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# Isolation, growth and characteristics of human ovarian surface epithelium

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**Abstract** The ovarian surface epithelium (OSE) is a key tissue in the pathogenesis of ovarian surface epithelial-stromal tumours and ovarian endometriosis, commonly encountered gynaecological diseases. Despite the high incidence of these diseases, experimental in vitro studies of OSE are few and so we used the scraping method with an enzymatic procedure to isolate human OSE and studied its characteristics in vitro. Nineteen normal ovaries were used. After incubation of the ovary for 40 min in collagenase type 1 solution (300 U/ml), the surface cells were removed by gentle scraping with a surgical blade. Cells obtained as a cluster after unit gravity sedimentation with 5% bovine serum albumin in medium 199 were cultured in medium 199 containing 15% fetal bovine serum. The viable cell number in a single ovary was $0.1–2.7 \times 10^6$. The outgrowth of cells started from a homogeneous population of single cells, and the cell population doubling time was between 7 and 10 days. Confluent monolayers were formed after 13–20 days and subcultured from one to three times. The monolayers mostly had a cobblestone appearance, and fusiform or polygonal cells were also observed. By cytochemistry, immunocytochemistry and scanning and transmission electron microscopy, the cells were shown to have characteristics of mesothelial OSE cells in short-term culture. This experimental approach was efficient in providing cultured human OSE, which can be utilized to investigate pathobiology and carcinogenesis.

**Key words** Human ovary · Ovarian surface epithelium · Tissue culture · Growth Characterization

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## Introduction

The incidence of malignant ovarian tumours appears to be increasing slowly and steadily in highly industrialized countries. Almost 90% of these tumours are considered to be derived from a single mesothelial layer of cells known as the ovarian surface epithelium (OSE; Scully 1977). However, the pathogenesis of endometriosis, a commonly encountered gynaecological disease, remains controversial. A metaplastic theory of origin of this disease, one of the two principal theories concerning its histogenesis, has not been widely supported. However, some investigators have recently proposed the importance of the "secondary müllerian system" (Lauchlan 1972) in the pathogenesis of endometriosis (Clement 1987; Fujii 1991; Nakamura et al. 1992), since the OSE and peritoneal mesothelium have a homologous embryology in common with müllerian-derived structures. Therefore, it is clinically and pathobiologically important to establish an experimental model for the study of human OSE.

During the past two decades, a few investigators have reported success in culturing normal OSE rabbit (Nicosia et al. 1984) and human (Auersperg et al. 1984; Kruk et al. 1990) cells. Also, human OSE have been studied morphologically and biochemically (Nicosia et al. 1985; Osterholzer et al. 1985; Siemens and Auersperg 1988). However, these culture systems have some problems; when applied to human OSE cells for example, the complexity of the procedure or difficulties in obtaining epithelial cells in sufficient quantities. In this study, we used Nicosia's method for the rabbit to isolate and purify human OSE, and the morphological characteristics of cells in vitro were defined by immunocytochemistry, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

## Materials and methods

Grossly normal ovaries were resected at laparotomy from 19 women undergoing total abdominal hysterectomy or radical hysterectomy for gynaecological disorders. The ovaries were all histologically normal. Ethical approval was sought and informed consent obtained in all cases. Their ages ranged from 36 to 55 years and included two menopausal women.

Under aseptic conditions, the specimen was immediately brought to the laboratory in calcium- and magnesium-free Hanks balanced salt solution (HBSS), then rinsed in HBSS containing 100 U/ml of penicillin and 100 μ g/ml of streptomycin (Gibco, Grand Island, N.Y.). The ovary was then incubated for 40 min in 20 ml of medium 199 (Gibco) containing *Clostridium histolyticum* collagenase type 1 (300 U/ml; Sigma, St. Louis, Mo.) at 37° C under a 5% carbon dioxide ($CO_2$): 95% air atmosphere with rotation at 100 revolutions per minute (rpm) by magnetic stirrer. Thereafter, the ovary was transferred into a 50 ml centrifuge tube (Corning, Corning, N.Y.) containing 10 ml of antibiotic-rich (penicillin, 100 U/ml; streptomycin, 100 μ g/ml) medium 199, and its surrounding medium was gently pipetted and vortexed at 30 rpm for 60 s. In a 60-mm plastic culture dish (Corning) with 10 ml of the same medium, the ovarian surface was scraped with a no.11 surgical blade (Futaba, Japan). The medium was collected and centrifuged at 1400 rpm for 5 min. Tissue resuspended in 3 ml of the medium was pipetted into medium 199 containing 5% bovine serum albumin (Sigma) and allowed to stand for 15 min at room temperature. The lower 5 ml was collected and cells were sedimented by centrifugation at 1400 rpm for 5 min. After incubation in 0.05% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA), cells were washed with HBSS and viability was assessed by trypan blue dye exclusion.

Cells were counted in a haemocytometer and seeded into four-well multidishes (Nunclon, Denmark) at $3.0 \times 10^4$ cells/1.9 $cm^2$/well containing a nutrient solution supplemented with antibiotics (penicillin, 100 U/ml; streptomycin, 100 μ g/ml), 85% medium 199 and 15% fetal bovine serum (FBS; Filtron, Brooklyn, Australia). Incubation proceeded at 37° C under a 5% $CO_2$: 95% air atmosphere and the medium was changed every other day. Cells were serially subcultured when they reached confluence. Throughout the culture, the cells were examined by phase-contrast microscopy using an IMT-2 inverted microscope (Olympus, Japan).

