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Identification of a High-yield Technique for Isolating Endometrial Epithelial Cells from the Mouse Uterus : A Comparison of Mechanical and Sedimentation-adherence Methods

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ABSTRACT

An *in vitro* assay following culture of endometrial epithelial cells is essential for understanding epithelial cell function in reproduction. Several diverse techniques have been developed for isolating endometrial epithelial cells, although an optimal technique has not been identified. In this study, we describe a sedimentation-adherence (S-A) isolation technique with a high-yield cell-separating ability to isolate endometrial epithelial cells from 8-week-old female C57BL/6 mice. We analyzed total cell number, viability, morphology, and expression of cytokeratin 18 as an endometrial epithelial cell-specific marker in cells isolated using a mechanical method compared to the S-A technique. There were no significant differences in the total number, viability, or morphology of the putative endometrial epithelial cells with either method. In contrast, significantly more endometrial epithelial cells harvested using the S-A method were positively stained for cytokeratin 18 than those isolated using the mechanical method. These results confirm that the S-A method is more efficient for retrieving endometrial epithelial cells than a mechanical method.

(Key words : mouse, sedimentation-adherence method, isolation, endometrial epithelial cells, uterus)

INTRODUCTION

The endometrium of the uterus consists of epithelial and stromal cells that undergo dynamic periodic changes in tissue differentiation, regeneration, and degeneration throughout the reproductive cycle (Deachapunva and O'Grady, 1998; Johnson *et al.*, 1999; Arnold *et al.*, 2001; Krikun *et al.*, 2009). These hormone-dependent changes subsequently influence the receptivity of the endometrium to embryos, which is accompanied by histological and functional remodeling (Hewitt *et al.*, 1979; Morris and Potter, 1984; Lindenberg *et al.*, 1988, 1989; Deachapunva and O'Grady, 1998; Srisuparp *et al.*, 2003; Hantak *et al.*, 2014), as well as abnormalities in endometrial thickness and endometriosis, which can cause pregnancy failure (Bongso *et al.*, 1988; Cha *et al.*, 2012; Kasius *et al.*, 2014). Thus, the endometrium is a key regulator of the microenvironment favorable for embryo implantation, and endometrial epithelial cell culture is the basis for analyzing endometrium function. There have been numerous attempts to investigate endometrium function during implantation (Tominaga, 1996; Thie *et al.*, 1998; Norwitz *et al.*, 2001; Spencer *et al.*, 2004; Cakmak and Taylor, 2011), the menstrual or estrus cycle (Bazer *et al.*, 1986; Bell and Dore-Green, 1987; Boron and Boulpaep, 2005), and the endometrium-embryo interaction (Genbacev *et al.*, 2003; Wang *et al.*, 2004; Wollenhaupt *et al.*, 2007; Margarit *et al.*, 2010). However, the reliability of experimental data has been hampered by the difficulty of successful isolation of endometrial cells.

Several techniques have been developed for the isolation of epithelial cells from the endometrium. These include mechanical procedures that scrape or squeeze endometrial tissue (Bigsby *et al.*, 1986; Lindenberg *et al.*, 1988; Cheng *et al.*, 2009; Eritja *et al.*, 2013; Janzen *et al.*, 2013), and a sedimentation-adherence (S-A) procedure that exploits differences in the weight and attachment capacity of endometrial epithelial and stromal cells

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(Riehl *et al.*, 1983; Fujiwara *et al.*, 2003; Azadbakht and Valojerdi, 2008). In addition, to improve the purity of endometrial cells, magnetic- (Chan *et al.*, 2004; Gargett *et al.*, 2004; Masuda *et al.*, 2012; Messier *et al.*, 2012) and fluorescence-activated (Chen *et al.*, 2013; Janzen *et al.*, 2013) cell sorting have been used after mechanical or enzymatic dissociation. However, to date, no studies have compared the purification yield among these isolation techniques, which have made it difficult to actively conduct endometrial epithelial cell research.

In this study, we investigated whether mechanical or S-A methods were more effective for the isolation of epithelial cells from endometrium by comparing the total yield, viability, morphology, phenotype, and cell-specific marker protein expression of epithelial cells.

