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Original Research

Feasibility of serum-free culture in isolating endometriotic stem cells

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Summary

Objective: To explore a novel approach for the isolation of human endometriotic stem cells for further study in the stem cell theory of endometriosis. *Materials and Methods:* A serum-free medium (SFM) was used to isolate the stem cells from ectopic endometrium, eutopic endometrium, and normal endometrium in vitro, and the biological properties including clonogenicity, multipotency, and invasive ability were examined and compared. *Results:* The ectopic endometrium-derived stem cells cultured in SFM showed higher clone-forming efficiency and sphere formation efficiency (SFE) than eutopic endometrium and normal endometrium, they all can differentiate to endothelial cell and fibroblast, and the ectopic endometrium-derived stem cells also have stronger invasive ability than eutopic endometrium-derived and normal endometrium-derived stem cells. *Conclusion:* According to the results of the present study, the authors can conclude that human endometriotic stem cells can be isolated in SFM effectively and ectopic endometrium-derived stem cells are probably associated with endometriosis.

Key words: Endometriosis; Stem cells; Cell culture techniques; Serum-free media.

Introduction

Endometriosis is a common gynecological disorder with the incidence of 10% to 15% of in reproductive women and 70% to 80% in the women with in fertility and chronic pelvic pain [1, 2], which is characterised by the presence of endometrial tissue outside the uterine cavity. The pathogenesis of endometriosis is yet not fully understood and the stem cell theory of endometriosis has obtained increasing focus with the rapid development in the stem cell research field [3, 4]. However it remains unclear how the ectopic endometrium-derived stem cells cause endometriosis and it is necessary to isolate the endometriotic stem cells effectively in order to explore the pathogenesis further. Nowadays the purified stem cell suspensions were obtained usually by selecting cells with magnetic bead sorting and colony-forming from endometriosis lesions with lower efficiency and complicated program, and it is very important to find a simple method with high efficiency for the study in the field of the stem cell theory in endometriosis. Serum-free suspension culture is a common method which is usually used to isolate stem cells without the application experience in endometriotic stem cell. The present study is to attempt to isolate human endometriotic stem cells using serum-free medium.

Materials and Methods

Three types of endometrial tissues were collected: ovarian endometrioma (ectopic endometrium) and endometrium from women with endometriosis (eutopic endometrium, autologous samples) (n=5) were collected from women aged 26 to 50 years undergoing ovarian cystectomy through laparoscopy, endometrium from women without endometriosis (normal endometrium) (n =5) were collected from reproductive women aged 25 to 52 years undergoing gynecology surgery for other disorders. All patients had not taken exogenous hormones for three months before surgery. Informed written consent was obtained from each patient and ethical approval was obtained from the Institutional Review Board of the Hospital.

The tissue samples were collected during operation and kept in the D-hanks solution. After the specimens were washed with PBS and the mucus on the surface was scraped off, the tissues were predigested with trypsin and collagenase at 37°C for one hour. The cyst walls were cut off and washed, followed by low-speed centrifugation. The supernatant was discarded and digestion was carried out at 37°C for one hour. The solution was then allowed to stand for two minutes and the undigested part was collected to repeat the steps above. The supernatant was transferred to another centrifuge tube and centrifugation was carried out at 1,200 rpm for five minutes. The supernatant was discarded and the left was cultured in serum free medium (SFM) and the culture medium was changed every two days. The cell clusters suspended in the SFM were collected to continue the next steps.

The cell clusters were digested with trypsin to single-cell suspensions and adjusted the cell concentration to 1×10^3 /ml, which were transferred into a 24-well plate with 1 ml per well. The cells were incubated at 37°C with 5%CO_2 and the medium was changed every two days. The numbers of whole cell clones and cell clones with the diameter more than 75 μm were recorded respectively and the clone-forming efficiency (clone numbers /cultured cell numbers×100%) and sphere formation efficiency (SFE, cell sphere numbers with the diameter more than 75 um / cultured

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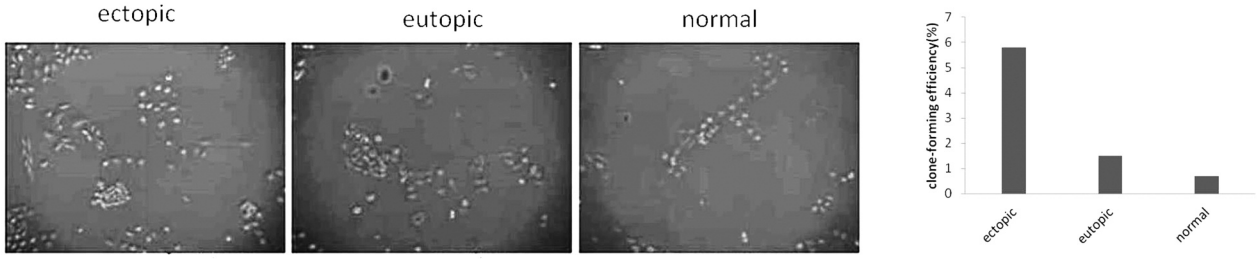

Figure 1. — The difference of clone-forming efficiency in ectopic, eutopic, and normal endometrium.

