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Immunomodulatory function of whole human umbilical cord derived mesenchymal stem cells

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ABSTRACT

Bone marrow derived mesenchymal stem cells (MSCs) play a critical role in immune modulation. However, immunomodulatory function of whole human umbilical cord derived mesenchymal stem cells (UC-MSCs) remains unclear. In this study, UC-MSCs were separated from whole umbilical cord using a single enzyme digestion. UC-MSCs ($CD73^+$, $CD90^+$, $CD105^+$, and $CD34^-$, $CD45^-$, $HLA\text{-}DR^-$) were differentiated into adipocytes, osteocytes and chondrocytes *in vitro* under specific stimulatory environments. UC-MSCs suppressed umbilical cord blood lymphocyte proliferation stimulated by mitogen, and ELISA showed that the secretion of INF-γ was downregulated, and the secretion of IL-4 was upregulated, with $CD8^+$ T cells markedly decreased and $CD4^+$ T cells changed lightly. Moreover, the infusion of UC-MSCs in recipient mice transplanted with donor bone marrow cells ameliorated acute graft-versus host disease (aGVHD) and extended survival. In conclusion, UC-MSCs might negatively modulate immunoreactions, and have application potential in the treatment of aGVHD caused by allogeneic stem cells transplantation.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an important therapeutic option for many malignant and non-malignant disorders. However, graft-versus-host disease (GVHD) remains the most frequent and serious complication following allogeneic HSCT and limits the broader application of this therapy (Choi et al., 2010). Since conventional immunosuppressive treatments cannot always control GVHD, many new strategies are investigated. In this respect, the infusion of human bone marrow derived mesenchymal stem cells (BM-MSCs) has been proposed as a new approach with a good clinical prospect (Horowitz et al., 1990; Paczesny et al., 2009; Ringden et al., 2006). BM-MSCs have been identified as non-hematopoietic pluripotent cells with the potentials of self-renewal and multi-differentiation, such as differentiation into adipocytes, osteoblasts and chondrocytes (Jiang et al., 2002; Prockop, 1997), and they have immunomodulatory effects on various immune cells, such as T and B lymphocytes, dendritic cells, and natural killer cells. Normal MSCs display suppressive effects on both innate and humoral immunity by inhibiting dendritic cell (DC) maturation, natural killer/B-cell activation and T cell proliferation, while simultaneously promote the regulatory T cell development (Corcione et al., 2006; Di Nicola et al., 2002; Ramasamy et al., 2007; Spaggiari et al., 2006), which are widely applied in clinical practice, such as GVHD and systemic lupus erythematosus (SLE) (Le Blanc et al., 2008; Liang et al., 2010; Sun et al., 2010). However, aspiration of bone marrow (BM) involves invasive procedures, and the frequency and differentiation potential of BM-MSCs decrease with donor age (Stenderup et al., 2003). MSCs have been obtained from multiple sources of the placenta including Wharton's jelly, umbilical cord vessels, amniotic membrane, chorionic membrane, decidua, and chorionic villi (Chen et al., 2015; Li et al., 2014). Alternative sources of

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MSCs have recently been concerned (Oliveira-Bravo et al., 2016; Shin et al., 2016). The umbilical cords were discarded as medical waste in the past. Human umbilical cord as an alternative source of MSCs has several advantages, including easy gain as a discarded tissue, a low risk of viral infection, and lack of ethical concerns (Macias et al., 2010). It was reported that MSCs from different compartments of placenta possessed immunoregulatory function *in vitro* (Li et al., 2007; Li et al., 2005; Magatti et al., 2009). However, it is unknown whether whole UC-MSCs possess immunomodulatory function as BM-MSCs, and further studies are required to investigate their immune properties *in vivo* and *in vitro*. In this study, the whole human placenta was selected as the origin of MSCs, their immunoregulatory properties *in vitro* were investigated and an experiment mouse model of aGVHD was developed to assess the treatment efficacy of UC-MSCs against aGVHD.

2. Materials and methods

2.1. Regents

PHA was purchased from Sigma and used at 10 μg/mL. Collagenase type II was purchased from Sigma and utilized at 0.1%. And TGF-β3 (Peprotech Inc.) was used at 10 ng/mL. High glucose DMEM, DF12 and RPMI-1640 were purchased from Invitrogen.

2.2. Umbilical cord and umbilical cord blood samples collection

The umbilical cord blood (UCB) and human umbilical cords (median gestational age, 39–40 weeks) were obtained from the Obstetric Department in Affiliated Hospital of Jining Medical University (Jining, China) after the normal delivery. All experiments were approved by the local institutional review board. Written informed consent was obtained from all newborns' mothers. Each sample kept in DF12 was processed within 24 h in the laboratory.