MATERIALS AND METHODS

1. Animals

Uterine epithelial cells were obtained from 8-week-old female C57BL/6 mice (Japan SLC, Inc., Hamamatsu, Japan) and total sixty mice were equally allocated to each experiment group, according to experimental design (Fig. 1). All animal housing, handling, and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (IACUC approval no. SNU-130225-1401) and conducted according to the Animal Care and Use Guidelines of Seoul National University.

2. Mechanical Isolation of Endometrial Epithelial Cells from the Uterus

Endometrial epithelial cells were isolated from the endometrium using a previously described mechanical method with some modifications (Bigsby *et al.*, 1986; Lindenberg *et al.*, 1988). Briefly, uterine horns were rinsed with Hank's balanced salt solution (HBSS; Gibco Invitrogen) supplemented with 2% (v/v) penicillin-streptomycin (Gibco Invitrogen) and 1.25 μg/ml Fungizone (Gibco Invitrogen) were dissected into 3～4 mm pieces and incubated in 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) at 4℃ for 1 h. After shaking for 30s, the epithelial tissue was separated from the endometrial tissue by mechanical scraping under a stereomicroscope (CK40M-32; Olympus, Tokyo, Japan). Subsequently, the separated endometrial epithelial tissues were dissociated by incubating in HBSS supplemented

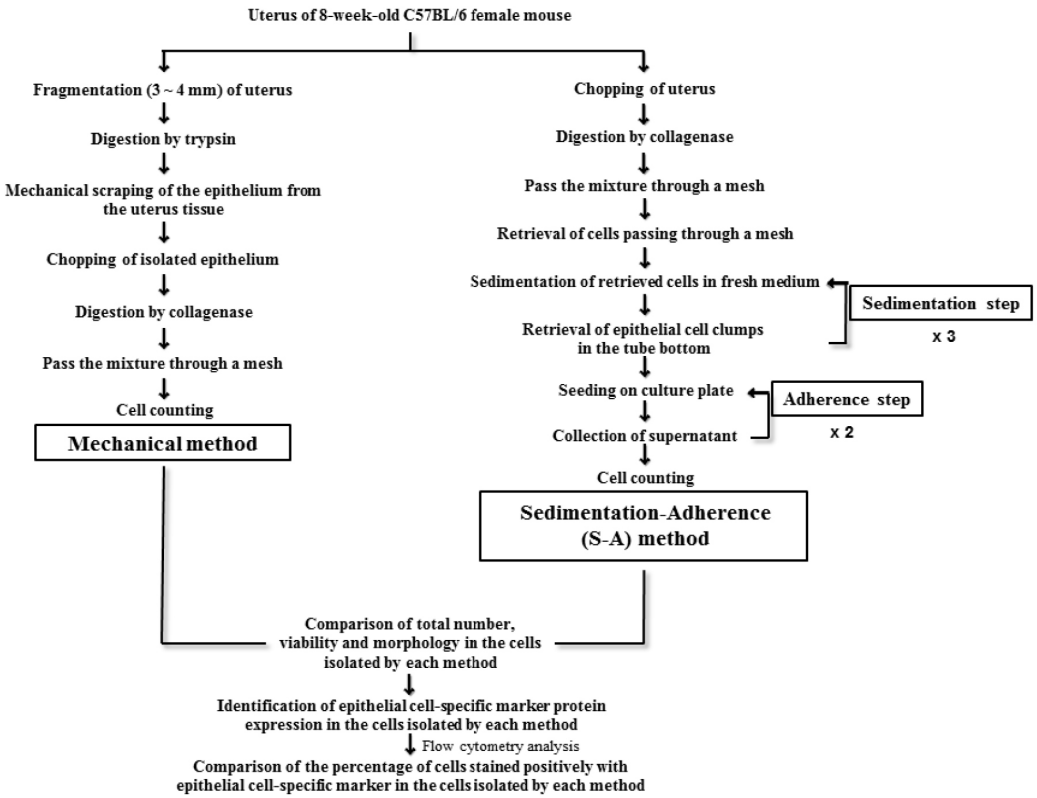

Fig. 1. Experimental design.

with 1.5 mg/ ml type I-S collagenase (Sigma-Aldrich) at 37℃ for 45 min and the dispersed cells were filtered through a 100 μm nylon mesh (Corning, Tewksbury, MA). Isolated endometrial epithelial cells were counted using a hemocytometer.

