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Journal of the Chinese Medical Association 81 (2018) 268–276

www.jcma-online.com

Original Article

Mesenchymal stem cells and their conditioned medium can enhance the repair of uterine defects in a rat model

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Received January 19, 2017; accepted March 18, 2017

Abstract

Background: Our aim was to examine the roles of mesenchymal stem cell (MSC) transplantation in the repair of large uterine defects.
Methods: Uterine defects were created in both uterine horns of female rats by a punch instrument, and bone marrow-derived MSCs, MSC-conditioned medium (MSC-CM) or vehicle were injected into the myometrium around the defect. The rate of uterine defect repair was monitored on day 2 and 4 after operation. Cytokine array of MSC-CM was performed, followed by neutralizing antibody experiments to clarify the exact cytokine participating in the MSC-CM-enhanced wound repair.
Results: Transplantation of MSCs, but not myometrial cells, significantly enhanced uterine defect repair. The transplanted MSCs were detected in the uterine horn with no signs of rejection on day 4 after transplantation, when the MSC-transplanted uterine wound was nearly healed. Moreover, uterine defect repair was also accelerated by injection of MSC-CM, indicating the paracrine effects of MSCs on uterine wound healing. Cytokine array analysis further revealed that MSC-CM contained abundant cytokines and chemokines, among which high levels of interleukin-6 (IL-6) were found. Additionally, antibodies against IL-6 were shown to block MSC-CM-enhanced uterine defect repair.
Conclusion: This study demonstrated that transplantation of MSCs could enhance uterine defect repair by paracrine effects involving IL-6, which are findings that may be applied to facilitate uterine wound healing in the removal of huge intramural masses.

Keywords: Mesenchymal stem cells; Paracrine effects; Transplantation; Uterine defect repair; Uterine surgery

1. Introduction

Extensive uterine surgery, such as removal of huge uterine myomas or adenomyomas, can occasionally result in uterine rupture during subsequent pregnancy, particularly when the surgery is performed laparoscopically.[1–8] The risks of uterine rupture after myomectomy depend on the size and location of the myomas, whether the endometrial cavity is entered, and whether the myometrial defect is adequately repaired.[9] While the incidence of uterine rupture in pregnancies following laparoscopic myomectomy is not high (0.26% reported in one series), uterine rupture can lead to catastrophic maternal death as well as fetal mortality and morbidity.[1,10] To minimize post-myomectomy complications, some adjuvant treatments have been proposed for the repair of myometrial defects. For example, fibrin glue has been used in the repair of uterine

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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http://dx.doi.org/10.1016/j.jcma.2017.03.013

defects; however, no benefit has been found.[11] Therefore, a new approach to facilitate uterine defect repair following extensive uterine surgery is warranted.

Mesenchymal stem cells (MSCs) are multipotent progenitors capable of differentiating into various lineages, including osteoblasts, chondrocytes, and neurons.[12–14] Sources of MSCs include bone marrow (BM), Wharton's jelly of umbilical cord, amniotic fluid, and decidua.[13,15–17] One of the advantages of BM-derived MSCs is that they can be of the patient's own origin, thereby avoiding immune rejection after transplantation. Transplantation of stem cells into recipients via local delivery or systemic injection may satisfy clinical application for tissue repair. Current therapeutic applications of MSCs include myocardial infarction,[18,19] stroke,[20] osteogenesis imperfect,[21] and tendon defect.[22]

Although transplanted MSCs *per se* can be induced to give rise to various differentiated cell types under both *in vivo* and *in vitro* conditions, mechanisms that transplanted MSCs may "drive" adjacent cells to proliferate and differentiate have been discussed. Prior studies have demonstrated that cardiac transfer of MSCs could favorably repair infarcted myocardium and nearly normalize cardiac performance[23]; however, the fact that functional improvement was observed as early as within 72 h has raised the question as to whether such early effects could be attributed to the myocardial differentiation from the transplanted cells.[24] It has been demonstrated that the enhanced tissue repair and functional improvement after MSC transplantation into infarcted hearts are mediated by paracrine action of MSCs.[24,25] Paracrine signaling molecules, such as cytokines and growth factors, released by transferred MSCs could stimulate resident stem cells near the injured tissue to repair the defect.[26]

The purpose of this study was to examine the roles of MSC transplantation in the repair of uterine defects. Uterine defects were made in both uterine horns of female rats by a punch instrument, followed by injection of BM-derived MSCs or vehicle (phosphate-buffered saline, PBS) into the myometrium around the defect. Thereafter, the rate of uterine defect repair was monitored. Furthermore, the effect of MSC-conditioned medium (MSC-CM) on uterine defect repair was studied to determine if paracrine signaling mediated the effects of transplanted MSCs on uterine defect healing.