2.3. Isolation and culture of MSC from the whole human umbilical cord

After drainage of umbilical cord blood, the umbilical cord was flushed extensively with phosphate-buffered saline (PBS) containing 2% gentamycin. Umbilical cord sections minced into 1 mm^3 size were digested with 0.1% collagenase type II for 1 h with gentle agitation at 37 °C. The digested cells were centrifuged at 1200 rpm for 15 min. The pellet was filtered through a 100 μm nylon strainer, and the filtered cells were then centrifuged at 1200 rpm for 15 min. The resuspended cells were plated at a density of 1–2 × $10^4/cm^2$ in culture flasks. Growth medium consisted of DF12 containing 10% fetal bovine serum (FBS, HyClone), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invtrogen). The cultures were maintained in a 95% humidified atmosphere with 5% CO_2 at 37 °C. After 3 days, the medium was replaced and non-adherent cells were removed. The medium was then changed twice weekly thereafter. The cells were harvested at subconfluence using Trypsin-EDTA (Invitrogen) and replated at a density of 1 × $10^4/cm^2$.

2.4. Immunophenotype analysis of UC-MSCs

For flow cytometry, UC-MSCs from P2 to P6 were harvested by treatment with Trypsin-EDTA. The detached cells were washed and resuspended in PBS in aliquots of 0.5 × 10^6 cells. They were stained with phycoerythrin (PE)-conjugated antibodies against CD73, CD105, HLA-DR, or fluorescein isothiocyanate (FITC)-conjugated antibodies against CD34, CD45, CD90, and incubated for 30 min at 4 °C in the dark. Mouse isotypic antibodies served as the control. All the antibodies were purchased from Becton Dickinson (San Diego, CA, USA). Cells were stained by a single label and then analyzed by flow cytometry with a FAC Scan (Becton Dickinson).

2.5. Adipogenic differentiation of UC-MSCs

To induce adipogenic differentiation, the cells were seeded in 24-well culture plates at a density of 2 × 10^3 cells/cm^2. The high-glucose DMEM was supplemented with 10% FBS, 100 μg/mL 1-methyl-3-isobutylxanthine (Sigma), 50 μg/mL ascorbic acid (Sigma), and 10^{-6} mol/L dexamethasone (Sigma), and half of the medium was replaced every 2–3 days. After 2 weeks, the cells were fixed in 10% formalin and stained with 0.5% fresh oil-red-O solution (Sigma).

2.6. Osteogenic differentiation of UC-MSCs

To induce osteogenic differentiation, the cells were seeded in a 24-well plate in at a density of 2 × 10^3 cells/cm^2. The high-glucose DMEM was supplemented with 10% FBS, 7.0 × 10^{-5} mol/L β-glycerophosphate (Sigma), 2.0 × 10^{-4} mol/L ascorbic acid (Sigma), and 10^{-8} mol/L dexamethasone (Sigma), and half the medium was replaced every 2–3 days. The cells were fixed with 10% formalin and subjected to Alizarin Red staining on day + 21.

2.7. Chondrogenic differentiation of UC-MSCs

To examine chondrogenic differentiation function of UC-MSCs, 4 × 10^5 cells were created in micromass pellets and placed in a cube with chondrogenic medium containing high-glucose DMEM supplemented with 1% ITS + 1(Sigma), 10 mM/L ascorbate-2-phosphate (Sigma), 10^{-7} mol/L dexamethasone (Sigma), and 10 ng/mL TGF-β1. The medium was changed 2–3 times per week, and cell pellets were harvested and fixed with 10% formalin, which were finally embedded in paraffin and stained with 1% Alcian Blue (Sigma) at day + 21.

2.8. Human mitogen proliferative assays

Umbilical cord blood mononuclear cells (UCB-MNCs) were obtained by using Ficoll-Hypaque (1.077 ± 0.001 g/mL; Pharmaccin) density-gradient centrifugation, and cultured in RPMI-1640 supplemented with 10% FBS. Mitogen proliferative assays were performed by incubating 1 × 10^5 UCB-MNCs with 10 μg/mL PHA in 96-well round-bottom plates (Nunc) and irradiating (15 Gy) UC-MSCs (1 × 10^4), which were cultured at 37 °C in a humidified 5% CO_2 atmosphere. BrdU was added to the plates on day + 3 at a final concentration of 10 μM. Cells were then harvested and treated according to the manufacturer's instructions of BrdU ELISA Kit (Roche).

