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ORGANOTYPIC CULTURE OF HUMAN OVARIAN SURFACE EPITHELIAL CELLS: A POTENTIAL MODEL FOR OVARIAN CARCINOGENESIS

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SUMMARY

The objective of this work was to establish an *in vitro* multidimensional culture system for human ovarian surface epithelial (HOSE) cells as a model for ovarian carcinogenesis. The epithelial origin of cell outgrowth from cells obtained from the ovarian surface was confirmed by keratin staining. Two cultures from two different patients were established, HOSE-A and HOSE-B. Cultures were infected with a retrovirus expressing human papillomavirus genes E6 and E7 to extend their life span. HOSE cells were seeded onto collagen gels containing NIH3T3-J2 fibroblasts as feeder cells and grown to confluence submerged in growth medium. The collagen bed was then raised to the air–medium interface for 7 d (organotypic culture). Microscopically, fixed cultures revealed a single layer of flat cells growing on the collagen surface, reminiscent of HOSE cells *in vivo*. Infected HOSE-A and HOSE-B cells exhibited aberrant growth because they stratified. In addition, established ovarian cancer lines grown in this fashion stratified and showed malignant phenotypes. Thus, cells grown in organotypic culture resemble their *in vivo* counterparts, providing a basis for establishing a system to study growth, proliferation, differential gene expression, and perhaps malignant transformation of HOSE cells.

Key words: ovarian epithelial cells; aberrant growth; multidimensional culture; extended life span.

INTRODUCTION

The lifetime risk for ovarian cancer among women with no family history of the disease is estimated to be 1 in 70. More than 27 000 new cases of ovarian cancer and 14 500 deaths were expected in 1997 (Parker et al., 1997); the vast majority of which would have been derived from the ovarian surface epithelium. The epithelial surface of the ovary is a continuation of the pelvic mesothelium and is the source of the common epithelial tumors of the ovary. Unlike other female genital tract cancers, the progression of ovarian carcinogenesis is not well understood.

It has been proposed that ovarian cancer develops as a multistage process through increasingly aggressive steps that result from the accumulation of mutations (Link et al., 1996). However, no model system has been developed to test this hypothesis. The culture of human ovarian surface epithelial (HOSE) cells has been well defined and cells can be subcultured through several population doublings (Auersperg et al., 1984; Kruk et al., 1990). The introduction of the simian virus 40 (SV40) large T antigen gene results in extended lifespan and increased growth rate, growth potential, and saturation density over normal HOSE cells grown as monolayers (Maines-Bandiera et al., 1992). In addition, these cells show aberrant growth and stratification when grown in a three-dimensional sponge culture system (Dyck et al., 1996).

Expression of human papillomavirus type 16 (HPV-16) E6 and E7 genes in human keratinocytes extends their life span and can immortalize the cells (Durst et al., 1987; Pirisi et al., 1987). Morphologically, HPV-16 immortalized human keratinocytes are similar to uninfected controls when maintained as monolayers. However, their culture characteristics change when monolayers are grown in organotypic culture at the air–medium interface (McCance et al., 1988). Uninfected keratinocytes stratify and display the characteristic layers of differentiating epithelium. Immortalized keratinocytes, on the other hand, although retaining the ability to stratify, display differentiation abnormalities histologically similar to intraepithelial neoplasia.

Expression of the HPV-16 E6 and E7 genes in HOSE cells, while not changing their morphology in monolayer culture, extends the life span of the cells (Tsao et al., 1995). It is unknown if this delay in senescence represents a change in cell growth patterns. To test whether HPV-16 early gene expression in HOSE cells does affect cell growth, we grew HOSE cells in organotypic cultures on collagen gels containing fibroblasts (Asselineau and Prunieras, 1984). The results show that normal HOSE cells form a single layer of cells on the collagen bed reminiscent of ovarian surface epithelium *in vivo*. On the other hand, cells expressing HPV-16 early genes showed atypical features compared to uninfected cells and stratified on the collagen, indicating loss of normal growth restraints, a property not seen in monolayer culture. This is the first demonstration of HOSE cells expressing HPV-16 early genes showing morphologically aberrant growth. For comparison, ovarian cancer cell lines grown in

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organotypic culture tended to exhibit histopathological characteristics similar to the tumor from which they were derived. These results may reflect the abnormal growth of ovarian surface epithelium observed *in vivo* and, for E6/E7-expressing HOSE cells, potentially an early step in neoplastic progression.

