

Review article

Human umbilical cord mesenchymal stem cells and the treatment of spinal cord injury

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Keywords: *umbilical cord; mesenchymal stem cells; spinal cord injuries*

Objective To review the recent studies about human umbilical cord mesenchymal stem cells (hUCMSCs) and advances in the treatment of spinal cord injury.

Data sources Published articles (1983–2007) about hUCMSCs and spinal cord injury were selected using Medline.

Study selection Articles selected were relevant to development of mesenchymal stem cells (MSCs) for transplantation in spinal cord injury therapy. Of 258 originally identified articles 51 were selected that specifically addressed the stated purpose.

Results Recent work has revealed that hUCMSCs share most of the characteristics with MSCs derived from bone marrow and are more appropriate to transplantation for cell based therapies.

Conclusions Human umbilical cord could be regarded as a source of MSCs for experimental and clinical needs. In addition, as a peculiar source of stem cells, hUCMSCs may play an important role in the treatment of spinal cord injury.

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Injury to the spinal cord initiates a series of biochemical events that are associated with a progressive decline in blood flow to the injured spinal cord and that exacerbate the extent of tissue damage. This problem is compounded by the poor regenerative response shown by axons in the central nervous system (CNS) and as a result, white matter tracts are permanently interrupted, causing paralysis. Enhancing the regenerative response of the CNS is a formidable challenge and requires an understanding of the barriers to repair at both the molecular and cellular levels. These obstacles have been assiduously outlined over many years and come in a variety of forms. The four greatest obstacles are: 1. proliferation of fibroblasts, astrocytes, microglia and endothelial cells at the lesion site forming a neuroglial scar that acts as a physical and/or chemical barrier; 2. an absence of Schwann cells, which help in guiding any regenerating axons; 3. the absence of neurotrophic factors to enhance axonal growth; and 4. inhibition of axonal growth by post-injury myelin-associated proteins such as Nogo-A,¹ myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein. A variety of cell transplantation approaches have been tested in an effort to replace lost tissue by grafting cells to repair areas with damaged myelin or provide a tissue bridge for nerve fiber growth.²⁻⁴ Mesenchymal stem cells (MSCs) have the capability for self-renewal and differentiation into various lineages of mesenchymal tissues,⁵⁻¹⁰ including osteocyte, chondrocyte, neurocyte (Figure). Moreover, these cells should not elicit graft versus host disease when transplanted into the injury site.^{11,12} These features of MSCs attract a lot of attention from investigators in the context of cell based therapies of several human diseases. Despite the fact that bone marrow represents the major source of MSCs, the use of bone marrow derived cells is not always acceptable due to the high degree of viral

infection and the significant drop in cell number and proliferative capacity with age.¹³ Furthermore, MSCs content of human marrow is scarce, up to 10 cells in every million monocytes.

UMBILICAL CORD BLOOD MESENCHYMAL STEM CELL

The blood remaining in the umbilical cord following birth contains haematopoietic precursors and this has become an important source for transplantation of haematopoietic stem cells.¹⁴⁻¹⁷ The presence of mesenchymal stem/progenitor cells in cord blood has recently been identified. However, there is controversy as to whether umbilical cord blood (UCB) contains MSCs that are capable of differentiating into cells of different connective tissue lineages such as bone, cartilage and adipose tissues and these cells are the best source for tissue engineering of musculoskeletal tissues.¹⁸ So far, little success has been reported about the isolation, characterization and differentiation of MSCs from UCB. Erices et al¹⁹ have reported that UCB derived, mononuclear cells gave rise to two adherent cell types and one of them expressed MSC related surface antigens. Mareschi et al²⁰ reported that under given conditions, it was possible to isolate

