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Enhanced hepatic differentiation of mesenchymal stem cells after pretreatment with injured liver tissue

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ABSTRACT

Liver failure represents a serious challenge for cell based therapies. Mesenchymal stem cells (MSCs) possess potential for regeneration of fibrotic liver; however, there is a dire need to improve their hepatic differentiation. This study examines a pretreatment strategy to augment the differentiation potential of MSCs towards hepatic lineage. MSCs were isolated from C57BL/6 wild type mice and were characterized by flow cytometry for CD44 (92.4%), CD90 (96.6%), CD105 (94.7%), CD45 (0.8%) and CD34 (1.4%) markers. To improve the differentiation potential of MSCs towards hepatic lineage, cells were pretreated with injured liver tissue in an in-vitro model, which resulted in high expression of albumin, cytokeratin 8, 18, TAT and HNF1α as compared to untreated MSCs. The efficacy of pretreated MSCs was evaluated by preparing in-vivo mouse model with liver fibrosis by intraperitoneal administration of CCl_4. Pretreated MSCs were transplanted in the left lateral lobe of mice with liver fibrosis and showed enhanced localization and differentiation abilities after 1 month. The expression for cytokeratin 8, 18, albumin and Bcl-xl was up-regulated and that of HGF, Bax and Caspase- 3 was down-regulated in animals transplanted with pretreated MSCs. Sirus red staining also confirmed a significant reduction in the fibrotic area in liver tissue transplanted with pretreated MSCs as compared to untreated MSCs and was concomitant with improved serum levels of bilirubin and alkaline phosphatase (ALP). Therefore, it was concluded that pretreatment with injured liver tissue augment homing and hepatic differentiation abilities of MSCs and provides an improved procedure for the treatment of liver fibrosis.

1. Introduction

Liver is one of the few organs of the body with immense regeneration potential that can often complicate prognosis of disease. The only suitable therapeutic option available for patients suffering from advanced or irreversible liver disease is liver transplantation (Dai et al., 2009). However, limited number of donors restricts the availability of this therapeutic option to most patients. As a result, over 10% of patients die while waiting for liver transplants (Freeman et al., 2008), meriting the urgent need for novel alternatives to treat patients with liver diseases.

Mesenchymal stem cells (MSCs) from bone marrow have gained significance because of their immense plasticity and easy availability (Jiang et al., 2002). Recent studies conducted by our group and others have demonstrated the therapeutic potency of MSCs for numerous disorders such as hearing (Sharif et al., 2007), myocardial infarction (Khan et al., 2009a), renal failure (Morigi et al., 2008), stroke (Hayase et al., 2009), neuronal defects (Keilhoff et al., 2006) and bone defects (Granero-Moltó et al., 2009). Liver diseases are also possible targets for stem cell therapy with the aim to reduce fibrosis induced cirrhosis and liver failure (Wynn, 2008). Several studies have suggested the ability of MSCs to differentiate towards hepatic lineages (Schwartz et al., 2002; Sato et al., 2005). Furthermore, it has been demonstrated that MSCs have a potential cytoprotective effect following transplantation mediated by the production of fibronectin (Kao et al., 2007).

More recently, it has been shown that MSCs can inhibit hepato-cellular death, augment liver regeneration and eventually improve survival in animals with hepatic failure (van Poll et al., 2008; Kuo et al., 2008; Oh et al., 2007). Improvement of survival, proliferation and differentiation of MSCs after transplantation in a fibrotic liver has emerged to be critical and studies have been conducted to show augmentation of liver repair by pretreatment with various agents including growth factors (Oyagi et al., 2006; Huh et al., 2004). Therefore, in the present study it was proposed and proved that pretreatment of MSCs with injured liver tissue augments their differentiation potential towards hepatic lineages.

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Furthermore, it was shown that the pretreated MSCs can also improve liver function and reduce liver fibrosis.

2. Materials and methods

2.1. Animals

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). All animals were treated according to procedures approved by the Institutional Review Board (IRB) at the National Center of Excellence in Molecular Biology, Lahore, Pakistan.

2.2. Cell isolation and culture

Mesenchymal stem cells (MSCs) were isolated according to the procedure described previously in our study (Khan et al., 2009b). MSCs were grown till second passage and were double labeled with PKH-26 for the cytoplasm according to the manufacturer's instructions (Sigma Aldrich, USA) and with 4-6-diamidino-2-phenylindole (DAPI) for the nuclei (Sigma Aldrich, USA).

2.3. Flow cytometry

MSCs were cultured till second passage and examined using flow cytometry. For this purpose, cells were trypsinized and incubated with antibodies CD44PE, CD90FITC, CD105FITC, CD45FITC and CD34PE (BD Biosciences, USA). The specific fluorescence of 10,000 cells was analyzed on FACScalibur (Becton Dickinson, USA) using Cell Quest Pro software.

