Effective Treatment of Severe Steroid-Resistant Acute Graft-Versus-Host Disease With Umbilical Cord-Derived Mesenchymal Stem Cells

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Background. Severe steroid-resistant acute graft-versus-host disease (aGVHD) is associated with high mortality. Bone marrow-derived mesenchymal stem cells (BMMSC) have been found to be immunosuppressive, and intravenous infusion of BMMSC is an effective therapy for steroid-resistant aGVHD. However, acquiring BMMSC requires an invasive procedure.

Methods. We compared umbilical cord-derived mesenchymal stem cells (UCMSC) and BMMSC for morphology, surface markers expression, differentiation, proliferative potential, and their suppressive effects on peripheral blood mononuclear cell proliferation. After institutional review board approved, we intravenously infused ex vivo expanded third-party UCMSC into two patients with severe steroid-resistant aGVHD. Adverse effects and patient responses of UCMSC were monitored. All procedures for UCMSC processing complied with current good tissue practice requirements.

Results. We found that UCMSC had superior proliferative potential and more suppressive effects on peripheral blood mononuclear cell proliferation compared with BMMSC. The aGVHD improved dramatically after each of four infusions of UCMSC into the two patients. No adverse effects were noted. Both patients are doing well now.

Conclusions. Considering that acquiring UCMSC is noninvasive, these cells would appear to be the ideal candidates for clinical cell-based therapies. This is the first report of UCMSC in a human clinical application, and this procedure seems both feasible and safe. These findings suggested that UCMSC were effective for treating aGVHD.

Keywords: Acute graft-versus-host disease, Umbilical cord, Mesenchymal stem cells.

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Although the mechanisms involved in the immunosuppressive effects of mesenchymal stem cells (MSC) have not been clearly defined, their immunosuppressive properties have already been exploited in the clinical setting (*1*). The bone marrow is the standard source of MSC for human clinical applications (*2–10*); however, their harvest involves an invasive and painful procedure. MSC can be acquired from fetal tissues without invasive procedures, thus avoiding risk to the donor. Umbilical cords are rich of MSC, and umbilical cord-derived MSC (UCMSC) have been shown to be easy to isolate, culture, and acquire (*11*). Therefore, the umbilical cord may be considered an alternative source of MSC for clinical applications.

Hematopoietic stem-cell transplantation is an effective therapeutic modality for a variety of disorders. However, severe acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem-cell transplantation is associated

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with a high mortality, especially in the case of steroid-resistant aGVHD (*12*). Because of the immunosuppressive properties of bone marrow-derived MSC (BMMSC) (*1*, *13*), intravenous infusion of BMMSC was found an effective therapy for severe steroid-resistant aGVHD (*2–4*).

In humans, no major adverse events have been reported after intravenous infusion of BMMSC (*2–10*). In our previous study, we intravenously transplanted UCMSC and cord blood to enhance hematopoiesis in seven patients. In that study (unpublished data), we found that intravenous infusion of UCMSC was feasible, and there were no side-effects during or after the infusion, even after several years of follow-up. Because of the safe use of UCMSC in our previous experience, in this study, we compared the characteristics of UCMSC with those of BMMSC and report the response in two patients with severe steroid-resistant aGVHD after infusion of UCMSC. This is the first reported use of UCMSC in human clinical applications.

RESULTS

Comparisons Between UCMSC and BMMSC

The UCMSC showed the same uniform spindle-shaped morphology as the BMMSC, and they were both positive for CD13, CD29, CD44, CD73, CD90, and CD105 but negative for CD14, CD31, CD34, CD45 and human leukocyte antigen (HLA)-DR. No significant difference was noted in the expression of any surface marker between the UCMSC and BMMSC. After specific induction, the UCMSC had a significantly stronger osteogenic potential but a lower capacity for adipogenic differentiation than the BMMSC (data not shown).