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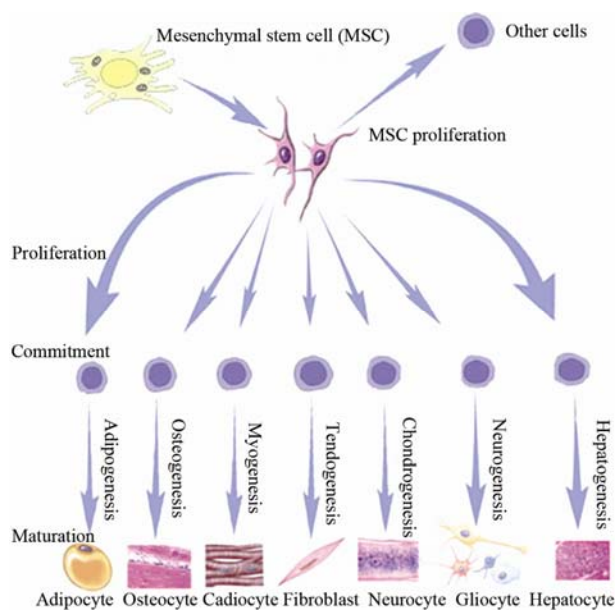


Figure. Mesenchymal stem cells have a multipotential differentiation capability and can replicate along distinctive lineage pathways to produce highly specialized phenotypes that fabricate bone, neuron and other connective tissues.

MSCs from bone marrow, but not from UCB. However, Goodwin et al²¹ have recently reported the multilineage differentiating capability of UCB isolated cells; cells that expressed bone, fat and neural markers. Kakinuma et al²² reported that they could differentiate UCB cells into hepatic progenitor cells. Neither of these reports provided sufficient evidence to fulfil the criteria for qualifying MSCs because both research groups found relatively heterogeneous cells. Wexler et al²³ have recently reported that UCB is not a rich source of human MSCs, while Romanov et al²⁴ also suggested using umbilical cord endothelial cells as a source of MSCs. Nevertheless, UCB cells have many advantages because of the immaturity of newborn cells compared with adult cells. Furthermore, UCB provides no ethical problems for basic studies and clinical applications. Musina et al²⁵ found that a specific feature of umbilical cord blood mesenchymal stem cells is their low count per volume of the initial material and very low proliferative activity. Therefore, UCB may not be a ideal source of MSC for clinical use. Thus, the search for other sources of MSC continues.

Another potential source of mesenchymal cells appeared with the report by McElreavey et al²⁶ of the culture of cells from Wharton's jelly (WJ) of human umbilical cord (UC) (hUCMSCs).

DESCRIPTION OF WHARTON'S JELLY

WJ is the mucoid connective tissue surrounding the two arteries and one vein of the umbilical cord. This gelatinous connective tissue of the UC was first described by Thomas Wharton in 1656.²⁷ Meyer et al²⁸ studied the network of glycoprotein microfibrils and collagen fibrils in WJ three and a half centuries later. WJ is composed of

myofibroblast like stromal cells, collagen fibres and proteoglycans. The interlaced collagen fibres and small, woven bundles are arranged to form a continuous soft skeleton that encases the umbilical vessels.²⁹ WJ has very little collagen, another indicator of the primitive state of this tissue. In WJ, the most abundant glycosaminoglycan is hyaluronic acid,³⁰ which forms a hydrated gel around the fibroblasts and collagen fibrils and maintains the tissue architecture of the UC by protecting it from pressure.³¹

METHODS OF ISOLATION AND hUCMSCs CULTURE

There are four methods for isolation of MSCs from UC: density gradient centrifugation, flow cytometer isolation, attachment screening and two step, enzymatic digestion.³²