2. Methods

2.1. Mesenchymal stem cell culture

Primary MSCs from BM of three normal human volunteers were obtained from the Tulane Center for Distribution of Adult Stem Cells (New Orleans, LA, USA) and prepared as previously described.[27] The cells were seeded at 100 cells per cm^2 and grown in a complete culture medium consisting of α-minimal essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 16.6% fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 2 mm L-glutamine (Invitrogen). The growth medium was changed every two days, and cells were subcultured by trypsinization (trypsin-EDTA solution; Invitrogen) when they reached approximately 80% confluence. Flow cytometry analysis for surface marker detection was performed as previously described.[13] Osteogenic, adipogenic and chondrogenic differentiation of MSCs was induced according to the protocols previously described.[13]

2.2. Preparation of MSC-conditioned medium

MSCs were seeded at 10,000 cells per cm^2 and incubated in a complete culture medium for 1 day. The attached cells were washed three times with PBS, and the medium was replaced with serum-free basal medium and incubated for 48 h. The MSC-CM was collected, centrifuged at 1500× *g* for 10 min to remove cell debris, and further concentrated 50× by ultrafiltration using centrifugal filter units with 5 kDa cut-off (Millipore, Billerica, MA, USA) following the manufacturer's instructions.

2.3. Myometrial cell culture

All animal experiments were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University. Uterine horns were isolated from 8 week-old female Sprague-Dawley rats (National Yang-Ming University, Taipei, Taiwan), and the endometrial tissue was scraped off. This was followed by enzymatic digestion of the remaining myometrial tissue by incubating in HBSS (Invitrogen) containing collagenase (0.1%; Sigma, St Louis, MO), DNAse I (0.02%; Sigma), protease and trypsin (0.01%; Sigma) for 10 min at 37 °C with shaking. After discarding the supernatant, the fresh enzyme solution was replaced and incubated for another 10 min, when the tissue pieces were gently broken up by drawing the mixture slowly through a large-bore siliconized pipette and this procedure was continued for 30 s every 3 min. Then the mixture was centrifuged at 20*g* for 5 min and supernatant was removed and saved, followed by the addition of fresh enzyme solution to the remaining pieces. The above procedures were repeated three times, and the combined supernatant solution containing myometrial cells was filtered through nylon mesh and centrifuged at 430*g* for 10 min. The cell pellet was resuspended in 1:1 DMEM:F12 nutrient medium (Invitrogen) containing 10% FBS, 1% antibiotics and sodium bicarbonate, and plated in 24-well plates. More than 95% of the cells in culture were identified as smooth muscle cells by the detection of smooth muscle actin (SMA) by immunocytochemistry (Abcam, Cambridge, MA, USA; Fig. 4A).

2.4. Cytokine array

Membranes from a human protein cytokine array kit (Proteome Profiler™ Array; R&D, Minneapolis, MN, USA) were used to assay relative levels of 36 cytokines/chemokines in the 50× concentrated MSC-conditioned medium according to the manufacturer's instructions.

2.5. Uterine defect model and MSC transplantation

After 8 week-old female Sprague-Dawley rats (National Yang-Ming University, Taipei, Taiwan) were anesthetized with Avertin (Sigma), the abdominal skin was disinfected and incised to enter the abdominal cavity, followed by exposure of bilateral uterine horns. Then a whole-layer punch wound with a diameter of 3 mm was created on the middle third of both uterine horns by the Ear Punch instrument (Shineteh, Taipei, Taiwan). Subsequently, a total of 2×10^6 MSCs or myometrial cells in 40 μL PBS were injected into the myometrium surrounding the defect in one uterine horn (4-spot injection, 5×10^5 cells in 10 μL PBS to each spot) by Hamilton syringe and needle (Hamilton, Reno, NV, USA). The uterine defect in the contralateral horn received an equal volume of PBS without cells. In experiments using MSC-CM, the defect in one uterine horn received 100 μL 50× MSC-CM; the defect in the contralateral uterine horn received serum-free basal medium. In experiments on the effects of interleukin-6 (IL-6) neutralizing antibody, the defect in one uterine horn received 100 μL 50× MSC-CM and 3 μg/ml non-specific control antibody and that in the contralateral uterine horn received 50× MSC-CM and 3 μg/ml IL-6 neutralizing antibody (R&D). Digital photographs of

