Effect of deforolimus and VEGF on angiogenesis in endometrial stromal cells following three-dimensional culture

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ABSTRACT
The presence of endometrial tissue outside of the uterine cavity is named endometriosis and is the most common gynecologic disorder in women. Determining the inhibitory effect of deforolimus on angiogenesis in a three-dimensional (3-D) culture of human endometrial stromal cells (hEnCs) in vitro. The important mechanism in the pathogenesis of endometriosis is angiogenesis, and deforolimus has been shown to have anti-angiogenic activity. This was an in vitro study of human endometrial stromal cells in 3-D culture of fibrin matrix. Endometrial stromal cells isolated and placed in a 3-D fibrin matrix culture system for angiogenesis with VEGF and inhibit angiogenesis by deforolimus. Finally these cells analyzed by CD31 antibodies. After 3 weeks, in cells treated with VEGF, endothelial cell branching was observed and rudimentary capillary-like structures formed. In the presence of 5 µM of deforolimus, angiogenesis was reduced. The deforolimus were shown to be effective in inhibiting the mechanisms of angiogenesis.

Keywords: Endometrial Stromal Cells; Endometrium; 3-D culture; Deforolimus; VEGF

1. INTRODUCTION
Endometriosis is a chronic benign gynecological disorder characterized by presence of endometrial cells throughout the uterus or peripheral organs and tissues. The rate of occurrence of endometriosis is about 11% in all women and is usually identified in women with pelvic pain and infertility. The etiology and pathogenesis of endometriosis is not clearly understood, but there are a number of theories. Historically theories have proposed different reasons for endometriosis including retrograde menstruation, coelomic metaplasia, embryonic rest and lymphovascular metastasis. However, several predisposing factors ranging from genetic to environmental factors as well as physiological and anatomical defects have been reported in correlation with it [1,2]. The human endometrium develops new capillaries from existing microvessels, i.e. angiogenesis, which then undergoes maturation and remodeling [3]. Excessive endometrial angiogenesis is suggested to be an important mechanism in the pathogenesis of endometriosis [4]. Recent progresses in the field of stem cells and evidences for in situ presence of endometrial stem cells or migratory stem cells from bone marrow have opened up a new way toward stem cell-based pathogenesis of endometriosis. Stem cells (SCs) are undifferentiated cells, which are can remain at this state for several generations following cell proliferations. SCs are also able to take part in natural in vivo phenomena such as wound repair or tissue regenerations after physical damages. Today, stem cells are being considered as an important tool in cell therapy which is a novel therapeutic method by clinical researchers [5,6]. On this basis, endometrial stem cells (EnSCs) with higher ability for proliferation, differentiation and fast angiogenesis during menstruation alongside with their immune tolerance for embryo during pregnancy have been considered as a valuable source of stem cell [7,8]. Additionally our previous studies demonstrated that EnSCs are exceptionally capable of participating in a phenomenon like angiogenesis in 3-D cultures which is similar to early stages of...
endometriosis [9-11] and may propose a novel mechanism in pathogenesis of endometriosis compared to the traditional theories that emphasize on retrograde menstruation as a possible factor. These studies are supported by others who demonstrated recruitment of bone marrow-derived mesenchymal stem cells to the endometrium [12] and these all may point to the role of stem cells in pathogenesis of endometriosis [13,14]. Therefore, endometrial stem cells could be considered as binary role-players in helping endometrium function naturally and also in endometriosis. They potentially could be used as targets in endometriosis therapy. For this, rapamycin was extracted from a bacterium, Streptomyces hygroscopicus, taken from the soil of Rapa Nui. Thereafter, there are three rapamycin analogs that are used as anti-tumor agents: CCI-779 (Temsirolimus), AP23573 and RAD001 (Everolimus) [15]. Rapamycin and its analogs bind to the cellular protein FK506 through binding protein 12 (FKBP12), and directly inhibit mTOR (mammalian Target of Rapamycin), a cellular enzyme that plays a key role in cell growth and proliferation [16,17]. Rapamycin appears to inhibit angiogenesis [18], which is consistent with the observation that PI3K signaling is required for expression and secretion of the pro-angiogenic vascular endothelial growth factor (VEGF) from endothelial cells [16]. Deforolimus has shown promise in treating a broad range of tumors, especially sarcomas. Deforolimus has been shown to inhibit the secretion of VEGF in cell lines, suggesting that it exerts some of its anti-tumor effects through inhibition of angiogenesis [19].

