In vivo hepatic differentiation potential of human cord blood-derived mesenchymal stem cells

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Abstract. Although recent studies have demonstrated the *in vitro* hepatic differentiation potential of mesenchymal stem cells (MSCs), the evidence supporting the *in vivo* engraftment of MSCs, hepatic differentiation and improvement of hepatic function is still lacking. We investigated *in vivo* hepatic differentiation potential and therapeutic effect of cord blood derived-MSCs (CBMSCs) transplantation in a cirrhotic rat model. CBMSCs ($2x10^6$) were infused in Wistar rats with thioacetamide-induced chronic liver injury. Biochemical markers, liver fibrosis and engraftment of CBMSCs were assessed. Infused CBMSCs were detected in the perivascular or fibrous region of the liver and did not acquire mature hepatic phenotypes. There was no difference in biochemical markers and in the area of liver fibrosis between the experimental and placebo groups. After infusion of CBMSCs in our experimental cirrhotic rat model we did not observe an improvement of liver function and liver fibrosis. Inversely, CBMSCs could have a pro-fibrogenic potential suggesting that a cautious approach is required in future research.

Introduction

Liver transplantation is currently the only successful treatment for acute hepatic failure or end stage liver disease. However, several problems are associated with liver transplantation: lack of donors, surgical complications, rejection, and high cost. Hepatocyte transplantation has been the alternative option for bridging patients to liver transplantation. But, primary hepatocytes are only available from a restricted number of donor organs, usually livers not allocated for organ transplantation (1). Also primary hepatocytes have a poor proliferative potential, which probably does not suffice to effectively repopulate the host liver and are difficult to maintain in an *in vitro* culture without loss of functions (2).

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Several studies have shown that hematopoietic stem cells from the bone marrow have the capacity to differentiate into a variety of non-hematopoietic cells such as liver, heart, and brain (3-6). Even multi-organ differentiation has been demonstrated from hematopoietic stem cells and from multipotent adult progenitor cells (7,8). However, hematopoietic stem cells contribute little to hepatocyte formation under either physiological or pathological conditions, although they provide cytokines and growth factors that promote hepatocyte functions by paracrine mechanisms (9).

The other potential candidate stem cells for therapy of an injured liver are mesenchymal stem cells (MSCs), which could be obtained from different sources such as the bone marrow, cord blood, amniotic fluid, placenta, or adipose tissue (10-14). Among the sources of MSCs, cord blood has a number of advantages in cell procurement, such as abundance, lack of donor attrition, and low risk of viral transmission such as transmission of herpes family viruses. Furthermore, stem cells in this neonatal blood are less mature than in other adult cells so that they do not produce a strong immune rejection. For these reasons, cord blood could be a prominent source of cells for transplantation in various diseases.

The hepatocyte differentiation potential of MSCs has been described (15-17). After *in vitro* stimulation with hepatocyte growth factor (HGF), dexamethasone, and oncostatin M (OSM), MSCs could differentiate into hepatocyte-like cells. Administration of MSCs from various sources has been shown to improve liver injury in a chronic model of liver cirrhosis (18,19). However, unequivocal evidence supporting the *in vivo* ability of transplanted human MSCs to enter liver parenchyma by circulation and to acquire markers of hepatocyte-like differentiation is still lacking (20).

The purpose of this study was to evaluate the *in vivo* hepatic differentiation potential of umbilical cord blood-derived MSCs (CBMSCs) and to assess whether CBMSCs are capable of reducing liver cirrhosis and improving hepatic function in a rat model of chronic liver injury.

Materials and methods

Isolation of CBMSCs. Cord blood samples from full-term deliveries were collected and mononuclear cells (MNCs) were separated within 6 h of collection using density gradient centrifugation. MNCs were washed three times with phosphate-buffered saline (PBS) and were set in culture at a

Table I. Biochemical markers of liver function in the control and experimental groups 1 and 4 weeks post-transplantation.

