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TOPIC HIGHLIGHT

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Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility

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Abstract

Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). Apart from their prominent advantages, such as a painless collection procedure and faster self-renewal, UC-MSCs have shown the ability to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining and subamnion and perivascular regions. In this review, we give a brief introduction to various compartments of UC as a source of MSCs and emphasize the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

Core tip: Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). UC-MSCs have shown the ability of faster self-renewal and to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining membrane and subamnion and perivascular regions. In this review, we introduce various compartments of UC and discuss the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

INTRODUCTION

Mesenchymal stem cells (MSCs) originate in the human embryo and are considered multipotent stem cells. MSCs are a heterogeneous subset of stromal stem cells, which can be isolated from the bone marrow[1], mobilized peripheral blood[2], cord blood[3], umbilical cord (UC)[4,5], placenta[6], adipose tissue[7], dental pulp[8], and even the fetal liver[9] and lungs[10]. UC contains two umbilical arteries (UCAs) and one umbilical vein (UCV), both embedded within a specific mucous connective tissue, known as Wharton's jelly (WJ), which is covered by amniotic epithelium (Figure 1). UC is considered medical waste and the collection of UC-MSCs is noninvasive; furthermore, the access to UC-MSCs has not been encumbered with ethical problems. UC-MSCs, similarly to MSCs derived

from other sources, have distinct capacity for self-renewal while maintaining their multipotency, *i.e.*, the ability to differentiate into adipocytes, osteocytes, chondrocytes, neurons and hepatocytes, although some differentiation abilities are known to be partial[11-13]. Moreover, UC-MSCs have also attracted great interest because of their immunomodulatory properties. Nowadays, UC-MSCs are proposed as a possible versatile tool for regenerative medicine and immunotherapy.

HISTORY OF UC-MSCs

During pregnancy, the fetus and mother are connected by UC. UC prevents umbilical vessels from compression, torsion and bending, while providing good blood circulation. McElreavey *et al*[4] for the first time reported isolation of fibroblast-like cells from WJ of human UC in 1991. The UC-derived cells have the similar surface phenotype, plastic adherence and multipotency as those of MSCs derived from other sources. It was 3 years earlier that the first cord blood (CB) transplantation was performed in France in 1988[14]. After that, together with the development of CB banking, CB transplantation has become the alternative source of hematopoietic stem cells. Although CB-derived MSCs cannot be consistently isolated[15-18], MSCs were considered to circulate in the blood of preterm fetuses and able to be successfully isolated and expanded[3]. Where these cells home at the end of gestation is not clear[13]. Thus, UC has inevitably become a focus of attention as a source of MSCs because it contains CB[18]. One key study appeared concerning CB-derived MSCs appeared around 2003[19]. Mitchell *et al*[20] successfully isolated matrix cells from UC WJ using explant culture and Romanov *et al*[19] isolated MSCs-like cells from the subendothelial layer of UCV.

ADVANTAGES OF UC-MSCs

Stem cell populations can be isolated from embryonic, fetal and adult tissues. Embryonic stem cells (ESCs) are a leading candidate for tissue engineering because of their high self-renewal capacity and pluripotency (ability to differentiate into all germ layers) *in vitro* and *in vivo*. Nonetheless, in addition to ethical restrictions, the clinical applications of ESCs are severely limited by technical difficulties with the depletion of immature cells that may result in the formation of a teratoma.

In contrast, adult stem cells, such as those in the skin, bone marrow (BM) and adipose tissue, may have wider clinical applications. BM-MSCs have been used for autologous and allogeneic purposes. Recently, successful clinical application of autologous BM-MSCs was reported for conditions such as cardiac infarction[21], graft-versus-host disease (GVHD)[22,23], Crohn's disease[24] and bone tissue engineering[25]. On the other hand, the autologous use was sometimes limited by cell numbers and age-related changes such as decreased growth and differentiation capacity[26,27].

Compared with BM-MSCs and ES cells, UC-MSCs show a gene expression profile more similar to that of ESCs and faster self-renewal rather than BM-MSCs[11,12].

It is easy to obtain a substantial number of UC-MSCs after several passages and extensive *ex vivo* expansion[28]. The most appreciable advantage is that the collection procedure is noninvasive and ethically acceptable.