A significant increase in cumulative population doubling from passage 2 to passage 5 was observed between the UCMSC (n=9) and the BMMSC (n=9) (6.2 ± 0.8 vs. 3.0 ± 0.7, respectively; $P<0.05$). The population doubling time was significantly shorter for the UCMSC than for the BMMSC (1.5 ± 0.2 days vs. 9.5 ± 1.1 days, respectively; $P<0.05$).

As shown in Figures 1 and 2, in mixed cultures of peripheral blood mononuclear cells (PBMC) stimulated by phytohemagglutinin (PHA) or irradiated PBMC and incubated in the presence of BMMSC or UCMSC at various cell dosages, the amount of PBMC decreased significantly ($P<0.01$ in all cases) compared with the co-cultures without BMMSC or UCMSC. The decrease of PBMC in the UCMSC co-cultures was significantly greater than that of the BMMSC co-cultures at each comparable dosage ($P<0.01$ in all cases; Figs. 1 and 2). In addition, the suppressive effects of UCMSC were dose dependent, as demonstrated by the decrease in PBMC proliferation with increasing doses of UCMSC. The suppressive effects on PBMC were still noted after progressive dilution, even as low as 100 cells. The suppressive effects of BMMSC or UCMSC on the PBMC were confirmed by the results of the carboxyfluorescein diacetate succinimidyl ester (CFSE) assays.

Patient Description

The first patient was a 4-year-old boy with severe aplastic anemia who had failed to respond to immunosuppressant therapy. He received peripheral blood stem-cell transplantation (PBSCT) from a high-resolution six of six HLA-matched unrelated donor. The conditioning regimen consisted of cyclophosphamide (120 mg/kg) and fludarabine (125 mg/m^2). GVHD prophylaxis included rabbit antithymoglobulin (10 mg/kg) and cyclosporine (3 mg/kg/day) combined with short-course methotrexate. On day 45 after PBSCT, the patient developed vomiting, hyperbilirubinemia, and maculopapular rash on both legs and arms. Methylprednisolone (2 mg/kg/day) was administered. Skin biopsy proved aGVHD. By day 50, because of progression to grade IV aGVHD with bloody diarrhea including severe abdominal pain, progres-

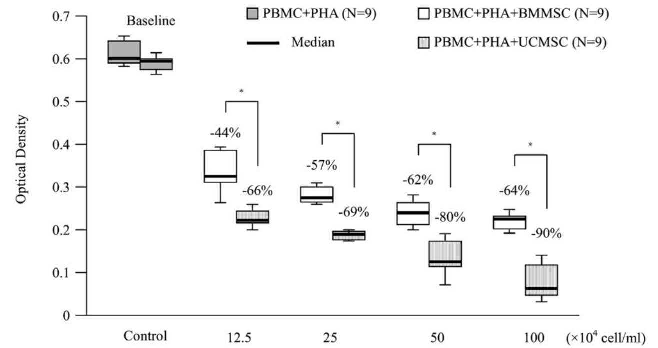

FIGURE 1. Suppressive effects of BMMSC and UCMSC on PBMC proliferation stimulated by PHA. The reduction in PBMC proliferation activated by PHA after co-culture with BMMSC or UCMSC at various dosages was compared with PBMC proliferation activated by PHA without BMMSC or UCMSC culture. The reduction in the number of PBMC between BMMSC and UCMSC at various cell dosages was also compared. The data are presented in a box plot (median and range) calculated from experiments performed in triplicates. **P* less than 0.05. BMMSC, bone marrow-derived mesenchymal stem cells; UCMSC, umbilical cord-derived mesenchymal stem cells; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