Ovaries after cell isolation were observed under light microscopy and EM and OSE cells were studied in vitro by immunocytochemistry and EM. For light microscopy, ovaries were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (HE). For immunocytochemistry, cultured cells were pelleted onto poly-*L*-lysine (Sigma) coated glass slides using a Cytospin 2 (Shandon, England) and immediately fixed with 95% ethanol. One slide was stained with Papanicolaou stain. After inhibition of endogenous peroxidase activity in methanolic hydrogen peroxidase (0.3%), the other slides were incubated with the following monoclonal antibodies: anti-human epithelial membrane antigen (EMA) mouse monoclonal antibody (1:400; Dako, Denmark), anti-human cytokeratin gp56kDa mouse monoclonal antibody (1:40; Immunotech, France) and anti-swine vimentin mouse monoclonal antibody (1:10; Dako). Immunoperoxidase staining was achieved by means of the avidin-biotin complex method using a Vectastain ABC kit (Vector, Burlingame, Calif.). Peroxidase activity was visualized by incubating the slides for 2–5 min with 3,3'-diaminobenzidine (Nakalai Tesque, Japan) in 0.05 mol/l of TRIS-hydrochlonic acid buffer (pH 7.6) containing 0.01% hydrogen peroxide. Positive and negative (normal serum) controls were included in all immunocytochemical reactions. Also, we examined these cells by the periodic acid-Schiff (PAS) reaction, diastase digestion and alcian blue staining.

For SEM, ovaries were cut into small blocks after cell isolation, which, as well as the cultured cells, were fixed in a chilled 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 2 h, washed in 0.05 mol/l cacodylate buffer and postfixed in 1% osmium tetroxide dissolved in 0.1 mol/l cacodylate buffer for 2 h, then dehydrated through a graded series of ethanol. After dehydration, the bottom of each culture dish was sawn into 0.8 $cm^2$ fragments. Ovaries and cultured cells were dried in a critical-point drier (HCP-2, Hitachi, Japan), coated with a layer of 30 nm gold with a sputtering device (IB-3, Eiko, Japan) and examined under a JSM-6400FK scanning electron microscope (JEOL, Japan). Cells in the culture dishes were also fixed in 2.5% glutaraldehyde, and postfixed in 1% osmium tetroxide, dehydrated through a graded ethanol series, then embedded in Epon 812 for TEM. Ultrathin sections were cut using an Ultratome MT6000-XL (RMC, Tucson, Aviz.) with a diamond knife (Diatome, Bienne, Switzerland), stained with lead citrate and uranyl acetate, then observed under an electron microscope (H-300, Hitachi).

OSE from six normal ovaries were observed as control by immunohistochemistry and EM. Formalin-fixed paraffin sections, 4 μm thick, were studied by immunohistochemistry and the antibodies were reacted as follows: EMA diluted 1:200, cytokeratin diluted 1:20 and vimentin diluted 1:5. Ovaries were cut into small pieces for TEM and SEM. These specimens were prepared as described above.

## Results

After collagenase digestion followed by gentle scraping, the sheets of surface epithelium detached at the level of the basement membrane (Fig. 1a, b). In the medium after unit gravity sedimentation, most of the surface epithelial cells formed a cluster (Fig. 2). At seeding after dispersal with trypsin-EDTA, the cell viability was about 50–90% and the viable cell number was $0.1–2.7 \times 10^6$ per ovary (Table 1).

The surface epithelial cells attached to the plastic dishes within 24–48 h of explantation. OSE outgrowths were initiated from a homogeneous population of single cells, after 13–20 days, forming confluent monolayers (Fig. 3a, b). The cell population doubling time was 7–10 days (Fig. 4). The cells from 11 specimens were subsequently subcultured from one to three times. However, confluent layers did not form in eight specimens since the cell growth was poor (Table 1). Among them, two samples (cases 6 and 15) were obtained from menopausal women and two (cases 11 and 16) were from patients who had pelvic adhesions except for the ovaries. There was no certain relationship between the initial viable cell number and cell growth.

Outgrowths of OSE cells were divided into three types. In the confluent monolayers, the cells had a cobblestone like appearance (Fig. 5a). Besides these, fusiform cells were occasionally dispersed (Fig. 5b), and subculturing enhanced the modulation of the cells to this type. It was difficult to distinguish fusiform cells from fibroblasts by light microscopy. Among the specimens that did not form confluent monolayers, the cell outgrowths were often flat. These cells appeared in loosely arranged networks of spindle-shaped or polygonal cells (Fig. 5c).

