Isolation and Monolayer Culture of Bovine Oviduct Epithelial Cells

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ABSTRACT. Oviduct epithelia obtained from 32 cows were cultured. The oviducts were classified into follicular and luteal phases and divided into ampulla and isthmus regions. The epithelial cells were dissociated by enzyme digestion and cultured in plastic dishes with Dulbecco's modified Eagle's medium/Ham's F12 (1:1) containing 10% calf serum. After enzyme treatment, the epithelial suspension showed free ciliated and non-ciliated cells, and cell mass. The non-ciliated cells contained secretory granules in the cytoplasm. The cell mass was composed of ciliated and secretory cells. The cell mass adhered to the dish within 12–24 hr, while the free ciliated cells attached on Day 2 of the culture. The cells grew into confluent monolayers on Day 4. The cell monolayers contained ciliated and non-ciliated cells. The monolayered non-ciliated cells showed a few secretory granules. When the cells were further cultured without subculturing, ciliary activity diminished on Day 5 and was rarely detected on Day 9. When the cells were subcultured on Day 3, ciliary movement was detected on the monolayers for only 2 days. Cell mass that did not adhere to the dish and remained floating in the medium formed ball-like structures on Day 2. Active ciliary beating was observed on the cells that were cultured in the medium supplemented with 10^{-5} and 10^{-9} M estradiol- 17β , however, the ciliary activity diminished on Day 5. No difference in the cell growth was observed between the follicular and luteal phases or between the ampulla and isthmus regions.—**KEY WORDS:** cow, monolayer culture, oviduct epithelium.

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Tissue culture of the epithelium of the female genital tract, i.e., vagina [4], uterus [3, 4, 13] and oviduct [16, 17], has been conducted in many laboratories. The objective of these studies was to investigate the hormone responsiveness of the epithelial cells *in vitro*. Recently, oviduct epithelium was used as a co-culture system with early stage embryos [5, 7, 12]. However, study of the monolayer culture of the oviduct epithelial cells is still limited. This may be due to the small size of the oviduct, the complexity of the luminal surface, which is composed of many mucosal folds, and the heterogenous population of the epithelial cells. Joshi [8, 9] reported that oviduct epithelial cells contained ciliated cells and non-ciliated secretory cells *in vitro*, and that the secretory cells maintained their secretory activity, while monolayered ciliated cells lost their cilia during culture. In the present study, the

monolayer culture of oviduct epithelial cells is described to confirm the characteristics of cell growth.

MATERIALS AND METHODS

Isolation and culture of epithelial cells: Reproductive tracts of 32 cows were obtained from a slaughterhouse. Thirteen oviducts in the follicular phase and 19 in the luteal phase of the reproductive cycle were used in this study. These oviducts were ipsilateral to the ovary with either a follicle of about 1 cm in diameter or a corpus luteum of over 2 cm in diameter. Two to 5 oviducts were pooled and treated in one experiment.

The oviducts were trimmed to remove the fat and connective tissue and then separated into ampulla and isthmus regions. The fimbriae were removed. The ampulla and

isthmus were flushed with Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate buffered saline (PBS) and cut open longitudinally on the margin of the mesosalpinx with fine dissecting scissors. The epithelial cells were then removed from the oviduct by enzyme digestion [15] as follows: the ampulla and isthmus were placed in a solution of PBS containing 0.5% trypsin (1:250, Difco Laboratories) and 2.5% pancreatin (Kanto Chemical Co., Inc.) for 2 hr at 4°C followed by 30 min at room temperature. They were then transferred to PBS. The oviduct epithelium was freed from the stroma of the mucosal folds in relatively large pieces with the careful use of dissecting forceps under a stereomicroscope. The removed epithelium was placed in PBS containing 0.05% trypsin and 0.02% EDTA-disodium salt and incubated for 5 min at 37°C. After epithelial cell dissociation, the cells were suspended in culture medium, i.e., Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd.)/Ham's F12 (Nissui), 1:1 (vol/vol; DMEM:F12) containing 10% heat-treated calf serum (Gibco Laboratories), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.5 $\mu\text{g}/\text{ml}$ fungizone. The epithelium was then sedimented and washed by centrifugation at $100 \times g$ for 5 min. The cells were incubated in a plastic dish to remove the stromal cells, which quickly attached to the plastic dish. After 2 hr at 37°C, the cells floating in the medium were collected. The cell viability was examined by the 0.5% trypan blue exclusion test. The cells were diluted with the culture medium to approximately 2×10^6 trypan blue-negative cells/ml. Then 1 ml of this suspension was seeded in a 35-mm plastic dish and cultured at 37°C under an atmosphere of 5% CO_2 in air. The day of seeding was designated as Day 0. The medium was changed every 2 days. For subculture, the cells were detached from the dish by 0.05% trypsin and 0.02% EDTA treatment for 5 min at 37°C. The cells were

then washed, diluted 1:5 in the medium and reseeded.

