An In Vitro Assay to Evaluate the Immunomodulatory Effects of Unrestricted Somatic Stem Cells

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ABSTRACT

Background: Unrestricted somatic stem cells (USSC) are cord blood stem cells that have been considered as candidates for the regulation of immune responses. Therefore, potential exists for their use in the suppression of immune response after transplantation surgery. **Objective:** The aim of this study was evaluation of the effect of USSC on mixed lymphocyte reaction (MLR) as a model for graft rejection. **Methods:** USSC and mesanchymal stem cells (MSC) were isolated and cultured from cord blood and bone morrow, respectively. The immunophenotypes of USSC and MSC were evaluated by flow cytometery and USSC and MSC were co-cultured with peripheral blood lymphocytes (PBL) in an MLR to evaluate the immunomodulatory effect of these cells as a percentage of the control response. **Results:** Current study demonstrated that proliferation of lymphocytes in the MLR was decreased after treatment with USSC, in a similar fashion to that seen with MSC. **Conclusion:** It can be concluded that USSC have similar regulatory effects as MSC on the MLR, which can be used as an indicator for potential organ rejection after transplantation. Therefore, the immunregulatory effect of these cells could be used in the clinic during organ transplantation and in the management of autoimmunity.

Keywords: Mesanchymal Stem Cells, Mixed Lymphocyte Reaction, Transplantation

INTRODUCTION

Although there is a wealth of knowledge and published data in the field of organ transplantation, the reaction of the immune system against transplanted tissue is a problem, which needs to be overcome (1). Several immune suppressive agents are industrially synthesized and are currently being used for prevention of graft versus host diseases, host

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versus graft diseases and graft rejection (2,3). Due to the known side effects of these agents, such as susceptibility to infections and cancers, researchers in the field have initiated programs to discover an appropriate mechanism for the regulation and management of the immune system against transplanted organs. Recent studies have revealed the important regulatory roles some stem cells have on modulating the immune system in response to transplanted grafts (4). In addition to immuno-suppressive reagents (2,3), mesanchymal stem cells (MSC) are also known as immune suppressive cells (4) and in some cases are used for prevention of autoimmunity or regulation of graft rejection (4,5). The use of unrestricted somatic stem cells (USSC) has also been reported to have potential in clinical applications. These cells are immature compared to MSC and do not express differentiation factors such as CD45 and class 2 HLA antigens and they possess the ability to differentiate toward MSCs (6). Moreover, some studies demonstrated that these cells not only produce anti-inflammatory cytokines *in vitro,* they also secret essential cytokines for differentiation and proliferation of bone marrow stem cells *ex vivo* (7). Due to the regulatory roles of these cells on the immune system, some investigators suggested that the presence of such cells in cord blood plays a supportive role in the regulation of the maternal immune response against embryonic tissues as a non-self tissue (8). Studies showed that mismatched HLA and gene polymorphisms are one of the main reasons for graft rejection (9,10). The mixed lymphocyte reaction (MLR) is a good test that evaluates the reaction of recipient lymphocytes against donor lymphocytes (11). The MLR test is dependant on mismatched HLA and gene polymorphisms to stimulate lymphocyte proliferation, which mimics the situation found in organ transplants between non matched donor and host. Therefore, any agent or cell system that can down-regulate the MLR can be assumed as a useful aid for graft acceptance in a patient.

Therefore, this study aimed to evaluate the suppressor effects of USSC on MLR. An additional aim of this study was to compare the effects of USSC and MSC on MLR.