3. S-A Method for the Isolation of Endometrial Epithelial Cells from the Uterus

The isolation of endometrial epithelial cells from the endometrium using a S-A method was conducted as previously described (Azadbakht and Valojerdi, 2008) with some modifications. Briefly, uterine horns were rinsed with HBSS containing 2% (v/v) penicillin-streptomycin and 1.25 μg/ml Fungizone, and split longitudinally to expose the luminal epithelium. The tissue was fragmented into fine pieces using surgical scissors and digested using 1.5 mg/ml type I-S collagenase in HBSS at 37℃ for 45 min, and the digested cells were filtered through 100 μm nylon mesh. A subsequent sedimentation step collected cell clumps in the tube bottom after separating the filtrated cells under unit gravity by incubating in a 15 ml tube at room temperature for 15 min. This procedure was repeated three times to remove stromal cells from cell clumps retrieved by sedimentation followed by an adherence step that retrieved the suspended cells after incubating cells clumps in a 100 mm culture plate at 37℃ for 10 min that was repeated twice. Purified endometrial epithelial cells were counted using a hemocytometer.

4. Endometrial Cell Phenotype

The isolated endometrial epithelial cells were cultured in standard culture medium consisting of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12; Gibco Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene, Daegu, Korea), 2% (v/v) penicillin-streptomycin, and 1.25 μg/ml Fungizone in a humidified atmosphere with 5% CO_2 at 37℃. The phenotype of the endometrial epithelial cells was observed at day 1 of culture under an inverted microscope (CKX41; Olympus) equipped with an EOS 600D digital camera (Canon, Tokyo, Japan).

5. Analysis of Cell Viability

Cell viability was assessed by incubating cells in 1 μg/ml propidium iodide (PI) staining solution (Sigma-Aldrich) dissolved in Dulbecco's phosphate-buffered saline (DPBS; Welgene) at 4℃ for 1 min in the dark, and PI fluorescence was determined using flow cytometry with the FL-2 channel of an FACS Calibur (Becton, Dickinson and Co., Franklin Lakes, NJ).

6. Immunocytochemistry

Cells fixed in 4% (v/v) formaldehyde (Sigma-Aldrich) for 20 min at room temperature were permeabilized in 0.1% (v/v) Triton X-100 (Sigma-Aldrich) solution for 10 min. After blocking with DPBS supplemented with 2% (v/v) heat-inactivated FBS for 30 min at 4℃, the cells were incubated with unconjugated mouse anti-Cytokeratin 18 IgG primary antibody (1:100; Santa Cruz Biotechnology, Dallas, TX) diluted in DPBS overnight at 4℃. Subsequently, primary antibody against cytokeratin 18 was detected using Texas Red-conjugated goat anti- mouse IgG secondary antibody (dilution rate = 1:100; Santa Cruz Biotechnology) for 1 h at 4℃. The stained cells were washed twice with DPBS, counterstained for 15 min with mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA), and observed under a fluorescence microscope (TE2000-U; Nikon, Tokyo, Japan) using NIS-Elements BR™ software (Nikon Instruments, New York, NY).

7. Flow Cytometry

Cells fixed with 0.01% (v/v) formaldehyde for 10 min at room temperature were washed with ice-cold HBSS and permeabilized by incubating in HBSS containing 2% (v/v) heat-inactivated FBS and 0.1% (v/v) Triton X-100 for 15 min. Then the cells were stained for 30 min at 4℃ with unconjugated mouse anti-Cytokeratin 18 IgG primary antibody (1:100) and primary antibodies were detected using FITC-conjugated goat anti-mouse IgG secondary antibody (1:100) for 30 min at 4℃. The stained cells were rinsed with DPBS and then sorted using a FACS Calibur (Becton, Dickinson and Co., Franklin Lakes, NJ). Analysis of data was performed using BD CellQuest Pro software (Becton, Dickinson and Co.).