OSE cells observed in the control specimen did not stain with anti-EMA antisera, but the suprabasal portion of them stained with anti-keratin antisera. In response to vimentin, they were weakly positive in the same portion of OSE cells beside the stroma (not shown). The cultured cells revealed strong reactions for

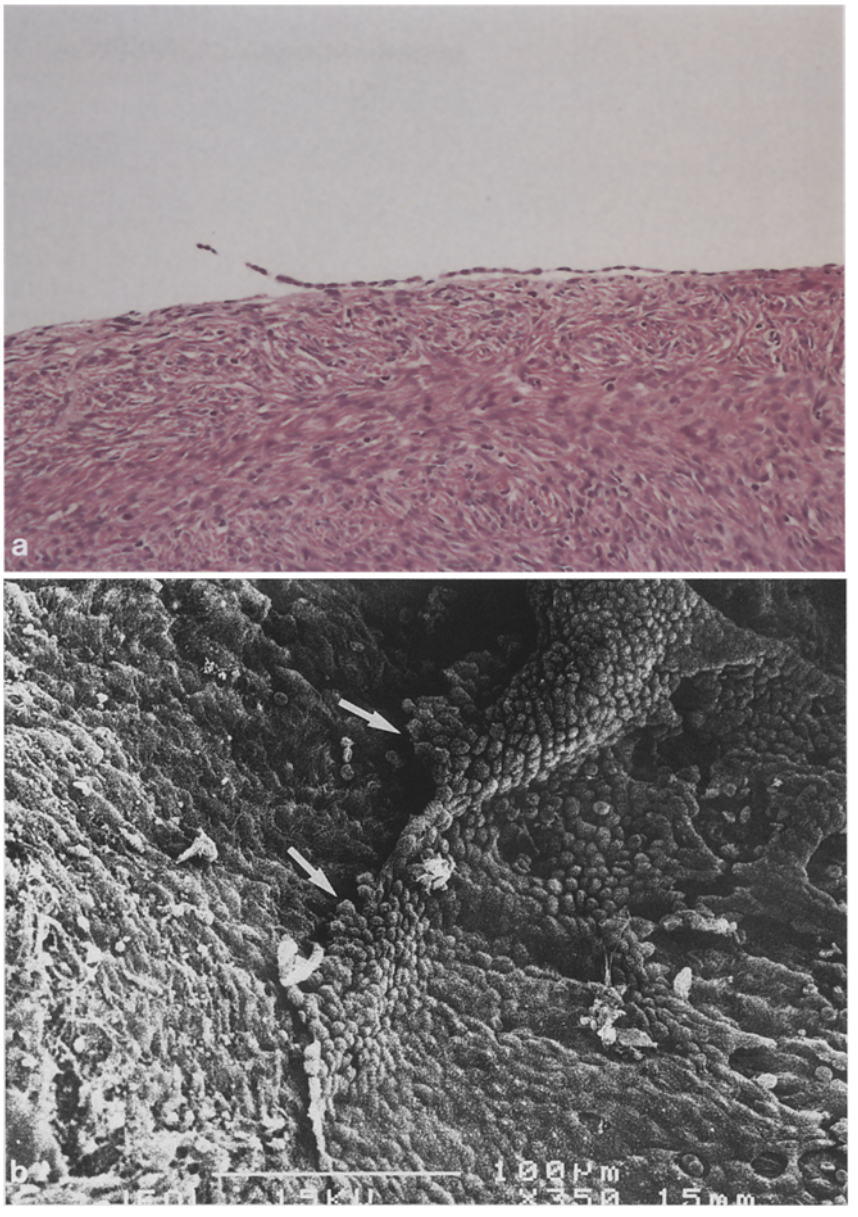



**Fig. 1** **a** Ovarian surface after exposure to collagenase type 1 followed by gentle scraping, H & E × 50. **b** The detachment of sheets of surface epithelium occurs at the level of the basement membrane (*arrows*), scanning electron micrograph

keratin and vimentin throughout the cytoplasm, but none for EMA (Fig. 6a–c). The subcultured cells immunoreacted the same way in each form of outgrowth. In addition, cultured cells were weakly positive for the PAS reaction, were digested with diastase and stained positively at the surface coat with alcian blue (Fig. 6d, e).

Ultrastructurally, the OSE cells in vivo were flat or cuboidal with abundant microvilli. Tight junctions, desmosomes, gap junctions, and narrow and dilated intercellular spaces were observed between cells. The nuclei were convoluted or indented with peripheral nucleoli. The cytoplasm contained well-developed organelles, including rough endoplasmic reticulum, mitochondria, Golgi complexes, lysosomes, and intermediate filaments (not shown). Under our culture conditions, the cells were round or spindle-shaped with numerous microvilli on their surfaces, and intercellular apposition was close with no overlap (Fig. 7a, b). The fusiform cells were also covered with numerous microvilli. Their cytoplasm contained rough endoplasmic reticulum, mitochondria, endocytotic vesicles and dense accumulation of intermediate filaments (Fig. 8a). The intermediate filaments were more prominent in vitro than in vivo, particularly at the cellular periphery (Fig. 8b). Intercellular contacts were desmosome-like structures but lacked tight junctions (Fig. 8c).

