Stem Cells and Endometrial Hyperplasia

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INTRODUCTION

Endometrial hyperplasia is characterized by a proliferation of endometrial glands that may progress to or coexist with endometrial carcinoma. Previous studies proved the presence of endometrial stem cells that may have a role in endometrial regeneration and may be responsible for proliferative disorders as in endometrial hyperplasia and endometrial carcinoma. The present study carried out to evaluate the potentiality of Umbilical cord blood Hematopoietic CD34+ and Mesenchymal stem cells to regress endometrial hyperplastic cells proliferation in vitro under certain condition. The Study carried out on 40 Umbilical cord blood samples collected from term delivering women, mononuclear cell were separated. Endometrial samples from the 40 cases (20 normal and 20 cases with endometrial hyperplasia) were collected by dilatation and curettage or by curetting endometrium after hysterectomy operation. Then treatment of the hyperplastic cells with the Mesenchymal stem cells extract and CD34+ Hematopoietic stem cells extract was done. Results showed that both CD34+ Hematopoietic stem cells extract and mesenchymal stem cells extract both had decreased the rate of growth of endometrial hyperplastic cell culture reaching near to the rate of growth of normal endometrial primary cell culture but the mesenchymal stem cells extract had a higher degree of control making endometrial growth rate nearer to the rate of growth of normal endometrial primary cell culture.

ABSTRACT

Endometrial hyperplasia is characterized by a proliferation of endometrial glands that may progress to or coexist with endometrial carcinoma. Previous studies proved the presence of endometrial stem cells that may have a role in endometrial regeneration and may be responsible for proliferative disorders as in endometrial hyperplasia and endometrial carcinoma [1]. It is hypothesized that endometrial stem or progenitor cells play key roles in the initiation of these endometrial proliferative disorders. The aim of the present study was to evaluate the potentiality of Umbilical cord blood CD34+ and mesenchymal stem cells to regress endometrial hyperplastic cells proliferation in vitro under certain condition.

MATERIALS AND METHODS

Umbilical Cord Blood Collection
Umbilical cord blood samples (n = 40) from delivering women at (37-40 weeks) were collected at department of Obstetrics and...
Gynecology, Faculty of Medicine Hospitals at Menoufia University. Umbilical cord blood samples were obtained from human healthy pregnant women after giving birth as fast as possible to preserve cell viability and the blood samples were processed by the Stem Cells Laboratory at Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City. The study started at January 2013 and ended June 2014. The volunteers’ age ranged from twenty five to thirty. They were not suffering from any chronic diseases and not taking any medications. UCB was collected from the umbilical cord vein with informed consent of the mother. A tube system containing 5 mL of citrate phosphate dextrose anticoagulant was used. All UCB samples were processed within 6h after deliveries.

Mononuclear cells separation
Fresh human cord blood samples treated with citrate phosphate dextrose anti-coagulant were diluted with 1-3 volumes phosphate buffer saline solution (PBS). 30 ml of diluted cell suspension were carefully layered over 10 ml Ficoll Hypaque (1.077 density) in a 50 ml conical tube and centrifuged at 1000 rpm for 30 minutes at -20 °C in a swinging-bucket rotor without brake. The upper layer was aspirated off leaving the mononuclear cell layer undisturbed at the interphase then the interphase cells (lymphocytes and monocytes) was carefully transferred to a new 50 ml conical tube. The conical tube was filled with PBS containing 2 mM EDTA, mixed well and centrifuged at 1000 rpm for 10 minutes at 20 °C then the supernatant was carefully removed completely. The pellet was resuspended in 50 ml of PBS containing 2 mM EDTA and centrifuged at 1000 rpm for 10-15 minutes at 20 °C then the supernatant was carefully removed completely [3].

Viability test of mononuclear cells was carried out by mixing one drop of cell suspension with one drop of 0.4% trypan blue vital stain in separate eppendorf tube and cell counting takes place using haemocytometer. The pellet was resuspended in a final volume of 300 µl/10⁶ total cells, then magnetic labeling was proceeded.