To estimate the effect of estradiol-17 β on primary culture, DMEM:F12+10% calf serum and serum-free medium (ASF medium 301, Ajinomoto Co., Inc.) supplemented with 10^{-5} or 10^{-9} M estradiol-17 β (Sigma Chemical Co.) were used as the growth medium. The calf serum that was added to the medium contained 10^{-10} M estradiol-17 β .

Histological preparations: The oviducts were fixed in 10% formalin solution and embedded in paraffin. Sections were stained with hematoxylin and eosin.

For histological examination, the cells were cultured in chamber slides (Lab-Tek, Nunc), fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide. For transmission electron microscopy, the specimens were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12A electron microscope. Semi-thin sections (0.5 μm) were stained with 0.5% toluidine blue (pH 7.5) and examined with a light microscope. For scanning electron microscopy, the specimens were critical-point dried, sputter-coated with gold and observed using a JEOL JSM-T200 electron microscope.

RESULTS

Isolation of epithelial cells: Histological sections of the oviducts during cell separation showed that the epithelium was removed from the mucosal folds. The connective tissue of the folds and the epithelium at the base of the folds, however, remained intact (Fig. 1A, B).

After trypsin-EDTA treatment, the epithelial suspension contained a large number of free cells and cell mass. The free cells were classified into three types; namely, spherical cells with ciliary activity (Fig. 2A),

spherical cells without cilia and high columnar cells with immotile cilia (Fig. 2B). The viabilities of these isolated cells were 93.6, 94.6 and 26.7%, respectively. The spherical cells with a ciliary beat spun actively in the medium. The columnar cells showed the nucleus in the cell base and were often vacuolated. The cell mass were composed of approximately 10–200 spherical cells and had cilia on the surface (Fig. 2C). The cell mass showed a tendency to rotate in the medium.

In the electron microscopic study, free ciliated cells showed polarity in the presence of cilia and basal bodies (Fig. 3A). Free non-ciliated cells showed secretory granules in the cytoplasm (Fig. 3B). Secretory granules had different sizes and density, and contained material possessing lamellar structures. The ultrastructure of cilia and secretory granule was similar to that previously described in the bovine oviduct [2]. Both ciliated and non-ciliated cells were identified in the cell mass (Fig. 4A). Ciliated cells showed cilia at the free surface. Non-ciliated secretory cells were recognized by the presence of dome-like projections of cytoplasm and secretory granules at the apical portion of the cytoplasm (Fig. 4B).

Culture of epithelial cells: After seeding of the isolated free cells and cell mass in the

plastic dish, the latter adhered to the dish within 12–24 hr, while the former floated in the medium. The free ciliated cells attached to the dish on Day 2 of the culture. The cells proliferated well and formed a large number of monolayered cell colonies composed of cohesive, polygonal cells. The epithelial cell monolayers showed 70% confluency on Day 3 and occupied the entire area of the dish on Day 4. The cell monolayers contained ciliated and non-ciliated epithelial cells without contamination of stromal cells. In the ciliated cells, rapid back-and-forth movement of cilia was observed, while the ciliary

Fig. 1. Histology of segments of the ampulla from a cow in luteal phase. $\times 70$. A) Before cell separation, the epithelium is intact. B) After cell separation, the epithelium has been removed and the mucosal folds remained.

Fig. 2. Epithelial cells immediately after isolation. $\times 200$. A) A spherical cell with cilia (arrow) and ciliated cells actively spinning in the medium (arrowheads). B) A high columnar cell with immotile cilia (arrow). C) Cell mass showing cilia on the surface (arrowheads).

Fig. 3. Transmission electron micrograph of free ciliated (A) and non-ciliated (B) cells showing cilia (c), basal bodies (b) and secretory granules (s). A, $\times 5,400$; B, $\times 6,000$.

Fig. 4. Scanning (A) and transmission (B) electron micrograph of the cell mass showing the cilia (c) and secretory granules (s). A, $\times 1,700$; B, $\times 48,000$.

Fig. 5. Scanning (A) and transmission (B) electron micrograph of monolayered ciliated cells. A) A bundle of cilia (c) on the cell surface. $\times 1,600$. B) Remnants of cilia (arrowheads) in the cytoplasm. $\times 16,000$.

movement was not coordinated in a specific direction.

In the electron microscopic study, numerous microvilli were present at the upper surface of the monolayered cell. The nuclear membrane was frequently infolded. Membrane interdigitations and junctional complexes were observed between cells in contact. Abundant rough endoplasmic reticulum, polysomes and mitochondria were distributed throughout the cytoplasm. Bundles of cilia were observed on the ciliated cells (Fig. 5A). However, the number of cilia varied from cell to cell. Remnants of

cilia and basal bodies were detected in the cytoplasm of both ciliated and non-ciliated cells (Fig. 5B). The secretory cells were flattened, and the dome-like projections of the cytoplasm were not detected. The secretory cells showed a few secretory granules in the cytoplasm. Granules with a homogeneous matrix as well as those possessing lamellar structures within the matrix were observed. The secretory granules emptied their lamellar contents by exocytosis into the medium.