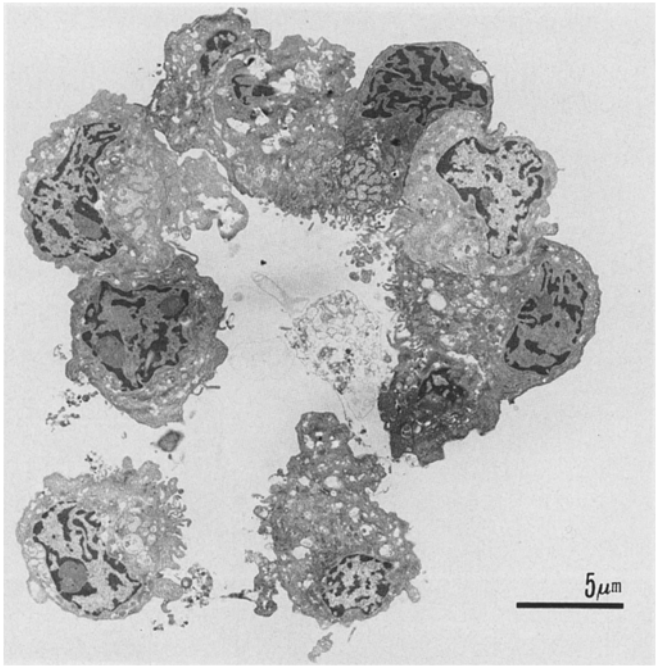



**Fig. 2** Clustered surface epithelial cells after unit gravity sedimentation. Lead citrate and uranyl acetate, transmission electron micrograph

## Discussion

OSE cells have important functions during reproductive life. It is proposed that they have secretory, transport, regenerative functions that are hormonally controlled (Van Blerkom and Motta 1979; Motta et al. 1980; Nicosia 1983). Furthermore, the OSE changes morphologically during prepubertal, reproductive, and postmenopausal periods (Van Blerkom and Motta 1979; Okamura et al. 1980; Nicosia 1983; Nicosia and Nicosia 1988). Clinically, ovarian cancer and ovarian endometriosis, commonly encountered gynaecological diseases, are closely related to OSE. In ovarian cancer, the rapid cycles of epithelial cell division associated with wound repair are considered to have an important role on the initiation of the disease (Godwin et al. 1992, 1993). It is also proposed that elevated gonadotropin levels increase ovarian cancer risk (Cramer and Welch 1982) and that the use of gonadotropins for ovulation induction and the multiple ovulations may add to the already increased risk of infertile women developing an ovarian malignancy (Atlas and Merczer 1982; Ben-Hur et al. 1986; Carter and Joyce 1987; Nijman et al. 1992). The metaplastic theory of endometriosis is supported by the fact that pelvic endometriosis is a result of a serial change from normal mesothelial cells containing OSE (Clement 1987; Fujii 1991; Nakamura et al. 1992). Therefore, the establishment of an experimental model for the study of human OSE would be useful.

A few methods of culturing human OSE have been reported, including explantation (Auersperg et al. 1984; Siemens and Auersperg 1988) and scraping (Kruk et al. 1990). However, some problems remained such as the complexity of the procedure or the low cell number of OSE cells acquired. Nicosia et al. (1984, 1985) established a method of isolating rabbit OSE cells, obtained good cell growth and studied their characteristics. We have adapted this method for human OSE, examined the growth of isolated OSE cells and studied their characteristics immunocytochemically and ultrastructurally.

An EMA immunoreaction is present in metaplastic OSE cells alongside ovarian surface epithelial tumours and mesothelial neoplasms. Of the two intermediate filaments used in our study, keratin is expressed by non-squamous epithelium, mesothelium and other mesodermal derivatives including the OSE. Vimentin is found in

**Table 1** Viable cell number and ovarian surface epithelial growth in culture in each case ( – growth did not reach confluence; * growth means last culture that could be subcultured)

| Case | Age (years) | Gravidity (G) and Parity (P) | Cycle date | Viable cell number $\times 10^6$ | Viability (%) | Growth* (number of subcultures) |
|---|---|---|---|---|---|---|
| 1 | 44 | 2G2P | 20 | 2.5 | 80 | 4 |
| 2 | 39 | 2G2P | 25 | 1.5 | 50 | – |
| 3 | 40 | 2G2P | 7 | 1.0 | 90 | 4 |
| 4 | 40 | 4G2P | Unclear | 0.7 | 65 | 2 |
| 5 | 52 | 4G4P | Unclear | 0.4 | 80 | – |
| 6 | 52 | 6G2P | Menopause | 0.9 | 78 | – |
| 7 | 43 | 3G1P | 24 | 1.0 | 90 | – |
| 8 | 45 | 2G1P | Unclear | 1.0 | 60 | 2 |
| 9 | 43 | 2G1P | 9 | 0.7 | 75 | 1 |
| 10 | 44 | 3G2P | 22 | 2.7 | 67 | 2 |
| 11 | 48 | 3G3P | 12 | 1.0 | 67 | – |
| 12 | 43 | 3G1P | 14 | 0.9 | 85 | 2 |
| 13 | 47 | 5G2P | 29 | 1.3 | 70 | – |
| 14 | 48 | 4G2P | 17 | 0.4 | 80 | 1 |
| 15 | 55 | 13G5P | Menopause | 0.6 | 90 | – |
| 16 | 36 | 2G1P | 10 | 0.2 | 75 | – |
| 17 | 51 | 5G3P | Unclear | 0.2 | 50 | 1 |
| 18 | 42 | 5G2P | Unclear | 0.3 | 50 | 3 |
| 19 | 40 | 0G0P | 31 | 0.1 | 60 | 3 |