Immunomagnetic labeling and separation of CD34+ / CD34- Progenitor Stem Cells
100µl FcR Blocking Reagent per 108 total cells was added to the cell suspension to inhibit unspecific or Fc-receptor mediated binding of CD34+ Micro Beads to non-target cells.100 µl CD34+ Micro Beads per 108 total cells was added for labeling cells, then mixed well and incubated for 30 minutes in the refrigerator at 6—12 °C. Cells was washed carefully and resuspended in appropriate amount of running buffer (Contains; Bovine serum albumin, EDTA, Phosphate Buffer Saline, 0.09% Sodium Azide); 500-1000 µl for MS- column (2x10⁵ cells/ml) then magnetic separation was preceded.

Cryopreservation
1x10⁶ viable cells resuspended in 1.5 ml cryoprotectant medium, slowly freeze at 1 °C / min for 24 hrs in -20 °C freezer then transferred to – 80 °C for further use.

Cell thawing
Cryoprotectant vials of CD34- and CD34+ transferred as fast to 37 °C water bath, cells thawed within 3-5 min. Cells are centrifuged at 1000 rpm for 10 min. then pellet resuspended in 25 ml DMEM growth medium supplemented with 20% calf serum and seeded in 75 cm² cell culture flasks with vented caps.

Separation of mesenchymal stem cells from CD34+
CD34+ cells were incubated in 5% CO₂ for three days, and then cells were investigated under inverted microscope to examine the fibroblast like appearance of mesenchymal stem cells. Floating cells containing other cell type were aspirated off leaving attached mesenchymal cells. DMEM growth medium was renewed with the same condition every three days to reach confluence.

Proliferation study
Culture of CD34+ hematopoietic progenitor cells were performed in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FCS (Fetal Calf Serum) inactivated at 56 °C for 30 min. Cells were incubated at 37 °C in a 95% humidified atmosphere of 5% carbon dioxide (CO₂) incubator. The growth medium was renewed every three days for two weeks.

Mesenchymal and CD34+ hematopoietic stem cells extract preparation
When cell of Mesenchymal and CD34+ Hematopoietic stem reach log phase of growth, cell culture flasks transferred from CO₂ incubator to -20 °C for freezing. Cell cultures were thawed at room temperature. All contents of cell flasks were collected into centrifuge tubes and centrifuged at 10000 rpm for 10 min. Supernatant of each cell type were collected into 1.5 Eppendorf tubes and kept in -20 °C for further use.

Endometrial sampling
Two groups of tissue sampling were taken; Normal endometrium (20 case) and Hyperplastic endometrium (20 case).

Cell cultures of human endometrial cells
Endometrial tissue samples were obtained at surgery from women (range 35–50 years) undergoing hysterectomy or dilatation and curettage. Written informed consent was obtained from each patient and the use of human tissue specimens was approved by the Ethics Committee of the Faculty of Medicine (Menoufia University). None of these cases had any previous history of uterine cancer, and all samples were confirmed by histopathological examination to confirm diagnosis. Tissue samples were collected in test tubes containing growth medium.

Cell culture of normal and hyperplastic tissues
Each type of tissue samples of both normal and hyperplastic tissues were put in sterile petri dish alone and cut into small pieces.10 ml of Collagenase type-III enzyme pre-warmed at 37 °C was added to each dish and incubated at 37 °C CO₂ incubator for 30 min and then examined under inverted microscope to see cell aggregation. Each sample was collected and passed into sterile mesh into clean 100 ml conical flask. The elute of each sample was collected into centrifuge tube and centrifuged at 1000 rpm for 10 min.

Supernatant was aspirated off and cell pellet was resuspended in DMEM fresh medium and seeded in 75 cm² cell culture flasks containing 30 ml DMEM supplemented with 10% FCS, 1 gm streptomycin, 1x10⁶ penicillin and garamycin 80/liter. Cell cultures of each cell type were incubated at 37 °C in a 95% humidified atmosphere of 5% carbon dioxide (CO₂) incubator to reach passage 1.

Experimental design
Two main Groups: I-Control groups containing; G1: Normal Endometrium primary culture untreated, G2: Hyperplastic Endometrium primary culture untreated; G3: CD34+
Hematopoietic stem cells primary culture and G4: Mesenchymal stem cell primary culture. II-Treated groups containing; G1: Hyperplastic Endometrium primary culture treated with Mesenchymal stem cells extract and G2: Hyperplastic Endometrium primary culture treated with CD34+ Hematopoietic stem cells extract. 3x10⁵ hyperplastic endometrial cells were resuspended in 3x10⁵/ml and 5 ml MEM growth medium containing 10% heat inactivated FCS and incubated at 5% CO₂ incubator for 24 hr. to reach complete monolayer, then growth medium renewed with fresh medium containing 2% FCS. The cell wells divided into two groups, group 1 treated with 200 µl hematopoietic cell extract and the second group treated with 200 µl mesenchymal stem cell extract.