	1 week		4 weeks	
	Control group (n=6)	Experimental group (n=10)	Control group (n=6)	Experimental group (n=8)
Ammonia (μmol/l)	107±20	114±41	112±22	128±34
Total protein (g/dl)	5.9±0.3	6±0.3	6±0.3	6±0.3
Albumin (g/dl)	2.5±0.2	2.6±0.1	2.6±0.2	2.6±0.1
Total bilirubin (mg/dl)	1.1±0.2	1±0.2	0.6±0.4	0.6±0.3
AST (IU/l)	100±29	94±16	85±8	104±13
ALT (IU/l)	52±18	44±9	70±14	71±17

density of $3x10^6$ cells/cm^2 into fibronectin (Sigma-Aldrich, St. Louis, MO) coated 6-well plates (Nunc, Rochester, NY) in endothelial growth medium-2 (EGM-2, Gibco, Grand Island, NY) or MSCGM medium (Cell Systems, St. Katharinen, Germany). Media were changed every 3 days. After colony formation between days 14 and 20, cells were harvested using 0.1% trypsin-EDTA solution (Gibco) and cultured thereafter in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS).

In vivo transplantation of CBMSCs. Six-week-old female Wistar rats (Oriental Bio, Seongnam, Korea) were used. All animal procedures were approved by the Institutional Animal Care and Use Committee. Chronic liver injury was induced by intraperitoneal administration of thioacetamide (TAA, Sigma-Aldrich), 300 mg/kg twice weekly for 6 weeks, as previously described (21). After 6 weeks of TAA injection, the rats were randomly divided into 2 groups: (i) a DMEM infused control group (n=12), and (ii) a CBMSC infused experimental group (n=18). In the experimental group, $2x10^6$ CBMSCs suspended in 400 μl of DMEM were injected into the tail vein. All rats of both groups were further administered an intraperitoneal injection of TAA at the same dose twice weekly until sacrificed. All rats were immunosuppressed with a daily intraperitoneal injection of 10 mg/kg/day of cyclosporine A (Cipol; Chong Kun Dang, Seoul, Korea), from 24 h before the transplantation to the day of sacrifice. Liver tissues and blood samples were obtained after 1 or 4 weeks of transplantation, after the animals were sacrificed.

Immunohistochemical analysis. Liver tissues were fixed in buffered formalin, embedded in paraffin. Liver sections (2 μm) were deparaffinized and rehydrated. Antigen retrieval was performed by incubating in boiling water containing 10 mM sodium citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide for 30 min. Non-specific staining was eliminated by an additional blocking step with 1% bovine serum albumin and 0.1% Tween-20 in phosphate buffer for 1 h at 37˚C. Rehydrated slides were then incubated overnight at 4˚C with primary antibodies as follows: anti-HepPar1 (1:100, M7158; DakoCytomation, Heverlee, Belgium), anti-human cytokeratin (CK)-18 (1:100, M7010; DakoCytomation), anti-human CK-8 (1:100, MAB3414; Chemicon, Hants, England), anti-human mitochondria (1:50, MAB1273; Chemicon), and anti-human nuclei (1:50, MAB1281; Chemicon). After unbound primary antibodies were washed off, the immunoreactivity was visualized using an EnVision detection kit (DakoCytomation) and diaminobenzidine. Sections were counterstained with hematoxylin and mounted for analysis.

Biochemical analysis. Blood samples were centrifuged at 3,500 x g for 10 min and then plasma was immediately separated. The following parameters were measured on a Hitachi 7600 automated chemistry analyzer (Hitachi, Tokyo, Japan): ammonia, total protein, albumin, total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

Quantitative analysis of liver fibrosis. For the evaluation of liver fibrosis, picrosirius red staining was performed using 0.1% picrosirius red solution as previously described (22). The area of liver fibrosis stained with picrosirius red was quantified using ImageJ software (4 randomly selected areas per slide, 4 slides for each rat).