2.9. Impact of UC-MSCs on T cells and quantification of human cytokines in PHA-stimulation assays

UBC-MNCs were obtained as described above. Mitogen proliferative assays were performed by incubating 1 × 10^6 UCB-MNCs with PHA in 1 mL RPMI-1640 at a final concentration of 10 μg/mL. 1 × 10^5 irradiated UC-MSCs were added at the beginning of the experiment. On day + 3, culture supernatants were harvested. T cells were analyzed by flow cytometry as described above. Interferon-γ and IL-4 were quantified by ELISA assay kits according to the protocol of the manufacturer (Jingmei, China).

2.10. Murine aGVHD models and treatment scheme

Male C57BL/6 mice (n = 10) and female BALB/c mice (n = 10) aged six to eight weeks were purchased from the Experimental

Animal Center of Academy of Military Medical Sciences (Beijing, China), and raised under pathogen-free environment. All the animal experiments were approved by the institutional review board. As previously described (Miao et al., 2006), we developed an MHC-mismatched murine transplantation model. Splenic lymphocytes and bone marrow (BM) cells from male C57BL/6 mouse donors were infused into the female BALB/c mice which received sublethal irradiation (7.5 cGy). On the first day of our experiment (day 0), UC-MSCs were administered intravenously. The survival and appearance of mice were monitored daily. Kaplan-Meier survival curves were plotted for each group. Three animals from each group were decapitated on day + 28 after transplantation. The gut, skin, and liver were fixed in 10% formalin, and the paraffin sections were stained with HChang et al., 2006).

2.11. Statistical analysis

Statistical analysis was performed using the SPSS10.0 software (SPSS, USA). Results were expressed as mean ± SEM. Most statistical comparisons were performed by using the two-sided Student *t*-test. Histopathology score were analyzed using Kruskal Wallis test. Kaplan-Meier survival curves were plotted and analyzed using log rank test. P value <0.05 indicated significant difference.

3. Results

3.1. Immunophenotype characteristics of UC-MSCs

These adherent cells exhibited the expected fibroblast-like morphology. The surface marker expression on UC-MSCs was not altered by passaging or by cryopreservation at least before passage 10 (data not shown), and the phenotypic markers were examined by flow cytometry. The results showed that UC-MSCs were positive for CD73, CD90 and CD105 expression, but negative for CD34, CD45 and HLA-DR expression (Fig. 1).

3.2. Differentiation of UC-MSCs

MSCs isolated from the whole human umbilical cord tissues were induced to differentiate into adipocytes, osteoblasts, chondroblasts by using selective culture media. UC-MSCs could undergo adipogenic differentiation, as demonstrated by positive staining for oil-red-O after 3-week culture in the adipogenic induction medium (Fig. 2A). After 3-week culture in the osteogenic induction medium, UC-MSCs showed calcium deposition, as was visualized by positive staining for Alizarin Red (Fig. 2B). The ability of UC-MSCs undergoing chondrogenic differentiation was shown by Alcian Blue staining after 3-week micromass culture (Fig. 2C). These results indicated that UC-MSCs possess multi-differentiation potential.