In our previous studies we showed that in vitro culture of human endometrial tissue in a 3-D fibrin matrix could proliferate and sprout new vessels [9-11]. In this study EnSCs were differentiated to endothelial cells (CD31+). The aim of the present study was to determine the potential inhibitory effect of a deforolimus (AP23573; Sigma) on angiogenesis in an in vitro three-dimensional fibrin matrix culture of human endometrial stromal cells treated with VEGF (CD31+).

2. MATERIALS AND METHODS

Endometrial biopsies were obtained from cases referred to the hospital for infertility treatments. A written informative consent form describing the procedures and aims of the study was taken from each donor in compliance with regulations concerning the use of human tissues. The biopsied tissues were washed in Dulbecco’s phosphate buffered saline (DPBS), minced and treated with collagenase I type A (2 mg/ml) for 30 - 45 min at 37°C with agitation. Following tissue digestion, epithelial and stromal cells were separated using filtration. The cells were then centrifuged and underwent Ficoll purification. The isolated cells were cultured in DMEM/F12 medium containing 10% FBS, 1% antibiotic penicillin/streptomycin and 1% Glutamine and then incubated at 37°C in 5% CO₂ [20].

2.1. Phenotypic Characterization

Having three passages after isolation, the cells were characterized by flow cytometry for cell surface markers. In order to do so, cells were washed with HBSS + 2% BSA two times and incubated with the specific antibody at the concentrations recommended by the manufacturers. Cells were incubated for 20 minutes and analyzed by flow cytometry. Following antibodies were used: FITC-conjugated anti-CD90, FITC-CD146, PE anti-human CD105 (mesenchymal markers), FITC-conjugated anti-CD34 (hematopoietic marker), PE anti-human CD31 (endothelial marker), FITC-conjugated mouse IgG1, and PE-conjugated mouse IgG1 were used for negative control, all from Santa Cruz. For intracellular staining by the antibody, cells were washed twice in PBS with 2% BSA and fixed with 4% paraformaldehyde (PFA) for 30 min. Subsequently cells were washed twice in 0.5% Tween 20 and 0.1% Triton X-100 in PBS (T-PBS). Primary antibodies were added and incubation was performed for 30 min. Cells were then washed twice in PBS. Secondary antibodies were subsequently diluted in PBS at the concentrations suggested by the manufacture instructions. Cells were analyzed using flow cytometry and OCT4 was used as the intracellular antibody (Abcam, USA).

2.2. Three-Dimensional in Vitro Culture of Endometrial Fragments

sEnSCs were examined to be free from endothelial cells using CD31 staining. The cells were then mixed with 1 ml/well fibrinogen solution (3 mg/ml in M199 culture medium), plated in culture dishes (24 wells) and 15 ul thrombin (50NIH unit/ml in 0.15 M NaCl) was added. After gel formation, each well was covered with 1 ml M199 supplemented with 5% fetal bovine serum (FBS), 0.1% e-amo-glutamine and 100× antibiotic solutions and VEGF 50 ng/ml. The cells were cultured at 37°C in 5.5% CO₂ and 95% humidity for time period up to 3 weeks. The medium was changed every 3 days and cultures were observed twice weekly with an inverted microscope for visual evidences of angiogenesis. After 3 weeks, we used EnSCs that were treated with VEGF before and the expression of CD31 was confirmed in these cells prior to being treated with 5 μM of deforolimus for 1 week in 3-D culture.

2.3. Morphological Observation

Cells were observed under a phase-contrast microscope in order to have their overall appearance be eva-
2.4. Immunocytochemistry Analysis

Day 21 post treatment (PT) cells for CD31 antibody as an endothelial cells marker were fixed with 4% PFA (Sigma-Aldrich) for 30 min at room temperature. After permeabilization with 0.2% Triton-X 100 (Sigma-Aldrich) for 10 min the cells were blocked with goat serum and incubated overnight with primary antibody CD31, mouse monoclonal anti-human, Abcam, 1:200 at 4°C and 2 h with secondary antibody (Alexa Fluor®488 donkey anti mouse IgG, at a 1:500 dilution; Abcam, USA) at 37°C. For negative controls, only the secondary antibody was used. Slides were washed with PBS between each step and nuclei staining were performed using 4’, 6-diamidino-2-phenylindole (DAPI, Sigma). Cells were examined by fluorescence microscope (Olympus BX51, Japan).

2.5. Flow Cytometry Analysis

After 3 weeks, EnSCs treated with VEGF were prepared for flow cytometry for CD31 (endothelial cells marker). Then VEGF treated EnSCs were treated with deforolimus after 3 weeks and were tested for CD31 by flow cytometry. Cells were trypsinized and washed with staining buffer, which contained PBS with 3% FBS and 0.05% sodium azide. Cell pellets were incubated on ice with CD31 conjugated directly with PE (Abcam) for 1 hour. Cells were washed 3 times by centrifugation at 400 g for 5 minutes and were resuspended in 500 µl to 1 ml of ice cold PBS, 10% FCS, 1% sodium azide. Cells were kept in the dark on ice or in a fridge at 4°C until analysis.