In 2003, Romanov et al using enzymatic digestion and centrifugation methods isolated well developed colonies of fibroblast like cells and further characterization revealed that these cells expressed MSC markers. However, the success rate of isolation, which is one of the most important factors regarding the clinical use, was not described. Lu et al³³ attempted to isolate MSC according to the protocol described in the report and obtained MSCs from three of ten UCs. Wang et al³⁴ centrifuged the mesenchymal tissue which had been scraped from the WJ, treated it with collagenase and 2.5% trypsin and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Finally, they obtained 25×10^3 cells per centimetre of umbilical cord. In 2006, Weiss et al³⁵ reported a more efficient method of starting the cultures via enzymatic degradation of the extracellular matrix to release the cells from the WJ. UCMSCs were isolated from 78% of the cords; including from one of the two cords refrigerated for 24 hours prior. Using this improved procedure, UCMSCs were isolated from every cord and up to 1.5×10^6 cells per cm of UC. In Fu's³⁶ study, approximately 1×10^6 hUCMSCs were collected from 20 cm of umbilical cord and the number of hUCMSCs doubled (2×10^6) in 10% FBSDMEM in 3 days. Fu et al³⁶ found that hUCMSCs in WJ of the UC can be easily obtained and processed compared with embryonic and bone marrow stem cells. Lu et al established a simple, two step enzymatic digestion, to isolate and culture hUCMSCs from each of 36 UCs, which may be the most efficient way to isolate MSCs from UC. Friedman et al³⁷ described a novel, simple method of obtaining and cryopreserving UCMSCs by extracting the WJ from a small piece of cord, followed by mincing the tissue and cryopreserving it in autologous cord plasma to prevent exposure to allogeneic or animal serum, thus showing that UCMSCs are a reliable, easily accessible, noncontroversial source of MSCs.

BIOLOGICAL CHARACTERS OF hUCMSCs

In vitro, hUCMSCs appear as spherical, star like or

elongated flat fibroblast like morphology with granules on the surfaces and cell bodies that have neither protrusions, nor networks between the cells.

Growth characteristics

hUCMSCs have the capability to become adherent cells. Besides the basic characteristics of stem cells, hUCMSCs are plastic and can be cloned under the influence of some signals. In 2006, Lu et al using the method of Romanov and Sarugaser,³⁸ as a starting point, have successfully isolated MSCs from UC and further confirmed a high capability for cloning *in vitro*. The mean of colony forming units of fibroblasts (CFUF) per million of nucleated cells was significantly high in UC (800, range 300 to 2000) as determined by limiting dilution assay. The mean doubling time of P1 cells was 24 hours and remained approximately constant until P10 when it increased to P30. The UC derived cells could be readily cloned *in vitro* by serial passage every 2–3 days for 30 passages, without visible changes in either the growth patterns or morphology, indicating the high proliferative potential of UC derived cells. Weiss et al also confirmed that the karyotype of the cells was stable through 13 passages (approximately 30 population doublings). WJ was previously shown to be composed of smooth muscle actin positive, myofibroblast like stromal cells. Mitchell et al³⁹ also measured smooth muscle actin expression by immunoblotting. Smooth muscle actin was expressed at similar levels in WJ cells grown on PDL/laminin matrix and in WJ cells grown on plastic. Thus, WJ cells that were maintained in culture for numerous doublings continued to express this myofibroblast marker. Cell cycle analysis demonstrated that more than 80% of UC derived cells (P2 to P6) were in G0G1 (median (90.0±0.4%); range, 82%–95%), but only a small proportion of cells was engaged in proliferation (G2-M phase: median (6.0±0.3%); range, 5%–10%; S phase: median (4.0±0.7%); range, 1%–8%).

In conclusion, in the course of differentiation, hUCMSCs maintain their morphology and high proliferative potential. These researches demonstrate that hUCMSCs could be more convenient for cell transplantation and be a more economical source of MSCs, than bone marrow MSC (BMMSC). That hUCMSCs are a more convenient source includes the fact of feasibility.

Immunophenotype of hUCMSCs

Although in 1992 Haynesworth et al⁴⁰ first identified specific antigens (SH2, SH3, and SH4) of hMSCs with murine antibodies, due to the lack of universally defined cell surface markers to characterize the MSCs,⁴¹ it remains enigmatic with regard to both their identity and qualification as a true stem cell.⁴² Recent studies show that hUCMSCs share most of their immunophenotype with BMMSC, including a cluster of differentiating makers, neural markers, extracellular adhesion molecules, genes of proteins shown to have a neurotrophic effect and the three germ layer derivatives. Perhaps more

significantly, hUCMSCs were negative for CD14, CD28, CD31, CD33, CD34, CD45, CD56, CD133, HLA-DR and for graft versus host disease (GVHD): CD80, CD86, CD40 and CD40L, which shows that hUCMSCs may be appropriate for transplantation (Table 1).

