Cell Therapy for the Treatment of Metabolic Liver Disease: An Update on the Umbilical Cord Derived Stem Cells Candidates

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Abstract: Cell therapy has emerged as an attractive alternative to orthotopic liver transplantation for the treatment of liver disease. Among the potential candidates, umbilical cord derived stem cells are of particular interest owing to greater proliferation potential and low immunoreactivity. Previous reports permit to distinguish different cell types that could be generated from cord blood, vessels and cord matrix itself. Wharton Jelly's derived umbilical cord stem cells and cord-blood derived mesenchymal stem cells have demonstrated a potential to differentiate into endodermal lineage, including hepatocyte-like cells. In addition, recent studies have underlined their potential to alleviate liver fibrosis and express liver metabolic functions in rodent models.

The present review focuses on the current knowledge on *in vitro* and *in vivo* use of these cells for liver cell therapy. We discuss the general characteristics homology between hepatic and umbilical cord derived stem cells and the results of hepatocyte-like differentiation attempts. We finally address the question of future application of these cells for the treatment of liver disease.

Keywords: Umbilical cord stem cells, liver regeneration, hepatocyte differentiation.

INTRODUCTION

Liver transplantation has long been the unique curative treatment for acute or chronic liver diseases. However, the shortage of donor organs and the invasiveness of the procedure in critically ill patients remain major limitations [1]. Recently, cell transplantation has offered a promising alternative approach for liver-based therapies [2-4]. Stem cells originating from various intra and extra-hepatic sources has been investigated for the treatment of hepatic diseases [5-15]. Stem cells are relatively easy to harvest, display an inherent ability to proliferate and demonstrate various differentiation potentials [16, 17]. Moreover, stem cells present immunotolerogenic features decreasing the risk for graft rejection [18]. Despite encouraging results, the technique is hampered by the restricted number of donors and invasive harvesting techniques. Furthermore, the stem cell yield, their differentiation capacity and *in vivo* repopulation potential decrease with aging [19]. Umbilical cord mesenchymal stem cells (UCMSC) are an attractive candidate for cell therapy because they are an unexhaustive cell source with a great proliferation potential [20-22]. In addition, because the umbilical cord is discarded at birth, their isolation raises few ethical concerns. These cells also express hepatocyte-like markers [10], which confer them a theoretical advantage for liver cell transplantation. *In vitro* data and animal studies on hepatocyte-like differentiation of UCMSC are very promising but the demonstration of liver specific functionality, a key task for regenerative medicine application, remains limited [10, 23, 24]. Further, the ability of UCMSC to repopulate the liver needs to be investigated.

In this review we discuss the general phenotype homology between hepatic and umbilical cord derived stem cells. Secondly, we evaluate the effect of various differentiation protocols on enhancing hepatocyte-like phenotype in UCMSC. Finally we will discuss the steps to be performed for future application of umbilical cord stem cells for the treatment of liver disease.

HEPATOCYTE-LIKE PHENOTYPE CHARACTERISTICS OF UCMSC

In response to various cytokines, the embryonic endoderm initiates its development toward a hepatic specification by generating the primary liver bud [25-27]. This tissue is mainly composed of liver precursors, the hepatoblasts. Early hepatoblasts progressively begin to express characteristic liver genes [28, 29] (such as albumin, alpha-fetoprotein and HNF-4) while undergoing important growth. From midgestation to perinatal life, hepatoblasts modulate the expression of specific genes to meet the specific needs of each developmental stage. Late hepatoblasts possess bipotential (hepatic and biliary) properties. The cells further differentiate and undergo metabolic and morphologic maturation to finally acquire mature hepatic function during infancy.