3. RESULTS

3.1. Characterization of Isolated Human EnSCs

The immunophenotype was based on the flow cytometry analysis of a subset of embryonic stem cell marker (OCT4), mesenchymal stem cell markers (CD90, CD146 and CD105), hematopoietic marker (CD34) and endothelial marker (CD31). The flow cytometric analysis showed that the EnSCs were positive for CD90, CD105, OCT4 and were negative for CD31, CD34 (Figure 1).

Figure 1. Flow cytometric analysis of isolated EnSCs for mesenchymal stem cell markers (CD90, CD146 and CD105), hematopoietic marker (CD34), endothelial marker (CD31) and embryonic stem cell marker (OCT4). As shown in figure 2 the isolated cells are positive for CD90, CD146, CD105, and OCT4 and are negative for CD31, CD34.
3.2. Morphological Differentiation, Immunocytochemistry and Flow Cytometry Analysis

The main cellular event observed during the first week of culture of EnSCs with VEGF was formation of sheets of cells in the fibrin matrix (Figure 2(A)). From the beginning of the third week, angiogenesis was observed as tube-like structures sprouting into the 3-D fibrin matrix and were observed in the outgrowths and grew independently until the end of culture period (Figure 2(A)). Immunocytochemistry analysis showed that endometrial stromal cells after treated with VEGF for 3 weeks express endothelial marker CD31 (Figure 3). Flow cytometry analysis of these structures was 15.57% positive for CD31 as a marker of endothelial cells (Figure 4(a)). During the 1st week of culture, invasion of deforolimus treated CD31 positive EnSCs occurred into the fibrin matrix in the control group and outgrowths were reduced to 11.64%. The effect of deforolimus was witnessed on angiogenesis in the experimental groups. In presence of 5 µM deforolimus, angiogenesis was markedly reduced compared to the control wells (Figure 2(C)). The results of flow cytometry for CD31, confirmed morphological results (11.64%, Figure 4(b)).
4. DISCUSSION

In the present study, in the first step, we have utilized 3-D in vitro fibrin matrix for culturing isolated endometrial stromal cells in presence of VEGF for angiogenesis. Using this model, we have demonstrated three-dimensional cell proliferation and invasion, resulted in generation of new vessels. These cells were 15.57% positive for CD31. In the second step, we treated these cells (CD31+) with deforolimus at concentration of 5 µM in 3D culture. The results show that deforolimus reduced tube-like structures and caused 11.64% decrease in CD31 expression. We found that deforolimus inhibited angiogenesis in these cells and can be used for endometriosis patients. The pathogenesis of endometriosis remains poorly understood, mostly because the initial stages of the disease are neither detectable nor observable in humans. By the time that endometriosis is brought to medical attention, the disease already is well established. In addition, this disease occurs only in humans and subhuman primates, since menstrual shedding is a requirement for its spontaneous development [1,2]. Endometrial angiogenesis suggests the possibility for novel medical treatments aimed at the inhibition of angiogenesis, which is similar to recently discussed concepts for cancer treatment [21,22]. Our findings suggest that deforolimus, an mTOR inhibitor, can negatively modulate both cell proliferation and angiogenesis in a concentration dependent manner. Deforolimus is a potent inhibitor of VEGF expression, which is most probably the mechanism behind the diminished blood-vessel formation. mTOR is a serine/threonine kinase that is a central regulator of cell growth, proliferation and metabolism in response to environmental and nutritional cues. It is a critical effector of signals mediated by the phosphatidylinositol 3-kinase (PI3K) pathway, a signaling cascade that is important in tumors [16]. Activation of PI3K by growth factors signals the pathway, a signaling cascade that is important in tumors [3].

5. CONCLUSION

In our previous studies we found, endometrial stem cells can differentiate to other cells such as endothelial cells, neuron, adipocyte, osteoblast and myoblast [26-30] and based on our findings, in this research, it seems that sloughed stromal cells as basic factor in angiogenesis causing pelvic fibrin adhesions in a retrograde manner would be able to proliferate as the first step of endometriosis. Furthermore, studying in vitro (3-D) models shows stromal cells proliferation and angiogenesis. These two phenomena are considered crucial for the development of the angiogenesis in endometriotic lesions and deforolimus were shown to be effective in inhibiting the mechanisms of angiogenesis.

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REFERENCES