Materials and Methods

Normal ovaries were obtained from postmenopausal women undergoing oophorectomy and/or hysterectomy for benign gynecologic disease. One-half of the ovary was placed in RPMI medium containing 10% fetal calf serum (FCS) and 50 μg gentamicin per ml and was used for cell culture; the other half was fixed in formalin for pathologic evaluation. Cells were gently scraped from the surface of the ovary with a cytobrush into the above medium containing 5 ng epidermal growth factor (EGF) per ml. Cultures were left undisturbed for 5 d in 5% CO_2 at 37° C and then washed with medium to remove unattached cells and debris. Cytospin preparations of trypsinized cells were stained for cytokeratins by standard immunocytochemistry with a cocktail of the monoclonal antibodies CAM5.2 (Becton Dickinson, Franklin Lakes, NJ), AE1/AE3 (Boehringer Mannheim, Indianapolis, IN) and CK904 (Enzo Diagnostics, Farmingdale, NY).

When the cultures reached 50% confluence, they were either passed or infected with an amphotrophic retrovirus containing vector LSXN. This vector can express the HPV16 E6 and E7 genes under the control of the 5′ long terminal repeat of Moloney leukemia virus and the neomycin resistance gene by the SV40 promoter (LSXN-16E6E7; kindly provided by D. Galloway) as described elsewhere (Tsao et al., 1995). Infected cultures were maintained in medium without EGF or followed by the addition of G418 (300 μg/ml) and maintained under selection. Cells were passed under continued antibiotic selection at a split ratio of 1:2 when reaching confluence for two additional passages. Uninfected cultures were initially passed at a split ratio of 1:2 when reaching confluence, at which time EGF was removed from the medium. Total RNA was extracted from cells with RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and Northern blots were hybridized to a radiolabeled full-length HPV-16 DNA probe to detect HPV-16-specific RNA.

The cervical cancer cell line HeLa was maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum and 50 μg gentamicin per ml, and ovarian cancer cell lines SK-OV-3, SW626, and MDAH2774 were grown in RPMI supplemented with 10% FCS and 50 μg gentamicin per ml. Cells lines were obtained from the American Type Culture Collection (Rockville, MD).

Organotypic cultures were grown on a collagen bed containing 10^5 mitomycin C-treated NIH3T3-J2 fibroblasts/ml (kindly provided by L. Laimins). Confluent cultures of fibroblasts were treated with 2 μg mitomycin C per ml overnight, washed, trypsinized, and resuspended in FCS. Treated cells were added to 200 m*M* HEPES, pH 7.5, and 10× Ham's F-12 medium at a ratio of 1:1:1 (vol:vol:vol) followed by the addition of 0.054 vol of 1 *N* NaOH and 7 vol of rat tail collagen (Type 1, Collaborative Biomedical Products, Bedford, MA). Two ml of the mixture was added to 30-mm culture dishes and incubated at 37° C for 1 h for the collagen to solidify. DMEM containing 10% FCS was added to prevent drying of the collagen. After overnight incubation, cells (2×10^5 in 2 ml medium containing 10% FCS and 50 μg gentamicin per ml) were added to the drained surface of the collagen bed. Cultures were incubated at 37° C in 5% CO_2 until confluent (2–4 d). A 1.5-cm^2 section of the collagen bed was cut from the culture dish and placed on a raised stainless steel grid (30 × 30 mesh) and culture medium added to just cover the top of the support. After 7–8 d of culture the grid and collagen bed were immersed in 10% buffered formalin for 1 h before overnight processing for routine histopathology. The collagen was then embedded in paraffin on edge and sections (5 μm) were stained with hematoxylin-eosin.