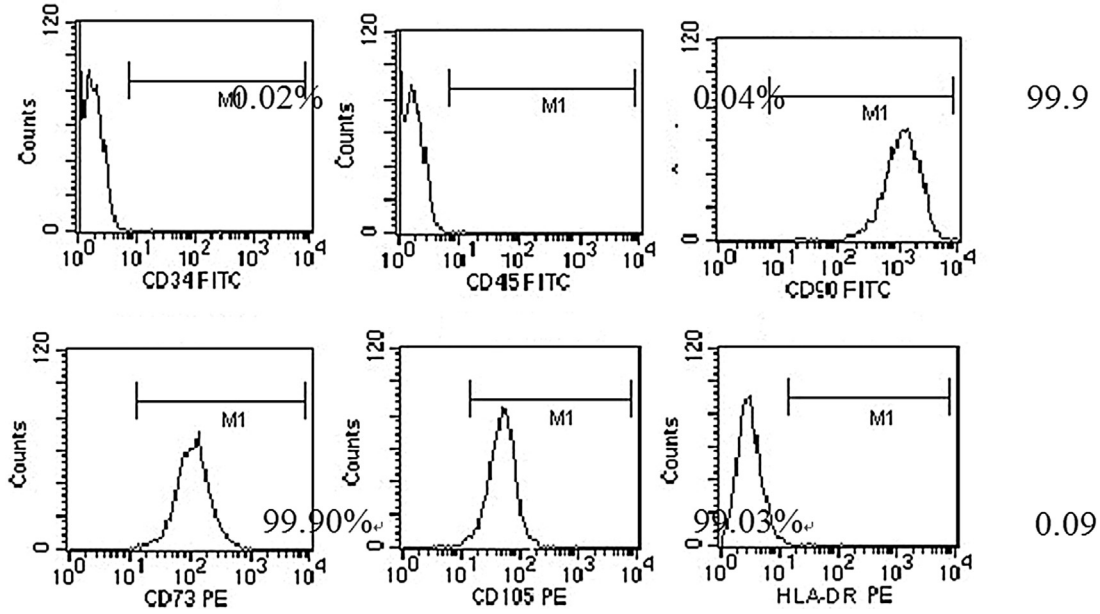

Fig. 1. Immunophenotype of UC-MSCs by FACS analysis. UC-MSCs were positive for CD73, CD90, and CD105, and they were negative for CD34, CD45 and HLA-DR.

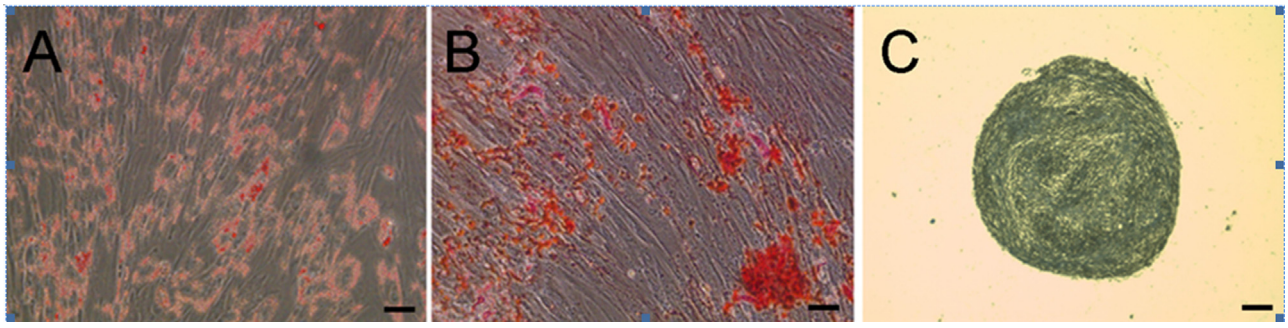

Fig. 2. Differentiation of PD-MSCs towards adipogenic, osteogenic and chondrogenic lineages. (A) Adipogenic differentiation was indicated by the accumulation of neutral lipid vacuoles by staining with oil-red-O. (B) Osteogenic differentiation was indicated by calcium deposition stained with Alizarin Red. (C) Chondrogenic differentiation was stained positive for glucosaminoglycans with Alcian Blue. Scale bars: 20 μm.

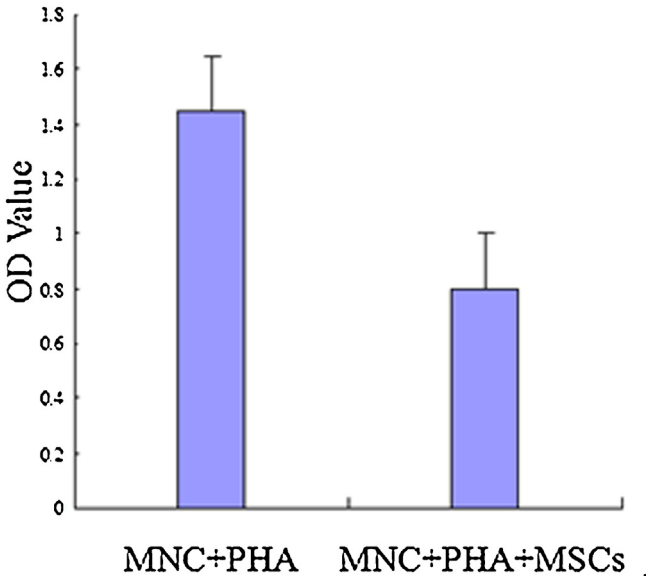

Fig. 3. The inhibitory effect of UC-MSCs on T lymphocyte proliferation. The lymphocyte activity was reduced by 44.82% in the presence of UC-MSCs. The experiments were performed for three times. Data were expressed as mean ± SEM.

3.3. Effects on T cell proliferation

Previous reports have demonstrated that BM-MSCs suppress T cell responses (Miao et al., 2006). To evaluate whether UC-MSCs also had a similar immunoregulatory effect on T-cell proliferation, umbilical cord blood mononuclear cells were stimulated with PHA in the presence or absence of allogeneic irradiated (15 cGy) UC-MSCs. It was demonstrated that UC-MSCs suppressed T cell proliferation to 44.82% of the control value (Fig. 3).