Statistical analysis. All results are expressed as mean ± standard deviation (SD). Statistical differences were assessed using the Mann-Whitney U test, where $p<0.05$ was considered statistically significant.

Results

In vivo hepatic differentiation of CBMSCs in the murine cirrhotic model. The engraftment and hepatic differentiation of CBMSCs in the liver of recipient rats were analyzed at 1 or 4 weeks after transplantation. Human hepatocytes (positive for anti-HepPar1 antibody) were very rarely detected as single cells located near portal veins. However, the HepPar1-positive cells did not stain for the human CK-18 and CK-8 antigen, as shown in human liver sections used as a positive control. (Fig. 1). These results do not amount to full evidence of the formation of mature human hepatocytes, but the lack of CK-18 and CK-8 could be explained by an immature phenotype of HepPar-1 positive cells or by cellular fusion that results in a cell type characterized by chimeric gene and protein expression. Tracing of human cells into recipient rat livers was detected by immunohistochemical detection of human

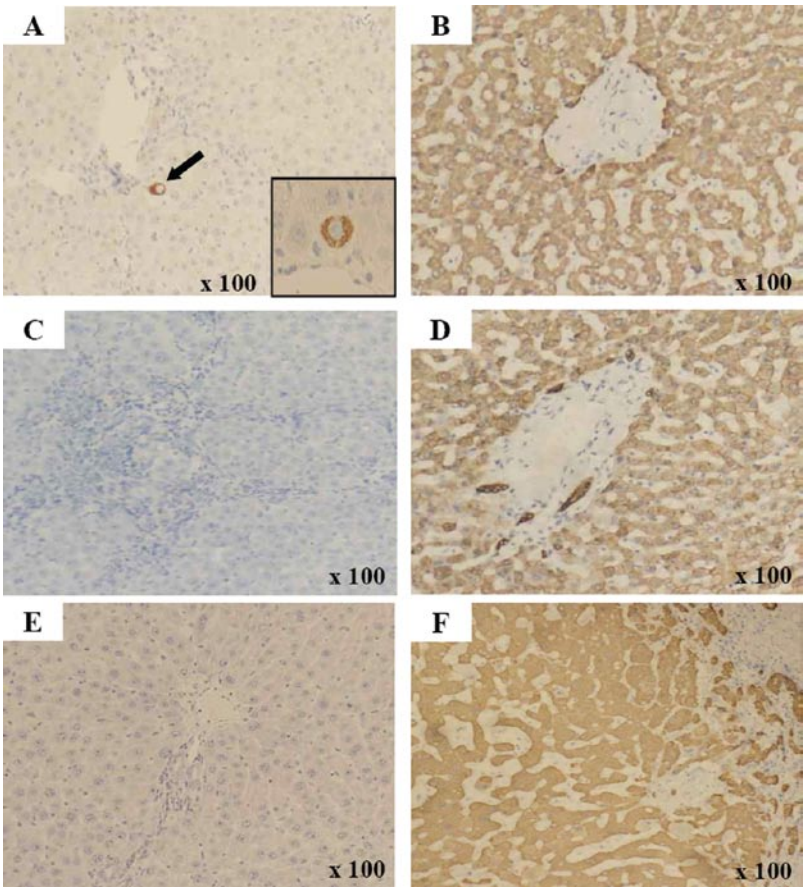

Figure 1. Representative figures showing minimal engraftment of cord blood derived-MSCs (CBMSCs) in the rat liver. (A) Infused CBMSCs were detected immunohistochemically by the expression of anti-human hepatocyte specific HepPar1 positive cells in the periportal area. There are no cells stained with anti-human CK18 (C) or anti-human CK8 (E) which detect human, but not rat, hepatocytes. Control human liver samples were stained with anti-HepPar 1 (B), anti-human CK18 (D), and anti-human CK8 (F) showing detection of the respective human antigens. Cells were counterstained with hematoxylin.

