Establishment of Human Tubal Epithelial Cells for Coculture in an IVF Program

D. J. WALKER,^{1,4} M. T. VLAD,² and C. R. KENNEDY³

Submitted: April 3, 1996

Accepted: September 25, 1996

Objective: Our goal was to develop a coculture system from human tubal epithelial cells for use in a human IVF program.

Design: Fallopian tubes were obtained from women undergoing hysterectomy for benign disease. Ampullary epithelial cells were cultured, passaged, and frozen and the karyotype and epithelial characteristics of later passages examined.

Setting: The study took place at the Assisted Conception Clinic, Walsgrave Hospital, and the Department of Biological Sciences, University of Warwick.

Results: Ampullary epithelial cells can be successfully cultured and maintained as diploid epithelial lines until the fifth passage. Freezing does not affect subsequent culture, the cytoskeleton, or the karyotype. In excess of 12×10^6 cells may be obtained from a pair of Fallopian tubes.

Conclusions: The ease of establishing and maintaining human tubal epithelial cell cultures justifies their use in clinical IVF for human embryo cocultures, and further investigations into their mode of action should be encouraged.

KEY WORDS: coculture; human; fallopian tubes; in vitro fertilization.

INTRODUCTION

The fallopian tube is the site for gamete transport, fertilization, and early embryo development. This environment presumably represents the optimal conditions for embryonic development before implantation.

Assisted reproductive techniques have contributed significantly to solving many of the problems of human subfertility. However, pregnancy and implantation rates have not been as high as those reported in farm animals, which are often greater than 60% per cycle

(1). The *in vitro* culture of human embryos up to the blastocyst stage is suboptimal under existing laboratory conditions, with only 17–25% of spare embryos cleaving normally to the blastocyst stage (2,3). Therefore, human embryos are replaced into the uterus quite early, at the four- to six-cell stage, before degenerative changes begin to occur *in vitro*.

Attempts to improve culture conditions have focused mainly on the formulation and selection of appropriate media and the supplementation of a variety of additives. Mimicking the tubal milieu with a human tubal coculture system may enhance this further. Studies have reported improved morphology (4), implantation (5), and pregnancy rates (6) following replacement of cocultured embryos.

MATERIALS AND METHODS

Fallopian tubes were obtained from women undergoing a total abdominal hysterectomy for nonmalignant gynecological disease. This study had obtained ethical approval from the hospital's medical ethics committee and informed written consent from the patients participating in the study.

Tubes were removed aseptically and placed in Hank's balanced salt solution (HBSS; GIBCO) in sterile containers and transported to the Department of Biological Sciences on ice. The HBSS contained streptomycin (50 mg/ml) and penicillin (100 U/ml) and was buffered with 10 mM Hepes buffer.

Tubes were washed in fresh HBSS and the ampullary region was cut off and opened. Small (0.5-cm²) areas of mucosa were dissected off and resuspended in fresh HBSS. Each mucosal strip was gently scraped and the resulting cell suspension was centrifuged at 6000g for 5 min. The cell pellet was resuspended in a Falcon 25-cm² culture flask containing prewarmed Chang's medium. Culture medium was prepared weekly and was supplemented with penicillin (50 U/ml), strepto-

¹ Royal United Hospital, Combe Park, Bath BA1 3NG, U.K.

² Department of Biological Sciences, University of Warwick, Warwick, U.K.

³ Assisted Conception Unit, Walsgrave Hospital, Coventry, U.K.

⁴ To whom correspondence should be addressed.

mycin (50 mg/ml), 1% antimycotic/antifungal solution (GIBCO), glutamine (2 mM), and 10% fetal calf serum.

The flasks were incubated undisturbed for 5 days in an atmosphere of 5% CO₂ in air at 37°C, after which time the medium was changed and the flasks were observed daily, until large colonies of cells were formed. Subculture was performed by detaching the cells in 0.05% trypsin-EDTA solution (GIBCO) and transferring to new culture flasks containing RPMI 1640 culture medium (GIBCO). The number of cells in each culture flask was assessed after trypsinization using a Makler counter.

Cells were frozen by suspending approximately 10⁶ cells in 1 ml of freezing mixture containing 50% fetal calf serum, 40% RPMI 1640, and 10% dimethyl sulfoxide (DMSO). The cell suspension was cooled overnight at -70°C before immersion in liquid nitrogen for long-term storage. Cells were rapidly thawed in a water bath at 37°C and were directly suspended into culture flasks containing prewarmed RPMI 1640 medium. Medium was changed after 6 hr to remove traces of the cryoprotectant DMSO.

Chromosomal analysis was performed by adding 0.02 mg/ml colchicine (Sigma) for a maximum of 14 hr. At the same time the fetal calf serum concentration was increased to 20%. The arrested cells were selectively harvested and resuspended in a 0.05 M KCl solution for 8 min at room temperature, followed by centrifugation and fixation in methanol:acetic acid (3:1). Chromosome preparations were obtained by spreading the cell suspension onto a microscope slide and were stained with Giemsa and viewed under light microscopy.