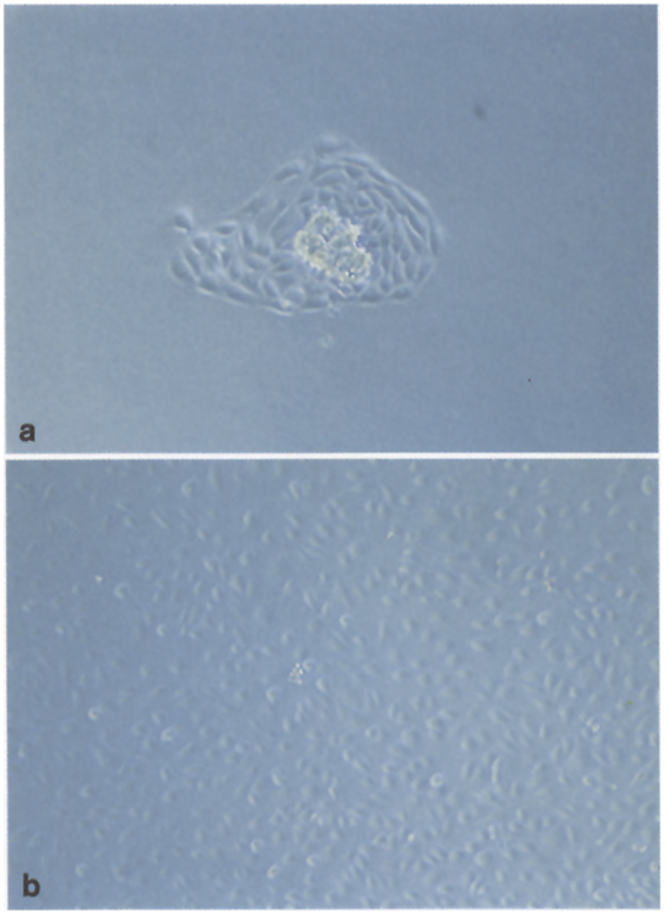



**Fig. 3a, b** The outgrowth of ovarian surface epithelium. **a** The growth occurred from homogeneous population of single cells, × 37.5. **b** A confluent epithelial-like monolayer in 14th day of culture, × 37.5