After the cells reached confluency, cell

vacuolation was found. The confluent-cell monolayers elevated partly from the dish and formed dome-like structures (Fig. 6A). The vertical sections of the dome-like structures revealed that the dome was composed of monolayered ciliated and non-ciliated cells and that liquid accumulated in the lumen (Fig. 6B). Some ciliated cells extended their cilia into the lumen of the dome (Fig. 6C). When the cells were further cultured without subculturing, the number of cells with ciliary activity decreased on Day 5 of the culture. Cell vacuolation was often detected, and ciliary beating was not found in the vacuolated cells. The dome-like structures increased in number and size. Several hundred domes were observed in a plastic dish on Day 8. On Day 9 of the culture, ciliary movement was rarely detected with the microscope.

Secondary cell culture was carried out before (Day 3) and after (Day 5) the cells reached confluency. The cells that were detached from the dish by trypsin-EDTA treatment and suspended in the medium revealed poor ciliary activity. The cells grew as monolayers on the next day. However, ciliary movement was not detected on the monolayers when subculture was conducted on Day 5. When the cells were subcultured

on Day 3, active ciliary beating was observed on the monolayers for 2 days. After the cells grew as confluent monolayers, the dome-like structures were detected. The cells were subcultured for at least 2 successive times.

The cells that did not adhere to the dish and remained floating in the medium were the free cells and the cell mass. Both types of cells were composed of ciliated and non-ciliated cells. The cell mass began to form cavities enclosed with the cells and grew into ball-like structures on Day 2 of the culture (Fig. 7A). Some ball-like structures expanded to over 700 μm in diameter. The histological section showed that the ball was composed of monolayered epithelial cells (Fig. 7B). The free ciliated cells showed poor ciliary activity on Day 6 and lost it on Day 9, while the cell mass still showed ciliary activity.

No difference in the appearance of cell growth was observed between the follicular and luteal phases or between the ampulla and isthmus regions.

When the cells were cultured in DMEM:F12+10% calf serum supplemented with 10^{-5} and 10^{-9} M estradiol- 17β , the cells proliferated well and active ciliary beating was observed on the mono-

Fig. 6. Dome-like structures on Day 4 of the culture. A) Differential-interference-contrast micrograph of monolayered epithelial cells. Arrow indicates the dome. $\times 150$. B) Vertical section of the dome. The cells are elevated from the dish. $\times 500$. C) Transmission electron micrograph of the dome showing microvilli (m) and cilia (c) on the upper surface of the cell and cilia extending to the lumen (arrow). $\times 4,600$.

Fig. 7. Ball-like structures on Day 5. A) Many balls floating in the medium. $\times 20$. B) Oblique section of the ball showing monolayers of epithelial cells. $\times 170$.

layers. However, the ciliary activity diminished on Day 5 of the culture and was rarely detected on Day 9. In serum-free medium, the cells adhered to the dish on Day 4, and the monolayered cells showed poor ciliary movement.

DISCUSSION

The present study describes a method for isolation of the bovine oviduct epithelial cells. The oviduct epithelium has been obtained by various methods, namely; scraping the luminal surface with the scalpel [12, 16, 17], flushing the oviduct with the medium [7] and enzyme digestion with 0.1% collagenase [9]. However, identification of the isolated cells by electron microscopy was very limited [9]. In this study, the light and electron microscopic observation revealed that the isolated cells obtained by trypsin-pancreatin and trypsin-EDTA treatment were composed of ciliated and non-ciliated epithelial cells without contamination by stromal cells. This result shows that the present method is effective for isolation of oviduct epithelial cells.

Enzyme digestion was conducted on the uterus to remove the epithelial cells from the connective tissue [3, 13, 15]. Histologic-

al examination of the uteri after trypsin-pancreatin treatment revealed that some small undissociated regions of endometrium still remained [13]. However, no histological observation on the oviducts during cell dissociation was reported in the previous studies. In the present study, oviduct epithelium was dissociated by trypsin-pancreatin treatment, while the epithelium remained intact at the base of the mucosal folds. This result suggests that epithelial-cell harvest is related to the feature of the luminal surface since the oviduct has many mucosal folds. When the oviduct is roughly treated, the epithelium is removed with the connective tissue of the mucosal folds, and this could result in the contamination of the epithelial cell culture by stromal cells.