MATERIALS AND METHODS

USSC Isolation and Culture. Processing of human full-term umbilical cord blood (UCB) samples and subsequent isolation of USSC from UCB were performed as previously described by Kogler et al., (6). Briefly, the mononuclear cell (MNC) fraction was obtained by a Ficoll (Pharmacia-Amersham, UK) gradient separation followed by ammonium chloride lysis of red blood cells. After two washes in phosphate-buffered saline (PBS), the collected mononuclear cells were resuspended in low-glucose Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL, USA) supplemented with 30% fetal calf serum (FCS) (GIBCO-BRL, USA), low dexamethasone (10^{-7} M) (Sigma, USA), 2 mM L-glutamine (GIBCO-BRL, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO-BRL, USA). Expansion of the cells was performed in the absence of dexamethasone in the same medium as described above. All cells were incubated at 37ºC in an atmosphere of 95% O2 and 5% CO2. For subculture, when the cells reached 80% confluence, they were removed from the flask with 0.25% trypsin (GIBCO-BRL, USA) and reseeded into a 50 ml Falcon flask. Medium was changed twice per week. Morphology and viability of isolated USSC were observed and evaluated by a light microscope. In order to test their adipogenic and osteogenic differentiation capabilities, induction medium was added and then differentiation was confirmed using alizarin red S and oil red O staining for osteogenesis and adipogenesis, respectively.

MSC Isolation and Culture. A volume of 10 ml of bone morrow (BM) was collected and then BM mononuclear cells were isolated and rinsed twice with a PBS-EDTA solution. Cells were counted and seeded into Falcon flasks at 1-2 × 10^6 cells per cm. The preferred culture medium was DMEM supplemented with 10% FBS (GIBCO-BRL, USA). Following 3 days of culture, non-adherent cells were discarded by washing with PBS. Every 3 days, the medium was replaced by a freshly prepared culture medium. Morphology, adipogenic and osteogenic differentiation capabilities of MSC were evaluated in the same way as USSC.

Low Cytometric Analysis. The phenotype of USSC and MSC were analyzed by flow cytometry. The expression of surface markers on USSC and MSC were separately evaluated using commercial mouse monoclonal antibodies against human CD105, CD106, CD90, CD117, CD54, CD166, CD10, CD45, HLA-ABC, CD31 (eBioscience, ESP), CD34 (Dako, USA) and CD133 (Milteny Biotech, Germany). CD10, CD44, CD166, HLA-ABC, CD90, CD54, and CD105 positive and CD34, CD45, CD117, CD133, CD106 and CD31 negative cells were considered as USSC, whereas CD10, CD44, CD166, HLA-ABC, CD90, CD54, CD106 and CD105 positive CD34, CD45, CD117, CD133 and CD31 negative cells were considered as MSC (Figure 1). The cells were detached by trypsin/EDTA and incubated with specific antibodies and/or isotype controls in 100 μl of PBS-BSA (3%) for 1 h at 4°C. Approximately 1×10^5–1×10^6 cells were used for each antibody. The cells were then fixed with 1% paraformaldehyde and analyzed with a periodic acid-schiff (PAS) flow cytometer using FloMax software (Partec, Münster, Germany).

MLR in the Presence of Various Concentrations of MSC and USSC. Purified peripheral blood lymphocytes (PBL) were prepared by centrifugation of heparinized blood on Ficoll–Isopaque (Lymphoprep, Nycomed, Oslo, Norway). Separated cells were cultured in RPMI-1640 medium. This method has been described in detail elsewhere (12). To study the regulatory effects of MSC and USSC on lymphocyte reactivity, irradiated PBL (3,000 cGy), in concentrations ranging from 0.1 to 40% of the total number of responder cells were incubated together in reaction wells. Typically 100–40,000 MSC were added at the beginning of the experiment, unless otherwise stated. Radiation was used as a method to inactivate proliferation of the stimulatory lymphocytes. Apart from the inactivation the irradiated cells met all conditions for survival. USSC and MSC were allogenic to responder or stimulatory lymphocytes. Lymphocytes were stimulated with irradiated lymphocytes from one or a pool of five donors as indicated. All experiments were run in triplicate. The standard deviation (SD) was approximately 10% and always less than 20%. ^{3}H thymidine with a specific activity of 5 Ci/mmol/l (Radiochemical Centre, Amersham, UK) in 0.02 ml PBS was added to each culture 24 h before cell harvesting. Cells were harvested automatically on a glass fibre filter using a Tomtec harvesting machine (Harvester 96, Tomtec, Orange, CT, USA). The radioactivity was measured by a micro β-liquid scintillation counter (Wallac, Turku, Finland). The effects of USSC and MSC on MLR were calculated as the percentage of the control response (100%): MLR = [(APx + MSC)/Apx] × 100, where A is the number of responding lymphocytes, P is the pool of lymphocytes from five donors, x is the irradiated stimulator cells and USSC and MSC in culture.