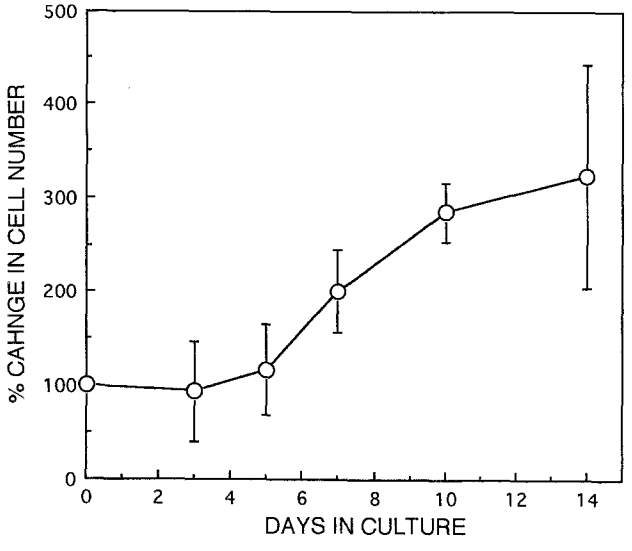



**Fig. 4** Ovarian surface epithelial cell growth in primary culture in the cases that reached confluence

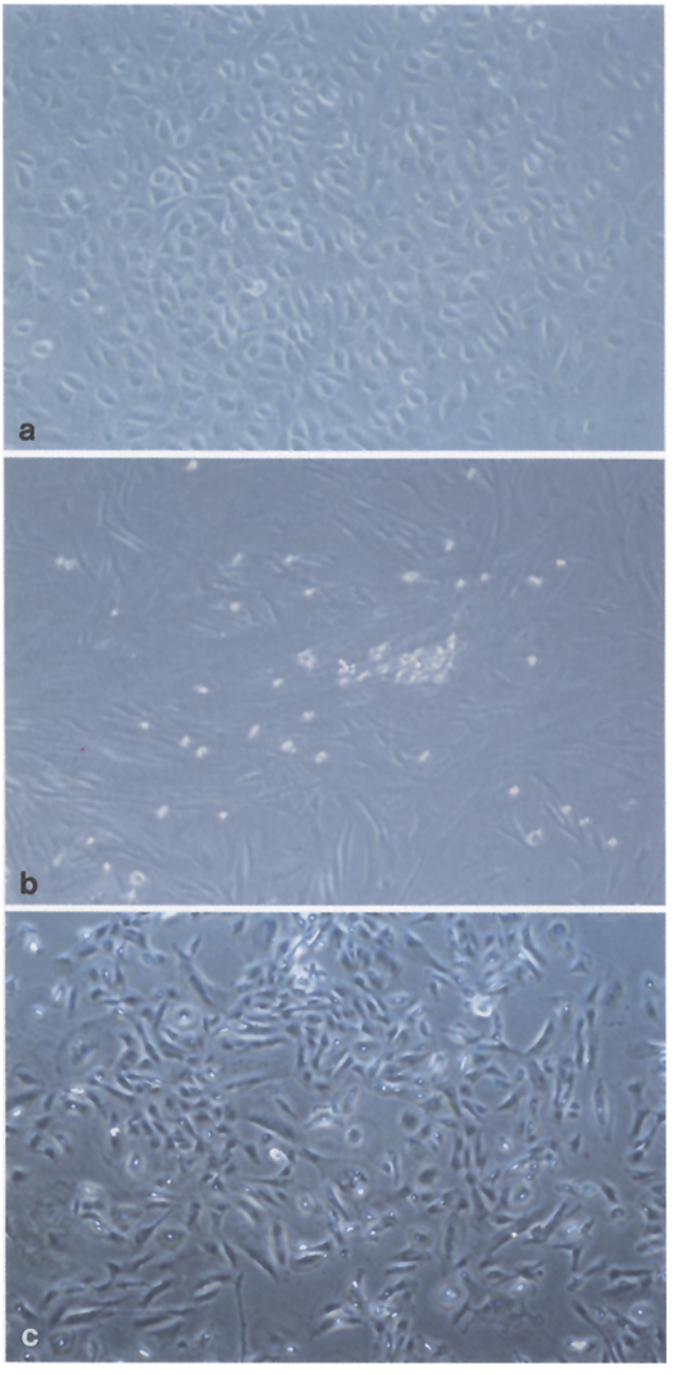



**Fig. 5** The morphology of OSE outgrowths. **a** Cobblestone like cells, × 37.5. **b** Fusiform cells, × 37.5. **c** Flat cells, × 37.5

both normal and neoplastic mesenchymal cells (Nicosia and Nicosia 1988). Normal mesothelial cells have a high keratin and