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Purified Culture Systems for Bovine Oviductal Stromal Cells

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Abstract. Isolated stromal cells from the ampullary and isthmic parts of bovine oviductal tissues were cultured in monolayer and spheroid (cell aggregate) systems. Prostaglandin F2α (PGF) plays a crucial role in oviductal contraction and is produced by oviductal epithelial cells in cattle. Since stromal cells of many organs produce PGF, PGF production by bovine oviductal stromal cells was investigated. After PGF synthesis was confirmed, the utility of isolation and culture methods for oviductal stromal cells was evaluated by PGF production in the present study. The homogeneity of the cells was > 99%. PGF production of the cells was increased by tumor necrosis factor-α. The stromal cells aggregated and formed a spheroid by the treatments with several reagents. PGF production was higher in the spheroid culture than in the monolayer culture. The isolation and culture methods described here will facilitate studies of the physiological function of bovine oviductal stromal cells.
Key words: Cell culture, Cell-to-cell interaction, Cow, Oviductal stromal cell, Spheroid

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Stromal cells play important roles including support of the function of epithelial cells [1] and production of hormones and cytokines [2–4] in the reproductive organs. However, the physiological function of oviductal stromal cells is not well understood in cattle, partly because cell isolation and culture systems are not available. A monolayer culture system is the most basic system for cell culture and has been used in many reports. In addition, in recent years, three-dimensional cell culture systems have been established for many kinds of cells [5–8]. Spheroid culture, the culture of cell aggregates, is one of these systems and is used for investigating cell-to-cell interactions [5, 7, 9]. The oviductal stromal layer is composed of stromal cells, blood vessels and other cells in the extracellular matrix (ECM) and has a multilayer constitution. Therefore, a three-dimensional cell culture system as well as monolayer culture is necessary for studying the function of oviductal stromal cells. Prostaglandin F2α (PGF) is known to affect oviductal contraction that is important for gamete and embryo transport in cattle [10]. A previous report demonstrated that bovine oviductal epithelial cells secreted PGF [10]. In addition, since PGF is produced by the stromal cells of several organs including the endometrium [1], corpus luteum [11] and kidney [12], it seems that bovine oviductal stromal cells produce PGF.

The objective of the present study was to establish systems for culturing cells in a monolayer and spheroid to investigate the physiological function of bovine oviductal stromal cells *in vitro*. First, PGF production in monolayer-cultured stromal cells was confirmed. After that, the utilities of the monolayer and spheroid culture methods for bovine oviductal stromal cells were evaluated by PGF production.

The homogeneities of stromal and epithelial cells, based on immunofluorescent staining, were higher than 99% in monolayer culture (Fig. 1). PGF production was detected in monolayer-cultured cells of the ampulla and the isthmus (Fig. 2). Tumor necrosis factor-α (TNF) significantly increased PGF production by the cultured stromal cells of the ampulla and the isthmus ($P<0.05$; Fig. 2A, B) and by ampullary epithelial cells ($P<0.05$; Fig. 2C), although a significant effect was not observed in isthmic epithelial cells ($P>0.05$; Fig. 2D) in monolayer culture. Figure 3 shows the stereoscopic observation (A, D) and hematoxylin and eosin-stained sections of spheroid formation. A folded sheet composed of stromal cells and pale pink-stained ECM was observed just after cell-sheet detachment from the plate (Fig. 3A, B, C). The spheroids (stromal cell aggregation) formed within 3 days (Fig. 3D), and the intercellular space was filled with abundant ECM (Fig. 3E, F). The cell viability was higher than 95% in 5-day spheroids. PGF production by 5-day spheroids was higher than that by monolayer cells ($P<0.05$; Fig. 4).

The present study demonstrated the development of monolayer and spheroid culture systems for bovine oviductal stromal cells. The immunocytochemical study showed that the oviductal stromal cells were purified by the current methods. The cultured stromal cells in the present study produced PGF and responded to TNF. TNF is known to stimulate PG production in oviductal epithelial cells [13, 14]. These results indicate that the isolation and culture methods performed in a monolayer are valuable for investigating the physiological function of bovine oviductal stromal cells.