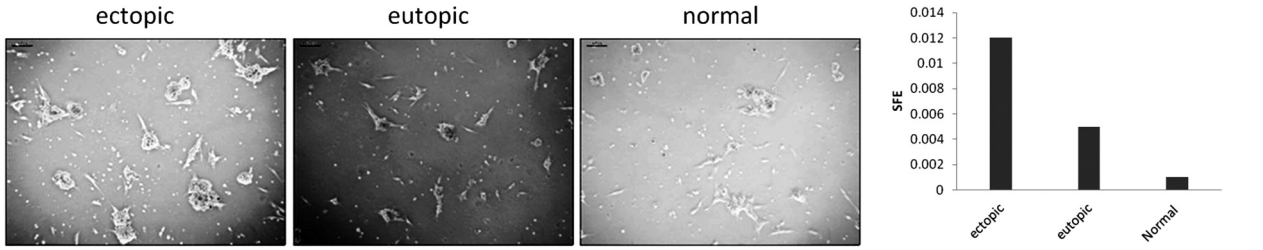

Figure 2. — The difference of SFE in ectopic, eutopic, and normal endometrium.

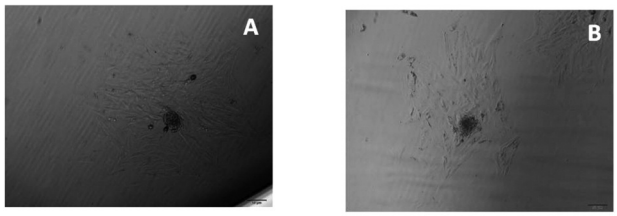

Figure 3. — The multipotency of ectopic endometrium with endothelial cell morphology (A) and fibroblast morphology (B).

cell numbers) were calculated.

The stem cell spheres were cultured in vascular endothelial induction medium and fibroblast induction medium to assess the multipotency and the medium was changed once every two days and the cultured time was about three weeks.

Transwell assay was used to detect cell invasive ability. Matrigel was diluted in serum-free RPMI 1,640 medium at a volume of 3:1.The Boyden chamber was placed in a 24-hole culture dish and formed two rooms up and down. One hundred μl of the prepared artificial basement membrane was added to the upper chamber of each Boyden chamber and incubated at 37°C for 2-6 hours to form a gel. When the cells were full, the cell concentration was adjusted and 300 μl cell suspension was added to the upper chamber, 600 μl of the chemotherapeutic agent-containing culture medium was added to the lower chamber, and placed in the cell incubator at 37°C, 5% CO_2 for 48 hours. The liquid in upper chamber was evacuated and wiped with a cotton swab carefully, rinsed twice with 37°C pre-warmed PBS solution, fixed for 30 minutes in 4% pre-cold paraformaldehyde. Then hematoxylin staining was carried out for five minutes. The polycarbonate film was carefully cut from the base of the upper chamber and the number of migrating cells were observed and counted under a microscope.

Data are expressed as mean ± standard deviation and the experiment was performed three times. Statistical analyses were performed with SPSS version 18.0. The analysis of variance and chi-square test were employed for comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Clones could form in each group after five days of cell culture. The cloning efficiency of ectopic, eutopic, and normal endometrium group was 5.8 ± 0.9%, 1.5 ± 0.3%, and 0.7 ± 0.1%, respectively. There were statistical significances among the three groups ($p < 0.05$). The cloning efficiency of eutopic endometrium group was slightly more than that of normal endometrium group and the cloning efficiency of ectopic endometrium group was significantly more than that of the other two groups.(Figure 1)

The SFE of ectopic, eutopic and normal endometrium group was 0.012 ± 0.004, 0.005 ± 0.001, and 0.001 ± 0.000, respectively. There were statistical significances among the three groups ($p < 0.05$). The SFE of eutopic endometrium group was slightly more than that of normal endometrium group and the SFE of ectopic endometrium group was significantly more than that of the other two groups (Figure 2).

