]. A direct cellular contact of MSCs with immune or inflammatory cells or liver cells was prevented by microencapsulation procedure in our experimental design, indicating that factors secreted by MSCs are responsible for the inhibitory effect on HSC activation.

We observed a reduced collagen deposition in the liver of mice treated with microencapsulated MSCs compared to mice treated with microencapsulated EDX cells or empty microspheres; this is in line with previous studies [10,11,42]. Further, reduced fibrosis could implicate matrix metalloproteinases, known to contribute to the matrix degradation during liver fibrosis and resolution of fibrosis [43]. The substantial increase of MMP-9 expression in the liver, induced by microencapsulated MSC, might represent a possible explanation for the reduced fibrosis in the mouse liver fibrosis model. Indeed, MMP-9 is overexpressed by lymphocytes, neutrophils, and Kupffer cells during chronic liver injury, to counteract massive extracellular matrix accumulation [43,44]; also, increased levels of MMP-9 have been associated with diminished liver fibrosis in rodents [12,45] or cardiac ventricular fibrosis [46].

The microencapsulation of human MSCs allows the study of the effect of human MSCs instead of murine MSCs, because the microsphere provides protection against the xeno-immune reaction. We sought to specifically analyze human MSC effects with the aim of applying our findings to clinical research. This is relevant because murine MSC isolation is challenging and homogenous populations showing similar properties compared to human MSCs are difficult to obtain, rendering the comparison between these two cell types difficult [47–50]. An alternative approach is to use immunodeficient mice unable to mount a xeno-immune reaction, but liver fibrosis may develop inadequately in such animals [51].

A clear advantageous observation in the present study was that microencapsulated human MSCs in immunocompetent mice induced minor inflammation, indicating that the observed effects on the liver are not likely to be due to a bystander effect of an activated immune system or inflammatory reaction. In order to exclude such an effect, we transplanted microencapsulated EDX cells as a control group. EDX cells had a marginal effect on fibrosis development in the BDL model; an intermediate effect was observed in the CCl_4 model. Albeit to a lesser extent compared to MSCs, fibrosis and transaminases were reduced, following encapsulated EDX cell transplantation. Of note, EDX had no effect on MMP-9 in either model. The effect of EDX cells might be

explained by their basal secretion of soluble molecules (Supplementary Fig. 4) that could be responsible for an anti-inflammatory effect [52]. Moreover, the differences between the two models may be explained by the fact that liver fibrosis, induced by the subcutaneous injection of CCl_4, is less severe and of different nature (mainly centrilobular *vs.* periportal) compared to BLD-induced fibrosis. This could explain why EDX cells have an intermediate effect in the CCl_4 model and a marginal effect in the BDL model. Nevertheless, a bystander effect caused by xenogeneic MSCs or EDX cannot be totally ruled out. Overall, the fact that MSCs have a stronger effect compared to EDX suggests that the anti-fibrotic effect is likely to be due to the secreted factors and not to a "deviation" of inflammation to the peritoneal cavity.

As stated above, MSCs are investigated in several clinical trials to treat end-stage liver diseases caused by hepatitis B, C or alcoholic hepatitis [5,30,31]. Overall, evidence for efficacy in most of these clinical studies is quite poor, and there were few indications of a safety concern. Factors contributing to a low efficacy might be that the cells are often poorly characterized; and improvements are claimed where there are insufficiently powered experimental/control groups or lack of randomization to make this claim. Based on the present experimental animal study, we suggest to test clinically microencapsulated MSCs for liver diseases, such as the group of patients with alcoholic steatohepatitis and underlying cirrhosis that do not respond to a short-term course of steroids. For these patients, no therapy is currently available and the mortality is over 50% at 6 months; essentially, there is an unmet medical need for alternative therapeutic options [53]. Microencapsulated MSCs might provide anti-inflammatory effects and prevent progression to liver failure.

In conclusion, we here show that secreted factors from human bone marrow-derived MSCs have a direct effect on HSC activation *in vitro*. We further demonstrate that microencapsulated MSCs show anti-fibrotic and anti-inflammatory effects in BDL-induced liver fibrosis in mice and that soluble factors secreted by the microencapsulated cells have a key role in this effect. Potential mechanisms may include increased secretion and activity of MMP-9, an anti-apoptotic effect mediated by IL-6 or IGFBP-2, and inhibition of inflammation by IL-1Ra.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

RPHM, RM, CW, CG, and LHB conceived and designed the experiments. RPHM, RM, JM, EM, and CG performed the experiments. RPHM, RM, JM, EM, YDM, CW, CG, and LHB analyzed the data. PM, PC, CW, CG, and LHB contributed reagents/materials/analysis tools. RPHM, RM, PM, JM, EM, YDM, PC, CW, CG, and LHB contributed to the writing of the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.10.030.

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