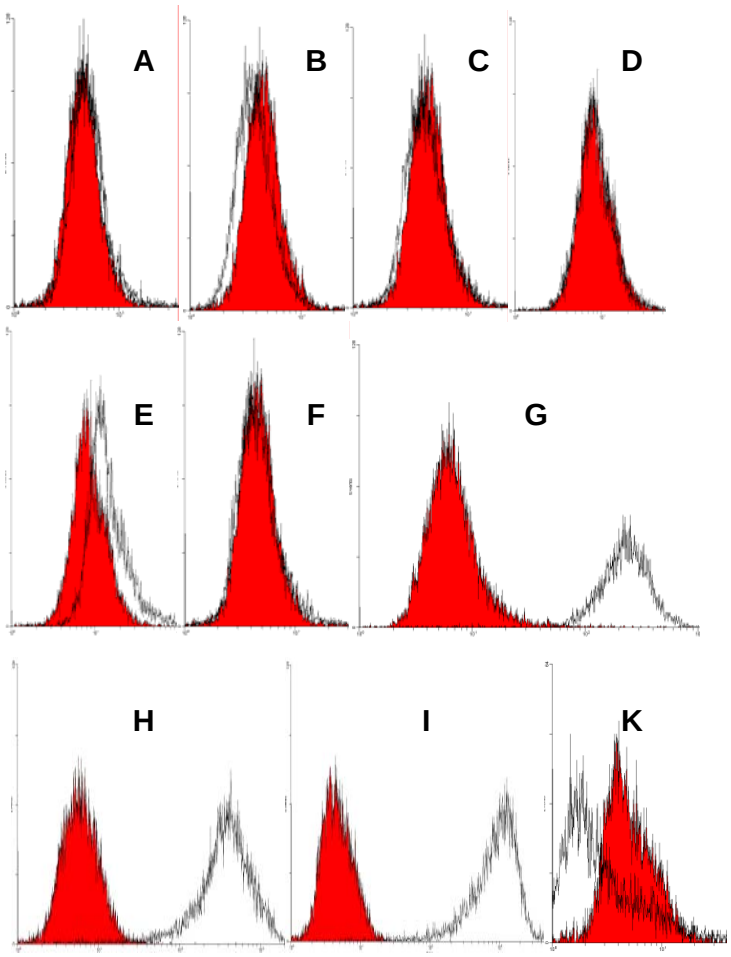

Figure 1. Flow cytometric analysis of MSC and USSC. Data demonstrates the expression of CD133 (A), CD34 (B), CD117 (C), CD45 (D), CD54 (E), CD31 (F), HLA-ABC (G), CD166 (H), CD44 (I) and CD106 (K) which were evaluated in MSC and USSC as described under materials and mothods.

Statistical Analysis. The differences in variables were analyzed by Student *t*-test, as appropriate. The p values of less than 0.05 were considered significant.

RESULTS

Differentiation and Culture of USSC. Our results demonstrated that the morphology of the USSC was similar to that of the MSC by observing spindle-shaped, fibroblast-like colonies adhering to the plastic surface. The results of this study also showed that the USSC were positive for CD10, CD44, CD166, HLA-ABC, CD90, CD54, and CD105, but not for CD34, CD45, CD117, CD133, CD106, and CD31.