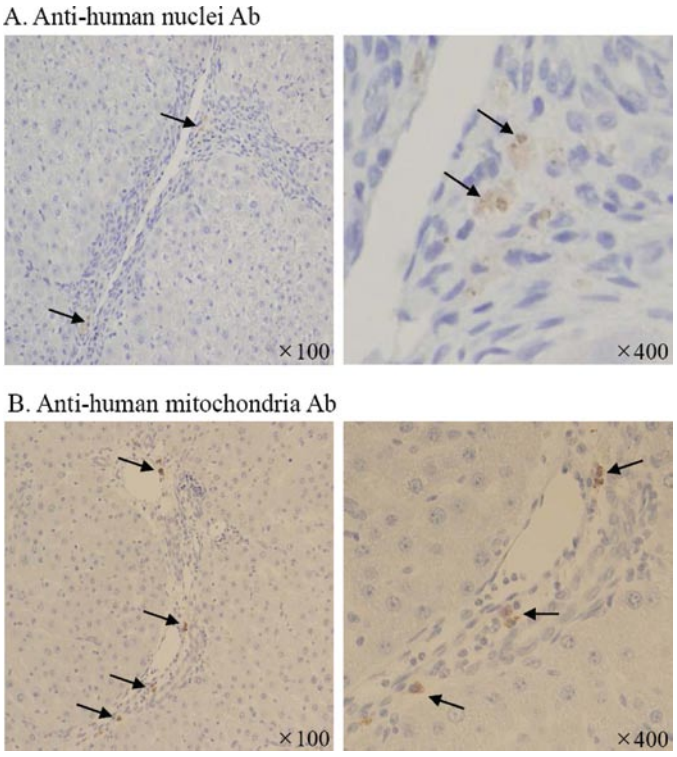

Figure 2. Detection of human-derived cells by immunohistochemistry of liver section with anti-human nuclei antibody (A) and anti-human mitochondria antibody (B). Cells were counterstained with hematoxylin.