3.4. UC-MSCs modulate the production of inflammatory cytokines in vitro

In order to identify factors that are involved in the immunomodulatory effects mediated by UC-MSCs, we investigated the production of proinflammatory cytokines generated during the incubation of UC-MSCs with PHA-stimulated T cells. When T cells were activated by PHA, the secretion of IFN-γ was significantly inhibited and IL-4 was unregulated by UC-MSCs ($P < 0.05$) (Fig. 4A, B). These results showed that UC-MSCs could modulate the production of inflammatory cytokines by activating T lymphocytes.

3.5. $CD8^+$ T cells are responsible for immunosuppression by flow cytometry

To investigate the mechanism involved in immunosuppression, we first determined whether proliferation of T cells was inhibited in the presence of UC-MSCs. The dynamic change of T cells was investigated during the incubation of the MSCs with PHA-stimulated T cells. When T cells were activated by PHA, the expression of $CD4^+$ T cells showed little change ($P > 0.05$) (Fig. 5), but that of $CD8^+$ T cells had been significantly induced by UC-MSCs ($P < 0.05$) (Fig. 5). These results suggested that UC-MSCs modulate the expression of $CD8^+$ T cells.

3.6. UC-MSCs ameliorate aGVHD in mice transplanted with allogeneic HSCT

Due to UC-MSCs' strong immunosuppressive properties *in vitro*, we further studied whether UC-MSCs changed the incidence of aGVHD among mice subjected to allogeneic HSCT *in vivo*. Lethally irradiated BALB/c mice were grafted with 1×10^7 C57BL/6 BM cells. Under these conditions, 100% of the grafted mice survived without any signs of GVHD, while all ungrafted animals died before day + 15 (data not shown). When 1×10^7 splenocytes from C57BL/6 donor mice were added to the BM transplant (BMT), all mice developed severe signs of GVHD as attested by hunched posture, dull fur, weight loss, and strong diarrhea, and died within 40 days (Fig. 6) after pretreatment. The infusion of UC-MSCs markedly decreased all clinical signs of aGVHD (data not shown), and mediated a significant increase in the survival of transplanted mice than aGVHD control group ($P < 0.05$) (Fig. 6). The severity of aGVHD histological signs in the gut, skin, and liver of GVHD group was worse compared to therapy group (Fig. 7). At day + 28, samples from gut, skin, and liver were studied to evaluate the severity of the GVHD (Fig. 7). The