In addition as shown in Figures 2 and 3, the results suggest that placenta-derived multi-potent cells have both adipogenic and osteogenic differentiation abilities.

Differentiation and Culture of MSC. Bone-marrow derived MSC from all donors were successfully cultured. They show a lag phase of about 5 days and give rise to a homogeneous population of fibroblast-like cells at a median period of 15 days.

Flow cytometric analysis showed that MSC were uniformly positive for CD10, CD44, CD166, HLA-ABC, CD90, CD106, CD54, and CD105, and were negative for CD34,

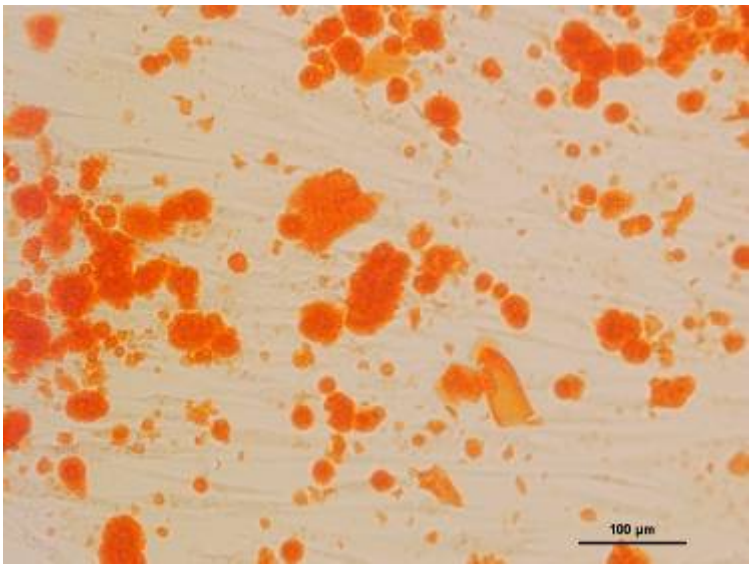

Figure 2. Production of osteogenic cells from USSC in the presence of induction medium. USSC were differentiated down an osteogenic linage and evaluated by staining with alizarin red to identify calcium deposition in bone nodules. Microscopic evaluation of the stained cells shows staining consistent with that expected for osteogenic cells.

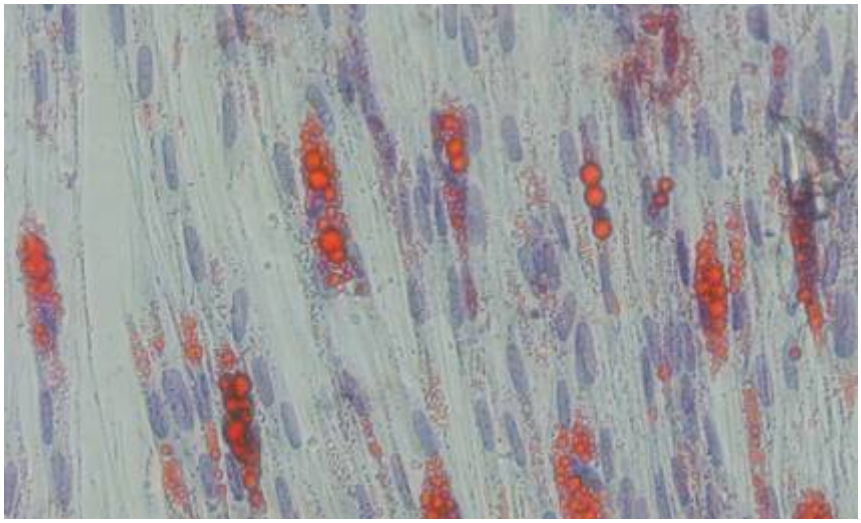

Figure 3. Production of adipogenic cells from USSC in the presence of induction medium. USSC were differentiated down an adipogenic linage and stained with oil red O to identify lipid storage. Microscopic evaluations of the differentiated cells show staining consistent with that expected of apidogenic differentiation.