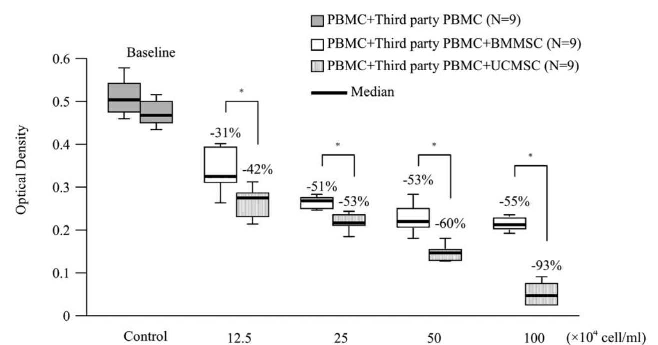

FIGURE 2. Suppressive effects of BMMSC and UCMSC on PBMC proliferation stimulated by third party PBMC. The reduction in PBMC proliferation activated by irradiated third party PBMC after co-culture with BMMSC or UCMSC at various dosages was compared with PBMC proliferation activated by third party PBMC without BMMSC or UCMSC co-culture. The reduction in the number of PBMC between BMMSC and UCMSC at various cell dosages was also compared. The data are presented in a box plot (median and range) calculated from experiments performed in triplicates. **P* less than 0.05. BMMSC, bone marrow-derived mesenchymal stem cells; UCMSC, umbilical cord-derived mesenchymal stem cells; PBMC, peripheral blood mononuclear cells.

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sion of skin rash, and elevation of bilirubin concentration (total bilirubin, 6.3 mg/dL), high-dose methylprednisolone (30 mg/kg/day) was administered for 3 days, but the treatment was ineffective.

After the institutional review board at the China Medical University Hospital approved the therapy and parental written informed consent was obtained, we requested suitable UCMSC from the UCMSC bank. One unit of UCMSC matching four of six HLAs with the patient was obtained. These cells were thawed and expanded in vitro to 6.6×10^7 cells (3.3×10^6 cells/kg body weight) at passage 3 and then cryopreserved again. After the UCMSC were shown to have a normal karyotype and the absence of pathogenic contamination, they were thawed, washed, and infused into the patient through a central venous catheter 13 days after our initial cell request. Before UCMSC infusion, the grade of aGVHD was IV (gut staging: +4; skin staging: +3; liver staging: +3) according to standard aGVHD criteria proposed by Glucksberg et al. (*14*). Two days after infusion, the severity of diarrhea improved and subsequently subsided. The total bilirubin concentration decreased to a normal range 6 days after UCMSC infusion, and the skin rash declined, scaled, and then disappeared 7 days after UCMSC infusion. No clinical manifestations of aGVHD in the gut, skin, or liver were noted 7 days after the UCMSC infusion.

Unfortunately, by day 28 after the infusion, the patient had recurrent diarrhea with abdominal pain, maculopapular rash, and an elevated bilirubin concentration of 6.3 mg/dL. He received the second infusion 12 days after the recurrence of aGVHD with three of six HLA-matched UCMSC expanded to 7.2×10^6 cells/kg at passage 3. Within 5 days, the diarrhea subsided, skin rash improved, and total bilirubin concentration normalized. Before the second UCMSC infusion, the grade of aGVHD was III (gut staging: +3; skin staging: +2; liver staging: +3). No clinical manifestations of aGVHD in the gut, skin, or liver were noted 6 days after the UCMSC infusion.

Despite continued cyclosporine administration, by day 25 after the second UCMSC infusion, the GVHD flared up again and the patient presented with skin rash, bloody diarrhea, severe abdomen pain, and hyperbilirubinemia (total bilirubin concentration of 5.1 mg/dL). He received the third infusion of UCMSC (8.0×10^6 cells/kg at passage 4) matching three of six HLAs on day 12 after the onset of GVHD. Before the third UCMSC infusion, the grade of aGVHD was IV (gut staging: +4; skin staging: +3; liver staging: +2). No clinical manifestations of aGVHD in the gut, skin, or liver were noted 6 days after the UCMSC infusion. We began to taper the cyclosporine dose slowly 2 months after the third infusion, and the GVHD did not present again. We discontinued all immunosuppressive drugs 12 months after PBSCT, and no chronic GVHD was observed. The patient is now well 15 months after PBSCT.

