Regenerative Potential of Menstrual Blood-Derived Stem Cells and Platelet-Derived Growth Factor in Endometrial Injury

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Background: Endometrial regeneration is essential for normal endometrial function; however, it is unclear whether and how menstrual blood-derived stem cells (MenSCs) and platelet-derived growth factor (PDGF) are associated with this phenomenon. The present study explored this topic.

Material/Methods: EM-E6/E7/hTERT cells were divided into 5 groups: control group, NC group, PDGF group, MenSCs group, and PDGF+MenSCs group. The effects of MenSCs and PDGF on cell proliferation, invasion, and microvascular formation of endometrial epithelium were investigated by CCK-8, Transwell, and tube formation assays, respectively. Mouse endometrial injury models were established and mice were randomly divided into control, model, PDGF, MenSCs, and PDGF+MenSCs groups. Pathological change was examined with hematoxylin and eosin (H&E) staining. Microvessel formation of endometrial epithelium was estimated by detecting the expression of CD34 protein with immunohistochemical (IHC) staining. Western blot analysis was used to detect the activation of Akt and Bad proteins in endometrial tissue.

Results: MenSCs, PDGF, and the combination treatments significantly promoted the proliferation, migration, and tube formation of endometrial epithelium compared to the control and NC group. The combination of MenSCs and PDGF remarkably promoted re-epithelialization and endometrial repair. IHC staining analysis showed significant increases in CD34 expression of the endometrial tissue following treatment with PDGF and MenSCs. The combination treatments also markedly enhanced the phosphorylation of Akt and Bad in endometrial tissue.

Conclusions: These results suggest that MenSCs and PDGF may be candidate substances for endometrial injury repair.

MeSH Keywords: Metrorrhagia • Receptors, Platelet-Derived Growth Factor • Uterine Diseases

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Background

Remodeling of the human uterus naturally occurs throughout menstruation and parturition [1]. During the remodeling period, the endometrium tissue undergoes a repeated process involving proliferation, differentiation, shedding, and repair in response to estrogen and progesterone [2]. Normal involution returns the uterus to the physiological homeostasis for the new menstrual cycle or for an optimal environment receptive to a new conceptus [3]. Although the endometrium has a strong regenerative ability, intrauterine surgery or infection can easily cause a poor or thin endometrium, leading to amenorrhea, infertility, and uterine adhesion [4–6]. Recent progress has been made in uterine or endometrial reconstruction in animal models with endometrial injury [7], but few methods are available to effectively restore the damaged uterus to normal structure and function.

The endometrium consists of columnar epithelium, stroma, and microvessels [7–9]. Remarkably, luminal epithelium is shed and microvessels are damaged at the site of disrupted endometrium [10]. Epithelial renewal or re-epithelialization has been described to be mediated by migration and proliferation of residual epithelial cells in the remaining basal layer, and by differentiation of endometrial stem/progenitor cells [10–13]. Re-epithelialization and re-vascularization of the denuded uterine surface are critical factors contributing to successful repair and remodeling of the endometrium [10,11,14]. However, this normal process is disrupted or hampered by clinically severe endometrial injury.

Endometrial stem cells (EnSCs) are identified in both the functional and basal layer of regenerative human endometrium [15,16], which can be used for the reconstruction of the human uterus and other organs [17,18]. They can be easily obtained from menstrual blood shedding without invasive surgical intervention or hospitalization, and the isolation is free of ethical concerns [12,19]. Furthermore, MenSCs demonstrate improved proliferation and differentiation potency [12,20]. Considering these advantages, MenSCs would be a good choice for use in cell-based therapy for tissue repair or regeneration. The regenerative potential of MenSCs has been demonstrated in preclinical studies of central nerve disorders [20], heart failure [21], limb ischemia [22], and endometrial injury [14]. However, the effect of MenSCs on endometrial epithelium has not been explored.