The stem cell balls slowly adhered to the wall after the cell spheres isolated from ectopic endometrium using serum-free medium and were cultured in endothelial induc-

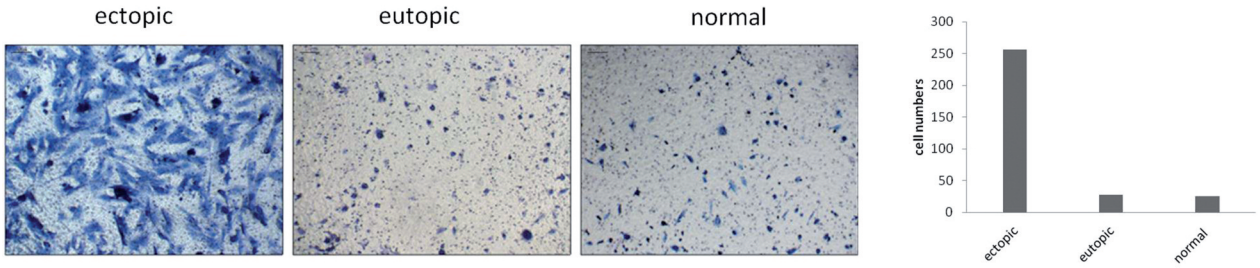

Figure 4. — The difference of invasive ability in ectopic, eutopic, and normal endometrium.

tion solution for three weeks. The adherent cells gradually extended from the cell balls and expanded to grow with endothelial cell morphology (Figure 3A).

Similarly, the stem cell balls slowly adhered to the wall after the cell spheres isolated from ectopic endometrium using serum-free medium were cultured in fibroblast induction solution for three weeks. The adherent cells gradually extended from the cell balls and expanded to grow with fibroblast cell morphology (Figure 3B).

Transwell results showed that the number of migrating cells was 256 ± 37 per high-power field, 26 ± 4 per high-power field and 28 ± 3 per high-power field in ectopic, eutopic and normal endometrium group, respectively. There are statistical significances among the three groups ($p < 0.05$). The invasive ability of the eutopic endometrium group was similar to that of the normal endometrium group and the invasive ability of the ectopic endometrium group was stronger and the ductility of the cells was better after the invasion (Figure 4).

Discussion

The pathogenesis of endometriosis remains unclear and there are different theories [5, 6] such as endometrium implant, coelomic epithelium metaplasia, immune or genetic factors, and so on, while none of them can explain the mechanism of endometriosis completely. In recent years more and more researchers have focused on the stem cell theory which hypothesizes that endometriosis is caused due to endometrial stem cells retrograde to the pelvic cavity to implant or the other stem cells that differentiate outside of endometrial cavity [7, 8]. Studies showed [9] appropriate microenvironment and concentration of 17β-estradiol can promote the differentiation of endometrial stem cells and bone marrow mesenchymal stem cells into the epithelial cells outside of the uterine cavity, which contributes to the genesis and development of endometriosis. While the characteristics of endometriotic stem cells are not clear and it should be considered to isolate endometriotic stem cells effectively for further study.

To date, the stem cells are commonly isolated by magnetic activated cell sorting and fluorescence activated cell sorting and the two methods take advantage of the surface markers to separate the stem cells. Studies indicate [10, 11] the endometriotic stem cells can express OCT4, SOX15, TWIST1, sal-like 4, CD133, and Musashi-1,and so on, but none was shown to be highly special for the endometriotic stem cells, which increases the difficulty of isolation.

The clone-forming method is usually used to isolate the endometriotic stem cells which has been proved to be mesenchymal stem cells (MSC) [11], while the procedures are complex with lower efficiency. In recent years, serum-free suspension culture has become a common method to isolate and enrich mesenchymal stem cells [12, 13] because the stem cells can be well tolerable to serum-free medium and can proliferate to form cell sphere in serum-free medium. The method is first used to isolate adult stem cells and has been always reported to be used to isolate tumor stem cells nowadays with the characteristics of self-renewal, multipotency, high tumorigenicity, and tolerance to radiotherapy and chemotherapy [14, 15]. Because endometriotic stem cells have been proved to be mesenchymal stem cells and endometriosis has similar biological behavior with malignant tumors, it is attempted to use serum-free suspension culture to isolate endometriotic MSCs which were examined by colony forming ability, multipotency differentiation, and invasion ability to determine whether serum-free medium can be effectively applied to isolate endometriotic MSCs. The present study showed that the cell spheres suspended from serum-free medium had stem cell characteristics with stronger ability of clone formation and invasion. As the literature has shown that endometriotic stem cells have osteogenic, chondrogenic, adipogenic capacity [11], on this basis, the present authors foucused on the differentiation potential of other directions and proved that they had the multipotency differentiation ability of endothelial cell and fibroblast. Due to the fact that survival and proliferation of endometriotic tissue depends on angiogenesis, which can promote MSCs to work as they should and the ectopic lesions fibrosis is an important feature of endometriosis, this suggests that the potency of endothelial cells and fibroblasts may be involved in the pathological process of endometriosis.

While some mechanism in stem cell theory of endometriosis are not fully understood, for example, migration conditions and microenvironment required for ectopic

differentiation of endometriotic stem cells, whether endometriosis lesions differentiated from bone marrow stem cells and endometrial stem cells have different tendency to form in different parts. The effective isolation of endometriosis stem cells will lay the foundation for further study in the fields of endometriosis stem cell theory. This finding also gives us a hope for future cell therapy studies with fewer concern about zoonotic infections from serum-containing medium.

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