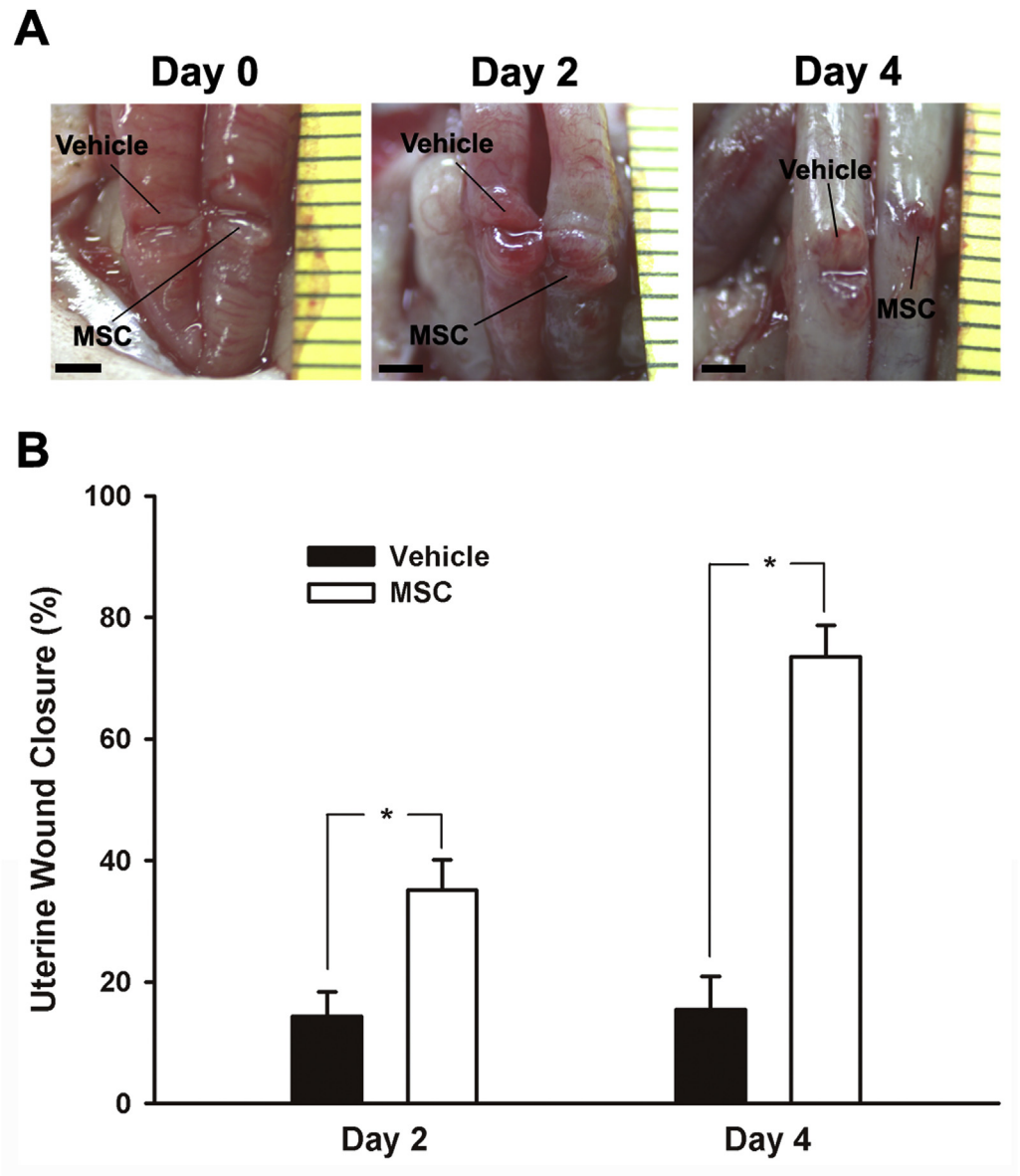

Fig. 1. Transplantation of mesenchymal stem cells (MSCs) could enhance the repair of uterine defects. (**A**) A whole-layer punch wound was created on each of the two uterine horns of the female rat, followed by injection of MSCs or vehicle into the myometrium surrounding the defect (day 0). The uterine wounds were monitored by laparotomy and photography on day 2 and 4 after the initial operation. As shown in a representative set of photographs from five independent experiments, uterine wounds receiving MSC transplantation were found to heal much more rapidly than those injected with vehicle; bar = 2 mm. (**B**) Quantitative analysis of the five independent experiments as performed in (A) demonstrated that MSC transplantation significantly increased the percentage of uterine wound closure [100 × (original wound area − wound area on the day of monitoring)/original wound area] on day 2 and 4 after the initial operation, as compared with the vehicle-injected controls (mean ± SEM, n = 5, $^*P < 0.05$).

original uterine wounds were taken (designated as day 0), followed by closure of abdominal skin. On day 2 and 4 after the initial operation, the extent of uterine wound healing in both uterine horns was examined by laparotomy and digital photographs were taken. Wound margin was traced in the photographs and wound area was calculated using Scion Image software system (Scion Corporation, Frederick, MD, USA). The percentage of wound repair was calculated as 100 × (original wound area − wound area on the day of monitoring)/original wound area.[28]

2.6. Histological examination and immunohistochemistry

Uterine tissue specimens were fixed in 4% paraformaldehyde for 24 h and embedded in OCT resin. Ten-micron-thick sections were washed with PBS, followed by blocking with 5% normal goat serum (NGS) for 30 min at room temperature. Then the tissues were incubated with mouse anti-human nuclei antibody (Millipore, 1:100 diluted in 5% NGS) or mouse anti-rat macrophages/monocytes antibody (ED1; Millipore, 1:500 diluted in 5% NGS) overnight at 4 °C. After they were washed with PBS, the tissues were incubated with biotin-conjugated rabbit anti-mouse immunoglobulins (Sigma, 1:250 diluted in 5% NGS) for 1 h at room temperature. Thereafter, the tissues were washed with PBS, followed by incubating with avidin-biotinylated horseradish peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Finally, the tissues were incubated with 3,3′-diaminobenzidine, and the slides were mounted and observed under Axioplan 2 Imaging microscope (ZEISS, Thorrwood, NY, USA).