Fibroblastoid morphology, absence of endothelial and leukocyte associated markers and expression of α -smooth muscle actin and cell adhesion molecules typical for myelosupportive stroma support classifying hUCMSCs as mesenchymal progenitors.

Table 1. Surface markers of hUCMSCs

CD markers	Adhesion molecules	Germ layer markers	Neural markers	Cytokines
CD10	Ncadherin	ACTG2	GDNF	SCF
CD13	Vcadherin	ACTA2	CNTF	LIF
CD29	Rcadherin	BMP1	VEGF	MSCF
CD44	Integrin- β 1	PDGFB	FGF20	Flt3
CD90 (Thy1)	Integrin- α 5	Keratin 8	TRKC	IL6
HLA1	VCAM1	SHH	NSE	GMCSF
CD49e	Integrin- α 2 (CD49b)	Insulin	TH	GCSF
CD105 (SH2)	Integrin- α V	-	NF	SDF1
CD73 (SH3)	Integrin- β 5	-	GFAP	VEGF
CD106	Integrin- α 4	-	-	-
CD166	Integrin- α 3	-	-	-
Ckit (CD117)	-	-	-	-

MULTILINEAGE DIFFERENTIATION CAPACITY OF hUCMSCs

Further characterizing studies reveal that hUCMSCs have strong cellular plasticity and could differentiate into many other cell types (Table 2).

Potential of hUCMSCs differentiating into osteoblasts

hUCMSCs *in vitro* presented osteoblastic phenotype under the culture condition of β -glycerophosphate, dexamethasone and ascorbic acid-2-phosphate. After osteogenic induction, osteogenic differentiation was detected by the calcification and expression of an osteogenic specific gene, osteopontin in the cells. In addition, the formation of alkaline phosphatase positive aggregates and von Kossa stain positive nodules were seen.

Potential of hUCMSCs differentiating into chondrocytes

Wang et al³⁴ cultured the hUCMSCs in serum free DMEM/LG containing insulin transferrin selenium, premix and transforming growth factor- β 1. The cell pellets developed chondrogenic characteristics after treatment. Alcine blue staining of an aggrecan rich extracellular matrix was evident in histological sections and a type II collagen rich extracellular matrix was demonstrated immunohistochemically, confirming the formation of normal chondrocytes.

Potential of hUCMSCs differentiating into adipocyte

Under the induction of dexamethasone, insulin,

Table 2. Differentiation ability of hUCMSCs *in vitro*

Cell types	Stimuli	Identification	References
Osteoblasts	β -glycerophosphate	Calcification	23,33,34
	Ascorbic acid-2-phosphate	Osteopontin	
	Dexamethasone	Alkaline phosphatase Bone nodules	
Chondrocytes	Insulin transferrin selenium	Type II collagen	33,34
	Premix transforming growth factor β 1	Aggrecan	
Adipocytes	Dexamethasone	Peroxisome proliferation activate receptor- γ 2	12,23,26,34
	Insulin	Lipoprotein lipase	
	Isobutylmethylxanthine		
Cardiomyocytes	Indomethacin	Troponin I	33,34
	5-azacytidine	N-cadherin	
Neurocytes	Fibroblast growth factor	NSE	33,35,36,43
	Butylated hydroxyanisole	III β tubulin	
	Dimethylsulfoxide	Neurofilament M	
	Composite salvia injection	Tyrosine hydroxylase	
		GAP43	
Gliocytes	Fibroblast growth factor	GFAP	39
	Butylated hydroxyanisole	CNPase	
	Dimethylsulfoxide		

isobutylmethylxanthine and indomethacin, hUCMSCs could differentiate into lipocytes. Oil red positive cells were found which expressed lipoprotein lipase and adipocyte marker peroxisome proliferation activate receptor- γ 2.