Similar to BM-MSCs, UC-MSCs can be considered for autologous and allogeneic use. Autologous UC-MSCs might be used as gene therapy for genetic diseases and as regenerative or anti-inflammatory therapy for neonatal injury, such as cerebral palsy or hypoxic brain damage. On the other hand, allogeneic UC-MSCs can be expanded and cryopreserved in a cell bank for patients in need. The only disadvantage is that physicians need to confirm the baby's health as a donor because it cannot be ascertained in advance whether the donor will grow normally without health problems; thus, genomic or chromosomal tests need to be performed. In contrast, in the case of a BM donor, the physician can directly see and examine the donor and then decide to collect BM. In the case of CB banking, many CB banks monitor the baby's health after birth. Thus, it is important to know the advantages and disadvantages of UC-MSCs for each clinical application.

DIFFERENT METHODS FOR ISOLATION OF MSCs FROM DIFFERENT COMPARTMENTS OF UC

There are two methods to obtain MSCs from various UC compartments or from the whole UC: the explant method and the enzymatic digestion method.

The explant method

UC, or its compartments, is manually minced into small fragments 1-2 mm^3. These fragments are aligned and seeded regularly on the tissue culture-treated dishes. After the tissue fragments are attached to the bottom of the dish, the culture medium is poured slowly and gently, so as not to detach the fragments, and the culture is started[29-31]. The culture medium is replaced every 3-7 d for 2-4 wk until fibroblast-like adherent cells reach 80%-90% confluence. The adherent cells and tissue fragments are rinsed once with PBS and detached using a trypsin solution, followed by washing with the medium. The cells and tissue fragments are filtered to remove the tissue fragments.

The disadvantage of this method is that the fragments often float in the medium, resulting in poor cell recovery. No MSCs can be obtained from the floating fragments. To collect a consistent number of MSCs each time, it is important to prevent the exfoliation of the tissue fragments from the bottom of plastic dishes.

The enzymatic digestion method

WJ is either directly exposed to enzymatic solutions to
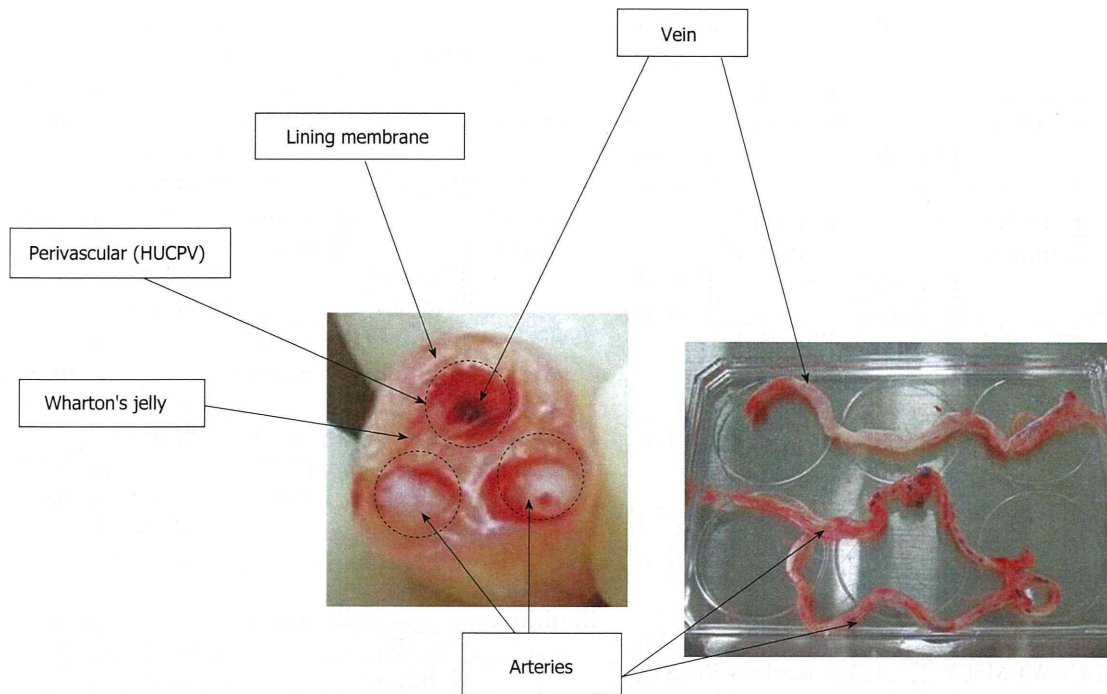
Figure 1 Various compartments of umbilical cord from which mesenchymal stem cells can be isolated. HUCPV: Human umbilical cord perivascular.

release the cells or it is cut into small pieces followed by enzymatic digestion. The enzymes used for digestion vary from simple collagenase[31,32] to a combination of either collagenase and hyaluronidase with or without trypsin[33,34] or collagenase, dispase II and hyaluronidase[33]. The digestion time and concentrations varied by researchers.