CD45, CD117, CD133, and CD31. As a result, a pure population of cells was obtained in the second and third passage. Pure-expanded MSC differentiated, into osteogenic, chondrogenic and adipogenic lineages as previously described (3). Briefly, for osteogenic differentiation DMEM supplemented with 10% FBS, 50 µg/ml ascorbate-2 phosphate, 10^{-8} M dexamethasone (Sigma, USA), and 10 mM β glycerophosphate (Sigma, USA) was used. Under these conditions MSC's form aggregate or form nod-

ules and increase their expression of alkaline phosphatase in which calcium accumulation can be seen over time. The bone nodules stain positively with alizarin red (Sigma, USA). Adipogenic differentiation was confirmed through DMEM supplementation with 10% FBS, 50 µg/ml ascorbate-2 phosphate, 10^{-7} M dexamethasone, and 50 µg/ml indomethacin (all from Sigma), Accumulation of lipid in the altered-MSC vacuoles is assayed histologically by oil red O (Sigma, USA) staining. Cell pellets in serum free DMEM were supplemented with ITS+ Premix (GIBCO-BRL, USA) and 10 ng/ml TGF-β1 (Sigma, USA). The cell pellets developed a multilayered, matrix-rich morphology, and histological analysis showed strong staining with toluidine blue (Sigma, USA), indicating for the presence of an abundance of glycosaminoglycans within the extracellular matrix.
The medium for all differentiation conditions was changed every 3 days.

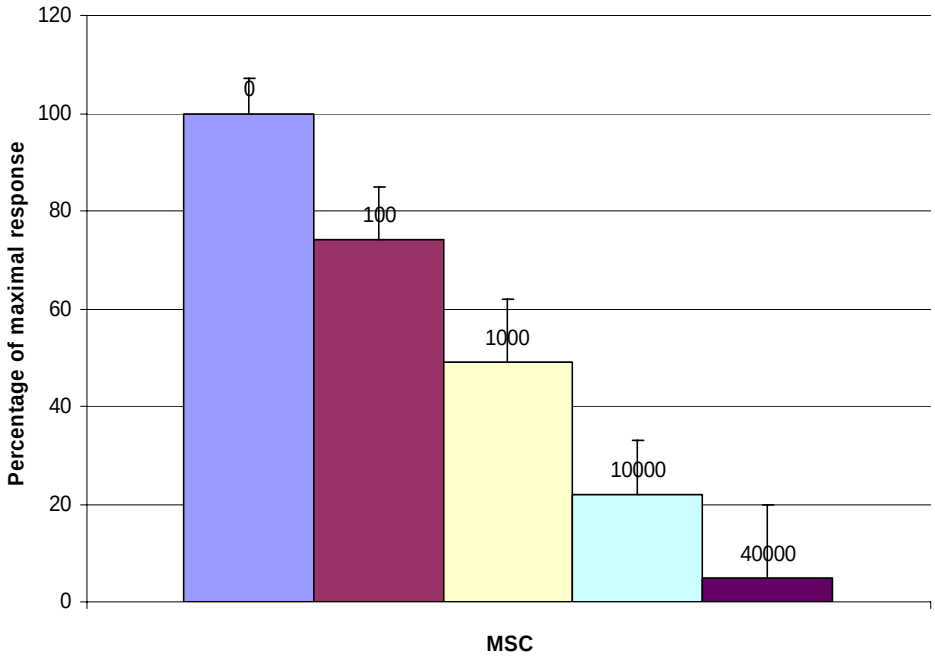

Figure 4. Percentage of maximum response in the MLR assay in the presence of different numbers of MSC. The figure shows the maximal MLR response in the first column as control (no MSC added, labeled 0 above the column). The MLR response was reduced as increasing number of MSC were added to the reaction (100, 1000, 10,000 and 40,000)