Results

Primary culture of human ovarian surface epithelial (HOSE) cells revealed colonies of cuboidal cells with a cobblestone appearance (Fig. 1) that eventually formed a confluent monolayer. These cultures could be split at a ratio of 1:2 for three to four passages for a period of 8 wk before the cells stopped replicating and senesced. Wright-Giemsa stain on cytospin preparations of trypsinized cells showed large polygonal cells. The antikeratin immunocytochemistry revealed a uniform cytoplasmic staining, confirming that the cells were of epithelial origin (Fig. 1 *b*).

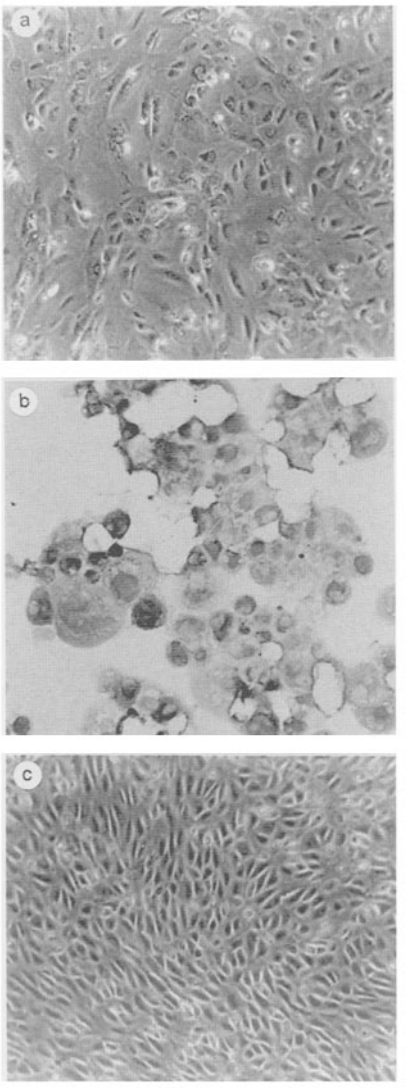

FIG. 1. Monolayer cultures of HOSE cells. (*a*) Primary culture of HOSE cells 14 d after seeding from cell scrapings of the surface of the ovary; (*b*) immunocytochemical detection of keratin in the cytoplasm of a cytospin preparation of trypsinized HOSE cells from a primary culture; (*c*) second passage of HOSE-A cells expressing HPV-16 E6/E7 genes. The *bar* below (*a*) is 5 μm.

Primary cultures infected with the amphotropic retrovirus showed no morphologic differences by phase-contrast microscopy or significant changes in growth rate at early passage compared to the parent cells. Northern blot analysis revealed a major HPV-16-specific RNA band at approximately 3.5 kilobases (data not shown). Two HOSE cultures from different patients have been established after infection with the retrovirus, HOSE-A and HOSE-B. Fig. 1 *c* shows HOSE-A cells at the second passage after infection. Although the cell morphology has remained cuboidal in later passages, the cells have become smaller and monolayers are more compact than uninfected cells. However, we have not determined the extent of increase in

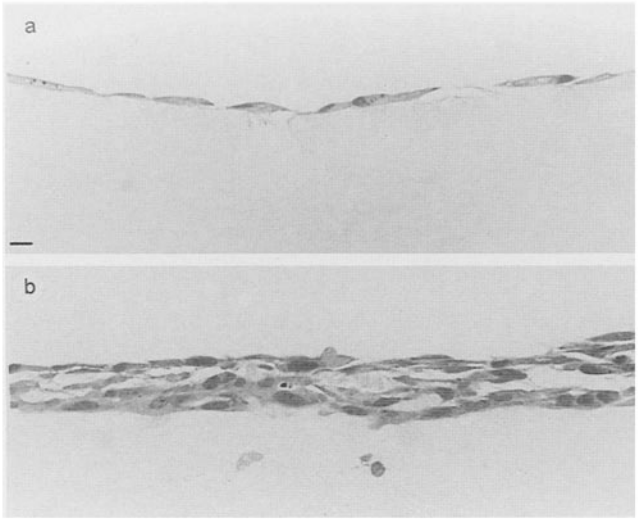

FIG. 2. Growth of HOSE cells in organotypic culture. (*a*) Second passage uninfected HOSE cells; (*b*) second passage HOSE-A cells expressing HPV-16 E6/E7 genes. The *bar* in (*a*) is 4 μm.

