**Explantation and Subculture of Epithelial Cells from Human Uterine Ectocervix**

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**ABSTRACT**

Human ectocervical explant cultures were grown in medium with d-valine substituted for L-valine. Pure epithelial cell monolayers were obtained with both dialyzed and undialyzed fetal calf serum. Epithelial cell explant colonies grown in d-valine medium supplemented with undialyzed serum could routinely be subcultured if plated at a density of \(1.5 \times 10^4\) cells/sq cm or higher. Such cultures could be passaged at least three times and could yield up to 21 population doublings per culture lifetime. Contaminating fibroblastic colonies were never detected in these cultures, which were free of surface-associated fibronectin as revealed by immunofluorescent tests. Both primary and passaged epithelial colonies retained many characteristic morphological features of ectocervical epithelium when examined by light and electron microscopy. Such cultures may be of use in investigating the action of viral and chemical carcinogenic agents upon epithelial cells in vitro.

**INTRODUCTION**

Recently, interest in the use of cell culture for study of oncogenesis has shifted from fibroblastic cells toward epithelial cells, since about 80% of all human tumors are epithelial (carcinomas) rather than fibroblastic (sarcomas) in origin. Carcinoma of the cervix is one of the most common of cancers. The uterine cervix is also frequently infected with various microbial agents, some of which may have an association with cervical carcinoma. Thus, pure monolayers of cervical epithelial cells which could be passaged would be useful for studies of in vitro transformation by biological and chemical agents.

Although methods have been developed to obtain epithelial outgrowths from cervical tissues (17, 23, 25), these methods have not proved entirely satisfactory. The epithelial monolayers obtained are subject to contamination by fibroblasts (9, 17, 22). In addition, few data have emerged that provide a basis for successful, reproducible protocols for the subculture of cervical epithelial cells (17).

Gilbert and Migeon (7) recently reported the successful selection of fibroblast-free kidney epithelial cells using culture medium in which D-valine was substituted for L-valine. This method is based upon the ability, lacking in fibroblasts, of many types of epithelial cells (7, 8, 24) to convert D-amino acids into their L enantiomers. Here, we report that D-valine medium can be used for the production and subculture of epithelial cells from human ectocervical explants.

**MATERIALS AND METHODS**

Cervical tissue samples were obtained from patients of childbearing age who had hysterectomies for benign uterine tumors at the North Carolina Memorial Hospital, Chapel Hill, N. C., under a protocol approved by the institutional committee for protection of human rights. Only patients with normal Papanicolaou smears were accepted.

Tissue strips were excised from the ectocervix, and the stroma was separated from the epithelial layer as described previously (23). Absence of atypia was confirmed histologically. Each strip was washed in phosphate-buffered saline solution (NaCl, 8.0 g; KCl, 0.20 g; KH2PO4, 0.12 g; Na2HPO4, 0.91 g; plus double-distilled water to 1000 ml) and cut into fragments measuring about 3 x 3 mm. To initiate explant cultures, 4 to 6 fragments were seeded into 60-mm plastic Petri dishes (22, 23) (Corning Tissue Culture Ware No. 25010; Corning Glass Works, Corning, N. Y.), which in some cases were coated with poly-L-lysine, as indicated (12, 13). The samples were incubated in 3 ml of growth medium: MEM (GIBCO), Ham's F-10 (GIBCO), or D-valine medium (GIBCO). Media were supplemented with 10% FCS. A single batch of serum (GIBCO) was used throughout these studies. All serum was heat inactivated and in some cases was dialyzed twice against 100 volumes of 0.85% NaCl solution for 48 hr at 5° and sterilized by filtering through a 0.45 µm filter before use. The growth medium was supplemented with 100 µg of streptomycin, 100 IU of penicillin, 1.5 µg of glutamine, and 5 µg of hydrocortisone (5, 16) per ml. The cultures were maintained in a humidified atmosphere with 5% CO2 at 37°, and the medium was changed twice a week.

Subcultures were initiated from actively growing cervical cell monolayers after disaggregation with 0.125% trypsin:0.015% EDTA.

All cultures were observed frequently under an inverted microscope. Methanol-fixed cultures for light microscopy were Giemsa stained.