RESULTS

I-Stem Cells Primary Culture
Cryopreserved umbilical cord blood HSCs in liquid nitrogen at -196 °C thawed in water bath at 37 °C within 3-5 min. resuspended in DMEM growth medium supplemented with 10% FCS and seeded in cell culture flasks 75 cm² and incubated in 5% CO₂ incubator at 37 °C. The growth medium renewed every 3 days. HSCs showed cluster formation a characteristic feature for HSCs (Fig 1). Cryopreserved umbilical cord blood MSCs in liquid nitrogen at -196 °C thawed in water bath at 37 °C within 3-5 min. resuspended in DMEM growth medium supplemented with 10% FCS and seeded in cell culture flasks 75 cm² and incubated in 5% CO₂ incubator at 37 °C. The growth medium was renewed every 3 days. MSCs showed fibroblast like cells formation a characteristic feature for MSCs (Fig 2).

Endometrial cells primary culture
Endometrial tissue samples of normal endometrium and endometrial hyperplasia disaggregated in collagenase enzyme type three for 1 hr. The collagenase enzyme deactivated by the addition of equal amounts of DMEM growth medium supplemented with 10% FCS. Cell suspension collected in centrifuge tubes and centrifuged at 1000 rpm for 10 min. The supernatant discarded off and cell pellet suspended in DME supplemented in 10% FCS and seeded in 75 cm² cell culture flasks with vented caps. Cell culture investigated under inverted microscope after three days of incubation to investigate cell behavior of normal endometrium (Fig 3), endometrial hyperplasia (Fig 4) and complex endometrial cells (Fig 5) in culture system.
Table 1. Distribution of the patients regarding their characteristics.

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Data presented in Table 1 shows no significant difference between normal Endometrium and Hyperplasia groups regarding to mean age, parity, medical history, examination and ultrasound findings. While there was highly significant difference regarding endometrial thickness between the two groups.

Table 2 shows equal number of cells (12×10^5 cells) in the four groups at start of proliferative study of normal endometrium, endometrial hyperplasia, endometrial hyperplasia with CD34+ hematopoietic stem cells extract and endometrial hyperplasia with mesenchymal stem cells extract. At day three of proliferative study, there was highly significant difference between growth of normal endometrium and endometrial hyperplasia with CD34+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was decrease in the rate of cellular growth and the decrease was significant in relation to endometrial hyperplasia group. While on the group where mesenchymal stem cells extract was added to endometrial hyperplastic cells there was highly significant decrease of the rate of growth of cells in relation to endometrial hyperplasia group.

At day six of proliferative study, there was highly significant difference between growth of normal endometrium and endometrial hyperplasia with CD34+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was significant difference in the rate of cellular growth between this group and endometrial hyperplastic group. While on the group where mesenchymal stem cells extract was added to endometrial hyperplastic cells there was also highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group. At day nine of proliferative study, there was highly significant difference between growth of normal endometrium and endometrial hyperplasia. With CD34+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was a highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group. While on the group where mesenchymal stem cells extract was added to endometrial hyperplastic cells there was also a highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group and the rate of growth of cells was near to normal rate as there was no significant difference in cellular growth rate between this group and normal endometrium. Also there was a highly significant difference between both treated groups at this day. At day twelve of proliferative study, there was a highly significant difference between growth of normal endometrium and endometrial hyperplasia. With CD34+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was a highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group, while on the group where mesenchymal stem cells extract was added to endometrial hyperplastic cells there was also a highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group and the rate of growth of cells was near to normal rate as there was no significant difference in cellular growth rate between this group and normal endometrium. A summary of all the data is graphically shown in Fig. 6.

Table 2. Distribution of the studied endometrial groups regarding their growth.