2.7. Statistical analysis

Data regarding percentage of wound repair were expressed as means ± SEM. Statistical significance between the groups was determined by paired *t*-test. All analyses were performed using the SPSS program (SPSS Inc., Chicago, IL, USA). A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of MSC transplantation on the repair of uterine defects

Primary BM-derived MSCs with multi-lineage differentiation ability and surface marker profile characteristic of MSCs were used in our transplantation experiments.[13,29,30] To examine the effects of MSC transplantation on uterine defect repair, a whole-layer punch wound was made on both

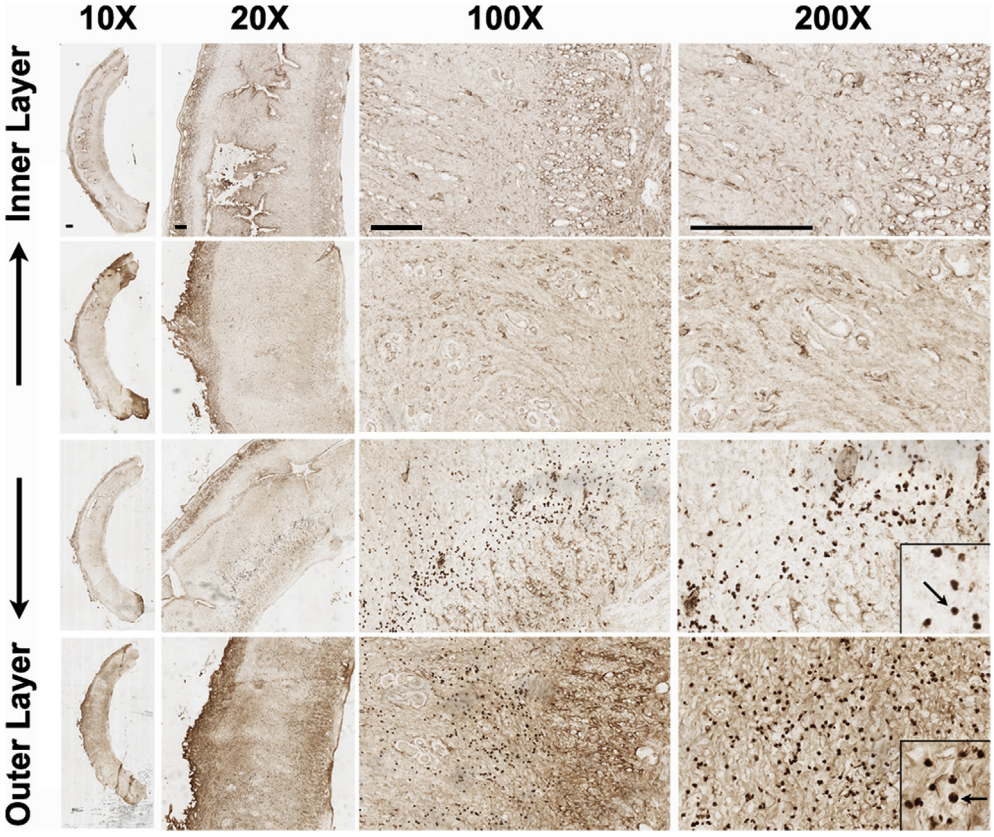

Fig. 2. Transplanted mesenchymal stem cells (MSCs) were detected in the uterine tissue on day 4 after transplantation. On day 4 after transplantation, rat uterine tissue surrounding the MSC-injected wound was harvested, sectioned and subjected to immunohistochemical staining of human-specific nuclear antigen to detect the presence of transplanted MSCs that were of human origin. Shown in the inset is the close-up view. Arrows indicate nuclei positively stained with human-specific nuclear antigen (dark brown); bar = 200 μm.

uterine horns of the female rat by the Ear Punch instrument, followed by injection of MSCs or vehicle into the myometrium surrounding the defect and photography of the uterine wounds (day 0). The uterine wounds were examined by laparotomy on day 2 and 4 after the initial operation and photographed. As shown in Fig. 1A, uterine wounds receiving MSC transplantation healed much faster than those injected with a vehicle. Quantitative analysis of wound areas showed that MSC transplantation significantly enhanced the percentage of uterine wound repair [100 × (original wound area − wound area on the day of monitoring)/original wound area] on day 2 and 4 after the initial operation, as compared with the vehicle-injected controls ($p < 0.05$) (Fig. 1B).

3.2. Detection of transplanted MSCs and evaluation of rejection

To detect the presence of transplanted MSCs on day 4 after transplantation, immunohistochemical staining of human-specific nuclear antigen was performed on sections of uterine tissue surrounding the MSC-injected wound, since the MSCs used in this study were of human origin. A significant number of human-specific nuclear antigen-positive cells were detected in the myometrium surrounding the wound, indicating presence of MSCs in the uterine tissue on day 4 after transplantation (Fig. 2, arrows). The MSCs were found in the outer half of the myometrium, which was compatible with their initial transplantation site (outer half).