**Immunofluorescence.** Immunofluorescent staining of the cultures for fibronectin (18) was kindly performed by Dr. Jorma Keski-Oja, Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Md. Cells on coverslips were fixed with 3% paraformaldehyde and stained indirectly. Rabbit antiserum against human plasma fibronectin was absorbed with FCS and

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4 The abbreviations used are: D-valine medium, minimum essential medium with D-valine substituted for L-valine; MEM, minimum essential medium; GIBCO, Grand Island Biological Co., Grand Island, N. Y.; FCS, fetal calf serum.
diluted 1:40 before use. Commercial fluorescein isothiocyanate-conjugated sheep anti-rabbitγ-globulin (Hyland Laboratories, Costa Mesa, Calif.) was used to visualize the rabbit anti-human fibronectin (15).

Electron Microscopy. For transmission electron microscopy, the monolayers were washed thoroughly and fixed with 2\% paraformaldehyde in 0.1 M Sorenson’s buffer for 2 to 6 hr at 4°C. After being washed in Sorenson’s buffer containing 6.8\% sucrose, postfixation of the monolayers was accomplished with 1\% osmium tetroxide in Sorenson’s buffer, pH 7.2, for 60 min at 25°C. The monolayers were then harvested with rubber policeman and embedded in agar. The samples were dehydrated in graded ethanols and embedded in Luft’s Epon. Thin sections were cut on an LKB ultramicrotome and poststained with saturated uranyl acetate for 20 min, followed by lead citrate for 4 min. All specimens were examined with an AEI electron microscope, accelerated at 60 kV, with a 30 μm objective aperture.

For scanning electron microscopy, the monolayers were washed, fixed, and postfixed as above and then dehydrated in graded ethanols. The monolayers were then critical point dried using Freon 13 and Freon 113 as transitional fluids. The cells were mounted on specimen stubs with silver paste, gold coated with a Polaron sputter coater, and viewed in an ETEC Autoscan electron microscope at 20 kV.

RESULTS

Growth Properties of Explant Cultures. A total of 211 human ectocervical explant cultures were initiated from 38 cervical samples. Initially, regular MEM or F-10 medium supplemented with undialyzed FCS, antibiotics, and glutamine was used to initiate and support epithelial growth from explants. Occasional fibroblastic colonies were observed in primary ectocervical cultures maintained in MEM, but fibroblasts were not seen in cultures grown under F-10 medium. After EDTA:trypsin disaggregation, however, contaminating fibroblastic cells appeared in about 10\% of the epithelial colonies grown in both these media.

During the course of these experiments, we also began to use MEM containing d-valine substituted for L-valine. d-Valine medium supplemented with dialyzed FCS yielded uniformly pavement-like monolayers, characteristic of epithelial cell cultures (17, 23, 25). The cells were polygonal and mononuclear with prominent nucleoli (Fig. 1). These explant cultures grew to densities up to 2.5 × 10^5 cells/colony (Table 1). Degenerative changes were observed, however, after about 6 to 8 weeks of culture, and gradually the cultures were lost. Cervical cell cultures grown in d-valine medium supplemented with undialyzed serum also displayed uniform morphological features typical of pure cervical epithelium as described above. Compared with cultures grown in the presence of dialyzed serum, these cultures expanded more rapidly (Table 1) and could be maintained for longer times. Of these cultures, 160 were followed up to 8 weeks, and 27 additional cultures survived up to 16 weeks. In no cases were contaminating fibroblastic colonies seen.

Subculture of Cervical Epithelial Cells. Cervical monolayers obtained from explant cultures were very adherent to the bottom of the culture vessel and to each other. After several attempts, a solution containing 0.015\% EDTA and 0.125\% trypsin in calcium- and magnesium-free Hanks’ balanced salt solution (GIBCO) was found effective to disaggregate the monolayers without damaging the cells. First the monolayers were washed 3 times with 2 ml of Ca^{++}- and Mg^{++}-free Hanks’ balanced salt solution. Then they were overlaid with 2 ml EDTA-trypsin solution which was removed after 1 min, leaving a thin film covering the cells. The culture dishes were then incubated at 37°C but were observed frequently under an inverted microscope. When the monolayers had disaggregated into small clusters and individual cells, a small volume of partially conditioned medium was added to the dishes and gently pipetted up and down several times. After a sample of these dispersed cells was counted in a hemocytometer, the cells were adjusted to the desired concentration with additional medium and plated into new 60- or 35-mm dishes. Occasionally, the cells were gently centrifuged (300 × g for 5 min) and resuspended in medium before plating. However, there were no apparent advantages or disadvantages to this procedure. In preliminary experiments, conditioned medium obtained from actively growing cervical cultures seemed to have a favorable effect on the initial attachment of subcultured cells. Therefore, conditioned medium mixed with fresh growth medium in a 1:2 ratio was used in all subsequent experiments. Actively growing cultures containing 10^5 to 5 × 10^6 cells were most suitable for serial cultivation in terms of plating efficiency and growth properties. Primary explants usually required approximately 4 to 8 weeks to grow to this density.