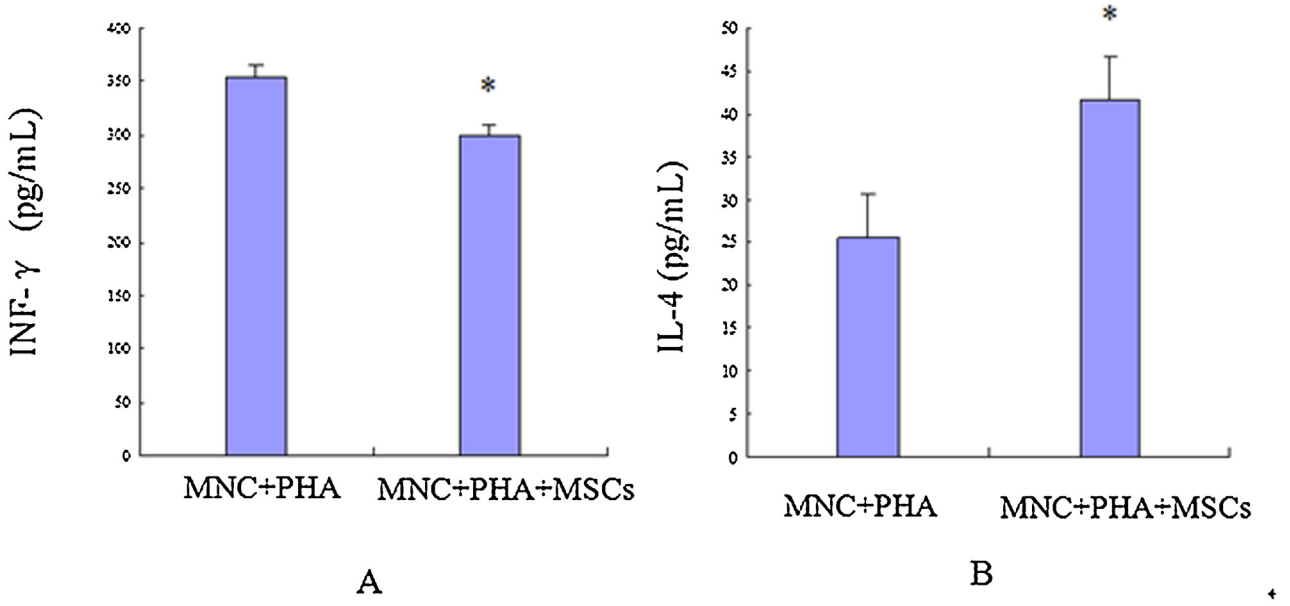

Fig. 4. The effect of UC-MSCs on INF-γ and IL-4 in PHA-stimulated cultures. Protein levels of INF-γ and IL-4 were measured in supernatants of PHA-stimulated cultures with and without irradiated UC-MSCs on day + 3, respectively. The experiments were performed for three times. Data were expressed as mean ± SEM. * $P < 0.05$.

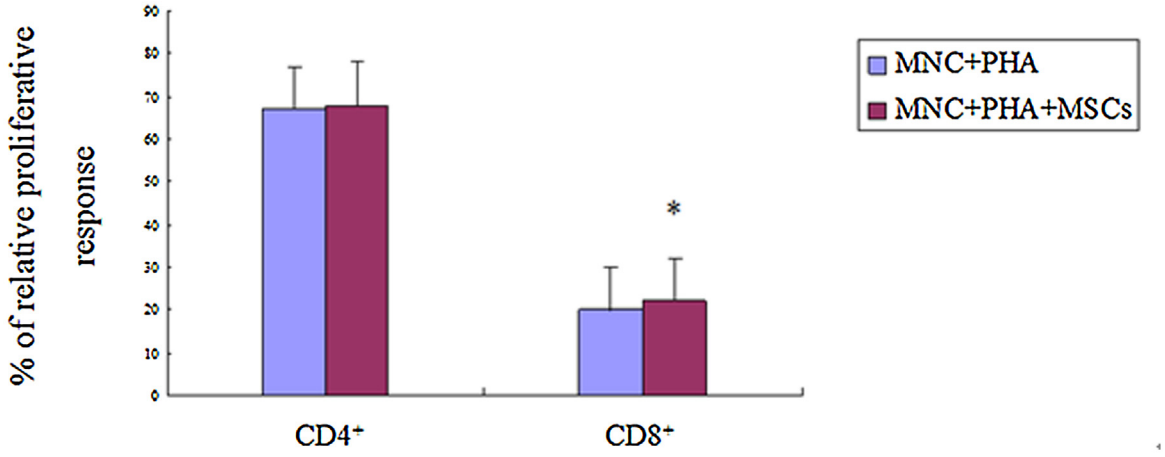

Fig. 5. UC-MSCs modulated T lymphocyte subsets in PHA-stimulated cultures on day + 3. UC-MSCs inhibited the proliferation of $CD8^+$ T cells and lightly decreased the proliferation of $CD4^+$ T cells in PHA-stimulated cultures with and without irradiated UC-MSCs. The experiments were performed for three times. Data were expressed as mean ± SEM. * $P < 0.05$.