There are four compartments of UC as a source of MSCs: (1) Whole UC-MSCs: the whole UC is cut into smaller pieces followed by either an explant procedure or enzyme digestion[30,35,36]; (2) UCWJ-, UCA- and UCV-MSCs: UCWJ-MSCs are obtained after removing umbilical vessels. Umbilical vessels [two arteries (UCAs) and one vein (UCV)] can also be minced into 1-2 mm^3 fragments. The fragments are aligned regularly on the plate and cultured until MSCs start growing; (3) UC lining and subamnion-derived MSCs: the subamnion region of UC lining membrane is removed with a razor blade and then cut into small pieces. These fragments are plated in plastic culture dishes until MSCs start growing (explant method). With this method, however, it might be difficult to remove the adjacent region underneath the amniotic epithelium completely[37,38]; and (4) Human UC perivascular stem cells (HUCPVC): the vessels are extracted from UC and tied at both ends into loops. The loops are then placed into an enzymatic solution for a defined period of time to allow the cells to separate from the perivascular region. The detached cells are cultured and collected as HUCPVCs[26,28,39].

It is still controversial whether the isolation of the cells from the whole or some compartment is superior to others with respect to their proliferation ability, differentiation ability and immunosuppressive capacity.

Proliferation assays
The frequency of colony-forming unit fibroblasts (CFU-F)

is significantly higher in whole UC-derived MSCs than in BM-MSCs with limiting dilution[26,30,40]. The authors first compared UCWJ-MSCs, UCA-MSCs and UCV-MSCs. UCV-MSCs exhibited a significantly higher frequency of CFU-F than UCWJ-MSCs and UCA-MSCs, but the doubling time was not different among these cell types[5]. The Mennan group also reported that there are no significant differences among the various compartments of UC, although the cells derived from any UC compartment proliferate significantly faster than BM-MSCs, with mean doubling times of 2-3 d at P0 through P3[41]. Depending on the purpose, researchers need to select either a compartment or the whole UC.

Biomarkers of UC-MSCs
The immunoprofile of UC-MSCs is analyzed using flow cytometry, according to the standard definitions for MSCs described by the position paper of the International Society for Cellular Therapy[42]. There are no single specific markers that can be used to identify multipotent MSCs. MSCs are positive for adhesion markers such as CD29 and CD44; mesenchymal markers such as CD90, CD73 and CD105; and human leukocyte differentiation antigen class I (HLA-ABC). MSCs are negative for endothelial cell marker CD31; hematopoietic cell markers such as CD34, CD45 and CD117; and human leukocyte differentiation antigen class II (HLA-DR)[43]. Among the different UC compartments, UCWJ-, UCV- and UCA-derived MSCs show a similar fibroblast-like spindle shape and the MSCs from these three types of tissues demonstrate no significant differences in the immunoprofile. These cells are positive for CD13, CD29 (integrin β1), CD73 (SH3), CD90 (Thy-1), CD105 (SH2; endogrin) and HLA-ABC at the cellular frequency greater than 90% and are negative

for CD34, CD45, CD133 and HLA-DR, with the cellular frequency less than 1%[5]. Mennan *et al*[41] also confirmed that MSC immunophenotypes showed no significant differences among different sources: BM, umbilical cord arteries, vein, WJ and UC lining membrane. Even although the authors could not find any major differences in their immunophenotypes, the cell populations derived from the different compartments may consist of different proportions of multipotent stem cells. Karahuseyinoglu *et al*[44] demonstrated that CD73 is expressed throughout the vessels and endothelium and is absent in the perivascular region, but the strongest expression is observed in the epithelial and subepithelial regions of WJ. CD90 is positive in most compartments but negative in the endothelial lumen lining. A high expression of vimentin, CKs (1, 4, 5, 6, 8, 10, 13, 18 and 19), desmin and SMA has been detected in the subamniotic layer and the perivascular region. Schugar *et al*[45] reported that CD146 (endothelial progenitor marker) is expressed in the vessel walls (100%) and the perivascular region of UC (62%) but is no longer expressed in UCWJ-MSCs[26,46]. These markers might aid in determining the multipotency of the isolated cell population. Phenotypic characterization of UC-MSCs might be influenced by the culture passage number, culture medium and culture method.