As a first proof of their endodermal commitment, UCMSC express some characteristic genes of the definitive endoderm phase (Table **1**) such as CXCR-4, Sox 17 and E-Cadherin. Furthermore, UCMSC express moderate basal mRNA and protein levels of albumin, alpha-foetoprotein, cytokeratin 19 and CD54 (I-CAM) with very low or negative

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1875-0435/11

levels of CD117 (c-kit), CD56 (N-CAM) and Cyp3A4. The combination of all these markers correlates well with the phenotype of early hepatoblasts. UCMSC also share more mature hepatic features such as cytokeratin 18 (CK18), glucose-6-phosphatase, alpha-1 antitrypsin, connexin-32, phosphoenolpyruvate carboxykinase (PEPCK) and tryptophan-2,3-dioxygenase (TDO). Despite an interesting phenotype, these cell characteristics have to be carefully analyzed, as they do not confer them the status of mature hepatocyte. In the perspective of regenerative medicine, commitment to hepatic lineage is not sufficient per se, as these cells have not yet demonstrated the ability to perform the various metabolic roles of the hepatocyte [41]. Indeed, neither cytochrome activity [10], nor bilirubin conjugation by uridine 5'-diphosphate-glucuronyltransferase could be detected in UCMSC [42]. Moreover, some of the above mentioned genes are not liver specific and the extent to which some of them are expressed by the liver alone is not clear. Finally, these cells also display mesodermal and ectodermal characteristics possibly associated with many other lineage differentiation potentials [22].

Taken together, UCMSC constitutively express some early stage or more mature hepatic markers and functions, giving them a theoretical advantage for liver tissue repair. Furthermore, their hepatic commitment could potentially be enhanced by *in vitro* or *in vivo* differentiation in response to specific growth factors or host liver environment.

ENHANCING HEPATIC COMMITMENT OF UCMSC AFTER *IN VITRO* DIFFERENTIATION

Differentiation Protocols

Hepatogenic differentiation protocols are performed using a cocktail of factors/cytokines known to be involved in the successive developmental stages leading to mature hepatocytes. To this day, no consensus has been put in place in the literature regarding the protocol that should be used to generate hepatocyte-like cells. The current majority of protocols are performed using fibrosblast growth factor (FGF) and hepatic growth factor (HGF) in early steps. During early embryogenesis, FGF induces competent endoderm cells to an hepatic fate [43] while HGF stimulate hepatoblast proliferation [44]. Later stages utilize Oncostatin M (OSM), which induces, via a STAT-3 mediated signaling cascade, terminal hepatocyte maturation [45]. It remains a matter of debate whether cells should be preferentially differentiated into mature hepatocytes or hepatic progenitors [46]. Indeed, these latter better resist transplantation and

Table 1. Expression of Developmental Hepatic Lineage Markers in UCMSC and Hepatocyte-like Differentiated UCMSC

	UCMSC	Differentiated UCMSC	Hepatic Stem Cell	Hepatoblast	Mature Hepatocyte	References
CXCR-4	+	ND	ND	ND	+	[30]
Sox17	+	ND	ND	ND	+	[30]
E-cadherin	+	+	+	+	+	UD, [31]
Albumin	+	+	+	++	+++	[10, 23, 24, 28]
Cytokeratin 18	+	+	++	++	+++	[10, 23, 29, 32]
Cytokeratin 19	+	+	+++	++//-	-	[10, 24, 29, 33-35]
Alpha Fetoprotein	+	+	-	+++	-	[10, 23, 24, 35, 36]
I-Cam (CD54)	+/-	ND	-	++	+++	[10, 35, 37]
N-Cam (CD56)	-	ND	+++	-	-	[35, 38]
Cyp3A4	-	+	-	-	++	[10, 35]
Connexin-32	+	+	+	++	+++	[10, 29]
Alpha-1antitrypsin	+	+	ND	++	+++	[10, 29, 36]
Glucose-6-phosphatase	+	+	ND	++	+++	[10, 24]
TAT	-	+	ND	ND	+++	[10]
TDO	+/-	+	ND	+/-	++	[10, 24, 39]
PEPCK	+	+	-	+	++	[10, 29]
HNF-4	+/-	-	+++	++	++	[10, 29, 32, 36, 40]

CYP: cytochrome P450, HNF4: hepatic nuclear factor 4, ND: no data, PEPCK: phosphoenolpyruvate carboxykinase, TAT: tyrosine aminotransferase, TDO: tryptophan 2,3-dioxygenase, UD: personnal unpublished data. Hepatoblast early // late markers. +/- controversial data in litterature.

maintain a greater proliferation and repopulation capacity than fully mature differentiated cells.