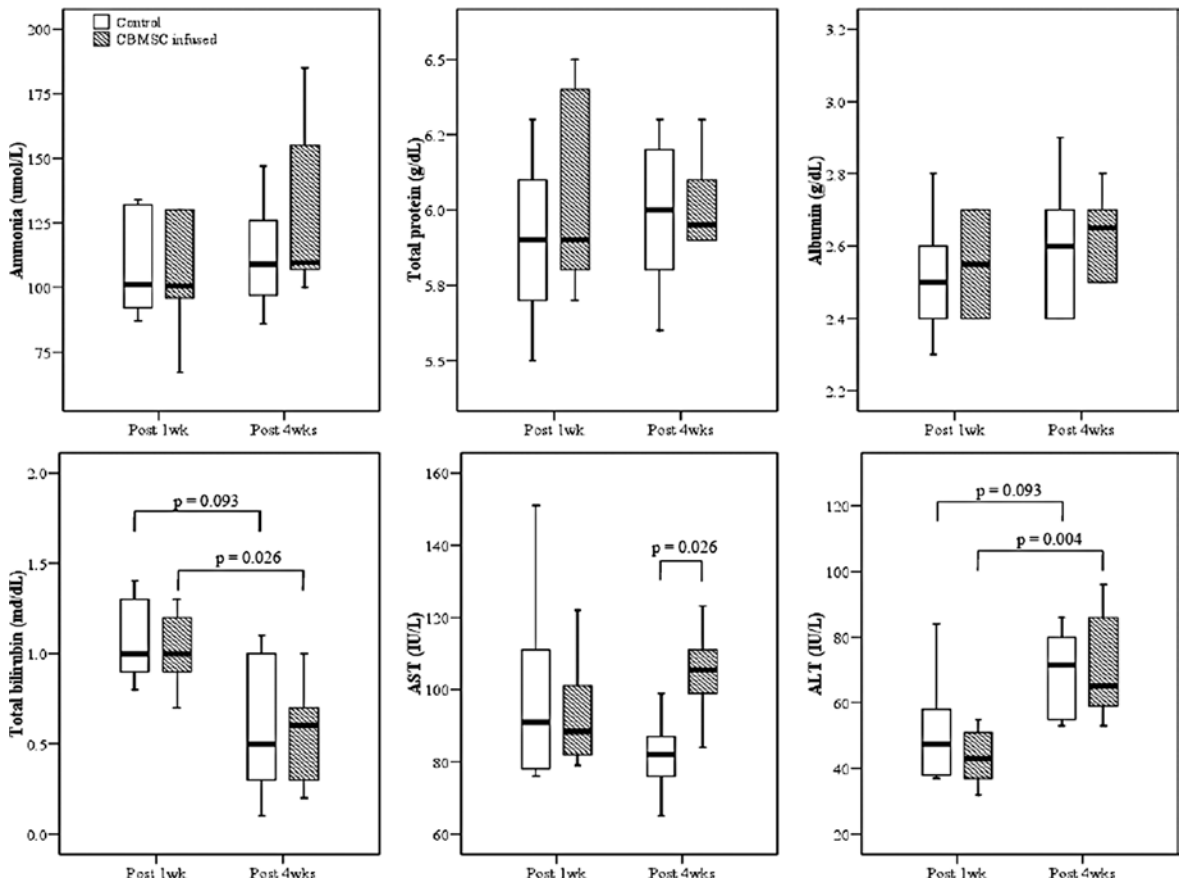

Figure 3. Biochemical markers of liver injury and function. Concentrations of ammonia, total protein, albumin, total bilirubin, AST and ALT in blood samples of the control and experimental groups. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

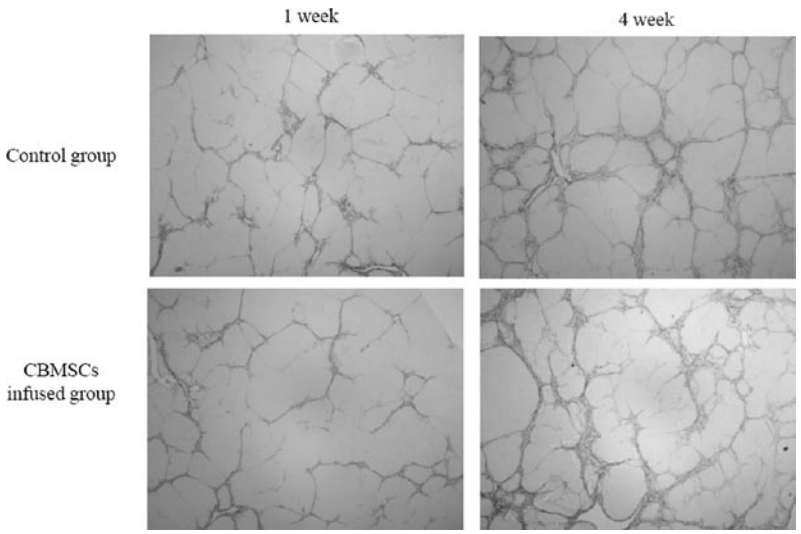

Figure 4. Fibrosis quantification in picrosirius red-stained field. Representative figures showing fibrosis area in the CBMSCs infused- and control groups after 1 or 4 weeks of transplantation. CBMSCs, cord blood derived-MSCs.

nuclei and human mitochondria. Some human CBMSCs with a fibroblast-like shape were incorporated in regions of the fibrous septal, perivascular and periportal areas (Fig. 2).

Alteration of liver function after transplantation of CBMSCs. Serum markers related to hepatic function, such as ammonia, total protein, albumin, total bilirubin, AST and ALT were measured after 1 or 4 weeks of transplantation. There were no statistically significant differences between the control group and the experimental group in terms of their ammonia, total protein and albumin levels (Table I, Fig. 3). The concentration of total bilirubin was significantly decreased with TAA administration; on the contrary, the activity of ALT was significantly increased.