Furthermore, ESC markers such as Oct4, Nanog, Sox-2 and KLF4 are expressed only at low levels in UC-MSCs[47]. This suggests that MSCs are primitive stem cells, somewhere between ESCs and mature adult stem cells. Nonetheless, a precise isolation of pluripotent MSCs using specific markers remains a challenge.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Gang *et al*[48] reported that SSEA4+ cells proliferate predominantly when the culture is initiated from primary BM cells in the medium supplemented with special cocktails of cytokines. In contrast, the authors failed to reproduce the same phenomena in UCWJ-MSCs in the medium consisting of α-MEM and 10% FBS. Furthermore, SSEA4 expression in UCWJ-MSCs significantly correlates with the FBS concentration in the culture medium, whereas SSEA3 expression was inversely correlated. We concluded that SSEA4 in UCWJ-MSCs is not a marker of either proliferation capacity or multipotency[31]. Schrobback *et al*[49] assessed SSEA4 expression in human articular chondrocytes, osteoblasts and BM-derived MSCs and characterized their differentiation potential. Their results showed that SSEA4 levels in these cells are not related to the capacity for chondrogenic and osteogenic differentiation and the proliferation potential *in vitro*[49].

THE ABILITY OF UC-MSCs TO DIFFERENTIATE INTO ADIPOGENIC, CHONDROGENIC AND OSTEOGENIC LINEAGES

UC-MSCs originating from the extraembryonic meso-

derm and their capacity for differentiation into adipogenic, chondrogenic and osteogenic lineages have been extensively studied[50]. Regarding the osteogenic differentiation ability, Hsieh *et al*[11] demonstrated that the gene profiles of UC-MSCs are close to ESCs; UC-MSCs show delayed and insufficient differentiation into osteocytes. On the other hand, BM-MSCs have already expressed an osteogenic gene profile and can easily differentiate into osteocytes. Among the three compartments, UCWJ, UCV and UCAs, UCWJ-MSCs demonstrate an obviously defective ability to differentiate into osteocytes, even although the expression of osteocyte-related genes is detected by reverse-transcriptase PCR, at levels similar to those in the other two tissues/compartments[5]. Mennan *et al*[41] compared the osteogenic differentiation among cord regions in six samples and found that the best differentiation is seen with UCWJ-MSCs and whole UC-derived MSCs, rather than with UCA-, UCV- and UC lining MSCs.

As for adipocytic differentiation, Mennan *et al*[41] reported that UC-MSCs produce small lipid vacuoles, whereas BM-MSCs produce more mature adipocytes (unilocular lipid vacuoles). UC-MSCs might maintain their multipotency for longer periods than BM-MSCs can[51], although there were no obvious differences among MSCs derived from UC compartments in our research[5].

With respect to chondrogenic differentiation, UC-MSCs show no apparent differences among the different cord regions (sources)[41]. Moreover, the comparison of the chondrogenic potential between BM-MSCs and UC-MSCs revealed that UC-MSCs produce thrice as much collagen as BM-MSCs; this finding indicates that the former may be a better option for fibrocartilage tissue engineering[52].

In relation to other differentiation abilities, UCWJ-MSCs are the most studied cell type among various UC compartments and many papers have been published[53,54]. In addition to differentiating into osteocytes, chondrocytes and adipocytes, UCWJ-MSCs can differentiate into cardiomyocytes (with the gene expression of N-cadherin and cardiac troponin I [55]), neurons and glia[20], oligodendrocytes[56] and hepatocytes[57]. Recently, clinical trials have been conducted using UC-MSCs for neurogenic disorders (spinocerebellar ataxia and multiple system atrophy of the cerebellar type)[58] and liver disorders[59,60].

IMMUNOSUPPRESSIVE PROPERTIES OF UC-MSCs

Immunosuppressive effects have now become the most popular property of MSCs for potential clinical use. First, MSCs themselves are weakly immunogenic owing to the lack of HLA-DR and low expression of MHC class I molecules. MSCs have been shown to have immunomodulatory properties *in vitro*[61]. Furthermore, MSCs lack both CD80 and CD86 proteins[36,62], which

are costimulatory molecules inducing T cell activation and survival. The lack of HLA-DR, CD80 and CD86 suggests that MSCs do not elicit acute rejection and are suitable for allogeneic cell-based therapy.

Second, UC-MSCs have immunosuppressive properties *in vitro* and *in vivo*. Many studies have been published about the immunosuppressive effect of UCWJ-MSCs[63], UC lining-MSCs[37,64], HUCPV[65] and whole UC-derived MSCs[66]. The immunosuppressive effect of UC-MSCs is mediated by soluble factors and cell-to-cell contacts. PGE2, galectin-1 and HLA-G5 are released from MSCs and serve as effective factors of immunosuppression[67]. Among these factors, indoleamine 2,3-dioxygenase (IDO) is one of the most relevant because it is inducible by IFN-γ and catalyzes conversion from tryptophan to kynurenine[62,68]. This depletion of tryptophan from the environment can suppress T cell proliferation. UCWJ-MSC-mediated immunosuppression may require preliminary activation by proinflammatory cytokines, such as IFN-γ, with or without TNF-α, IL-1α or IL-1β.

