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Adipose-derived mesenchymal stem cells slow disease progression of acute-on-chronic liver failure

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ABSTRACT

A serious complication of chronic hepatic insufficiency is acute-on-chronic liver failure, a recognized syndrome characterized by acute decompensation of cirrhosis and organ/system failure. We investigated the use of adipose-derived mesenchymal stem cells (AD-MSCs) in an experimental model of acute-on-chronic liver failure, developed by microsurgical extrahepatic cholestasis in rats. Rats undergoing microsurgical extrahepatic cholestasis were treated by intraparenchymal liver injection of human or rat AD-MSCs, undifferentiated or previously differentiated *in vitro* toward the hepatocyte lineage. The groups treated with rat AD-MSCs showed less ascites, lower hepato- and splenomegaly, less testicular atrophy, and an improvement in serum biochemical hepatic parameters. There was also an improvement in histological liver changes, in which the area of fibrosis and bile duct proliferation were significantly decreased in the group treated with predifferentiated rat AD-MSCs. In conclusion, an isograft of hepatocyte-predifferentiated AD-MSCs injected intraparenchymally 2 weeks after microsurgery in extrahepatic cholestatic rats prevents secondary complications of acute-on-chronic hepatic failure. These data support the potential use of autologous AD-MSCs in the treatment of human cholestasis, and specifically of newborn biliary atresia, which could be beneficial for patients awaiting transplant.
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1. Introduction

A serious complication of chronic hepatic insufficiency is acute-on-chronic liver failure, a recognized syndrome characterized by acute decompensation of cirrhosis, organ/system failure and extremely poor survival [1,2], requiring early liver transplantation [3,4]. Because the number of donors is limited, new approaches are needed in order to extend the time frame for transplantation [5].

Significant progress has been made in treating liver diseases by taking advantage of the immunomodulation and regenerative properties of mesenchymal stem cells (MSCs) [6]; for a review see [7]. Adipose-derived mesenchymal stem cells (AD-MSCs) are an attractive source, given elective liposuction yields high quantities of MSCs with local anesthesia [8].

Several animal models of both acute and chronic cirrhosis and treatment with MSCs and pluripotent stem cells have shown benefits [9–12]; however, these models fail to mimic acute-on-chronic liver disease with early ascites and death. MSCs improve liver function in the treatment of patients with chronic liver cirrhosis due to various causes [13–18]; other studies, however, have shown no benefit [19]. It is not clear whether MSCs diminish or contribute to fibrogenesis in the liver, and whether this process is dependent on the route and time frame of administration [20–22]; thus, more research is needed before MSC therapy as a mainstream treatment for liver failure can be established (for a concise review see [6]).

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2. Materials and methods

2.1. Experimental animals

A total of 239 male Wistar rats weighing 200–400 g were used. All the procedures were approved by the La Paz University Hospital Animal Welfare Committee and were in accordance with ethical guidelines from the European Community Council Directive (86/609/EEC).

2.2. Experimental design

For the preliminary studies, 84 rats were divided into 5 groups: a cholestatic control group that was euthanized 2 weeks after surgery to establish that the surgery had produced cholestasis ($n = 8$, results not shown); and 4 groups of cholestatic rats treated with predifferentiated human hepatocyte AD-MSCs or vehicle and euthanized at 5 weeks ($n = 15$ in each group) or 8 weeks ($n = 16$ for vehicle-treated rats and $n = 30$ for cell-treated rats) to determine the progress of the disease at those times.

For the formal studies, 155 rats were divided into 5 groups: a sham group ($n = 20$) that underwent only a laparotomy; a control group ($n = 20$) that underwent a microsurgical cholestasis and was administered intrahepatic vehicle injection 2 weeks after the procedure; and 3 experimental groups: the undifferentiated rat AD-MSC-treated group ($n = 47$); the predifferentiated hepatocyte rat AD-MSC-treated group ($n = 30$); and the predifferentiated hepatocyte human AD-MSC-treated group ($n = 38$).

2.3. Surgical procedure

The rats were anesthetized with 2% isoflurane (TEC 4, Ohemeda). In the sham group, the bile duct and its lobular branches were dissected without resection. In the remaining groups, an extrahepatic biliary tract resection was performed as previously described [23,24].

The rats were administered with buprenorphine (0.05 mg/kg/12 h) for the first 24 h after the procedure, and ceftazidime (50 mg/kg, twice a week) and vitamin K1 (phytomenadione) (8 mg/kg, once a week) throughout the postoperative evolution.