saturation density or changes in serum requirements. HOSE-A and HOSE-B have been in culture for 21 and 10 mo., respectively.

Hematoxylin-eosin staining of organotypic culture of normal HOSE cells is shown in Fig. 2 *a*. These cells formed a continuous sheet only one cell thick, reminiscent of the flat to low cuboidal cells forming the surface epithelium of the ovary. HOSE-A cells expressing the HPV-16 early genes, on the other hand, behaved quite differently two passages after G418 selection. They formed a multilayer consisting of atypical cells arranged in 3–4 layers (Fig. 2 *b*). Nuclei were somewhat larger and more hyperchromatic with a higher nuclear/cytoplasmic ratio than uninfected cells. Some nuclei contained small nucleoli. The cells showed a uniform morphology throughout the stratification. Infected and uninfected HOSE-B cells grew in an identical fashion to the HOSE-A counterpart in organotypic culture (data not shown).

Expression of HPV-16 E6 and E7 genes in HOSE cells compared to uninfected cells had little effect on their growth behavior in monolayer culture at early passage. However, their ability to stratify in organotypic culture indicated an aberration in growth control. In our hands, carcinoma cell lines HeLa (cervix) and MDAH2774, SK-OV-3, and SW626 (ovary) do not stratify in monolayer culture. These cells lines were grown in organotypic culture with feeder cells to determine their growth characteristics.

HeLa cells showed stratification of malignant pleomorphic cells with many mitotic figures (Fig. 3 *a*). The nuclei were large, hyperchromatic with prominent nucleoli, and a high nuclear/cytoplasmic ratio. Although HeLa cells were established from an adenocarcinoma, they stratify as usually seen in squamous cell carcinoma in this culture system. MDAH2774 cells also stratified and formed small projections (Fig. 3 *b*). The cells were pleomorphic with many mitotic figures. SK-OV-3 stratified consisting of 3–4 layers of atypical cells with hyperchromatic nuclei (Fig. 3 *c*). Mitotic figures were moderate. The cytoplasm was moderately vacuolated. SW626 showed a patchy distribution with some areas showing stratification of 3–4 layers (Fig. 3 *d*) and others of a monolayer of cuboidal cells with a high