Cultured cells from different passages were stained with antibodies to confirm the epithelial nature of the cells. Monolayers were established on slide flasks (Nunc), fixed in 100% methanol for 5 min, and air-dried. Slides were incubated with either monoclonal anticytokeratin antibody followed by anti-mouse IgG TRITC conjugate and viewed under a rhodamine filter or with monoclonal antivimentin followed by anti-mouse IgM FITC conjugate and viewed under a FITC filter.

RESULTS

Thirty pairs of Fallopian tubes were obtained, 13 of which produced a successful primary culture (43%). When evaluated according to the stage of the menstrual cycle, seven of nine (78%) tissues obtained in the follicular phase and two of six (33%) in the luteal phase

proved successful. Fallopian tubes were obtained from two patients taking the antigonadotropin hormone, Danazol. Cells from both samples failed to grow *in vitro* and were not passaged. The age of patients ranged from 34 to 47. No difference in successful cultures was noticed in tubes obtained from patients under or over the age of 40 (46 vs 40%).

After the first passage there was no difference in the time taken for the cells to reach confluence in either Chang's or RPMI 1640 medium. As RPMI 1640 is a fraction of the cost of Chang's medium, it was used for culturing cells after the first passage.

Epithelial cells established round colonies after 5 days in culture (Fig. 1). Nuclei were located centrally and cells were polygonal in shape. Active ciliated cells were observed until the first passage. Some ciliated cells did not attach to the base of the flask and instead floated above the base of the flask in sheets of cells. This was best avoided by gentle but thorough dissociation of cells with a pipette prior to their being added to the culture flask. After 10–12 days from the initial seeding, when monolayers covered at least 20% of the base of the flask, the cells were passaged for the first time. Cell monolayers in the first and second passages continued to show the characteristic epithelial cell growth pattern in large swirls and rounded colonies (Fig. 2). However, from the third passage a greater predominance of fibroblast cells was observed (Fig. 3), and by the fifth passage, cell senescence occurred in the "epithelioid" cells. Fibroblasts were evident by their spindle shape and long, drawn-out cytoplasmic processes. Confluent monolayers were formed within 5 days in the first three passages; beyond this, the rate of cell growth was markedly reduced, taking 8–10 days to reach confluence. The cells frozen after the

Fig. 1. Phase-contrast photograph of epithelial cells culture 5 days after initial seeding. Original magnification, $\times 200$.

Fig. 2. Phase-contrast photograph of cells 2 days after the first cell passage. Epithelial appearance is maintained. Original magnification, $\times 100$.

second passage took a similar time to reach confluence and cells retained their epithelial appearance.

Cell counts obtained after initial seeding ranged from 0.5 to $0.85 \times 10^6/25\text{-cm}^2$ flask. Cells from one flask were split into two flasks at the first passage and into three flasks for later passages. Each flask contained a mean of 0.7×10^6 cells (range, $0.5\text{--}1.2 \times 10^6$) at confluence. From one successful primary culture, a total of 18 flasks of confluent cells was obtained by the end of the third passage, taking approximately 3–4 weeks and containing a mean total of 12×10^6 cells. The number of cells obtained from one culture has relevance when discussing the role of cocultures in an IVF program.

Chromosomal studies were performed in fresh cultures at the second and fifth passage and on frozen cells at the fourth passage. Giemsa staining revealed 46 chromosomes for 15 cells analyzed in each sample. Banding was not sufficiently intense to identify minor

Fig. 3. Phase-contrast photograph of cells 5 days after the third passage, at flask confluence. Original magnification, $\times 100$.

chromosomal abnormalities but examination of the whole karyotype excluded the possibility of large chromosomal rearrangements.

Epithelial cells stained positively with anticytokeratin (Fig. 4) and negatively with antivimentin. In contrast, fibroblasts did not stain with anticytokeratin but exhibited notable antivimentin staining (Fig. 5). Similar results were obtained with frozen–thawed samples. These results confirmed the epithelial appearance of cells under phase-contrast light microscopy. There was an increasing number of vimentin-positive cells in later passages, correlating with the increased number of fibroblast cells.

DISCUSSION

The small number of published reports on human fallopian tube epithelium in culture reflects the difficulty in the isolation and long-term growth of fallopian tube epithelium cells. Although several studies (5,6) have highlighted the improved blastocyst formation and subsequent pregnancy rates from replacement of cocultured embryos, the widespread use of a human epithelial coculture system has never materialized due to the reported complexity of establishing a cell line. This study shows that ampullary cells can be cultured to form monolayers which maintain their epithelial morphology when passaged over 4–5 weeks.