8. Statistical Analysis

Statistical Analysis System software was used to analyze all numerical data. Comparisons among treatment groups were conducted using the Duncan's method or the least-squares difference test, and the significance of main effects was evaluated by one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered a statistically significant difference.

RESULTS

To elucidate a technique with high retrieval efficiency and low cytotoxicity, we compared mechanical and S-A methods by assessing total cell number and viability of putative endometrial epithelial cells. No significant differences in the total cell number (Fig. 2) and cell viability (Fig. 3) were found with these isolation techniques. However, cells isolated using the S-A method showed a 10% increase in viability compared to those isolated with the mechanical method (66.15±2.86 vs. 55.48±12.34, respectively).

We characterized the putative endometrial epithelial cells isolated with each method by assessing morphology and the expression of endometrial epithelial cell-specific marker protein. Both isolation methods resulted in cells with typical epithelial cell-like morphology, described a polygonal in shape with more regular dimensions and growing attached to a substrate in discrete patches (Fig. 4) expressing cytoplasmic cytokeratin 18 (Fig. 5). The percentage of cells expressing cytokeratin 18 was significantly increased in cells isolated with the S-A method compared to the mechanical method (53.67±5.83% vs. 5.39± 2.69%, respectively). Moreover, the purity of cells harvested by the S-A method was significantly higher than those harvested by the mechanical method (Fig. 6). Thus, these results demonstrate that the S-A method can effectively isolate endometrial epithelial cells.

DISCUSSION

We investigated the most effective method for isolating endometrial epithelial cells from the mouse uterus from among those previously described (Bigsby *et al.*, 1986; Lindenberg *et al.*, 1988; Azadbakht and Valojerdi, 2008). Analyses revealed that 53.67% of the putative endometrial epithelial cells isolated using the S-A method expressed cytokeratin 18 as an endometrial epithelial cell-specific marker protein. Furthermore, the S-A method did not result in any significant reduction in total number or viability of the retrieved cells compared to the other methods. Thus, the S-A method produced the highest yield of purified cells.

As shown in Fig. 3 and 4, final viability and morphology of endometrial epithelial cells retrieved by each isolation method didn't differ significantly and greatly, indicating that enzymatic and mechanical steps during entire isolation process don't induce alteration of their characteristics. Accordingly, we can speculate that endometrial epithelial cells have strong resistant and

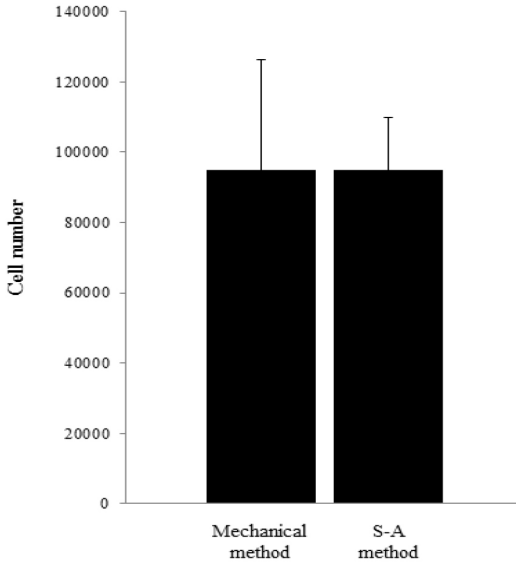

Fig. 2. Comparison of total number of putative endometrial epithelial cells isolated from the mouse uterus with different isolation techniques. Putative cell populations were retrieved by mechanical or S-A dissociation methods. Total cell number was determined using a hemocytometer. No significant difference in the total number of cells was detected. All data represent means±SE (standard error) of three independent experiments.