Effects of MSC on MLR. The results of this study showed that when increasing numbers of MSC were added to a MLR, there was a dose-dependent suppressive effect. The number of lymphocytes was $153\times10^3 \pm 11.232\times10^3$ without MSC, and $113.5\times10^3 \pm 12.5\times10^3$ when incubated with 100 MSC. Additional reactions contained $75.833\times10^3 \pm 10.103\times10^3$, $33.5\times10^3 \pm 3.77\times10^3$ lymphocytes after mixing with $8\times10^3 \pm 1.2\times10^3$ in 1×10^3, 10×10^3 and 40×10^3 MSC, respectively (Figure 4 and Table 1).

Table 1. Inhibitory effects of MSC and USSC on MLR.

The absolute number of allogeneic USSC or MSC added	Lymphocyte number in MLR using MSC (Mean ± SD)	Inhibition percent of maximal response for reactions containing MSC	Lymphocyte number in MLR using USSC (Mean ± SD)	Inhibition expressed as a Percentage of the maximal response for reactions containing USSC (%)
40000	$8\times10^3 \pm 1.2\times10^3$	5	$26.5\times10^3 \pm 2.121\times10^3$	19
10000	$33.5\times10^3 \pm 3.77\times10^3$	21	$31.750\times10^3 \pm 1.06\times10^3$	23
1000	$75.833\times10^3 \pm 10.103\times10^3$	49	$77\times10^3 \pm 11.532\times10^3$	56
100	$113.5\times10^3 \pm 12.5\times10^3$	74	$119.166\times10^3 \pm 23.464\times10^3$	86
0	$153\times10^3 \pm 11.232\times10^3$	100	$137.5\times10^3 \pm 12.5\times10^3$	100

Effect of USSC on MLR. Our results showed that after treatment with USSC the numbers of lymphocytes were 137.5×103 ± 12.5×103 in the absence of USSC, and $119.166\times10^3 \pm 23.464\times10^3$ in the presence of 100 MSC. Final lymphocyte numbers were $77\times10^3 \pm 11.532\times10^3$, $31.750\times10^3 \pm 1.06\times10^3$ and $26.5\times10^3 \pm 2.121\times10^3$ in the presence of 1×10^3, 10×10^3 and 40×10^3 MSC, respectively (Figure 5 and Table 1).