The platelet-derived growth factor (PDGF) family consists of 4 homodimeric proteins (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD) and 1 heterodimeric protein (PDGF-AB) [23,24]. PDGF-mediated signaling plays an essential role in cellular proliferation, migration, angiogenesis, and tissue injury and in its repair [25]. Distinct PDGF isoforms, especially PDGF-BB, have been shown to stimulate proliferation and migration of endometrial stromal cells in vitro, indicating that PDGF can help promote endometrial tissue repair [23,26]. The effect of PDGF on endometrial epithelial cells has not been reported. It was recently reported that platelet-derived soluble factors can promote migration and proliferation of endometrial epithelial cells in vitro [10]. Therefore, we speculated that PDGF can exert a similar effect on re-epithelialization of endometrial epithelial cells.

This study aimed to evaluate the effect of MenSCs, PDGF-BB, and their combination on the regeneration of endometrial epithelium in vitro and in vivo and to elucidate the underlying mechanisms. Our findings may provide a novel therapeutic strategy for endometrial injury.

Material and Methods

Cell culture and treatment

The human endometrial epithelial cell line EM-E6/E7/hTERT was immortalized by the expression of HPV16 E6/E7 and telomerase reverse transcriptase (TERT). Stably established EM-E6/E7/hTERT cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA), 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 25 μg/mL insulin-transferrin-selenium (Invitrogen, USA). MenSCs were purchased from Cryo-Cell International, Inc. (Oldsmar, USA) and maintained in DMEM containing 10% FBS and 1% (v/v) penicillin and streptomycin. All the cells were kept in a 37°C incubator with 5% CO₂. Cells were passaged when reaching 80–90% confluence, and cells in logarithmic growth phase were used for further experiments. Human umbilical vein endothelial cells (HUVECs) have the potential of stem cells, so they are often selected for angiogenesis experiments. HUVECs were purchased from ATCC (Manassas, VA, USA). HUVECs was cultured in DMEM medium (1X, Gibco, Invitrogen, USA) containing 4.5 g/L D-Glucose, L-Glutamine, and 110 mg/L sodium pyruvate and placed in a 37°C, 5% CO₂ incubator. Cells in logarithmic growth phase were selected for experimentation after the cells grew to 80% confluence, and the cells were collected for detection.

EM-E6/E7/hTERT cells were divided into 5 groups: no treatment (control group), phosphate-buffered saline (PBS) treatment (negative control group, NC group), PDGF (5 μg/mL) treatment (PDGF group), MenSCs (10 cells/mL) co-culture (MenSCs group), and the combined MenSCs and PDGF treatment (MenSCs + PDGF group).

Proliferation assay

Cell proliferation was measured using CCK-8 assay (Bio-Rad, USA). Cells (2×10⁴) were cultured for 24, 48, 72, and 96 h.
We added 10 μL of CCK-8 solution (DojinDo, Shanghai, China) to each well. After incubation for 4 h at 37°C, the medium was discarded and the cells were incubated with 150 μL of DMSAO (Bio-Rad, USA). The plate was shaken on a micro-vibrator for 10 min, and the optical density (OD) of each well was measured using an ELISA reader (Bio-Rad, USA) at 490 nm.

**Transwell assay**

Cell invasion and migration were assessed by Transwell assay performed using 24-well Transwell filters (8 μm pores, BD Biosciences, USA). Briefly, a mixture of 50 μL Matrigel was added to the upper chamber and cured in a 37°C incubator for 30 min. A total of 5×10⁴ cells were added to the upper chamber in serum-free media, while the lower chamber was filled with DMEM containing 20% FBS. After 24-h incubation, the cells that penetrated into the lower chamber were fixed and stained with 0.5% crystal violet for 20 min.

**Tube formation assay**

Fifty microliters of Matrigel (BD Bioscience, USA) was plated into 96-well plates at a horizontal level and allowed to polymerize at 37°C for 1 h. HUVEC cells were digested, centrifuged at 1000 rpm for 5 min, and resuspended. The cell suspension was seeded on the top of the polymerized Matrigel layer at a density of 4×10⁴ cells/well. The cells were treated with either MenSCs, PDGF, or the combination. Following incubation at 37°C for 48 h, tube formation was assessed.