Table II. Quantitative measurement of liver fibrosis expressed as % collagen deposited area/total area.

	Control group		Experimental group	
1 week post-transplantation	2.05±1.37	(n=73)	2.25±2.01	(n=141)
4 weeks post-transplantation	3.26±2.76	(n=80)	3.58±2.24	(n=81)

n, number of examined fields.

In order to quantify the amount of fibrosis over time comparing the control and experimental group, picrosirius red stained areas, representing collagen deposition, were measured. After 1 week of transplantation, experimental group had 2.25±2.01% of collagen in the liver, compared to 2.05±1.37% of control group (Table II). These results are not significantly different. After 4 weeks of transplantation, the CBMSC infused group revealed a fibrosis area of 3.58±2.24%, compared to 3.26±2.76% in the control group. The area of liver fibrosis after 4 weeks of CBMSCs transplantation was significantly more than that in the control group (p=0.021, Fig. 4).

Discussion

The potential to differentiate into endodermal cells, such as hepatocytes has been investigated *in vitro* with human MSCs from the bone marrow (8,15,23), umbilical cord blood (23,24), and adipose tissue (19). Initially, Schwartz *et al* induced multipotent adult progenitor cells (MAPCs) into cells with morphological, phenotypical and functional characteristics of hepatocytes *in vitro* upon exposure to well-defined hepatogenic factors (8). However, these culture conditions yielded a mixture of epithelioid cells and other cell types. Therefore, attempts were made to improve the hepatic differentiation process through exposure to liver-specific factors in a sequential time-dependent manner (25) or via epigenetic modifications using histone deacetylase inhibitors, such as DMSO, sodium butyrate or trichostatin A.

Even though the differentiation potential of MSCs into hepatocytes *in vitro* has been demonstrated, there are pros and cons regarding the therapeutic efficacy of MSCs in acute or chronic liver injury. Sakaida *et al* (22) demonstrated a significant reduction of liver fibrosis and an increase of albumin concentration by transplanting mononuclear cells in mice. Similarly, Oyagi *et al* (26) have reported that MSCs cultured with HGF are capable of increasing albumin concentration and reducing fibrosis in rats, an effect that was not observed in MSCs cultured without HGF. On the other hand, in murine models involving bone marrow ablation, transplanted bone marrow cells were reported to engraft to the liver during chronic liver diseases and to significantly contribute to liver fibrosis by differentiating into pro-fibrogenic myofibroblast-like cells, with hepatocyte-like transdifferentiation again being a rare event (27).

In this study, *ex vivo* expanded CBMSCs were transplanted into TAA-injured rat to assess the hepatic differentiation potential of CBMSCs and therapeutic efficacy in a chronic liver injury model. Although the experimental animals were immunosuppressed by cyclosporin A, effective repopulation by differentiated CBMSCs was not achieved. This extremely low repopulation efficiency might be a consequence of the xenotransplantation, with the rat liver failing to provide the proper cell-cell or cell-matrix communications required for efficient integration of the human cells. In chronic liver injury, *in vivo* differentiation of intravenously transplanted CBMSCs into hepatocyte-like cells represents a relatively rare and quantitatively unsatisfactory rare event.

Along with the low efficiency of *in vivo* hepatic differentiation, liver function measured by blood parameters was not significantly improved. It is widely recognized that experimental models of liver fibrosis have a major problem: the degree of fibrosis in each animal is highly heterogeneous. Whereas some animals show intense extracellular matrix components deposition, others are only mildly affected. Therefore, the appropriate selection of experimental animals would be an important factor for future studies. In agreement with the biochemical results, we found that CBMSCs did not reduce liver fibrosis. Given the findings of this study, if CBMSCs could have a pro-fibrogenic potential, a possible outcome may be enhanced fibrosis of the liver, suggesting that a cautious approach is required.

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