The success of primary fallopian tube cell culture is in accordance with other workers (7,8). The method described to obtain the primary culture was found to be the most successful of several tried in preliminary studies and in accordance with other methods reported by previous authors (9,10). Bongso *et al.* (9) reported that the presence of ciliated cells at the time of setting up the primary cultures corresponded to the estrogenic phase of the patient's menstrual cycle. This was similar to the findings of Verhage *et al.* (11), who described an *in vivo* ciliogenesis cycle where ciliated cells were maximal in the presence of high estrogen levels and reduced in the presence of progesterone. No benefit could be determined from obtaining specimens from a particular phase of the menstrual cycle, nor did the age of the patient have an effect, although the numbers of samples obtained at the different stages of the menstrual cycle were small. The ideal supply of fallopian tubes would be from healthy women having bilateral salpingectomy for fertility control.

Immunocytochemical staining has confirmed the epithelial appearances of cell cultures when observed by phase-contrast microscopy. There are conflicting

Fig. 4. Immunofluorescent staining for cytokeratin showing positive staining of epithelial cells at the second passage. Original magnification, $\times 200$.

reports in the literature on the ability successfully to passage human tubal epithelial cells over several passages. Henriksen *et al.* (12) reported unsuccessful attempts, whereas Ouhibi *et al.* (7) were successful.

In this study, we were able to subculture tubal epithelial cells up to the fifth passage and successfully freeze and culture cells after the second passage. The chromosomal content of cells remains diploid until the fifth

Fig. 5. Immunofluorescent staining for vimentin showing positive staining of fibroblast cells. Original magnification, $\times 200$.

passage. It was not possible to analyze cells after this passage due to cell senescence. Further studies are being undertaken to analyze the protein secretion of cell cultures *in vitro*.

The total number of cells obtained from one successful seeding at the end of the third passage is 12×10^6 . One hundred thousand cells plated in cell wells are sufficient to culture at least 10 embryos to the blastocyst stage *in vitro*. The number of cells obtained from one successful culture would support the development of in excess of 1000 embryos to the blastocyst stage. The time and cost of establishing and maintaining cell lines will be more than offset by the improved pregnancy rates and the reduction of multiple pregnancies by selective transfer of two embryos.

This is the first study reporting the practical implications of establishing a human coculture system. It clearly shows the ease with which a cell culture may be established and the viability of cells after freezing. The argument that a human coculture system has no practical use in human IVF is not valid based on the results of this study and more research should be done to establish the efficacy of such a system in early embryonic development.

REFERENCES

1. Iritani A: Current status of biotechnological studies in mammalian reproduction. *Fertil Steril* 1988;50:543-551
2. Fehilly CB, Cohen J, Simons RF, Fishel SB, Edwards RG: Cryopreservation of cleaving embryos and expanded blastocysts in the human: A comparative study. *Fertil Steril* 1985;44:638-652
3. Bolton VM, Hawes SM, Taylor CT, Parsons JH: Development of spare human preimplantation embryos in vitro: An analysis of the correlations among gross morphology, cleavage rates and development to the blastocyst. *J Vitro Fert Embryo Transfer* 1989;6:30-35
4. Menezo Y, Guerin JF, Guerin JF, Czyba B: Improvement of early human embryo development in vitro by co-culture on monolayers of Vero cells. *Biol Reprod* 1990;42:301-306
5. Wiemer KE, Hoffman DI, Maxson WS, Eager S, Muhlberger B, Fiore I, Cuervo M: Embryonic morphology and rate of implantation of human embryos following co-culture on bovine oviductal epithelial cells. *Hum Reprod* 1993;8:97-101
6. Bongso A, Ng SC, Ratnam S, Chan C, Anandakumar C, Wong YC, Goh V, Devendra S, Soon SB, Fong CY, Vasuthevan S: Pregnancies after fertilisation and cleavage in human co-culture. *J Vitro Fert Embryo Transfer* 1991;8:116-117
7. Ouhibi N, Menezo Y, Benet G, Nicollet B: Culture of epithelial cells derived from the oviduct of different species. *Hum Reprod* 1989;4:229-235
8. Takeuchi K, Maruyama I, Yamamoto S, Oki T, Nagata Y: Isolation and monolayer culture of human Fallopian tube epithelial cells. *In Vitro Cell Dev Biol* 1991;27A:720-724
9. Bongso A, Chye NS, Sathananthan H, Lian NP, Rauff M, Ratnam SS: Establishment of human ampullary cell cultures. *Hum Reprod* 1989;4:486-494
10. Matthews CJ, Redfern CPF, Hirst BH, Thomas EJ: Characterisation of human purified epithelial and stromal cells from endometrium and endometriosis in tissue culture. *Fertil Steril* 1992;57:990-997
11. Verhage HG, Bareither ML, Jaffe RC, Akbar M: Cyclic changes in ciliation, secretion and cell height of the oviductal epithelium in women. *Am J Anat* 1979;156:505-508
12. Henriksen T, Tanbo T, Abyholm T, Oppedal BR, Claussen OP, Hovig T: Epithelial cells from human Fallopian tube in culture. *Hum Reprod* 1990;5:25-31