**Experimental animals and ethics statement**

We purchased 150 female ICR mice, weighing 18–22 g, from Jinan Pengyu Experimental Animal Breeding Co. (Shandong, China, License Number: SCXK (Lu) 2014007). The mice were kept in a well-ventilated and quiet animal house at our hospital for 1 week of acclimation prior to the experiments. The room temperature was 22–24°C and the relative humidity was 50–60%. The mice were given free access to food and water during the entire experimental period.

All experiments were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and all protocols were approved by the Animal Care and Use Committee of our hospital.

**Animal models and experiments**

After intraperitoneal injection of 0.6% sodium pentobarbital (40 mg/kg), the mice were anesthetized and placed on a 37°C thermostatically controlled heated pad. The cortex was lifted with ophthalmic forceps at about 0.5 cm above the urethral orifice and cut layer-by-layer with ophthalmic scissors to expose the Y-shaped uterus behind the bladder. The right uterine horn was gently picked out. Then, the second venous indwelling needle was inserted from the vagina of the uterine cavity and out of the ovary to connect with a catheter connected to a water source at 85°C while the other end was connected to a 50-mL syringe. Then, the hot water was pumped out at a relatively slow and uniform speed. The hot water passed through the indwelling needle for 80 s. After that, the indwelling needle tube was taken out. The uterus and intestinal tube were reset, and the abdomen was washed with 20000 U/mL penicillin. The skin was sterilized, and the abdomen was closed by sutures layer-by-layer.

Mice were randomly divided into 5 groups: Model group, NC group (an intraperitoneal injection of 100 μL PBS), MenSCs group (an intraperitoneal injection of 1 × 10⁶ cells in 100 μL PBS), PDGF group (an intraperitoneal injection of 500 ng PDGF in 100 μL PBS), and MenSCs+PDGF group (the combined injection of MenSCs and PDGF). Six mice in each group were sacrificed on the 0th, 5th, 10th, 15th day after injection, respectively, for Western blot assay. The remaining mice were sacrificed at 8 weeks and the uteruses were taken surgically for H&E staining and immunohistochemistry assays.

**Histology and immunohistochemistry**

The mouse uterus was removed surgically and fixed in 4% paraformaldehyde. The samples were embedded in paraffin and cut into 4-μm sections. The paraffin sections were dehydrated with serial dilutions of ethanol, and stained with hematoxylin and eosin (H&E) (Soelab, China). CD34 expression was analyzed using immunohistochemistry assay. The sections were incubated with rabbit anti-CD34 monoclonal antibody (1: 100, Abcam, UK) overnight at 4°C. The sections were washed and incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (1: 1000, Abcam, UK) for 40 min at room temperature. The cells were visualized using diaminobenzidine (DAB) substrate (Maixin Biotech, China) and counterstained with hematoxylin (Beyotime Biotechnology, China) for 3 min.

**Western blot**

Total protein was separated in 10% sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Darmstadt, Germany). The membranes were blocked in Tris-buffered saline Tween 20 (TBST) solution containing 5% skim milk at room temperature for 2 h, and then probed overnight with primary antibodies at 4°C. The following antibodies were used: Rabbit anti-Akt antibody (1: 2000, Abcam, UK), rabbit anti-p-Akt antibody (1: 2000, Abcam, UK), rabbit anti-Bad antibody (1: 1000, Abcam, UK), rabbit anti-p-Bad (1: 1000, Abcam, UK), and...
Effect of PDGF and MenSCs on EM-E6/E7/hTERT cells migration and invasion

To evaluate the effect of PDGF and MenSCs on cell migration and invasion, Transwell assay was used (Figure 2). The PDGF+MenSCs group presented a higher invasive potential, compared with the NC or control group (p<0.05). Compared with the PDGF or MenSCs group, the PDGF+MenSCs group also showed a significant increase in migration and invasion (p<0.05). Our data suggest that PDGF and MenSCs markedly enhance the ability of endometrial epithelial cells to migrate in vitro in a synergistic fashion.