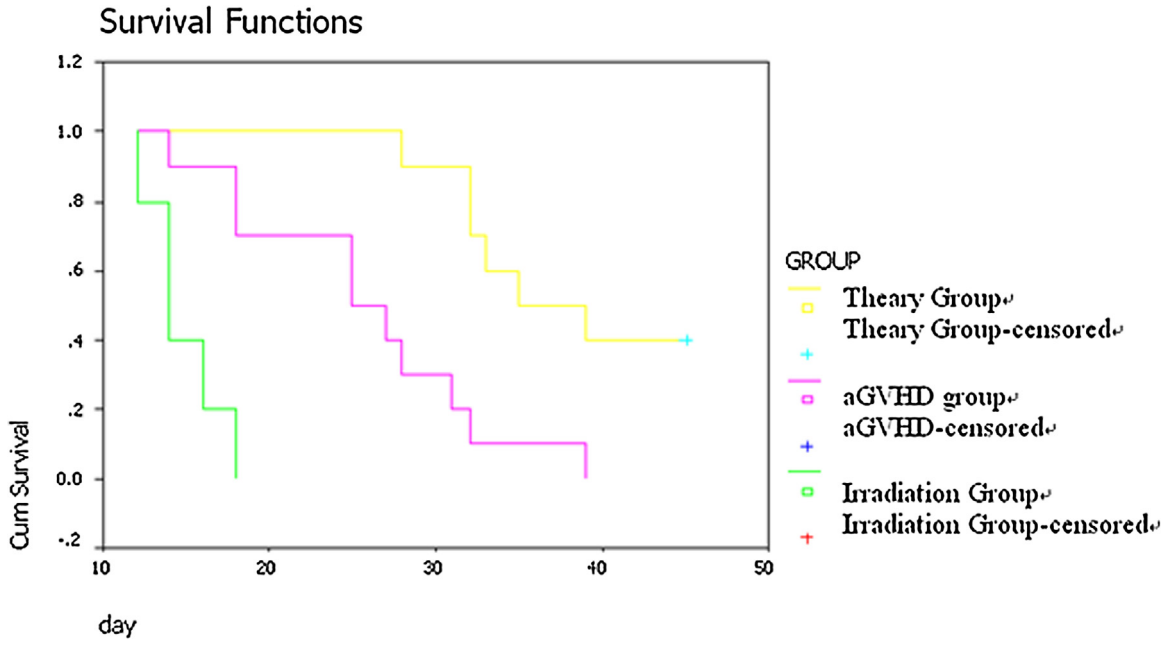

Fig. 6. Kaplan-Meier survival curve of mice with allogeneic transplantation within 40 days after transplantation. UC-MSCs significantly enhanced the survival of the GVHD mice ($P < 0.05$).

Table 1
Effects of UC-MSCs on the incidence and severity of aGVHD.

Group	No.	Grade		
		Skin*	Liver**	Gut**
aGVHD Group	#1	1	2	1
	#2	2	2	2
	#3	1	1	1
Therapy Group	#1	0	1	1
	#2	0	1	0
	#3	0	0	0

The severity of histopathologic changes observed was scored 28 days after transplantation. The skin, liver and gut were obtained from 3 recipients in each group. *$P < 0.05$, **$P > 0.05$, compared between two groups.

aGVHD grade in the skin was notably diminished when UC-MSCs were infused into recipient mice after transplantation ($P < 0.05$), but not in the gut and liver ($P > 0.05$) (Table 1). Thus, UC-MSCs could prevent and lessen aGVHD in allogeneic BMT setting.

4. Discussion

As MSCs from BM are more and more widely applied in clinical practice, such as GVHD and SLE, it is worthwhile to explore an alternative source of MSCs which is readily available and ethically acceptable. Then the placenta is being regarded as a feasible source of MSCs (Parolini et al., 2008). Many researchers have recently reported that different sections from human placenta contain stem cells with phenotypic, functional, and immunomodulatory characteristics similar to those described in MSCs derived from other sources, such as bone marrow and cord blood (Hwang et al., 2009; Li et al., 2007; Li et al., 2005; Miao et al., 2006; Shetty et al., 2010). In the present study, MSCs were successfully isolated from the whole human umbilical cord and their immunomodulatory function was demonstrated by analyzing their morphology, immunophenotype, three-lineage differentiation potentials, PHA-stimulation assays, cytokine spectrum, T cells, and the efficacy of aGVHD treated by UC-MSCs.