The stromal cells formed a spheroid, a rounded cellular aggregation, in the present spheroid culture method. The stromal cells in spheroid culture produced more PGF than did the cells in monolayer culture. This is probably because the greater cell-to-cell contact in spheroid cells restores the cell-to-cell interactions that normally occur *in vivo* [5, 7, 9]. In addition, ascorbic acid, which stimulates collagen secretion by the fibroblasts in stromal cells [15], was used for the preparation of spheroids in the present study. The oviductal stromal cells *in vivo* show a multilayer constitution and are surrounded by abundant ECM composed mainly from collagen. The ECM affects

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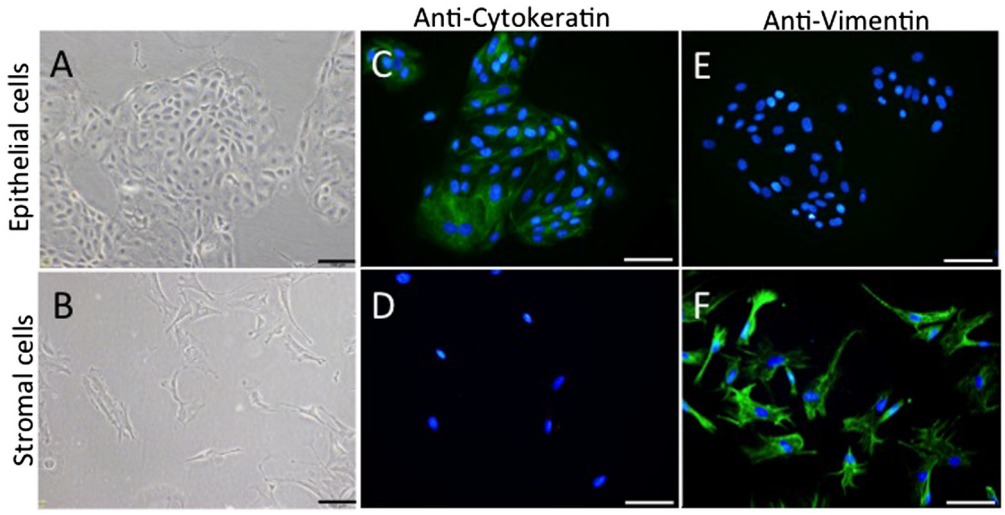

Fig. 1. Micrographs of oviductal epithelial (A) and stromal cells (B) in monolayer culture and immunostaining by anti-cytokeratin and anti-vimentin antibodies in ampullary oviductal epithelial cells (C, E) and stromal cells (D, F). Anti-mouse-IgG conjugate-donkey (green) was used for staining cytokeratin and vimentin as the secondary antibody. DAPI (blue) was used to visualize nuclei. Staining in the isthmus was virtually the same as that in the ampulla. Each scale bar indicates 100 μm.