Assessment of Hepatocyte Differentiation *in Vitro*

A critical point in using stem cells for liver cell therapy lies in the acquisition of a sufficient extent of hepatic differentiation to display mature metabolic functions required for effective liver replacement. Classical characterization of differentiated cells includes a comparison of morphological features and the evaluation of mRNA transcript or protein expression. UCMSC, incubated in a specific differentiation medium, display morphological changes evocative of the acquisition of hepatocyte-like features [10]. Spindle shaped UCMSC progressively become more polyhedric, but ultrastructural data is required to further confirm these changes. In most studies, the description of an acquired hepatocyte-like phenotype is based on a restricted panel of markers (Table **1**). The interpretation of data concerning the extent of hepatic differentiation is difficult using these markers, as authors sometimes observed a dissociation between mRNA or protein expression and maturation of related metabolic function [47]. More interesting -and mandatory- are the concomitant analysis of liver specific functional activities. Altogether, the results of different studies con-firmed that the acquisition of hepatocyte-like phenotype was only partial. Indeed, differentiated cells displayed some early or more mature hepatic markers/functions (such as Cyp3A4, UGT1 and TAT), but lacked expression of molecules such as HNF-4. Moreover, UCMSC maintained native mesenchymal markers, suggesting the persistence of a chimerical phenotype after differentiation.

IN VIVO USE OF UCMSC FOR THE TREATMENT OF LIVER DISEASES

In Vivo Hepatocyte Differentiation Potential of UCMSC

While *in vitro* testings may suggest an hepatocyte-like differentiation potential of UCMSC, the ultimate proof of this capacity relies on the demonstration that cells can repopulate and rescue the liver function in an animal model that mimics human liver disease. Studies analyzing the repopulation and differentiation capacity of UCMSC *in vivo* are scarce. To our knowledge, no study has used rodent models of metabolic liver failure (Gunn, Nagase Rats; FAH mice,…) to specifically asses the potential of UCMSC to restore liver function. Instead, studies used immunocompromised models (SCID) without liver injury or selective advantage for the injected cells (Table **2**). Finally, others used models with induced liver damage (CCl4, …). The data obtained using these xenogenic models would further benefit from being complemented by studies using humanized models or human subjects, since cell adhesion, cell-to-cell interactions and differentiation may vary between species [48].

Cell engraftment can be evaluated *in vivo* by different methods such as fluorescent in situ hybridization, immunostaining, Real Time PCR, etc. As all these techniques carry specific pitfalls, the use of combined methods is largely recommended for accurate evaluation. Thus far, engraftment rates remain low, below 5%, especially in models that do not provide space or selective advantage for eventual implantation of injected cells (unpublished data). These engraftment numbers seem in most cases insufficient to provide a definitive curative treatment. Different approaches can be considered to favor cell implantation. Regenerative stimulus is very efficient in animal models and clearly was associated with significant improved repopulation. However, the translation of such techniques to humans is difficult. Some methods have already been described in human, such as hepatic irradiation [49], partial hepatectomy or ischemia/reperfusion injury [50]. Finally, successful hepatic repopulation can be hampered by graft rejection. Recent studies underlined the ability of UCMSC to induce tolerance [51].

Effect of UCMSC on Liver Fibrosis

Liver fibrosis occurs in response to chronic liver damage induced by a variety of conditions including liver metabolic diseases and autoimmune or viral hepatitis. Chronic hepatic injury of any etiology leads to persistent activation of tissue repair mechanisms mediated by various cytokines and growth factors. Ultimately, this condition drives the progressive accumulation of extracellular matrix (ECM) components [52]. The propensity of MSC to alleviate fibrosis in injured liver has first been described in mice transplanted with bone marrow stem cells [53]. Likewise, rodents in which liver damage was established using CCl4 or TAA injections demonstrated reduced hepatic inflammation and ECM deposition after UCMSC transplantation [39, 54, 55]. Mechanisms mediating these effects are thought to imply the modulation by UCMSC of endogenous secreted factors such as metalloproteinases [56, 57]. These findings however remain controversial, as other authors could not find any effect on hepatic function when rodents were maintained alive for longer periods or in case of extended liver injury (association of hepatotoxic chemicals) [58]. In addition, studies have demonstrated that mesenchymal stem cells display a profibrogenic propensity [59]. Yan *et al.* [54] have also suggested this fact for UCMSC. Indeed, intravenously infused UCMSC contributed to the myofibroblast population (αSMA and fibroblast secretory protein-1 positive cells) in mice with CCl4 induced liver injury.