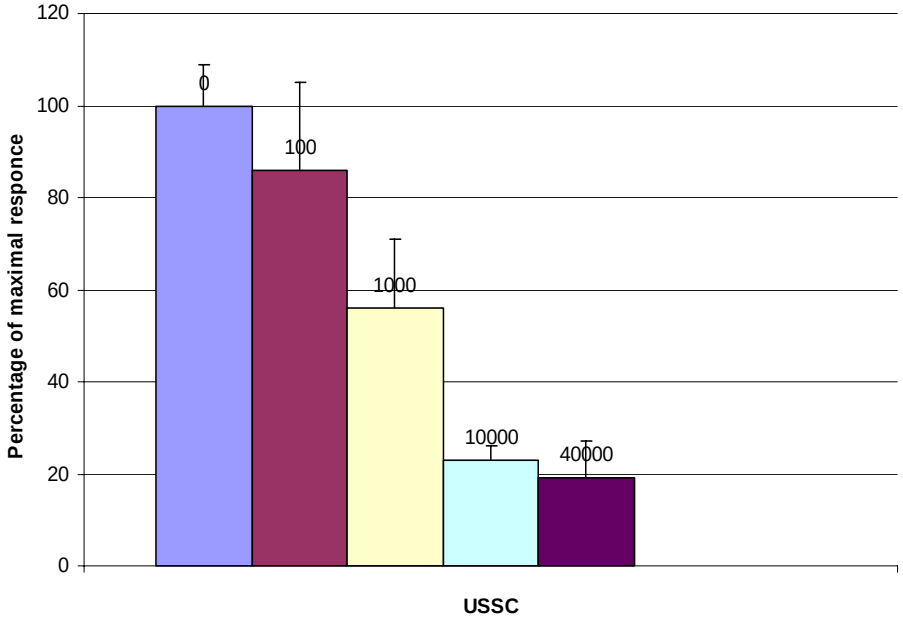

Figure 5. Percentage of maximum response in the MLR assay in the presence of different numbers of USSC. The figure shows the maximal MLR response in the first column as control (no USSC added, labeled 0 above the column). The MLR response was reduced as increasing numbers of USSC were added to the reaction (100, 1000, 10,000 and 40,000).

DISCUSSION

Stem cells are found in most multi-cellular organisms. So far several different sources of stem cells such as cord blood, bone marrow (BM) and adult tissue-specific stem cells have been identified. They represent immature pluripotent cells that can differentiate into various cell types (13). Some immunoregulatory effect of these cells has been reported (14,17). Previous studies showed that these properties are more relevant to USSC

and therapeutic approaches can be performed in the clinic using allogeneic sources. This study aimed to examine the influences of USSC, as potential source of regulatory cells, on the inhibition of graft rejection, and lymphocyte proliferation in MLR. Here, we showed that USSC had inhibitory effect on MLR. On the other hand, the immunoregulatory effects of USSC, like MSC, are improved when increasing numbers of these cells are used in the MLR (Table 1).

The results presented here confirmed that cord blood (CB) stem cells have several advantages in comparison to cells of similar pluripotency taken from other sources. For instance, cord blood is reasonably easily available and can be processed within a short course of three weeks in comparison to BM for which processing takes several months (i.e. up to 4 months). Cord blood collection is not a particularly complicated method and is free of surgery, painless and without side effects. Due to the immunoregulatory effects of USSC and based on the information presented above, it seems that these cells are a suitable candidate for use in immune system disorders such as autoimmune diseases as well as for the inhibition of graft rejection.

Based on the findings of this study we concluded that USSC can suppress the immune response against different HLA classes and gene polymorphisms typically seen in transplantation surgery. It seems that USSC facilitate these functions through several mechanisms. For instance, USSC may produce some immunoregulatory cytokines such as IL-10 and IL-4. Expression of surface anti-inflammatory molecules may also contribute to this activity. In agreement with our findings, previous studies also demonstrated that MSC have regulatory effects on immune cells and they are appropriate candidates for the control of the immune system. In bone marrow transplantation, MSC play an important role in graft acceptance and/or inhibition of graft versus host disease (GVHD) (13). MSC also regulate the behavior of NK cells and cytotoxic T lymphocytes by IL-10 expression (14). Clinically based studies demonstrated that application of MSC in kidney transplantation increases the recipient's survival as well as decreasing alloreactivity (15).

Hong et al., (2009), reported that MSC have the capacity to reduce MLR responses (14). Due to the similar actions of MSC and USSC and the fact that USSC are obtained from cord blood and posses the advantages of cord blood stem cells, scientists prefer to use them in the field of transplantation research. Another advantage of USSC cells that makes them valuable for transplantation management is their property to lower allogenisity. These cells are unable to activate HLA non-matched lymphocytes of recipients following irradiation. Riordam and colleagues demonstrated that USSC facilitate the processes of graft acceptance, GVHD inhibition and homing of stem cells (8) in a cell-dose manner. Jager et al., also showed, in a rat model, that USSC aided in the healing of critical femor injuries. In their experiments they were able to demonstrate that xenograph cells were maintained longer and aided the healing process in the presence of USSC but not in their absence (17). Finally, based on the results of the current study and other related ones regarding the suppression of MLR by USSC, it can be concluded that USSC act in a similar fashion as MSC and have regulatory roles in MLR and immune responses. Therefore, these cells show promise as a potential therapeutic candidate for use in the clinic for the treatment of immunological disorders, such as autoimmune diseases, and also in the post operative management of organ transplantation.

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