low vimentin content in vivo and they co-express keratin and vimentin in culture (Connell and Rheinwald 1983; LaRocca and Rheinwald 1984). Also, histochemical studies have shown that OSE cells have PAS-positive material and that the surface of these cells was stained with alcian blue (Blaustein and Lee 1979). Compared with these reports, the cells cultured in this

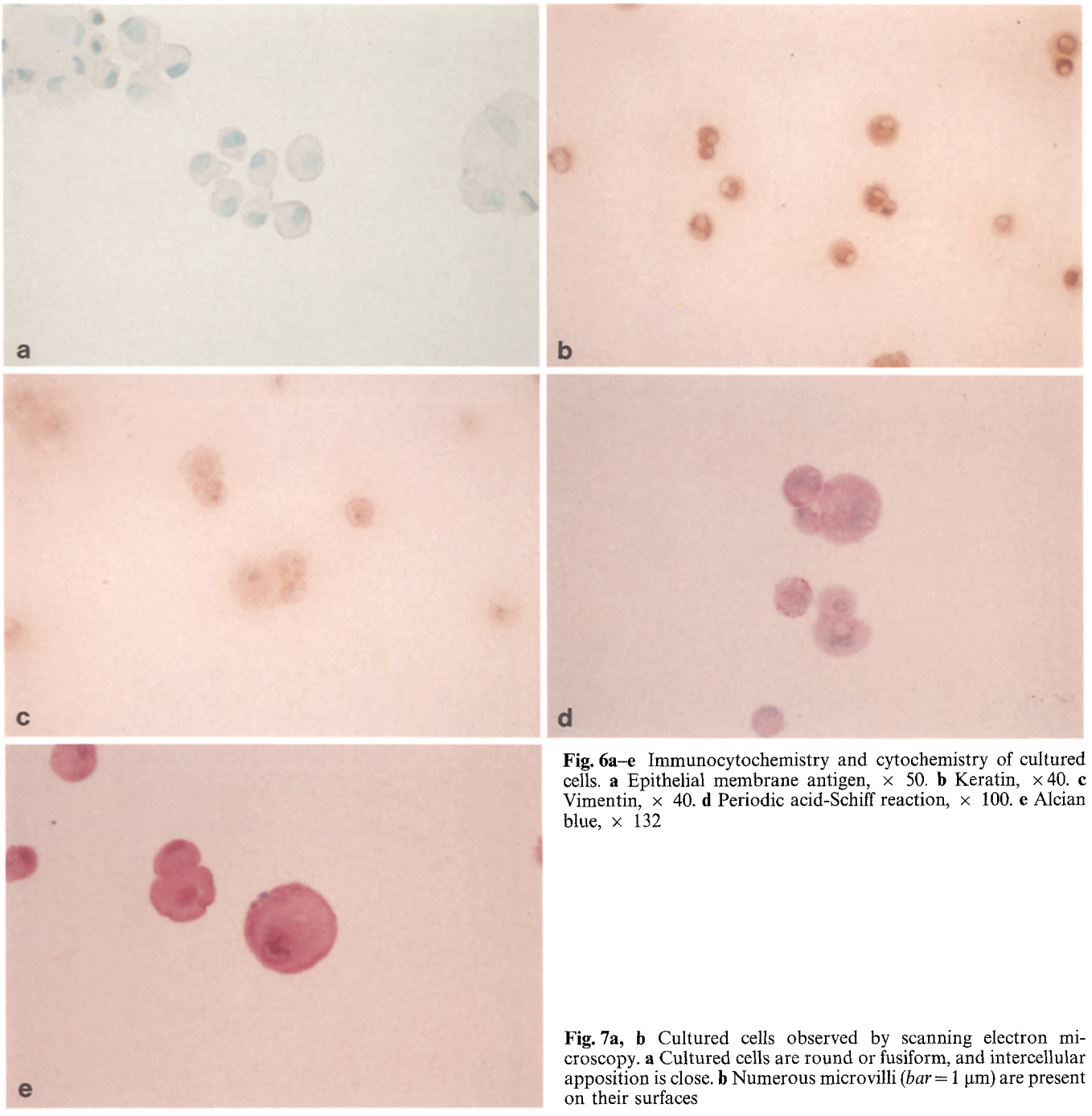
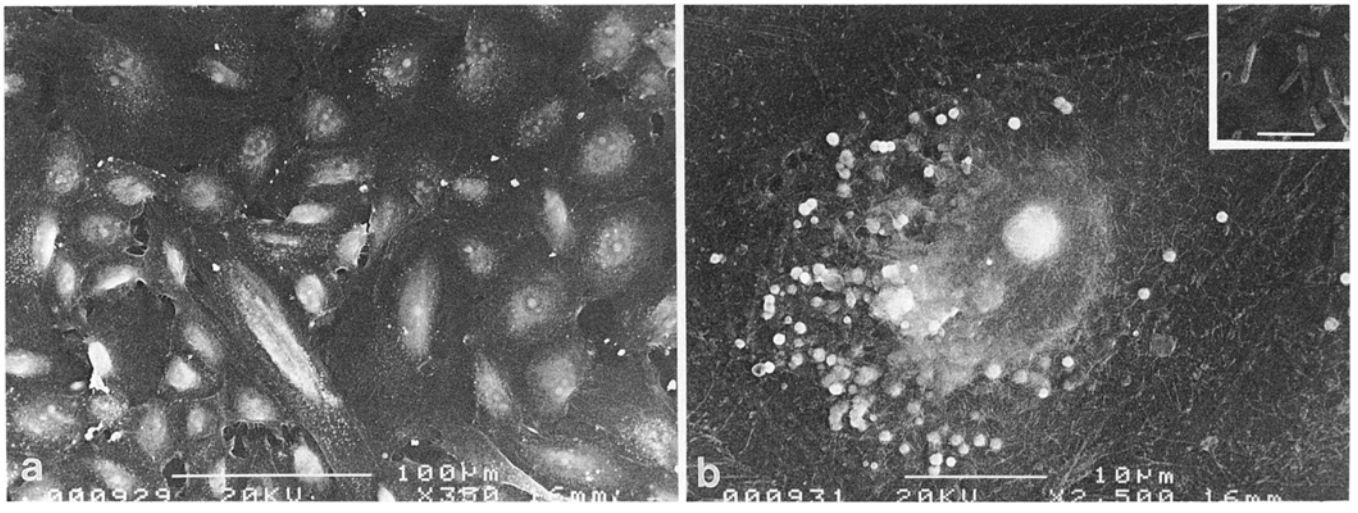