It was recently suggested that the inflammatory environment produced by the upregulation of cytokines such as IFN-γ and TNF-α might alter the biological activity of MSCs from immunosuppression to immuno-stimulation[68]. In this case, UC-MSCs maynot prevent GVHD *in vivo*. It is known that upon stimulation by activated immune cells or cytokines (priming), MSCs are primed and become functional immunosuppressors. The extent of immunosuppression is greater with UCWJ-MSCs than with BM-MSCs[62]. Polchert *et al*[68] demonstrated that MSCs primed with IFN-γ are effective in a mouse GVHD model despite upregulated MHC class II molecules. In order to ensure the effective and safe therapeutic use of UC-MSCs, more *in vivo* experiments need to be conducted because of the many discrepancies with *in vitro* data.

CONCLUSIONS

Compared with the counterparts of other origins, UC-MSCs have attractive advantages as MSCs and as UC-derived cells: (1) a noninvasive collection procedure for autologous or allogeneic use; (2) a lower risk of infection; (3) a low risk of teratoma; (4) multipotency; and (5) low immunogenicity with a good immunosuppressive ability. It is still unclear which compartment in UC is the best for clinical use; nonetheless, the era of the clinical use of UC-MSCs is approaching quickly.

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Improved Explant Method to Isolate Umbilical Cord-Derived Mesenchymal Stem Cells and Their Immunosuppressive Properties

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The umbilical cord (UC) has become one of the major sources of mesenchymal stem cells (MSCs). The common explant method of isolating UC-derived MSCs (UC-MSCs) involves mincing the UCs into small fragments, which are then attached to a culture dish bottom from which the MSCs migrate. However, the fragments frequently float up from the bottom of the dish, thereby reducing the cell recovery rate. To overcome this problem, we demonstrate an improved explant method for UC-MSC isolation, which involves the use of a stainless steel mesh (Cellamigo®; Tsubakimoto Chain Co.), to protect the tissue from floating after the minced fragments are aligned at regular intervals in culture dishes. The culture medium was refreshed every 3 days and the adherent cells and tissue fragments were harvested using trypsin. The number of UC-MSCs isolated from 1 g of UC using the explant method with Cellamigo was $2.9 \pm 1.4 \times 10^6$/g, which was significantly higher than that obtained without Cellamigo ($0.66 \pm 0.53 \times 10^6$/g) ($n = 6$, $p < 0.01$) when cells reached 80–90% confluence. In addition, the processing and incubation time required to reach 80–90% confluence was reduced in the improved explant method compared with the conventional method. The UC-MSCs isolated using the improved method were positive for CD105, CD73, CD90, and HLA class I expression and negative for CD45 and HLA class II expression. The isolated UC-MSCs efficiently inhibited the responder T cells induced by allogeneic dendritic cells in a mixed lymphocyte reaction. Conclusively, we demonstrated that the use of Cellamigo improves the explant method for isolating UC-MSCs.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are considered to possess a multipotent differentiating ability and tend to accumulate in the inflamed regions or damaged tissues. At the regions, they suppress the inflammatory response or accelerate the repair process. MSCs can be isolated from several sources, including the bone marrow,[1] cord blood (CB),[2] umbilical cord (UC), placenta,[3] and adipose tissue.[4] Among them, UC-derived MSCs (UC-MSCs), which originate in the human embryo, have become one of the major MSC sources for clinical and research applications because of their prominent advantages with respect to painless collection and faster self-renewal properties; therefore, these cells were chosen as the model for the current study. There are two main methods used to isolate UC-MSCs: one is an enzyme digestion method using enzymes such as collagenase, hyaluronidase, or trypsin,[5–7] and the other is an explant method.[8–10] We recently reported that there were no significant differences in the number and characteristics between Wharton's jelly (WJ)-MSCs isolated using the collagenase and conventional explant methods.[10] However, the collagenase digestion method was associated with some problems, including decreased cell viability due to lytic activity and different sensitivities to collagenase. Collagenase is produced by *Clostridium histolyticum*, and not by humans, and it is difficult to obtain clinical-grade collagenase. In addition, while it takes several hours to lyse the UC tissue with collagenase, the explant method can be used for culturing soon after mincing the UC. Therefore, we focused on the explant method because of its increased simplicity and feasibility compared to the enzyme digestion method. In the explant method, the UC is minced into small fragments, which are then attached to the culture dish bottom from which the MSCs migrate. However, the fragments frequently do not

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