Potential of hUCMSCs differentiating into cardiomyocyte

hUCMSCs can differentiate into cardiomyocytes. After exposure of these cells to cardiomyocyte conditioned medium or 5-azacytidine, they expressed cardiac troponin I and N-cadherin, indicating differentiation into cardiomyocytes.

Potential of hUCMSCs differentiating into neurocyte

Wang et al³⁴ treated hUCMSCs with basic fibroblast growth factor and low serum media plus butylated hydroxyanisole and dimethylsulfoxide. WJ cells developed rounded cell bodies with bipolar or multipolar neurite like extensions, similar to the morphology of neural stem cells. Neuron specific enolase (NSE, a neural stem cell marker) was expressed in these cells. The neuron like cells in these cultures also stained positively for several neuronal proteins, including neuron specific class III β -tubulin, neurofilament M, an axonal growth cone associated protein and tyrosine hydroxylase. Ma et al⁴³ claimed that hUCMSCs were capable of differentiating into neuron like cells with the treatment of composite salvia injection. The induced MSCs not only underwent nerve cell like morphological changes and mature ultramicrostructure with increasing cell organelles, but also expressed the neuronal cell markers (nestin and tyrosine hydroxylase). Weiss et al³⁵ used the protocol described by Woodbury⁴⁴ to induce hUCMSCs to differentiate into neurocytes and their results showed a lower number of cells expressing the marker for early neural progenitors (nestin) and a greater number of cells expressing the mature neural marker for catecholaminergic cells, tyrosine hydroxylase.

POSSIBLE MECHANISMS OF MULTIPOTENTIAL DIFFERENTIATION OF MSCs

Through their capability for a broad range of differentiation, potential MSCs are a possibility for a range of therapeutic applications, but the precise signalling pathways that determine their differentiated fate are not fully understood. Evidence is emerging that developmental signalling cues may be important in regulating stem cell selfrenewal and differentiation programmes.^{45,46} Etheridge et al⁴⁷ identified a consistent expression profile of Wnt signalling molecules in MSCs and provide evidence for a Wnt pathway in these cells. Wnts bind to Frizzled receptors and subsequent signalling inhibits glycogen synthase kinase-3 β , causing β -catenin translocation into the nucleus to induce target gene expression. Mosimann et al⁴⁸ claimed that Wnt signalling proteins control many cellular events during development, tissue homeostasis and tumorigenesis. In most cases, the effects of these signals are brought about by transcriptional changes of target genes. It is thought that Wnt targets are activated by β -catenin mediated involvement of auxiliary factors to TCF/LEF DNA binding proteins.⁴⁹ Weiss et al³⁵ suggests that gene silencing or DNA methylation may be common mechanisms to regulate differentiation of hUCMSCs. These data indicate that further studies concerning the MSC will greatly enhance our current understanding of the signalling mechanisms involved in maintenance of stem cells and mesenchymal differentiation.

COMPARISON OF hUCMSCs AND BMMSCs

Not surprisingly, hUCMSCs shared most of the characteristics with BMMSCs, including fibroblastic morphology, typical immunophenotypic markers, cell cycle status, adipogenic and osteogenic differentiation capacity, range of cytokines as well as haematopoietic supportive function (Table 3). However, recent studies show that there are still several differences between

them.⁵⁰ Firstly, the CFUF frequency was significantly higher in UC derived nucleated cells than in bone marrow (BM) derived nucleated cells. Since CFUF represents the mesenchymal progenitor cell, this suggested a higher frequency of MSC in the nucleated cells of UC than in those of BM. Secondly, hUCMSCs have a faster doubling time. Such proliferative characteristics did not change even after 30 passages.