PERSPECTIVES AND CONCLUSIONS

Over the last few years, major advances have allowed a better understanding of the different steps and pathways involved in hepatogenesis. In parallel, a great amount of work has been performed in the field of stem cell therapy for regenerative medicine. Umbilical cord mesenchymal stem cells certainly represent a very attractive cell source for liver based treatments as they display several hepatic markers characterizing the sequential steps of liver development. In addition, their differentiation ability to hepatic lineage can be enhanced *in vivo* and *in vitro* after culture with hepatogenic factors. A better understanding of the temporal sequence of hepatic differentiation steps will permit the amelioration of *in vitro* differentiation protocols in the future.

Generating fully mature hepatocytes from stem cells may be an idealistic, extremely difficult task. The extent of hepatic maturation needed for effective cell transplantation greatly depends on the pathology we want to cure. Some conditions such as inborn errors of metabolism are related to

Table 2. Representative Studies Investigating *in Vitro* and/or *in Vivo* Hepatocyte-like Differentiation Ability of UCMSC

Protocol	*In vitro* characterization Before differentiation	After differentiation	*In vivo* characterization	
4 steps - 5 weeks IMDM supplemented with: step 1: EGF, FGF (2days) step 2: FGF, HGF, Nico, ITS (10days) step 3: HGF, OSM, Nico, ITS (10days) step 4: OSM, Dexa, ITS (10days)	RNA: (+) ALB, AFP, CX-32, A1AT, CK18, CK19, G6P, PEPCK, TDO (-) CYP, HNF4, TAT Protein: (IF) (+) ALB, AFP, CX-32, CK18, CK19, DPPIV Function: (+) G6P, Urea, Cyp3A4, Glycogen storage (-) ALB	RNA: (+) ALB, AFP, CX-32, A1AT, CK18, CK19, G6P, PEPCK, TDO, **CYP**, **TAT** (-) HNF4 Protein: (+) ALB, AFP, CX-32, CK18, CK19, DPPIV Function: (+) G6P, Urea, Cyp3A4, Glycogen storage (-) ALB	Host: SCID Injury: none Time: TRP: - intrasplenic - 10^6 UD cells Characterization: RNA: Not tested Protein: ALB, AFP, CK19 Function: Not tested	Campard [10]
1 step - 3 weeks IMDM supplemented with: step 1: HGF, FGF-4, (21days)	RNA: (+) ALB, AFP, CK18 Protein: (-) ALB, AFP, CK18 Function: (-) Glycogen storage, LDL uptake	RNA: (+) ALB, AFP, CK18 Protein: (+) **ALB**, **AFP**, **CK18** Function: (+) **Glycogen storage**, **LDL uptake**	Not tested	Zhang [23]
2 steps - 5 weeks DMEM-F12 supplemented with: step 1: HGF, FGF, ITS, Dexa (16days) step 2: OSM, Dexa, ITS (16days)	RNA: (PCR) (+) ALB, AFP, CK19, G6P (-) TDO Protein: (IF) (+) ALB, AFP, CK19 Function: (-) LDL uptake, Albumin secretion, Urea	RNA: (+) ALB, AFP, CK19, **G6P**, **TDO** (-) / Protein: (IF) (+) ALB, AFP, CK19 Function: (+) LDL uptake, Albumin secretion, Urea	Host: NOD-SCID Injury: CCl4 TRP: - tail vein - 5.10^5 D cells Characterization: RNA: Not tested Protein: ALB Function: Not tested	Zhao [24]
Co-culture with C57BL/6 mice liver tissue exposed to thioacetamide (TAA) 24h earlier (2-4days)	RNA: (PCR) (-) ALB,CK18, TDO, AFP, CYP7A1 Protein: Not tested Function: Not tested	RNA: (PCR) (+) **ALB**, **CK18**, **TDO**, **AFP**, **CYP7A1** Protein: Not tested Function: Not tested	Host: Wistar Kyoto Injury: TAA Time: 64 days TRP: - portal vein - 1.10^6 UD cells Characterization: RNA: Not tested Protein: ALB Function: Not tested	Lin [39]
4 steps - 5 weeks IMDM supplemented with: step 1: EGF, FGF (2days) step 2: FGF, HGF, Nico, ITS (10days) step 3: HGF, OSM, Nico, ITS (10days) step 4: OSM, Dexa, ITS (10days)	RNA: (+) ALB, AFP, CX-32, A1AT, CK19, G6P, PEPCK, TDO, UGT1A1 (-) CYP3A4, HNF4, TAT Protein: (IF-WB) (+) ALB, AFP, CX-32, CK19, DPPIV (-) UGT1 Function: (+) G6P, Urea, Cyp3A4, Glycogen storage	RNA: (+) ALB, AFP, CX-32, A1AT, CK19, G6P, PEPCK, TDO, UGT1A1, **CYP3A4**, **TAT** (-) HNF4 Protein: (+) ALB, AFP, CX-32, CK19, DPPIV, **UGT1** (-) / Function: (+) **G6P**, **Urea**, **Cyp3A4**, **Glycogen storage**	Not tested	Scheers [42]