Effect of PDGF and MenSCs on tube formation in vitro

We examined whether PDGF and MenSCs could regulate the differentiation of endometrial endothelial cells into tube-like structures using HUVECs cells plated on a Matrigel matrix. As shown in Figure 3, cells formed tube-like structures when cultured alone on Matrigel in the control or NC group; however, the addition of PDGF or MenSCs led to a significant increase in the number of tube-like structures at similar level as in the control or NC group (p<0.05). We next assessed whether PDGF and MenSCs play a synergistic role of tube formation. We found that the combination treatment with PDGF and MenSCs significantly enhanced the number of tube-like structures relative to PDGF or MenSCs treatment alone (p<0.05).

Effect of PDGF and MenSCs endometrial morphology

Histopathologically, endometrial tissues in the NC group were clearly stratified and showed normal architecture (Figure 4A). Obvious histomorphological changes were observed in the endometrial lesions of the model group, which simultaneously included mononuclear cellular infiltration, numerous dilated and congested blood vessels, and interstitial edema, together with loss of luminal and glandular epithelium (Figure 4B). PDGF or MenSCs treatment significantly promoted repair of the damaged endometrial tissues in the animal models, inducing the reappearance of clearly stratified tissue structure and regeneration of stromal cells and epithelial cells (Figure 4C, 4D). Similar results were observed in the PDGF+MenSCs group, in which the endometrial tissue regained its normal appearance and structure (Figure 4E). Histological results supported a synergistic regenerative effect of a combination of PDGF and MenSCs in the endometrial damage mouse models.

Effect of PDGF and MenSCs on microvessel formation in vivo

CD34 is regarded as the most sensitive marker for detection of microvessels. Accordingly, we examined CD34 expression in the endometrial tissue using IHC analysis. There were significant...
Figure 2. Effect of PDGF and MenSCs on EM-E6/E7/hTERT cells invasion and migration. (A) Invasion assay. (B) Migration assay. Photographs were taken at ×400 original magnification at 24 h after scratch injury and quantified using Image J software. All data are shown as the mean±SD (n=6). * p<0.05 as compared with control, # p<0.05 as compared with NC group, ^ p<0.05 as compared with PDGF group, & p<0.05 as compared with MenSCs group.

Figure 3. Effect of PDGF and MenSCs on tube formation in vitro. (A) Tube formation photomicrographs were taken at 200× magnification. (B) Quantitative results of tube formation are shown as mean±SD (n=6). * p<0.05 as compared with control, # p<0.05 as compared with NC group, ^ p<0.05 as compared with PDGF group, & p<0.05 as compared with MenSCs group.

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increases in CD34 expression in the endometrial tissue following treatment with PDGF (p<0.05) or MenSCs (p<0.05) compared with the Model group (Figure 5). Furthermore, the PDGF+MenSCs group showed remarkably higher upregulation of CD34 expression than in the PDGF or MenSCs group alone (p<0.05). Our data indicated that PDGF or MenSCs significantly promoted microvessel formation of the endometrial lesion of the mouse models, and the combination of PDGF and MenSCs exerted a more pronounced effect on tube formation and endometrial repair.

**Figure 4.** Effect of PDGF and MenSCs on endometrial morphology. Endometrial tissue sections were stained with H&E. Photomicrographs showing histopathology of endometrial tissue were taken at 200×magnification from control group (A), model (B), PDGF group (C), MenSCs group (D), and PDGF+MenSCs group (E).

**Figure 5.** Effect of PDGF and MenSCs on microvessel formation in vivo. Endometrial tissue sections were incubated with anti-CD34 antibody. (A) Photomicrographs were taken under 400×magnification. (B) The number of CD34 positive cells was counted from 5 random fields. All data are shown as the mean±SD (n=5). *p<0.05 as compared with control, @p<0.05 as compared with Model group, ^p<0.05 as compared with PDGF group, &p<0.05 as compared with MenSCs group.