2.4. In vitro co-culture model

Livers were harvested from mice (2 months old, $n=8$) injected intraperitonealy with carbon tetrachloride (CCl_4) (1 ml/Kg CCl_4 dissolved in olive oil in 1:1 ratio) single dose, 1 day prior to co-culturing. MSCs from normal animals were cultured till the second passage and then 1×10^4 cells/well were plated in a 6-well plate (Corning Inc, USA). Livers from CCl_4 treated and non-treated animals were weighed and 60 mg piece of liver was placed on transwell culture inserts (Millipore, USA) within wells containing MSCs. Animals without CCl_4 treatment were used as controls ($n=8$).

2.5. Gene expression profiling of MSCs

RNA was extracted from both groups of MSCs (normal and CCl_4 treated liver) 2 weeks after treatment using trizol reagent (Invitrogen, Inc. USA). cDNA synthesis was carried out from 1 μg of RNA sample using M-MLV reverse transcriptase (Invitrogen, Inc. USA).

For analysis of gene expression in MSCs after various treatments, real time RT-PCR was carried out using SYBR Green PCR Super Mix (BioRad Lab, CA, USA), 8 μM of each primer and 100–500 ng/μl of template cDNA on BioRad System iQ5. The relative ratio and standard deviation between the normal and treated samples were calculated using the comparative C_τ method ($\Delta\Delta$ C_τ value), as recommended by the BioRad iQ5system. The expression of albumin, cytokeratin 8, 18, TAT, HNF1α was estimated. B-actin was used as internal control.

Analysis of gene expression (albumin, cytokeratin 8, 18, HGF, Bax, Bcl-xl, capsase 3) in livers transplanted with pretreated MSCs was carried out by RT-PCR. All primer sequences have been mentioned in Table 1.

Table 1
Primer sequences.

Gene	Product size (bp)	Primer sequence
Albumin (F)	196	GCTGTAGTGGATCCCTGGTG
Albumin (R)		GCTGTAGCCTTGGGCTTG
Cyt-18 (F)	118	CACACTCACGGAGCTGAGAC
Cyt-18 (R)		GCCAGCTCTGACTCCAGATG
Cyt-8 (F)	232	CTCACTAGCCCTGGCTTCAG
Cyt-8 (R)		ACAGCTGTCTCCCCGTGA
Cyt-19 (F)	157	GCCTGGCTGCAGATGACT
Cyt-19 (R)		AGCTCCTCCTTCAGGCTCTC
HGF (F)	167	TCACACAGAATCAGGCAAGACT
HGF (R)		AAGGGGTGTCAGGGTCAA
Caspase-3 (F)	220	TGTCATCTCGCTCTGGTACG
Caspase-3 (R)		AAATGACCCCTTCATCACCA
Bax (F)	152	TGGAGATGAACTGGACAGCA
Bax (R)		CAAAGTAGAAGAGGGCAACCAC
Bcl-xl (F)	150	TTCGGGATGGAGTAAACTGG
Bcl-xl (R)		AAGGCTCTAGGTGGTCATTCAG
GAPDH (F)	372	CTCTTGCTCTCAGTATCCTTG
GAPDH (R)		GCTCACTGGCATGGCCTTCCG
TAT (F)	185	TGCGGTGCAAATAAATACCA
TAT (R)		GACAGCAAAAGTGGCAATGA
HNF-1 (F)	179	CTAGTGTAGGTCTTGGGACACG
HNF-1 (R)		CTCCTCTCCCCACATTTGTC

2.6. Periodic acid schiff staining

Periodic acid Schiff (PAS) (Sigma Aldrich, Germany) staining was done as described by the manufacturer's protocol on MSCs from both groups (normal and CCl_4 treated liver) to demonstrate hepatocyte like function.

2.7. Preparation of liver fibrosis model and cell transplantation

Liver fibrosis was produced in female wild type C57BL/6 (2 months old) mice by intraperitoneal injection of CCl_4 as described previously (Sakaida et al., 2004). Animals were divided into four groups ($n=10$) each. Group I includes normal mice injected with saline and Group II (control) includes CCl_4 treated mice injected with saline. Groups III and IV represent CCl_4 treated mice transplanted with untreated MSCs and MSCs pretreated with injured liver tissue, respectively. MSCs were transplanted at two different points in the left lateral lobe of the liver directly at a concentration of 1×10^6 cells/100 μl/animal.

2.8. Assessment of liver function

Blood samples were taken from all experimental groups at the start of the treatment (day 0) and after 15 and 30 days of treatment. Serum was isolated and the amount of bilirubin (Diazyme Europe, Gmbh) and alkaline phosphatase (Bioassay System, USA) was estimated using commercial kits according to the manufacturer's protocol. PAS staining was done on liver sections as described by the manufacturer's protocol; three different slides from each animal in a group were used to estimate the hepatocyte like function of liver cells.

2.9. Measurement of liver fibrosis

Fixed livers were embedded in paraffin and sections were cut from different lobes of the liver. Sections were stained with sirius red stain (Moriya et al., 2008). Images of the fibrotic area of each