Data presented in Table 1 shows no significant difference between normal Endometrium and Hyperplasia groups regarding to mean age, parity, medical history, examination and ultrasound findings. While there was highly significant difference regarding endometrial thickness between the two groups.
DISCUSSION

This study was designed to evaluate the ability of CD 34+ hematopoietic stem cells and mesenchymal stem cells extracts generated from umbilical cord blood to change the growth kinetics of endometrial hyperplastic cells to normal endometrium in vitro. The study was based on previous studies that proved the presence of stem cells in the endometrium. These cells may have a role in endometrial proliferation [3]. Also these cells may play a role in proliferative disorders of endometrium as in cases of endometriosis, endometrial hyperplasia and endometrial carcinoma [3]. In this study, 40 Umbilical cord blood samples were collected from term delivering women. UCB was collected from the umbilical cord vein with informed consent of the mother. Mononuclear cells were separated, cultured, and there was continuous growth of both CD34+ hematopoietic stem cells and mesenchymal stem cells on culture media and there was no significant difference regarding the mean number of cells in the two groups (CD34+ hematopoietic stem cells and mesenchymal stem cells) all over the proliferative study period.

Recent advances in stem cell research have brought about the possibility of stem cell therapies [4] and new approaches using human genetics have been developed to validate therapeutic targets [5]. The regulation of differentiation in stem cells involves the expression of several genes; for example, myogenic differentiation 1 induction in immature human iPSCs leads to the differentiation of these cells into mature myocytes [6].

MSCs are known to be involved in the immune response during several processes such as the allogenic transplantation of bone marrow, which mainly causes an immunosuppressive effect. A MSC-induced immunosuppressive effect might be caused by the down-regulation of T-cell differentiation into T helper 17 (Th17) cells and of the function of mature Th17 cells. This inhibition of mature Th17 cells could occur via the cell-to-cell contact mechanism of MSCs and may be mediated by the programmed death-1 pathway [7]. Allogenic MSCs have also been demonstrated to regulate the function of Th17 cells derived from rheumatoid arthritis patients. Co-cultures of MSCs with peripheral blood mononuclear cells (PBMCs) cause a decrease in orphan nuclear receptor gamma (ORR-γ), which is involved in Th17 differentiation and is expressed in endometrial CD34+ cells as well as in endometrial mesenchymal cells [18]. MSCs produce TGF-β and interleukin-6 (IL-6) and regulate the differentiation of T cells into regulatory T cells or Th17 cells [9]. Therefore, the immune response may be regulated by MSCs via Th17 signaling.

MSCs have also been applied as novel vaccine platforms. MSC vaccination strategies include the modified application of MSCs in anti-microbial or cancer immunization [10]. Genetically modified MSCs may act as antigen presenters or mediators as well as suppliers of immune-related cytokine [10]. MSCs from the placenta are known to suppress allogeneic umbilical cord blood lymphocyte proliferation and it has been suggested that placenta-derived MSCs may be applied in allograft transplantations [11]. The immunomodulatory properties of equine adult-derived MSCs derived from bone marrow, adipose tissue, umbilical cord blood and umbilical cord tissue have been compared, and it was revealed that lymphocyte proliferation is suppressed by MSCs and secretion of prostaglandin E2 and IL-6 is increased upon allogenic PBMC or phytotrimacillin stimulation [12]. MSCs also decrease the production of tumor necrosis factor-α and interferon-γ [12]. The immunomodulatory effect of MSCs on B and T cells have also been studied [13]. One of the main roles of MSCs on B cells is the inhibition of B cell proliferation, but their effects on B cells are still controversial [13]. The MSC-induced regulation of the proliferative response of lymphocytes has been reported to be independent of the major histocompatibility complex, although MSCs mainly induce inhibition and sometimes cause enhancement of the mixed-lymphocyte reaction [14].