Then, we evaluated if rejection occurred after MSC transplantation. Acute rejection is an acute inflammatory process that destructs the transplanted organ or tissue, and infiltration of lymphocytes and macrophages is a prominent feature of acute rejection.[31] Acute rejection episodes frequently initiate within the first days or weeks after engraftment, and the immunohistochemical staining of mononuclear cell infiltration and activation is responsible for acute graft destruction in many transplantation models, among which ED1 is frequently used as a marker for activated mononuclear cells.[31–33] Serial sections from MSC/vehicle injection sites on day 4 after the initial operation were

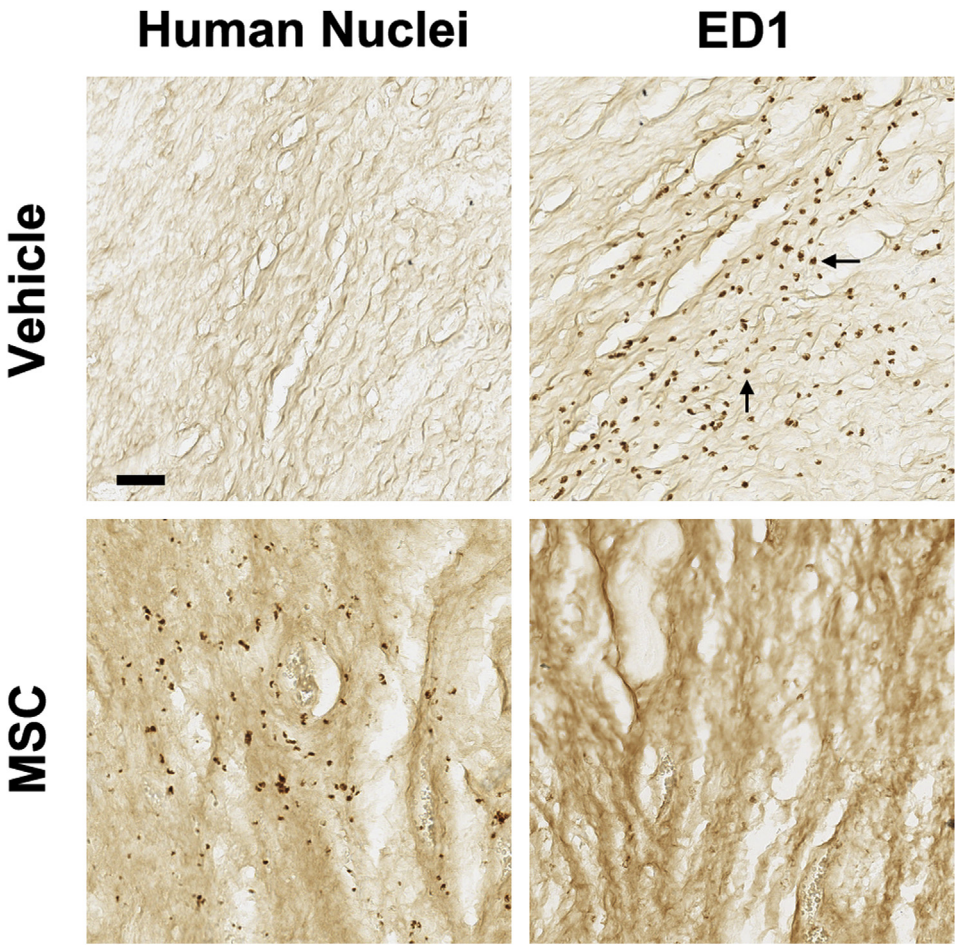

Fig. 3. Transplantation of mesenchymal stem cells (MSCs) did not induce acute rejection. On day 4 after the initial operation, rat uterine tissue surrounding the injection sites of MSCs or control vehicle was collected and sectioned. The consecutive sections were immunostained for human-specific nuclear antigen (designed as 'Human Nuclei') or ED1, a marker specific for activated rat monocytes and macrophages, since mononuclear cell infiltration and activation were responsible for acute rejection. The number of $ED1^+$ cells is decreased in uterine tissue surrounding MSC-injected sites as compared with that in uterine tissue surrounding vehicle-injected sites. Arrows indicate $ED1^+$ cells; bar = 50 μm.

immunostained for ED1. In the rats receiving vehicle injection into the wound edge, a significant number of $ED1^+$ cells were detected in the myometrium surrounding the wound (Fig. 3). On the contrary, the number of $ED1^+$ cells in the myometrium near the grafted MSCs was markedly reduced, indicating no signs of acute rejection at day 4 after MSCs transplantation.

3.3. Effect of myometrial cell transplantation on the repair of uterine defects

Myometrial cell transplantation was performed to determine if cell transplantation other than MSCs could enhance the repair of uterine defects. Myometrial cells were isolated from female rats, and cultured. Immunostaining with SMA showed that more than 95% of the cells in culture were of smooth muscle origin (Fig. 4A). As shown in Fig. 4B and C, myometrial cell transplantation (2×10^6 myometrial cells in 40 μL PBS) did not enhance the repair of uterine defects, as compared with injection of vehicle controls.