Effect of MenSCs and PDGF on activation of Akt and Bad in endometrial tissue

The expression and phosphorylation of Akt and Bad in the endometrial tissue were assessed by Western blot analysis at different time points (0, 5, 10, 15 days) after surgery (Figure 6). However, the extent of phosphorylation of both Akt and Bad was significantly higher in the PDGF or MenSCs group compared to the Model group (p<0.05). Combination treatment with PDGF and MenSCs resulted in significantly enhanced...
phosphorylation of Akt and Bad compared with PDGF or MenSCs (p<0.05). These data predicted the importance of Akt and Bad signaling for re-epithelialization and suggested that the positive effect of PDGF and MenSCs on endometrial repair following surgery-induced endometrial damage was partially dependent on Akt and Bad activity.

Discussion

The present study is the first to provide the evidence that MenSCs and PDGF might be candidate substances for endometrial injury repair through contributing to EM-E6/E7/hTERT cells proliferation, migration, and invasion. Further, the in vivo data confirmed that MenSCs and PDGF can promote vascular formation of endometrial epithelial cells and phosphorylation of Akt and Bad in endometrium.

Adult stem cells have proven to be an attractive cell type for tissue repair therapeutics due to their remarkable regenerative capacity. Studies have shown that stem cells can also migrate to the injured site, where they promote cell migration, proliferation, and differentiation and play critical roles in re-epithelialization and tissue repair [27]. To substantiate the possible

Figure 6. Activation of Akt and Bad in endometrial tissue. Representative images of expression and phosphorylation of Akt (A) and Bad (B) are shown. β-actin was used as the loading control. Quantitative analysis of relative changes in phosphorylated Akt and Bad was performed. Relative phosphorylation levels were normalized to total protein signal. Results are presented as mean±SD. * p<0.05 as compared with control, @ p<0.05 as compared with Model group, ^ p<0.05 as compared with PDGF group, & p<0.05 as compared with MenSCs group.
roles of MenSCs and PDGF in the endometrial re-epithelialization and re-vascularization process, our data from in vitro experiments demonstrated that the combination of MenSCs and PDGF significantly promoted cellular migration and proliferation, and promoted the differentiation of these cells into tube-like structures. Reconstruction of the vascular capillary network is also crucial to endometrial repair. Therefore, the formation of new vessels was examined by detecting the microvessel marker CD34 using IHC assay. Dilated blood vessels were identified and the number of vessels was markedly reduced during endometrial injury. MenSCs and PDGF treatment induced significant infiltration of new microvessels into the glandular layer.

Akt signaling plays a critical role in various cellular events such as cell proliferation, migration, apoptosis, and angiogenesis [14,28]. It has been documented that Akt prevents apoptosis in cells through phosphorylation and inhibition of pro-apoptotic mediators such as Bad [28]. Phosphorylation of Bad induced by Akt causes a conformation change and subsequently prevents the interaction with anti-apoptotic Bcl-2 protein; the free anti-apoptotic Bcl-2 protein then inhibits Bax-triggered apoptosis [27,28]. Both MenSCs and PDGF have been previously found to activate Akt in human endometrial cells, and are required for MenSCs- or PDGF-induced cell proliferation, migration, and angiogenesis [14,26]. Our present findings confirmed that PDGF and MenSCs can activate Akt in endometrial tissue. Phosphorylation of Bad was also observed following either PDGF, or MenSCs, or the combination treatment, suggesting that Bad phosphorylation was dependent on Akt activation. These results could partially clarify the mechanisms of improved re-epithelialization and angiogenesis induced by MenSCs and PDGF.

Conclusions

Our study is the first to show the potential roles of MenSCs and PDGF in the regulation of biological functions of endometrial epithelium and, subsequently, in endometrial repair and regeneration. Our combined data from in vitro and in vivo experiments show that the combination of MenSCs and PDGF synergistically promoted the formation of new vessels and re-epithelialization of endometrium. This two-agent combination might therefore represent a novel candidate substance for endometrial injury repair.

Conflict of interests

None.

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