Ciliary movement was observed on the monolayered cells. In organ cultures of rabbit and pig oviducts, the cilia in the ampulla beat in a downward direction [6]. However, the present study revealed that the cilia did not beat in one direction. The ciliated cells might lose coordination of ciliary movement by cell dissociation. The cell monolayers reached confluency on Day 4 of the culture, and the number of the ciliated cells decreased on Day 5. Neither the cell subculture nor a supplement of estradiol-17 β in the medium showed a substantial effect on maintaining the ciliary activity. Electron microscopy of the monolayered cells revealed remnants of cilia in the cytoplasm of both ciliated and non-ciliated cells. Joshi [9] reported that the monolayered ciliated cells lost their cilia after 4–5 days of culture and that the cilia seemed to be replaced by microvilli. These results suggest that Day 4–5 of the culture is a critical period for the ciliary movement. Furthermore, the ciliary activity markedly decreased after trypsin-EDTA treatment which suggests that the ciliary movement may be sensitive to the enzyme digestion. The ciliary movement almost disappeared in

the monolayered cells and floating individual cells on Day 9, but was maintained in the floating cell mass. It was previously reported that the ciliated cells in floating colonies maintained ciliary movement for 9–10 days [9]. In the cell mass, the cells were not as flattened as in the monolayers and could contact with many cells. The ciliary activity might be affected by some cell-to-cell interactions.

The characteristics of the secretory cells also changed during the culture. The monolayered secretory cells lost their dome-like projections of cytoplasm and had a few secretory granules. Two types of secretory granules, namely, those with the homogeneous matrix and those possessing lamellar structures within the matrix, were observed in the cells. Joshi [9] reported a few secretory granules with lamellar inclusions by electron microscopy of the monolayered secretory cells and suggested the elaboration of specific secretory products by immunocytochemical tracing of the secretory proteins in cultured cells. In the present study, it was observed that the monolayered secretory cells released the lamellar contents by the exocytosis. However, in order to estimate the secretory activity of the cultured cells, biochemical studies should be conducted on cell supernatants. The amounts of lipid and glycogen and the activities of enzymes in the monolayered epithelial cells of bovine oviducts were changed by supplementation with estradiol-17 β to the culture medium [16, 17]. The secretory activity might be affected by the presence of estradiol-17 β in the medium.

The dome-like structures of the monolayered cells and the ball-like structures of the floating cells contained a liquid in the lumen. The dome was also reported in the monolayer culture of mammary epithelial cells [10]. Liquid accumulation in the dome is thought to be caused by the intracellular liquid transport of secretory cells. The

presence of the dome- and ball-like structures might indicate that the secretory activity was maintained in cultured epithelial cells.

The oviduct and uterine epithelia have been used in co-culture with embryos to enhance embryo development *in vitro* [1, 5, 7, 10, 12]. In the endometrial cell co-culture, the embryos induce the secretion of specific proteins by the epithelial cells [14], and the embryos take up proteins synthesized by the endometrial cells [11]. However, the mechanism of the co-culture is still not clear. In oviduct cell co-cultures, the epithelial cells have been cultured for 1 to 30 days prior to egg culture [5, 7, 12]. The results of the present study revealed that the characteristics of the cells change during culture. Therefore, the duration of the cell culture should be considered when oviduct cells are to be utilized in a co-culture system.

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要 約

ウシ卵管上皮細胞の分離と単層培養：菱沼 貢・高橋芳幸・金川弘司（北海道大学獣医学部家畜臨床繁殖学講座）——ウシ32頭から採取した卵管上皮細胞の培養を実施した。卵管は卵胞期と黄体期および膨大部と峡部に分けて、酵素処理によりそれぞれの部位から上皮細胞を分離した。培養液は、Dulbeccoの修正Eagle培地とHam F12（1：1）混合液に10%子ウシ血清を加えて使用した。酵素処理後の細胞は、単独の細胞と細胞塊から構成された。単独の細胞は線毛を有するものと有しないものに分類され、線毛を有しない細胞には分泌顆粒が認められた。細胞塊には、線毛細胞と分泌細胞が認められた。培養12～24時間後には細胞塊がプラスチックシャーレに付着し、2日目には単独の線毛細胞にも付着がみられた。4日目には上皮細胞がシャーレ一面に単層を形成した。単層を形成した細胞には線毛細胞と分泌細胞が認められたが、分泌顆粒の数は減少していた。そのまま培養を継続した場合には、5日目から線毛の運動性が低下し、9日目にはほとんど認められなくなった。培養3日目に継代を行った場合でもその後2日間しか線毛運動はみられなかった。シャーレに付着せず培養液中に浮遊している細胞塊は、培養後2日目から球状を示した。10⁻⁵あるいは10⁻⁹Mのestradiol-17βを含む培養液で細胞を培養した場合でも、線毛運動の消長に変化はみられなかった。培養細胞の発育については、卵胞期と黄体期および膨大部と峡部との間に差は認められなかった。