3.4. Effect of MSC-conditioned medium on the repair of uterine defects

To determine if paracrine signaling mediated the enhancing effect of MSC transplantation on uterine defect repair, MSC-conditioned medium (MSC-CM) was prepared by incubating MSCs in serum-free basal medium for 48 h, collecting the resulting supernatants and concentrating 50× by ultrafiltration. MSC-CM was injected into the myometrium surrounding the defect in one uterine horn, and the uterine defect in the contralateral horn received injection of basal medium to serve as the control (Fig. 5A). MSC-CM was found to enhance uterine wound healing as compared with basal medium (Fig. 5A). Quantitative analysis of wound areas confirmed that the percentages of uterine wound repair on both day 2 and 4 were significantly higher in the MSC-CM group than in the basal medium group ($P < 0.05$)(Fig. 5B). These findings indicated that transplanted MSCs could enhance uterine defect repair by paracrine effects. Cytokine array analysis revealed that MSC-CM was abundant in cytokines and chemokines, among which plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), and IL-6 ranked highest in levels (Fig. 5C). Since IL-6 is known to play an important role in the wound healing process, the involvement of IL-6 in MSC-CM-enhanced uterine defect repair was examined. Neutralizing antibody against IL-6 was shown to block the enhancing effects of MSC-CM on uterine defect repair (Fig. 5D and E).

4. Discussion

Laparoscopic removal of huge uterine masses and suturing of the large defects are a challenging operation that may increase the risk of uterine rupture in subsequent pregnancies.[2,5,7] Transplantation of MSCs has been shown to promote skin wound healing[34]; however, the effects of MSC transplantation on uterine defect repair have not been examined. Using a uterine defect model in rats, we found that transplantation of BM-derived MSCs, but not myometrial cells, significantly enhanced uterine defect repair. The transplanted MSCs were detected in the uterine horn with no signs

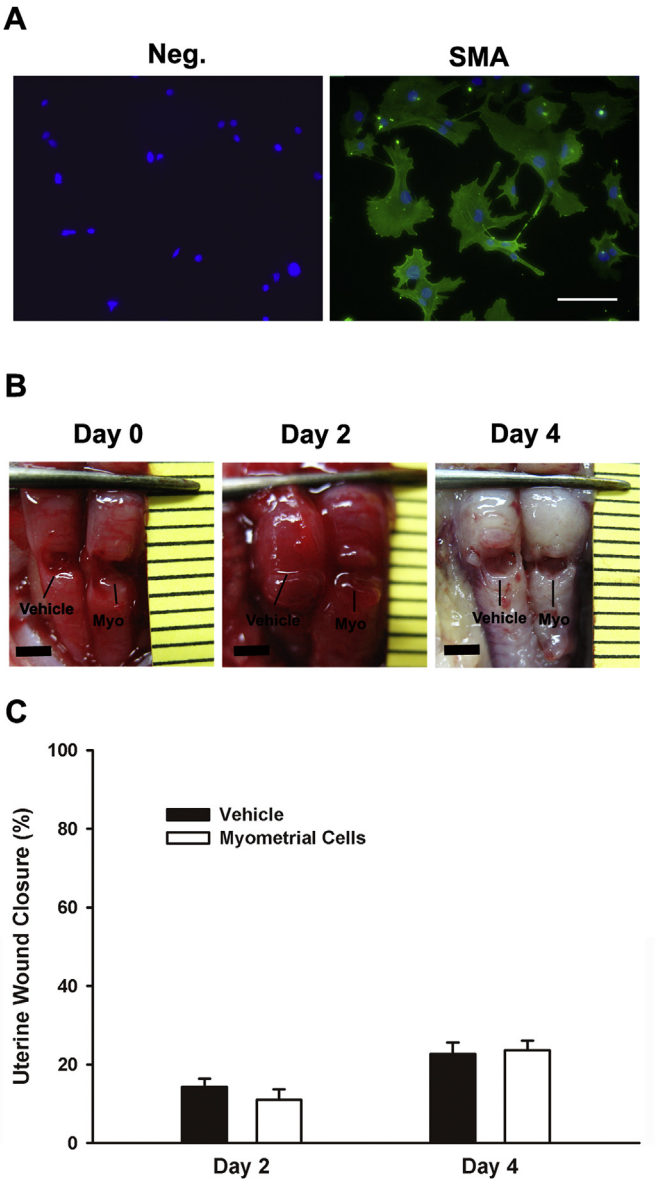

Fig. 4. Transplantation of myometrial cells did not enhance the repair of uterine defects. (**A**) Myometrial cells were immunostained with smooth muscle actin (SMA; FITC-labeled green fluorescence) and counterstained with Hoechst 33,258 (blue fluorescence) for nuclear localization. Neg., negative control in which the primary antibody was deleted. Bar = 50 μm. (**B**) A whole-layer punch wound was created on each of the two uterine horns of the female rat, followed by injection of myometrial cells (Myo) or vehicle into the myometrium surrounding the defect (day 0). The uterine wounds were monitored by laparotomy and photography on day 2 and 4 after the initial operation. As shown in a representative set of photographs from three independent experiments, myometrial cell transplantation did not enhance the repair of uterine defects; bar = 2 mm. (**C**) Quantitative analysis of the three independent experiments as performed in (B) demonstrated that myometrial cell transplantation did not increase the percentage of uterine wound closure on day 2 and 4 after the initial operation, as compared with the vehicle-injected controls (mean ± SEM, n = 3).