This study included two groups; first group included 20 normal patients as control group. The second group included 20 patients with endometrial hyperplasia, there was no significant difference between the 2 groups regarding to age, parity, history of medical disorders or type of operation done while there was highly significant difference regarding endometrial thickness between the two groups. Endometrial samples from the 40 cases were collected by dilatation and curettage or by curetting endometrium after hysterectomy operation. Cell cultures of each cell type were incubated at 37 °C in a 95% humidified atmosphere of 5% carbon dioxide (CO2) incubator. Then treatment of the hyperplastic cells with the Mesenchymal stem cells extract and CD34+ Hematopoietic stem cells extract was done. So 4 groups were formed; Control groups contains G1: Normal Endometrium primary culture untreated and G2: Hyperplastic Endometrium primary culture untreated and Treated groups contains also G1: Hyperplastic Endometrium primary culture treated with Mesenchymal stem cell extract and G 2: Hyperplastic Endometrium primary culture treated with CD34+ Hematopoietic stem cells extract. The growth kinetics of cells growing in cell culture in both treated groups was carefully monitored and compared to the growth kinetics of both control groups. Viability test was made by mixing one drop of cell suspension and one drop of 0.4% Trypan blue stain, the viable cells stained brilliant blue and the unviable cells stained dark blue. Then cell counting was done using THOMA Haemocytometer.

This study revealed that growth kinetics characteristics of normal endometrial primary cultures was as follow: on the start of study the mean number of cells in culture was 1.2x10^5 cells, then cells growth was as follow, 1.3x10^5, 18.2x10^5, 26.2x10^5 and 33.5 x10^5 for days 3, 6, 9 and 12 respectively. Growth kinetics characteristics of hyperplastic endometrial primary cultures was as follow: on the start of study mean number of cells in culture was 1.2x10^5 cells, then cells growth was as follow, 16.4x10^5, 32.8x10^5, 54.2x10^5 and 62.3 x10^5 cells for days 3, 6, 9 and 12 respectively.

Growth kinetics characteristics of hyperplastic endometrial primary cultures treated with CD34+ Hematopoietic stem cells extract was as follow: on the start of study mean number of cells in culture was 1.2x10^5 cells, then cells growth was as follow, 15.1x10^5, 28.2x10^5, 36.2x10^5 and 40.0 x10^5 cells for days 3, 6, 9 and 12 respectively. Growth kinetics characteristics of hyperplastic endometrial primary cultures treated with mesenchymal stem cells extract was as follow: on the start of study mean number of cells in culture was 12x10^5 cells, then cells growth was as follow, 14.3x10^5, 23.4x10^5, 28.0x10^5 and 34.2x10^5 cells for days 3, 6, 9 and 12 respectively. Day analysis of these data, the study started with equal number of cells (12x10^5 cells) in the four groups at start of proliferative study. On day three, there was highly significant difference between growth of normal endometrium and endometrial hyperplasia. With CD34+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was decrease in the rate of cellular growth and the decrease was significant in relation to endometrial hyperplasia group. While on the group where mesenchymal stem cells extract was added to
endometrial hyperplastic cells there was a highly significant decrease of the rate of growth of cells in relation to endometrial hyperplasia group. On day six, there was a highly significant difference between growth of normal endometrium and endometrial hyperplasia. With CD34^+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was significant difference in the rate of cellular growth between this group and endometrial hyperplastic group. While on the group where mesenchymal stem cells extract was added to endometrial hyperplastic cells there was also a highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group. On day nine, there was a highly significant difference between growth of normal endometrium and endometrial hyperplasia. With CD34^+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was a highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group, while on the group where mesenchymal stem cells extract was added to endometrial hyperplastic cells there was also a highly significant difference in the rate of cellular growth between this group and normal endometrium. Also there was a highly significant difference between both treated groups at this day. On day twelve, there was a highly significant difference between growth of normal endometrium and endometrial hyperplasia. With CD34^+ hematopoietic stem cells extract added to endometrial hyperplastic cells there was a highly significant difference in the rate of cellular growth between this group and endometrial hyperplastic group, while on the group where mesenchymal stem cells extract was added to endometrial hyperplastic cells the rate of growth of cells was nearer to normal rate as there was no significant difference in cellular growth rate between this group and normal endometrium. The growth curve of the four groups all over the entire period of proliferative study revealed that both treated groups curves showed a lower rate of growth than endometrial hyperplastic curve rate but the mesenchymal group curve was nearer to normal endometrial growth curve.

CONCLUSION

These data revealed that both CD34^+ Hematopoietic stem cells extract and mesenchymal stem cells extract both had decreased the rate of growth of endometrial hyperplastic cells cultured reaching near to the rate of growth of normal endometrial primary cell culture but the mesenchymal stem cells extract had a higher degree of control making endometrial growth rate nearer to the rate of growth of normal endometrial primary cell culture.

REFERENCES