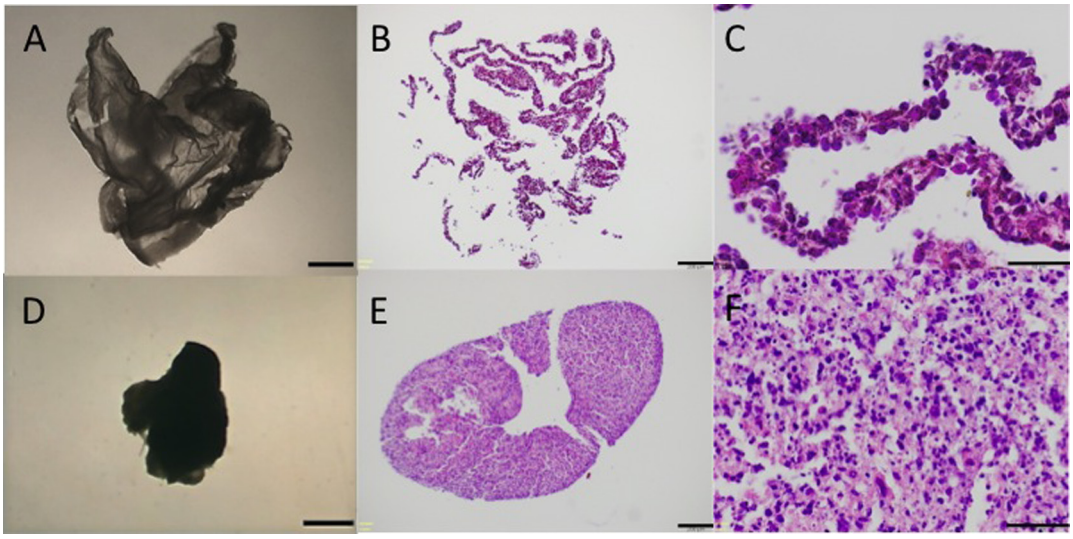

Fig. 3. Stereoscopic observation (A, D) and sections of spheroid from the bovine oviductal ampullary part stained with hematoxylin and eosin (B, C, E, F). Spheroid just after detachment (A, B, C) and after culture for 3 days (D, E, F). Scale bars indicate 1 mm in A and D, 200 μm in B and E, and 50 μm in C and F. The magnification in C and F is 3.6 times greater than that in B and E, respectively.

cellular proliferation, function and cell-to-cell interaction [16]. Our spheroid culture system seems to mimic *in vivo* cellular conditions and should be valuable for studying the function of oviductal stromal cells with the cell-to-cell interaction.

From these results, it was shown that bovine oviductal stromal cells could produce PGF and that PGF production was stimulated by TNF, although its physiological function was not clear. Therefore, further studies are required to clarify the function of oviductal stromal cells in cattle.

In summary, we developed methods of isolating and culturing bovine oviductal stromal cells in a monolayer and spheroid. These methods should facilitate studies of the specific function and cell-to-cell interactions of bovine oviductal stromal cells *in vitro*.

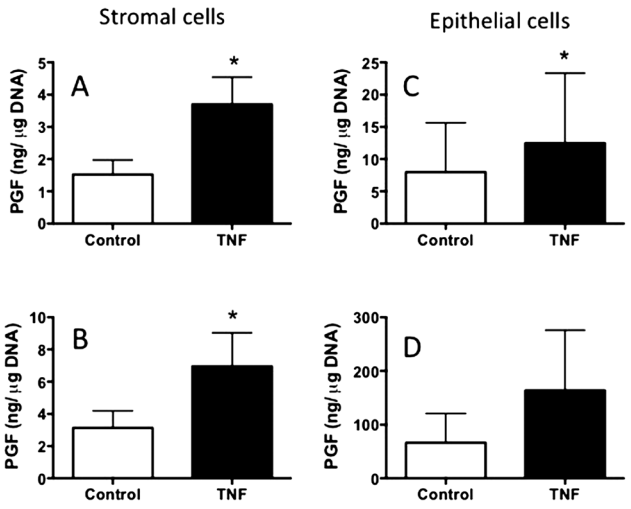

Fig. 2. Effects of tumor necrosis factor alpha on prostaglandin F2α production in the bovine oviductal stromal (A, B) and epithelial (C, D) cells from ampullary (A, C) and isthmic (B, D) parts (mean ± SEM, n=3–10 oviducts). An asterisk (*) indicates a significant difference (P<0.05).

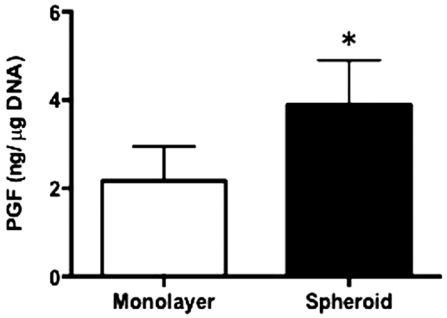

Fig. 4. Prostaglandin F2α production in monolayer and 5-day spheroid culture of oviductal stromal cells (mean ± SEM, n=7 oviducts). An asterisk (*) indicates a significant difference (P<0.05).