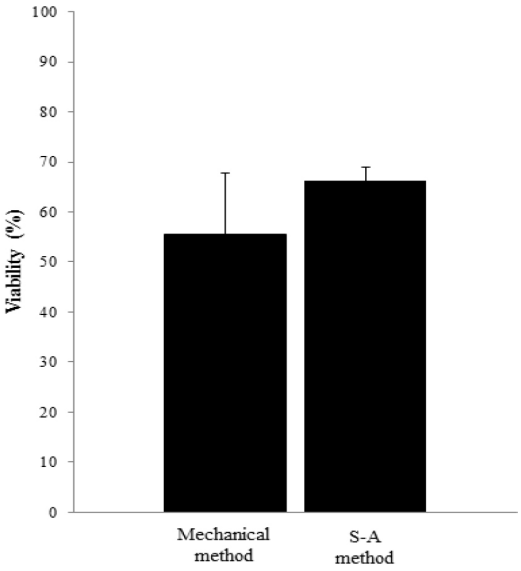

Fig. 3. Comparison of putative endometrial epithelial cell viability using different isolation techniques. Putative cell populations were retrieved by mechanical or S-A dissociation methods. The viability of isolated cells was analyzed by flow cytometry using propidium iodide. No significant difference in cell viability was observed. All data represent means±SE (standard error) of three independent experiments.

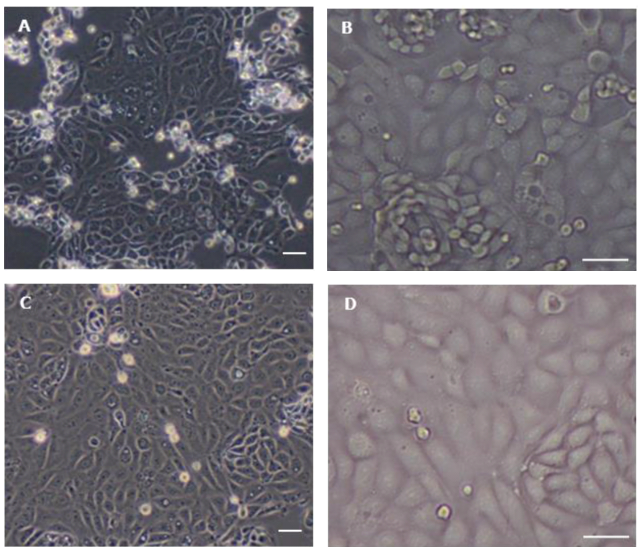

Fig. 4. Morphology of putative endometrial epithelial cells isolated with mechanical (A, B) and S-A methods (C, D). Retrieved cell populations were cultured for 1 day in standard epithelial cell culture medium. Cell morphology was observed by microscopy. Most of the isolated cells populations were polygonal in shape with regular dimensions and had grown attached to a substrate in discrete patches, n=3. Scale bars =50 μm.

recovery capability against external stimulation, supported by the facts that they experience periodically dynamic alterations in structure and function during estrus cycle and preparation of blastocyst implantation *in vivo* (Valdez *et al.*, 2015; Jeong et al, 2016).

During mechanical dissociation, a massive loss of endometrial epithelial tissue or acquisition of stromal tissue adhering to endometrial epithelial tissue can occur when removing stromal fractions from the uterus after enzymatic digestion. This can reduce the number of putative endometrial epithelial cells derived from small pieces of endometrial epithelial tissue. However, no significant decrease in the total number of the retrieved cells was observed, suggesting that there was a massive acquisition of stromal tissue adhering to endometrial epithelial tissue. This explains the lower proportion of purified endometrial epithelial cells in the cell suspension isolated using a mechanical method.