**Fig. 6a–e** Immunocytochemistry and cytochemistry of cultured cells. **a** Epithelial membrane antigen, × 50. **b** Keratin, ×40. **c** Vimentin, × 40. **d** Periodic acid-Schiff reaction, × 100. **e** Alcian blue, × 132

**Fig. 7a, b** Cultured cells observed by scanning electron microscopy. **a** Cultured cells are round or fusiform, and intercellular apposition is close. **b** Numerous microvilli (*bar* = 1 μm) are present on their surfaces



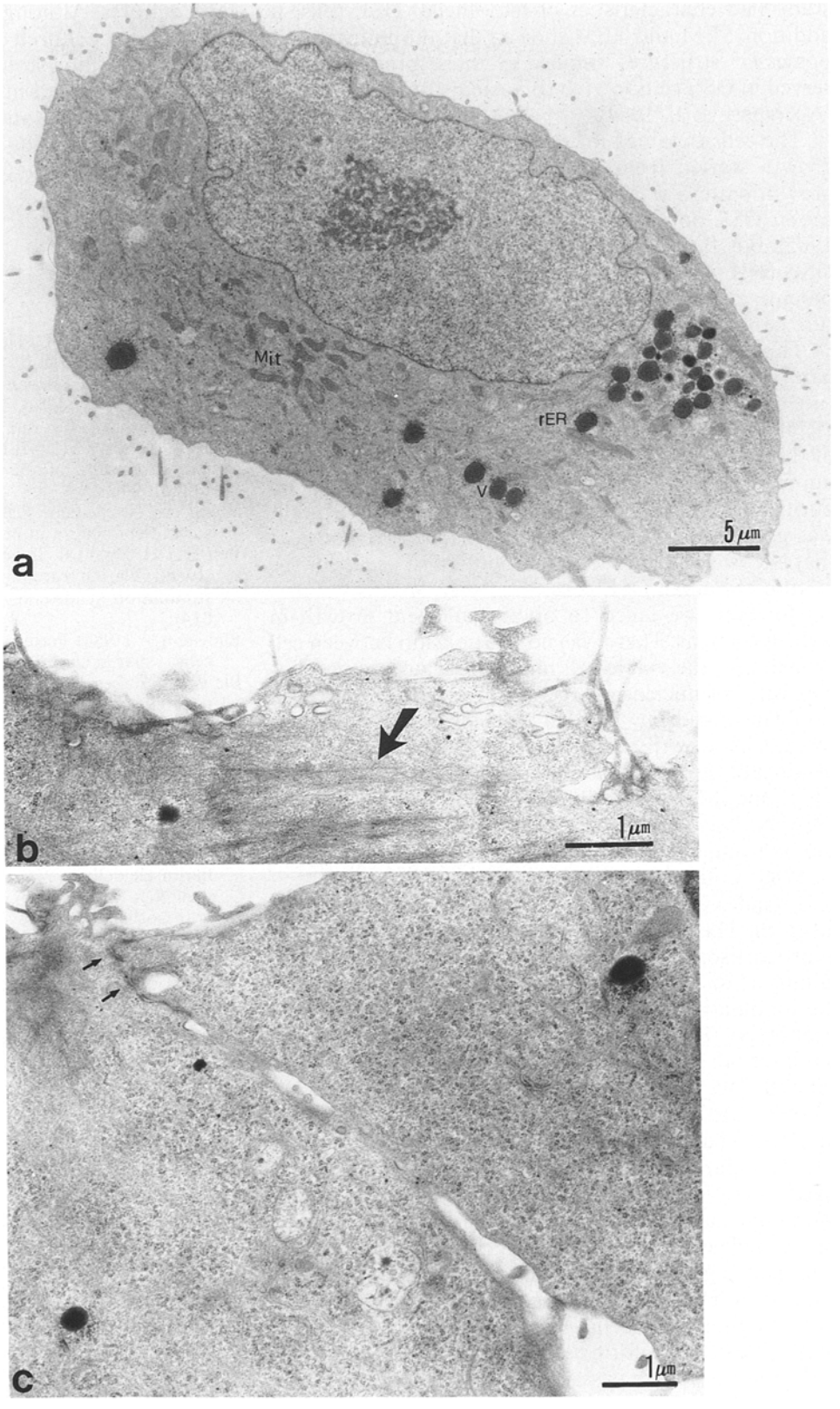



**Fig. 8a–c** Cultured cells observed by transmission electron microscopy. **a** Rough endoplasmic reticulum (*rER*), mitochondoria (*Mit*) and endocytotic vesicles (*V*). **b** Dense accumulation of intermediate filaments at the cellular periphery (*arrow*). **c** Desmosome-like junctions (*arrows*). Lead citrate and uranyl acetate

study had characteristics of mesothelial OSE cells. In addition, SEM and TEM showed that the cultured cells possessed structures similar to those previously observed in OSE cells in vivo (Blaustein 1984) and in vitro (Auersperg et al. 1984).

The cells obtained formed a cluster initially and cell growth started from the edge of these clusters. This growth pattern was similar to that of rabbit OSE. The rabbit OSE doubling time was 30–36 h (Nicosia et al. 1985), but that of the human was 7–10 days. Human OSE cells were considered to have much less growth potential than those of the rabbit under the same culture conditions.

The method and medium conditions used to propagate animal OSE are reportedly inadequate for the culture of human OSE (Auersperg et al. 1984; Kruk et al. 1990). However, the total cell number and the viability of those grown were not mentioned. In our study, the number of human OSE cells acquired after isolation and purification was adequate and the viability of OSE cells was good enough for subsequent studies. We consider that Nicosia's method for the rabbit is also useful for the culture of human OSE cells.

However, we failed to obtain sufficient growth in eight specimens. There was no relationship between cell growth and the viable cell number because some samples with a sufficiently large cell number did not reach confluence, and low numbers of cells could be subcultured. The growth potential in culture may not be dependent of the isolation method, but may be affected by endocrine (Makabe et al. 1980; Nicosia and Saunders 1986; Nicosia and Nicosia 1988) and/or the growth factor environment (Adashi et al. 1989; Gospodarowicz et al. 1989; Lobb et al. 1989) in vivo.

Subculture increased the tendency of cells to become fusiform. The addition of epidermal growth factor and hydrocortisone to medium 199 and MCDB 202 containing 15% FBS increases the growth rate, but causes the modulation of human OSE cells to a fibroblastic phenotype (Siemens and Auersperg 1988). However, Kruk et al. (1990) reported that medium 199 and MCDB 105 containing 15% FBS supported rapid growth and maintained the epithelial phenotype. MCDB 105 and 202 are both modifications of medium F12. Compared with MCDB 202, MCDB 105 has high levels of glutamine and proline which may enhance extracellular matrix synthesis (McKeehan et al. 1978) and has suitable cysteine and calcium concentrations to encourage the growth of other epithelial cells (McKeehan et al. 1984). Therefore, MCDB 105 may be more efficient in maintaining epithelial-like morphology. Since the keratin-positive fusiform cells more frequently observed at the third or fourth passage in our culture system were considered to have the characteristics of epithelial cells, we are now studying the effect of MCDB on our culture system in order to harvest OSE with more of the characteristics of epithelial cells.

Godwin et al. (1992, 1993) reported that OSE in rats showed spontaneous malignant transformation in long-term cultures. Although in our culture system the OSE cells were subcultured only one to three times, the possibility of these morphological and genetic changes occurring may be present in long-term cultures. The effect of long-term culture and various agents such as gonadotropins, steroids and growth factors on their growth and differentiation will be studied.

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