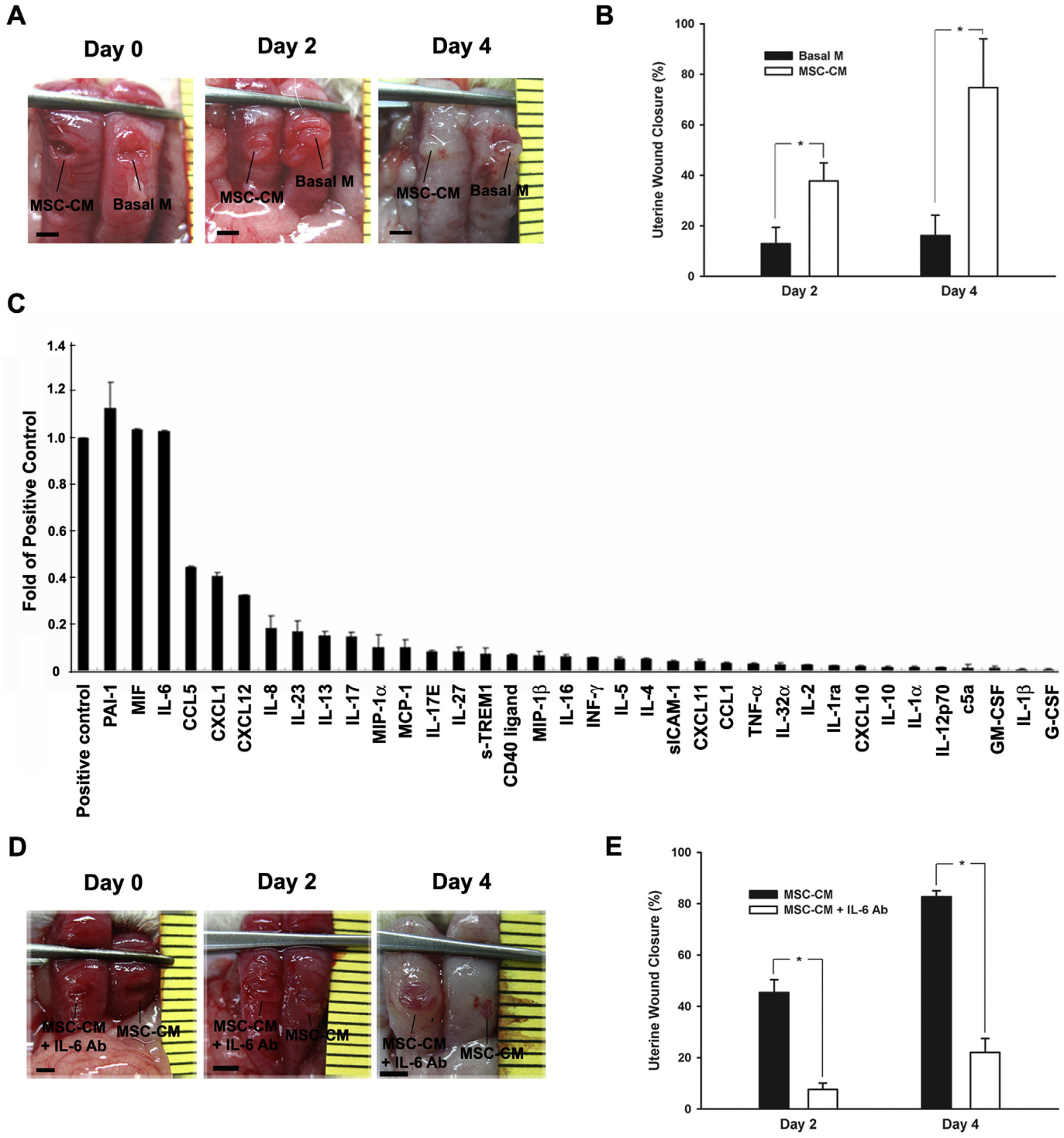

Fig. 5. Mesenchymal stem cell-conditioned medium (MSC-CM) promoted the healing of uterine defects. (**A**) A whole-layer punch wound was made on each of the two uterine horns of the female rat, followed by injection of 50× MSC-conditioned medium (designated as 'MSC-CM') or control basal medium (designated as 'Basal M') into the myometrium surrounding the defect (day 0). The uterine wounds were monitored on day 2 and 4 after the initial operation. As shown in a representative set of photographs from three independent experiments, uterine defects receiving injection of MSC-CM were found to heal much faster than those injected with control basal medium; bar = 2 mm. (**B**) Quantitative analysis of the three independent experiments as performed in (A) showed that injection of MSC-CM significantly increased the percentage of uterine wound closure on day 2 and 4 after the initial operation, as compared with the basal medium-injected controls (mean ± SEM, n = 3, $*p < 0.05$). (**C**) Cytokine profile of 50× MSC-conditioned medium was analyzed by Human protein cytokine array. The results were normalized by the positive controls (mean ± SEM, n = 3). (**D**) Neutralizing antibodies against IL-6 (designated as 'IL-6 Ab') could block the enhancing effects of MSC-CM on uterine defect repair. 50× MSC-CM was injected into uterine horn defects with or without 3 μg/ml neutralizing antibodies against IL-6. As shown in a representative set of photographs from three independent experiments, IL-6 Ab effectively blocked the enhancing effect of MSC-CM on uterine defect repair; bar = 2 mm. (**E**) Quantitative analysis of the three independent experiments as performed in (D) (mean ± SEM, n = 3, $*p < 0.005$).