Methods

Collection of bovine oviductal tissues

Oviducts from Holstein cows were collected at a local abattoir within 10–20 min after exsanguination. The stages of the estrous cycle were determined based on macroscopic observations of the ovary and the uterus [2, 17]. Oviductal tissues ipsilateral to the corpus luteum were collected at days 0-3 after ovulation and utilized for cell culture.

Isolation of oviductal cells

Epithelial cells: Epithelial cells were isolated separately from the ampullary and the isthmic sections of the oviduct by perfusion with 20 ml Hank's balanced salt solution (HBSS) containing 0.25% (wt/vol) bovine trypsin (>7500 BAEE units/mg solid; Sigma-Aldrich, St. Louis, MO, USA), 0.02% (wt/vol) EDTA2Na (Sigma-Aldrich), 0.1% (wt/vol) BSA (Roche, Manheim, Germany), 100 IU/ml penicillin (Meiji Seika Pharma, Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika Pharma) at a flow rate of 10 ml/min and a temperature of 38 C for 30 min as described previously [10].

Stromal cells: After the epithelial cells were dissociated, the oviductal tissues were perfused again with 40 ml HBSS containing 0.125% (wt/vol) bovine trypsin, 0.01% (wt/vol) EDTA2Na, 0.1% (wt/vol) BSA, 100 IU/ml penicillin (Meiji Seika Pharma) and 100 μg/ml streptomycin at a flow rate of 20 ml/min and a temperature of 38 C for 30 min. After the second perfusion, the oviducts were cut open, and the outer and the inner surfaces of the tissue were wiped to remove remaining epithelial cells. The tissues were minced into pieces smaller than 1 mm^3, and the pieces were digested by stirring for 30 min in 10 ml of HBSS containing 66 units/ml collagenase type I (Worthington Biochemical, Lakewood, NJ, USA), 80 units/ml deoxyribonuclease I (BBI Enzymes, Cardiff, UK), 0.1% (wt/vol) BSA, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Monolayer culture methods of oviductal cells

The dissociated epithelial and stromal cells were filtered through metal meshes (150 μm and 77 μm) to remove undissociated tissue fragments. The filtrates were washed by centrifugation (180 × *g* for 10 min at 4 C) with Tris-buffered ammonium chloride (pH 7.5) to remove hemocytes and then washed with Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 0.1% (wt/vol) BSA, 100 IU/ml penicillin and 100 μg/ml streptomycin. After the washing, the final pellets were resuspended by DF (DMEM/Ham's F-12; 1:1 (vol/vol) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (vol/vol) bovine calf serum (Invitrogen), 20 mg/ml gentamicin (Invitrogen) and 2 mg/ml amphotericin B (Sigma-Aldrich)). Cell viability was higher than 95% as assessed by 0.5% (wt/vol) trypan blue dye exclusion. The cells were seeded at a density of 1.0×10^5 viable cells/ml into 25-cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany) and cultured at 38.5 C in a humidified atmosphere of 5% CO_2 in air.

A property of stromal cells is that they are the only cells that attach to a plate within 2 h. The medium and unattached cells in the culture flasks for the stromal cell were removed 2 h after seeding, and new medium was added. The medium in both of the stromal and epithelial cell culture was changed every 48 h for 5–10 days until the cells reached 80–90% confluence.

Purification of the oviductal cells

Epithelial cells: After reaching 80–90% confluence, the epithelial cells were trypsinized using 0.02% porcine trypsin and 0.02% bovine trypsin for purification as described previously [10]. The cells were placed in fresh DF to adjust them to a density of 1.0×10^5 viable cells/ml after trypsinizing. These cells were seeded on 24-well plates (Greiner Bio-One) for monolayer culture and incubated at 38.5 C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 48 h until the cells reached confluence.