Table 3. Surface markers expressed by hUCMSCs and BMMSCs

Surface markers	hUC	BM
CD13	++++	++++
CD14	—	—
CD29	++++	++++
CD31	—	—
CD34	—	—
CD38	—	—
CD44	++++	++++
CD45	—	—
CD73	++++	++++
CD90	++++	++++
CD105	++++	++++
CD106	+	+++
CD166	+++	+++
CD146	++++	+++
HLA-ABC	+++	++++
HLA-DR	—	—

In contrast, BMMSC showed a significantly slower doubling time, which became even longer after P6. These indicate a higher proliferative capacity of hUCMSCs in comparison with BMMSC. In addition, hUCMSCs showed lower expression of CD106 and HLA-DR in comparison with BMMSC. The different expression of CD106 in hUCMSCs and BMMSCs may represent a specific indicator for identifying peripheral MSCs from BMMSCs because low expression of CD106 has also been identified in adipocyte derived MSCs. Furthermore, Lu et al³³ noted low expression of HLA-ABC on hUCMSCs in addition to the absence of HLA-DR expression. This observation is consistent with the findings of Sarugaser et al.³⁸ Because HLA-ABC could be a hurdle for allogeneic cell therapies, the lower expression of HLA-ABC may favour the use of hUCMSCs for allogeneic cell therapy.

In addition, unlike mesenchymal stem cells derived from adult bone marrow stromal cells, small populations of hUCMSCs express endoglin (SH2 and CD105) and CD49e at passage 8 and express higher levels of CD146, a putative MSC marker. hUCMCS cells also express growth factors and angiogenic factors, suggesting that they can be used to treat neurodegenerative disease.

EXPERIMENTAL RESEARCH ON hUCMSCs TRANSPLANTATION IN SPINAL CORD INJURY

Ideal donor cells for therapy of spinal cord injuries should be easily available, capable of rapid cloning in culture, immunologically compatible, capable of long-term survival and integration into the spinal environment, amenable to stable transfection and long-term expression

of exogenous genes such as tyrosine hydroxylase. hUCMSCs have five properties that make them of interest as a source of cells for therapeutic use: they can be isolated in large numbers, are negative for CD34 and CD45, grow robustly and can be frozen and thawed, can be cloned, and can easily be engineered to express exogenous proteins. Transplantation of hUCMSCs into the injured spinal cord may have the following functions: compensation for demyelination; removal of inhibition; promotion of axonal regeneration; direction of axons to appropriate targets; creation of bridges; replacement of lost cells. Weiss et al³⁵ has treated Parkinson's disease with hUCMSCs transplantation: the results demonstrated that the hUCMSCs produce significant amounts of glial cell line-derived neurotrophic factor (GDNF), one of the most potent trophic factors for dopaminergic neurons and fibroblast growth factor, the animals with transplanted cells showed a significant recovery in behaviour. Although the above data from hUCMSCs indicate that these cells may be therapeutically useful in treating CNS disorders, transplantation of hUCMSCs for treatment of spinal cord injury is just the beginning. Wang et al⁵¹ transplanted hUCMSCs into spinal cord hemisection, murine model and the results revealed that transplanted hUCMSCs survived and were MAB1281 positive in the injured spinal cord and migrated to the injured site. Furthermore, NSE or glial fibrillary acidic protein (GFAP) was detected in some MAB1281 positive cells with double immunostaining. Obvious dendritic growth was noted in MAB1281 GFAP positive cells. The behavioural tests showed significant improvements with time in rats with hUCMSCs transplantation. Therefore, as a source of stem cells, hUCMSCs may play an important role in the treatment of spinal cord injury. These researches indicate the prospect of wide application of hUCMSCs. Although the study on hUCMSCs is still on the threshold, more and more exciting results will come to us in future.

In conclusion, hUCMSCs could replicate stably in culture. Possessing the capability of differentiating into nerve like cells, these cells could represent a readily available source of stem cells for transplantation into CNS. hUCMSCs hold great promise as tools for understanding development and as therapeutic agents.

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