In contrast, we expected that there would be minimal loss of endometrial epithelial tissues using the S-A method due to enzymatic digestion of the entire uterus. Moreover, the increased density of cell clusters and slow adhesion of cells to the bottom of the culture dish (Azadbakht and Valojerdi, 2008)

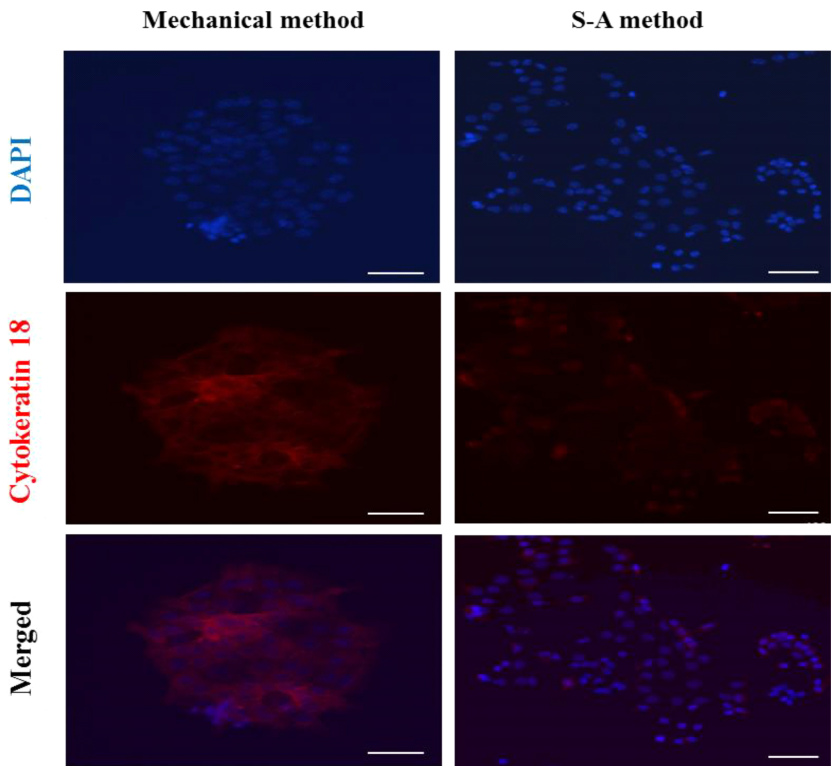

Fig. 5. Translational expression of cytokeratin 18 as an endometrial epithelial cell-specific marker protein (red) in putative endometrial epithelial cell populations isolated with different isolation techniques, n=3. Scale bars=50 μm.

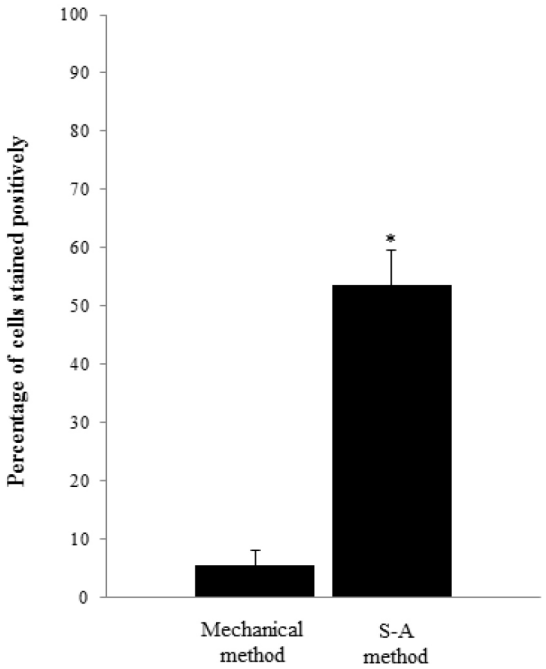

Fig. 6. The percentage of cytokeratin 18-expressing cells in the putative endometrial epithelial cell populations isolated using the mechanical or S-A method as analyzed by flow cytometry. The yield of cytokeratin 18-positive cells in cells retrieved through the S-A method was significantly higher than that retrieved through the mechanical method. All data represent means±SE (standard error) of three independent experiments. * $p<0.05$.

may play a significant role in improving stromal cell removal from dissociated uterine cells, resulting in an increase in the proportion of real endometrial epithelial cells compared to putative cells.

In conclusion, the S-A method was an efficient and convenient technique for the isolation of endometrial epithelial cells from the mouse uterus. It can contribute significantly to future endometrial epithelial cell researches related with implantation of blastocysts.

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