Table 1

<table>
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<tr>
<th>Type of medium</th>
<th>No. of cells/colony at</th>
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<tbody>
<tr>
<td></td>
<td>14 days</td>
</tr>
<tr>
<td>MEM</td>
<td>5.0 × 10^4 ± 1.7 × 10^4</td>
</tr>
<tr>
<td>d-Valine medium and undialyzed serum</td>
<td>5.0 × 10^4 ± 1.7 × 10^4</td>
</tr>
<tr>
<td>d-Valine medium and dialyzed serum</td>
<td>4.0 × 10^5 ± 1.0 × 10^4</td>
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*Mean ± S.D.*
further experimental manipulation. Junctional complexes (Fig. 4b) were frequently observed in these cells. Occasionally, cytoplasmic lysosome-like lamellar bodies were observed (Fig. 4a) as well. The nuclei were round or oblong, but irregular lobulated nuclei were also seen (Fig. 4a).

By scanning electron microscopy, the typical mosaic-like arrangement of an epithelial monolayer was observed. The cells were flat and polygonal, and there were raised terminal bars between adjacent cells. Both the nuclei and prominent nucleoli could easily be distinguished (Fig. 5a). Solitary cells assumed a more or less elongated appearance with long filopodia (Fig. 5b). Micronidges characteristic of ectocervical epithelial cells were also frequently observed in primary and passaged cells (Fig. 5) [10].

### DISCUSSION

The cell selection methods used in this work are based on the D-amino acid oxidase positivity of epithelial cells, which allows them to convert D-valine to L-valine (1, 7, 11, 14, 24). While not all epithelial cell types can grow in D-valine medium (2, 6, 8), human cervical cell explants and subcultures grew well and formed pure epithelial cell monolayers in this medium. These cultures could be grown in medium containing dialyzed serum, but significantly better growth resulted with undialyzed serum. Although the undialyzed serum probably contained a small amount of L-valine (4), it apparently was not enough to allow fibroblastic contamination of cervical cell cultures. This may be based on the fact that fibroblastic overgrowth was not as much of a problem in cervical cell culture as it appears to be for renal (7, 8) or human skin cell (16) cultures. The ability to use undialyzed serum is important, since dialyzing of serum leads to undesirable loss of small molecules necessary for long-term proliferation and subculture (3, 8).

Subcultures of ectocervical epithelial cells could be regularly initiated from colonies growing in medium with undialyzed serum. An important stage in subculturing was to disaggregate the cells without overtrypsinizing them. This was accomplished with an EDTA-trypsin solution and careful monitoring of the disaggregation procedure. A second important condition for successful passage of epithelial explant cultures was plating the cells at a density of 1.5 x 10^4 cells/sq cm or higher on plastic vessels in partly conditioned medium. A very recent paper (20) has described the use of irradiated feeder cells to apparently enhance colony formation in cervical epithelial cell cultures. Perhaps epithelial cell densities of cervical cell inoculum reported here provide an important feeder effect necessary for colony initiation and subsequent cell growth.

Both the primary and subcultured cervical cells exhibited many characteristic morphological features of epithelial cells (17, 23, 25). The well-developed micronidge pattern on the surface of these cells indicated active, differentiated cell populations (10). The typical hallmarks of epithelial cells were observed in both primary and subcultured cells, showing that cervical cells do not lose their differentiated morphology when passaged. These cervical cell monolayers were also consistently free of surface-associated fibronectin, a major component of the extracellular matrix of fibroblasts grown in vitro (21). It is noteworthy that fibronectin, abundant in cervical fibroblasts, is absent also from another cervical epithelium type, the human endocervical epithelial cell (23).

In conclusion, we have reported here methods for the successful propagation of pure ectocervical epithelial cell mono-
layers in vitro. Such actively growing cultures may serve as an important source of cells for various experimental studies, including attachment, growth, and possible transformation of differentiated human epithelial cells in vitro.

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REFERENCES

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