The second patient was a 6-year-old boy with acute lymphoblastic leukemia in second remission. He received a transplant of 2 units of umbilical cord blood (UCB). The 2 units of UCB were matched with each other and the recipient at 4 of 6 HLA antigens based on allele-level HLA-A, -B, and -DRB1 typing. The conditioning regimen consisted of cyclophosphamide (120 mg/kg), etoposide (30 mg/kg), and fractionated total body irradiation (12 Gy). GVHD prophylaxis included rabbit antithymoglobulin (10 mg/kg) and cyclosporine (3 mg/kg/day) combined with mycophenolate mofetil (30 mg/kg/day). On day 21 after UCB transplantation, the patient developed hyperbilirubinemia and maculopapular rash on the trunk. Methylprednisolone (2 mg/kg/day) was given. Skin biopsy proved aGVHD. By day 25, because of progression to grade IV aGVHD, high-dose methylprednisolone (30 mg/kg/day for 3 days) and oral mycophenolate mofetil (45 mg/kg/day) were given. However, the treatment was ineffective. Six days after we requested cells from the UCMSC bank, he received a UCMSC infusion with UCMSC matching three of six HLAs expanded to 4.1×10^6/kg at passage 3. Before the UCMSC infusion, the grade of aGVHD was IV (gut staging: +4; skin staging: +3; liver staging: +3). No clinical manifestations of aGVHD in the gut, skin, or liver were noted 5 days after the UCMSC infusion. We began to taper the cyclosporine dose slowly 2 months after the UCMSC infusion. We discontinued all immunosuppressive drugs 10 months after UCB transplantation and no chronic GVHD was observed. The leukemia is in complete remission, and the patient is now well 18 months after UCB transplantation.

No severe infections were noted in the two patients during the treatment period with UCMSC. There were no side effects during or after each UCMSC infusion. We maintained cyclosporine at trough levels of 200 to 400 ng/mL and neither added other immunosuppressive therapies nor increased the immunosuppressant dosages during the period of UCMSC treatment. The characteristics of the patients and UCMSC are summarized in Table 1. The clinical course of the first patient to the UCMSC infusions is summarized in Figure 3. In our previous experience at our children's hospital, 12 patients died within 2 months of the onset of severe, steroid-resistant aGVHD.

DISCUSSION

In addition to BMMSC, MSC derived from adipose tissue (*15*) and fetal tissues, such as umbilical cord (*16*), UCB (*17*), and placenta (*18*), have been found to have immunosuppressive properties. The mechanisms involved in the immunosuppressive effects of MSC may be associated with T lymphocytes, dendritic cells, natural killer cells, and B lymphocytes (*1*). In this study, because PBMC contain all of the cells involved in the complicated immune interactions, we used PBMC as responder cells. This is the first report to compare the suppressive effects on PBMC between UCMSC and BMMSC, and we found that UCMSC have superior suppressive effects in vitro on PBMC proliferation compared with BMMSC.

MSC have been used for tissue repair and regeneration (*19*). Although MSC are easy to obtain from umbilical cords, to our knowledge, UCMSC have not been used in human clinical applications. In this study, we infused UCMSC four times into two human recipients with severe steroid-resistant aGVHD. After each UCMSC infusions, the manifestations of aGVHD dramatically improved, even without additional immunosuppressive therapies or increased immunosuppressant dosages, indicating the in vivo immunosuppressive effects of UCMSC and their potential role for the treatment of severe aGVHD.

Over time, donor MSC are eliminated in the recipients after infusion (*20*). Le Blanc et al. (*4*) reported that half of

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TABLE 1. Characteristics of patients and UCMSC

Patient	1			2
Age (yr)/gender	4/male			6/male
Diagnosis	SAA			ALL
HLA match of HSC	6/6 matched unrelated donor			4/6 mismatched unrelated donors
HSC source	PBSC			Two units of UCB
Conditioning regimen	Flu+Cy			Cy+VP16+TBI
GVHD prophylaxis	CsA+MTX+ATG			CsA+MMF+ATG
	First infusion	**Second infusion**	**Third infusion**	**First infusion**
UCMSC infusion				
HLA match of UCMSC	4/6	3/6	3/6	3/6
Duration of UCMSC expansion (days)	8	10	12	9
Cell dose, $\times 10^6$/kg	3.3	7.2	8.0	4.1
Cell passage	3	3	4	3
Outcome	Alive 15 mo after PBSC transplantation			Alive 18 mo after UCB transplantation