of rejection at day 4 after transplantation, when 70−80% uterine wound repair was achieved. Moreover, uterine defect repair was also promoted by injection of MSC-CM, indicating paracrine effects of MSCs on uterine wound healing. Neutralizing antibody against IL-6 could block MSC-CM-enhanced uterine defect repair. Our results demonstrated that MSC transplantation could enhanced the repair of uterine wound by paracrine effects involving IL-6, suggesting that MSCs or MSC-conditioned medium may be utilized to promote the repair of uterine defects during laparoscopic operations on huge uterine masses.

Stem cell therapy holds great promise for regenerative medicine.[35−38] In contrast to embryonic stem cells, MSCs have fewer ethical concerns and are free from teratoma formation after transplantation.[39] Moreover, transplantation of autologous BM-derived MSCs has the advantage of being immunorejection-free. In this study, we first demonstrated that transplantation of BM-derived MSCs could significantly enhance the repair of uterine defects. The enhancing effect of transplanted MSCs on uterine defect repair was mainly mediated by MSC-secreted paracrine factors, since injection of MSC-conditioned medium could promote uterine defect repair to a degree similar to that achieved by MSC transplantation. Direct differentiation of transplanted MSCs into myometrial cells was less likely to contribute to enhanced uterine wound repair because engrafted MSCs on day 4 after transplantation did not exhibit smooth muscle cell morphology (data not shown). Moreover, transplantation of myometrial cells *per se* did not accelerate the repair uterine defects (Fig. 4). Our findings were supported by previous reports that MSCs accelerated the regenerative process of skin and myocardium injuries by secreting growth factors, cytokines and chemokines.[26,40] Cytokine array analysis showed that our MSC-conditioned medium was rich in cytokines and chemokines, especially PAI-1, MIF, and IL-6, which may participate in optimal wound repair by promoting cell migration, cell proliferation or angiogenesis.[41−43] Using neutralizing antibodies against IL-6, we demonstrated that the enhancing effect of MSC-CM on uterine defect repair was mediated, as least in part, by IL-6 (Fig. 5D and E).

Previous studies have demonstrated that transplanted allogeneic or xenogeneic MSCs can be detected in recipients at extended time points, indicating a lack of immune recognition and clearance.[16,44,45] In the present study, xenogeneic MSCs were also detected in the myometrium surrounding the almost healed wound at day 4 after transplantation. Moreover, the number of $ED1^{+}$ monocytes/macrophages, a marker of acute rejection and inflammation, was markedly decreased in the myometrium surrounding the MSC-transplanted wound, as compared with the vehicle-injected wound. Our data were consistent with previous studies showing that transplanted MSCs could exert powerful immunomodulatory effects.[46] The anti-inflammatory effects of MSCs have been applied to the treatment of Alzheimer's disease and rheumatoid arthritis.[47,48] Mechanisms by which MSCs modulate the immune response remain to be explored, but they most likely involve expression of local factors and interaction with dendritic or antigen-presenting cells.[49]

Clinical trials of MSC transplantation for diseases like osteogenesis imperfecta and myocardial infarction have shown some promise demonstrating the effectiveness and safety of both allogeneic and autologous cells.[50] Before application of MSC transplantation to laparoscopic removal of huge uterine mass, functional tests are required to determine if MSC transplantation into uterine defects really improves the strength of uterine scar in our animal model. Punch wounds may be made in both uterine horns of female rats, transplanted with or without MSCs, and allowed to heal, followed by burst pressure determination as described by Peacock et al.[11] or analysis of pregnancy-related uterine rupture rate after mating with male rats.

In conclusion, this study demonstrated that transplantation of BM-derived MSCs could enhance the repair of uterine defects by paracrine effects involving IL-6. We propose that MSCs or MSC-conditioned medium may be applied to promote healing of uterine defects in laparoscopic removal of huge intramural masses. Further investigation is required to confirm the effectiveness of MSCs or MSC-CM in the prevention of uterine rupture in patients receiving laparoscopic uterine surgery who subsequently become pregnant.

Acknowledgments

This study was supported by the National Science Council, Taipei, Taiwan (NSC 97-2321-B-010-004-MY3 and NSC 101-2314-B-010-031-MY3) and the Taipei Veterans General Hospital, Taipei, Taiwan (V102C-142 and V102E1-009).

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