Abbreviations: A1AT: alpha 1 antitrypsin, AFP: alphafetoprotein, ALB: albumin, CK19: cytokeratin 19, CCl4: carbon tetrachloride, CX-32: connexin 32, CYP: cytochrome P450, D: differentiated cells, Dexa: dexamethasone, DPPIV: dipeptidylpeptidase IV, EGF: epidermal growth factor, FGF: fibroblast growth factor, G6P: glucose-6-phosphatase, HGF: hepatocyte growth factor, HNF4: hepatic nuclear factor 4, IF: immunofluorescence, ITS: insulin-transferrin-selenium, LDL: low density lipoprotein, Nico: nicotinamide, NOD: non obese diabetic, OSM: oncostatin M, PEPCK: phosphoenolpyruvate carboxykinase, SCID: severe combined immunodeficiency, TAT: tyrosine aminotransferase, TDO: tryptophan 2,3-dioxygenase, UD: undifferentiated cells, UGT1: UDP-glucuronyltransferase, WB: western blot.

the deficiency of only one enzyme. In those cases, cells expressing mature hepatic levels of the particular enzyme could be used even in the absence of other hepatic metabolic functions. However, for the treatment of other conditions such as acute hepatic failure, advanced hepatic differentiation will be needed.

Enhancing cell engraftment and survival will be another critical point on the road to effective clinical application. Study results converge to the conclusion that less than 5% functional cells should be enough to correct metabolic disease [60]. However, this repopulation rate could be insufficient in case of hepatic failure. Animal models will be useful to better examine UCMSC engraftment in specific conditions. Furthermore, it remains unclear to which extent UCMSC are involved in the reconstitution of hepatic non-parenchymal and metabolically active cells. *In vivo* UCMSC engraftment and functionality in animal models presenting a metabolic disease remains an unanswered question. Finally, reducing graft rejection can enhance cell survival. Although UCMSC present a poor immunogenic profile [24], it is unclear if immunosuppressive therapy is needed to avoid graft rejection.

Recent research has demonstrated the long-term *in vitro* and *in vivo* tumorigenic safety of UCMSC. Extensive analysis of cell karyotype and modulation of cell cycle regulation genes during culture remained normal over a long period of time [42]. However, other safety issues concerning cell administration route, clotting risk, etc. have to be further assessed.

Finally, studies performed on liver cirrhosis models have demonstrated promising anti-inflammatory and anti-fibrogenic properties of UCMSC. In depth studies of the mechanisms involved in these processes will certainly bring new insights for the targeted treatment of liver cirrhosis. Although previous work has underlined the ability of UCMSC to differentiate into hepatocyte-like cells *in vivo*, further investigations should be performed to better understand the effective role of these cells in helping functional liver repair.

Overall, UCMSC present interesting characteristics for cell based therapies. Further improvements regarding cell distribution, engraftment and differentiation capacity with demonstration of acquired metabolic functions *in vivo* will help to unveil the real potential and place of UCMSC in clinical liver based therapies.

ACKNOWLEDGEMENTS

IS: drafting the manuscript

LC, MN, ES: revising it critically for important intellectual content

CONFLICT OF INTEREST

The authors report that no conflict of interest exists.

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Received: February 28, 2011 Revised: May 13, 2011 Accepted: June 12, 2011