UCMSC, umbilical cord-derived mesenchymal stem cells; SAA, severe aplastic anemia; ALL, acute lymphoblastic leukemia; HLA, human leukocyte antigen; HSC, hematopoietic stem cell; PBSC, peripheral blood stem cells; UCB, umbilical cord blood; Flu, fludarabine; Cy, cyclophosphamide; VP16, etoposide; TBI, total body irradiation; GVHD, graft-versus-host disease; CsA, cyclosporine; MTX, methotrexate; ATG, rabbit antithymoglobulin; MMF, mycophenolate mofetil.

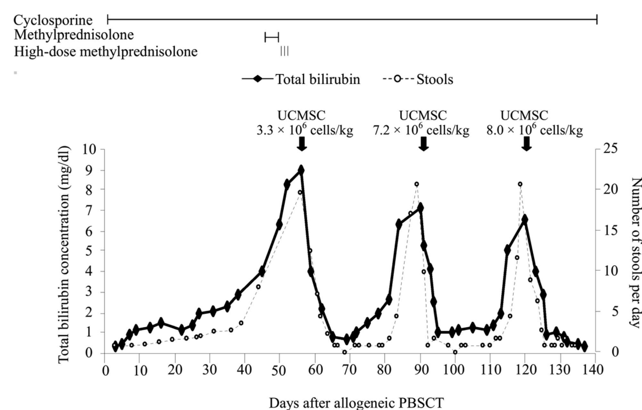

FIGURE 3. Clinical course of the first patient and responses to UCMSC. UCMSC, umbilical cord-derived mesenchymal stem cells; PBSCT, peripheral blood stem-cell transplantation.

patients required more than one BMMSC infusion to control severe aGVHD. In this study, we needed to infuse UCMSC three times to control aGVHD in the first patient. Therefore, multiple doses of MSC may be needed for some severe immune disorders, such as GVHD, in some patients until immune tolerance develops.

There are some limitations to this study. The responses and results of BMMSC for treatment of aGVHD were found to be better in children than in adults (*4*). In this study, we found that UCMSC were effective in the treatment of aGVHD; however, both patients were children. Further studies are needed to confirm the effectiveness of UCMSC, especially in adult patients. It is important to determine whether treatment with MSC, including BMMSC or UCMSC, would further aggravate immune incompetence and increases the risk of infection. Although no severe infections were noted in the two patients treated with UCMSC, whether UCMSC treatments increase the risk of infection require further study.

UCMSC are easier to obtain than BMMSC and cause no suffering to the donor, indicating that they might be the ideal candidates for cell-based therapy. The umbilical cord may be an alternative MSC source, similar to UCB as a good source of hematopoietic stem cells. This is the first reported use of UCMSC in a human clinical application, and this procedure seems both feasible and safe. UCMSC were effective against aGVHD in our patients, but prospective, controlled studies with UCMSC are warranted.

MATERIALS AND METHODS

Collection and Cryopreservation of UCMSC

All eligible umbilical cord donors had tested negative for HIV-I, HIV-II, human T-lymphotropic virus-I, human T-lymphotropic virus-II, hepatitis B and C, cytomegalovirus, and syphilis and were free of active infection during UCMSC harvesting. The umbilical cords were collected immediately after parturition and express shipped to an UCMSC bank for MSC processing. UCMSC were harvested from Wharton's jelly as previously described (*21*). In brief, the umbilical cord was sectioned, and the main vessels were carefully removed to acquire Wharton's jelly. Wharton's jelly was digested in 1 mg/mL of collagenase (Sigma, St. Louis, MO) and plated in α-MEM (Gibco; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco; Invitrogen) and penicillin–streptomycin (Gibco; Invitrogen) at 37°C with 5% CO_2 in a humidified atmosphere. After 48 hr, the medium with suspended nonadherent cells was discarded and fresh medium was added. When 80% to 90% confluence was reached, the cells were detached with 0.25% trypsin-EDTA (Gibco; Invitrogen) and subcultured for further expansion. After UCMSC were identified and proven negative for bacteria, fungi, mycoplasma, and endotoxins, the cells were formulated in 10% dimethyl sulfoxide (WAK-Chemie Medical GmbH, Steinbach, Germany), cryopreserved by using a controlled-rate freezer, and maintained in liquid nitrogen at the UCMSC bank. All procedures for UCMSC processing complied with the current good tissue practice requirements.