The groups of rats that were administered human cells were given the immunosuppressor tacrolimus (0.05 mg/kg intramuscular, 3 times a week) from 24 h prior to the injection of the stem cells until euthanasia.

2.4. Preparation of the adipose-derived mesenchymal stem cells

2.4.1. Isolation and culture of human and rat processed lipoaspirate-derived cells

Lipoaspirate from 6 female donor patients who provided informed written consent and underwent elective liposuction was obtained by a plastic surgeon. The isolation protocols were approved by the Institutional Review Board of La Paz University Hospital (Madrid, Spain) and were performed in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The lipoaspirates obtained were processed as reported by Zuk et al. [25].

Mesenchymal stem cell marker characterization was performed by fluorescence-activated cell sorting analysis with anti-CD34, CD45, CD90, and CD105 [26–28]. Functional characterization was performed by *in vitro* differentiation into chondrogenic, adipogenic, and osteogenic lineages, as in [25,27,28] (not shown).

For the isolation of rat AD-MSCs, 3 Wistar rats were euthanized under anesthesia by intracardiac injection of potassium chloride. The adipose tissue obtained from the retroperitoneal fat pads was processed and cultured as reported for human AD-MSCs.

2.4.2. In vitro *predifferentiation of AD-MSCs into hepatocytes*

The AD-MSCs isolated from human adipose tissue were predifferentiated into hepatocytes, according to the technique described by Seo et al. [29]. For predifferentiation of rat cells, they were grown for 10 days on collagen (0.1%) with rat hepatogenic medium: Dulbecco's Modified Eagle Medium-low glucose (Gibco); 0.5% fetal bovine serum; 10^{-9} M dexamethasone (Sigma); 20 ng/ml recombinant human hepatocyte growth factor (R&D Systems); 10 ng/ml fibroblast growth factor 4 (FGF-4, R&D Systems); 1% insulin-transferrin-selenium; 1% GlutaMAX; and 1% penicillin/streptomycin.

After 10 days, differentiation toward the hepatocyte lineage was confirmed by albumin immunocytochemistry (Dako), as described by [27].

2.5. Cell transplantation into the liver

Twenty-four hours or 2 weeks after microsurgical cholestasis, the rats were transplanted with cells or administered with vehicle. Before their transplantation, the AD-MSC intracellular membranes were labeled with the dialkylcarbocyanine fluorescent solution chloromethyl-benzamide (Vybrant CM-DiI, Molecular Probes) (1:200) for 10 min at 37 °C.

In the preliminary studies, rats were injected once with 4×10^6 cells in a volume of 1 ml Hanks' balanced salt solution (HBSS) (Gibco) distributed in the parenchyma of the middle hepatic lobe, the right lateral hepatic lobe, and the left lateral hepatic lobe; n was 15 rats per group.

In the formal studies, the control group was injected only in the middle hepatic lobe with 300 μl of vehicle (HBSS) once. The other 3 groups were injected once with 2.5×10^6 AD-MSCs in the same fashion. The n-value was variable depending on the survival of each group in order to obtain end-point data for statistical comparison: 20 rats in the sham group, 20 rats in the vehicle injected group, 38 rats in the predifferentiated human cell injected group, 47 rats in the undifferentiated rat cell group, and 30 rats in the predifferentiated rat cell group.

The animals were euthanized by anesthetic overdose.

2.6. Analysis of liver disease progression

2.6.1. Rat-adapted MELD score

The Model for End-Stage Liver Disease (MELD) is a scale system that scores the severity of liver disease and gives a prediction of the probability of death in patients [30]. MELD is routinely used in human clinics, and it was developed to predict death in order to prioritize patients for hepatic transplant. A higher MELD score indicates higher probability of death. For rats, the MELD was adapted based on the following formula: Rat adapted MELD Score = 9.57 ln creatinine (Cr) + 3.78 ln (bilirubin) + 11.2 ln international normalized ratio (INR) + 6.43 [30]. The n-value was 4 rats in the sham group, 12 in the vehicle group, 12 in the human cell group, 19 in the undifferentiated rat cell group, and 16 in the predifferentiated rat cell group.

2.6.2. Portosystemic collateral circulation

Portosystemic collateral circulation was studied by the presence of increased collateral veins [31].

2.6.3. Serum biochemical tests

Blood levels of hepatobiliary metabolites: total and direct bilirubin (TB and DB), alkaline phosphatase (AP), gamma glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total proteins (TP) and albumin, urea and Cr, INR, prothrombin time (PTT), activated prothrombin time (APTT), fibrinogen, and cephalin time (Tceph) were determined by

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