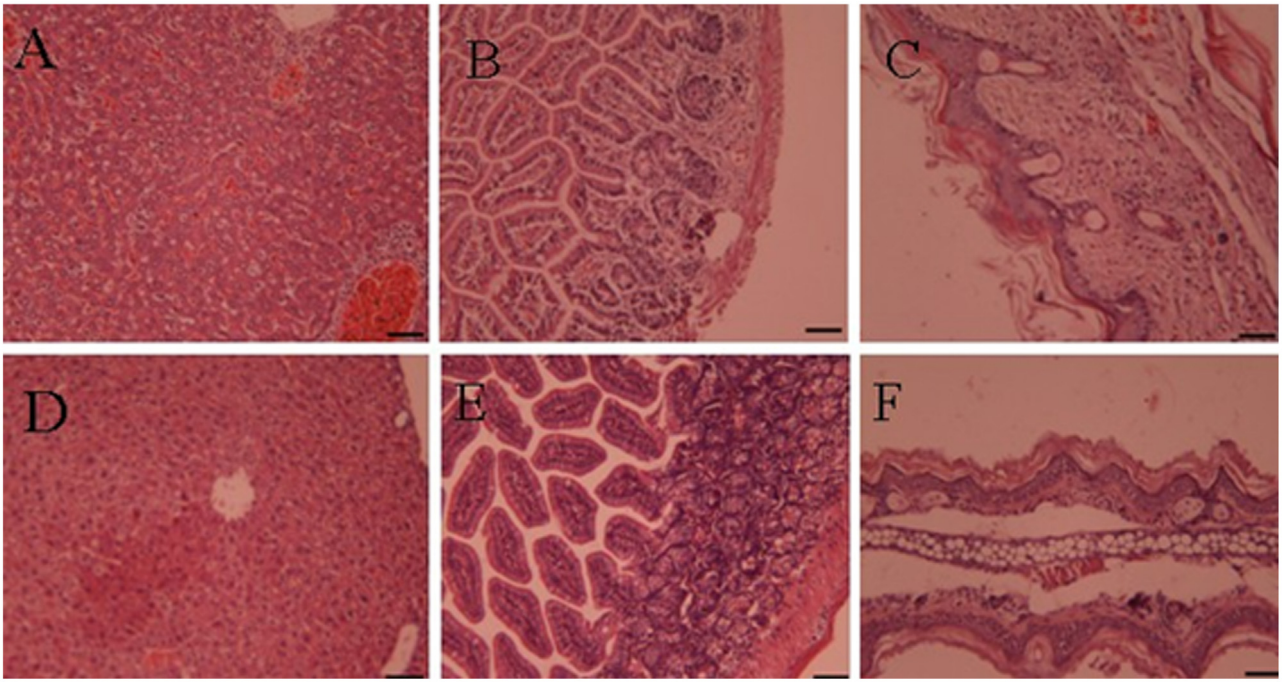

Fig. 7. Histopathology of the liver, intestinal tract, paw skin from mice in aGVHD and therapy group 28 days after transplantation. The liver (A, D), gut (B, E) and skin (C, F). (A) The liver specimen from aGVHD Group showed that the portal area was surrounded by remarkable hepatic lymphocyte infiltration and local hemorrhage. (B) Gut biopsy from aGVHD Group demonstrated severe atrophic mucosa and severe inflammatory cell infiltration. (C) The biopsy specimen of paw skin from aGVHD Group showed parakeratosis, severe lymphocytes infiltration into the superficial layer of the derma, with of basal cell liquefaction degeneration. (D) The liver specimen from Therapy Group showed little lymphocyte infiltration and hemorrhage. (E) There were moderate lymphocyte infiltrations and no crypt abscess in the gut from Therapy Group. (F) The biopsy specimen of paw skin was characterized by mild lymphocyte infiltration, little parakeratosis and basal cell liquefaction degeneration in the superficial layer of the derma in the Therapy Group. Scale bars: 100 μm.

In this study, it was considered that MSCs could be directly obtained from the whole umbilical cord with a single enzyme method. UC-MSCs appeared to share the morphology, phenotype and multi-differentiation with MSCs from adult bone marrow and other fetal tissues (Lu et al., 2006). Some reports demonstrated that BM-MSCs had significant immune modulatory effects on mitogenic stimulation of human peripheral blood lymphocytes (Di Nicola et al., 2002). In this respect, we found for the first time that the whole UC-MSCs had immunomodulatory function. First, UC-MSCs significantly inhibited the proliferation of UCB-MNCs stimulated by PHA. Similarly, MSCs from perfusate of the placenta in Li' report had such immune function (Li et al., 2007). Second, our results indicated that UC-MSCs had exerted immunoregulation partly by affecting T cell subsets, with most reduction in $CD8^+$ T lymphocytes, which may be one of the immunomodulation mechanisms, while the previous report also obtained the same results (Djouad et al., 2003), unlike CD4 and CD8 cells equally inhibited by MSCs from Glennie and Chang's reports (Chang et al., 2006; Glennie et al., 2005). The difference was probably caused by species, culture condition, isolation methods, etc.

Several studies have shown that the inhibition elicited by MSCs is mediated by soluble factors (Rasmusson et al., 2005). We found an increase in IL-4 levels and a reduction in INF-γ levels in mitogenic stimulation assays, which were consistent with previous results on the immunosuppressive effect of MSCs from adipose tissue (Chang et al., 2006). In this connection, we studied whether UC-MSCs could potentially control aGVHD by developing mouse models for xenogenic HSCT. Our findings clearly indicated that UC-MSCs led to a reduction of aGVHD responses, and extended the survival of mice in therapy group. Skin histological grade was significantly different between the two groups, but not in the liver and gut, which owed to tissue variability and the sample number. This study showed that UC-MSCs could efficiently control aGVHD associated with allogeneic HSCT, supported by previous reports on adipose tissue-derived MSCs (Chang et al., 2006). Therefore, our present study provided the basic information that UC-MSCs might act as a good strategy of treating aGVHD.

5. Conclusion

Our study showed for the first time that MSCs obtained from the whole human umbilical cord exerted immunoregulatory properties *in vitro* and *in vivo*, and they can treat aGVHD, opening a new window to the application of UC-MSCs. In this regard, further studies are needed to confirm and expand our preliminary findings in the clinical setting after the safety of UC-MSCs infusion is well-defined.

Acknowledgements

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