Expansion, Thawing, and Infusion of UCMSC

On a physician's request, we selected a unit of banked UCMSC matching HLA based on antigen-level HLA-A, HLA-B, and HLA-DR typing with the

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recipient. Then, the selected unit of UCMSC was thawed, expanded with subculture to adequate cell dosages, and cryopreserved again. After UCMSC expansion and before cryopreservation again, karyotyping was performed. After the cells were shown to have a normal karyotype and be negative for bacteria, fungi, mycoplasma, and endotoxins, the cells were thawed, washed, and intravenously infused into the patient with aGVHD.

Identification of MSC

BMMSC were harvested from the bone marrow of healthy donors as previously described (*22*). BMMSC and UCMSC were immunolabeled with the following mouse anti-human antibodies: CD14, CD34, CD45, CD13, CD29, CD31, CD44, CD90, and HLA-DR (BD Biosciences, San Jose, CA); CD105 (AbD Serotec, Oxford, United Kingdom); and CD73 (BD Pharmingen, San Diego, CA). The cells were incubated with a secondary antibody, anti-mouse IgG-fluorescein isothiocyanate or IgG-phycoerythrin, and analyzed by flow cytometry (BD Biosciences). The MSC underwent osteogenic, adipogenic, and chondrogenic differentiation under specific induction conditions (*19*, *21*, *22*).

Proliferative Potential Assay

The population doubling of the cultured MSC was calculated according to the previous report using the following equation: population doubling$=\log_2$ (number of viable cells at harvest/number of seeded cells) (*22*). The population doubling time was derived from the time interval divided by cumulative population doubling from passage 2 to passage 5.

PBMC Proliferation Assay

We used the PBMC proliferation assay to assess the suppressive effects of BMMSC and UCMSC (*13*). In brief, different cell dosages of BMMSC or UCMSC were plated into 96-well plates containing responder cells (PBMC, 5×10^5 cells/mL) and the stimulator. In the mitogen proliferative assays, PBMC were stimulated using the nonspecific mitogenic stimuli, 5 μg/mL PHA (Sigma, St. Louis). In mixed lymphocyte cultures, irradiated (30 Gy) third-party PBMC (5×10^5 cells/mL) were used as the stimulator. We irradiated the BMMSC and UCMSC (25 Gy) to prevent their proliferation. After 72 hr of co-culture, 200 μL of the cells from each well were transferred to new 96-well plates with 10 μL of WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). After incubation for 1 to 4 hr at 37°C, the absorbance was measured at 450 μm with a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) using a reference wavelength of 600 nm.

We also used the CFSE assay to confirm the results. BMMSC or UCMSC were plated into 24-well plates containing CFSE-labeled PBMC (5×10^5 cells/mL) with a CD3/CD28 antibody in RPMI-1640 (Invitrogen). Analysis of cell division was performed by flow cytometry.

Statistical Analysis

All experiments were performed in triplicate, and the data are presented as median and range. A box-plot was also made to represent the suppressive effects of BMMSC and UCMSC. The reduction in PBMC proliferation activated by the stimulator (PHA or irradiated PBMC) after co-culture with BMMSC or UCMSC at various cell dosages was compared with PBMC proliferation activated by the stimulator (PHA or irradiated PBMC) without co-culture with BMMSC or UCMSC using the Kruskal–Wallis test with Bonferroni adjustment for multiple testing. Comparisons of the reduction in the number of PBMC between BMMSC and UCMSC at various cell dosages were analyzed using the Wilcoxon rank sum test. A P value of less than 0.05 was considered to indicate a significant difference.

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