Stromal cells: The stromal cells were purified by exploiting their greater sensitivity to trypsin as compared with the epithelial cells. When the stromal cells reached 80–90% confluence, the cells in the culture flask were washed with 0.1 M phosphate buffer saline (PBS)

(–) twice. After washing, 0.02% (wt/vol) porcine trypsin (1000–2000 BAEE units/mg solid; Sigma-Aldrich) with 0.008% (wt/vol) EDTA2Na in PBS was added to the flask, and the cells were incubated for 5 min at 38.5 C to detach the stromal cells. After that, the solution containing the stromal cells was washed by centrifugation (180 × *g* for 10 min at 4 C) with culture medium. The cells were placed in fresh DF to adjust them to a density of 1.0×10^5 viable cells/ml. These cells were seeded on 24-well plates for monolayer culture and on 6-well plates (Greiner Bio-One) for spheroid culture and incubated at 38.5 C in a humidified atmosphere of 5% CO_2 in air. The medium was changed 2 h after seeding to remove nonattached cells, and the culture medium was changed every 48 h until the cells reached confluence. Stromal cells seeded on 6-well plates for spheroid preparation were cultured in a medium containing 250 μM ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan) to stimulate collagen production.

Evaluation of cell homogeneity

The homogeneity of the epithelial cells and stromal cells was evaluated by immunofluorescent staining using anti-cytokeratin IgG produced in the mouse (Sigma-Aldrich) and anti-vimentin IgG produced in the mouse (Sigma-Aldrich) as the primary antibodies, anti-mouse IgG (FITC) conjugate-donkey (Sigma-Aldrich) as the secondary antibody, and ProLong Gold Antifade Reagent with DAPI (Invitrogen) as described previously with our modification [18, 19]. The cells were observed with a fluorescence microscope (Olympus, Tokyo, Japan).

Evaluation of PGF production by the epithelial and stromal cells in monolayer culture

When the cells reached confluence, monolayer-cultured epithelial and stromal cells were treated with 10 ng/ml TNF (kindly donated by Dainippon Sumitomo Pharmaceutical, Osaka, Japan) in fresh DF supplemented with 0.1% BSA, 500 μM ascorbic acid, 5 μg/ml holotransferrin (Sigma-Aldrich), 5 μg/ml sodium selenite (Sigma-Aldrich) and 2 μg/ml insulin (Sigma-Aldrich). Since TNF is known to enhance PGF production by oviductal epithelial cells [13], it was utilized to evaluate reactivity of the stromal cells. After 24 h incubation, the media were collected for determination of PGF.

Spheroid culture method for oviductal stromal cells

The spheroids were prepared using ascorbic acid as described previously [5]. After reaching confluence, the cells seeded on 6-well plates were washed with PBS (–) twice and treated with 5 mM EDTA2Na to detach the cells from the culture plates. The cells detached in the form of a sheet of cells. The sheets of cells floating in the media were washed with DMEM twice and transferred by using a 1,000 μl micropipette to 1.5% agarose-coated dishes followed by addition of DF containing 250 μM ascorbic acid. Culture media were changed every 48 h. Spheroids formed on days 3–5 after cell detachment from the plates. The culture media for spheroids were replaced with fresh DF on day 5 after cell detachment and incubated for 24 h. Cell viability in spheroids was evaluated using a TdT-mediated dUTP-biotin nick end labeling assay kit (MEBSTAIN, Medical & Biological Laboratories, Aichi, Japan). Monolayer cells seeded on 6-well plates were also incubated with the DF for 24 h as a control after the cells reached confluence. After incubation, the culture media were collected for measurement of PGF.

Determination of PGF production

The concentrations of PGF in the culture media were determined by enzyme immunoassay as described previously [20]. The media were diluted to 1:10 or 1:100, or not diluted, which depended on the sample. The range of sensitivity of the assay was 15.6 to 2,000 pg/ml PGF. The intra- and inter-assay coefficients of variation were on average 2.9% and 4.9%, respectively. Cellular DNA content was measured spectrophotometrically [21] to standardize the results. All experimental data are shown as the mean ± SEM. The statistical significance of differences was assessed by paired *t*-tests.

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