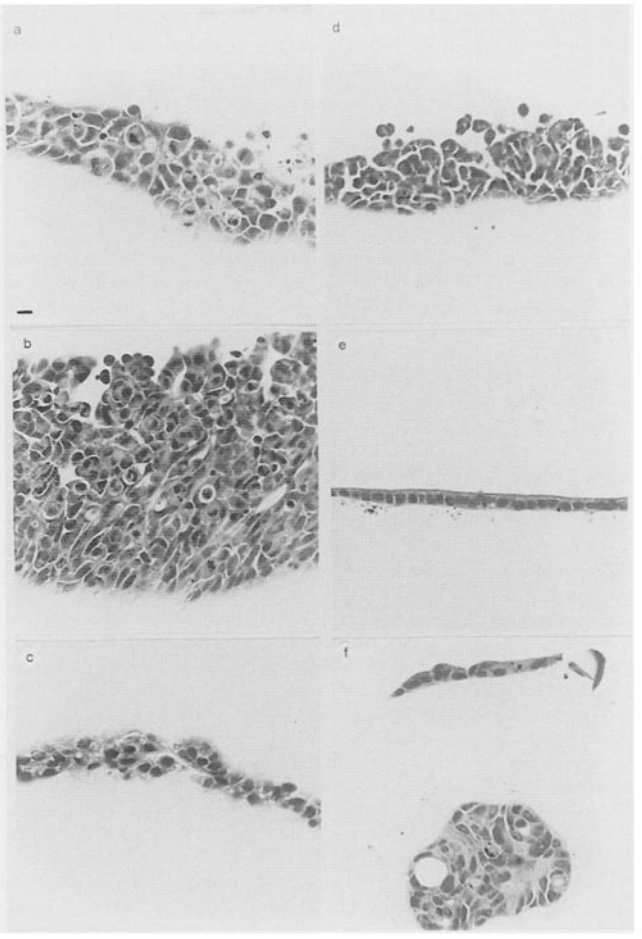

FIG. 3. Growth of human cancer cell lines in organotypic culture. (*a*) HeLa cells; (*b*) SK-OV-3 cells; (*c*) MDAH2774 cells; (*d*) SW626 stratified area; (*e*) SW626 monolayer area; (*f*) SW626 colony growing within collagen. The *bar* in (*a*) is 4 μm.

nuclear/cytoplasmic ratio (Fig. 3 *e*). In the stratified areas there were numerous mitotic figures with cells forming papillary projections. In addition, this cell line showed a tendency for downward invasion of the collagen (Fig. 3 *f*). Organotypic cultures of SK-OV-3 and MDAH2774, but not SW626, stained positive for mucin (data not shown).

DISCUSSION

HOSE cells grown in a three-dimensional organotypic culture form only a single layer of cells over the surface of the collagen gel with (this paper) or without (Kruk et al., 1994) feeder cells, and this cell layer is similar to the surface epithelium of the ovary. However, if cell cycle checkpoints are perturbed by expression of HPV type 16 E6 and E7 early genes, HOSE cells form a multilayered structure of atypical cells on the collagen. These results suggest that these cells have acquired changes in their growth control which is likely a reflection of p53 degradation and pRb inactivation through interactions with HPV-16 E6 and E7, respectively (Munger et al., 1989; Scheffner et al., 1990).

Phase-contrast microscopy can distinguish between monolayer cultures of ovarian cancer cell lines because of subtleties in culture morphology but cannot provide information about the tumor from which the cell line was derived. Cell line SK-OV-3 was originally derived from an ovarian adenocarcinoma (Fogh et al., 1977) and in organotypic culture the cells stratify and stain positive for mucin. Cell line MDAH2774, which originally was derived from a well-differentiated endometrioid ovarian cancer (Freedman et al., 1978), displays stratification and stains positive for mucin. These cells have been reported to produce poorly differentiated, papillary serous cystadenocarcinomas in nude mice. SW626 was originally derived from a cystadenocarcinoma (Fogh et al., 1977) and produces well-differentiated papillary adenocarcinoma in nude mice. In organotypic culture, this cell line stratifies and fails to stain for mucin. The ability of these cell lines to produce stratifications in organotypic culture that mimic their progenitors indicates that the cells have retained the potential to express genes necessary to produce unique growth phenotypes.

Cells from a number of epithelial tissues (liver, mammary epithelium, colon) when grown in organotypic culture, show the morphological characteristics comparable to the *in vivo* state of differentiation (Michalopoulos and Pitot, 1975; Emerman and Pitelka, 1977; Paraskeva et al., 1984). Furthermore, cells from premalignant and malignant lesions of the cervix cultivated in raft culture form stratifications that closely resemble the lesions from which they were derived (Rader et al., 1990). Thus, organotypic culture provides a tool to study differentiation and oncogenesis in epithelial cells.

The growth response of normal HOSE cells and ovarian cancer cell lines in organotypic culture suggests that these cells mimic the *in vivo* state. Whether the stratification of atypical HOSE cells expressing the HPV-16 E6 and E7 genes represents an early step in the neoplastic process is unknown. However, it would be interesting to speculate that these cells may have an analogous relationship to a putative neoplastic precursor (pseudostratification) frequently seen in nonneoplastic ovaries contralateral to ovarian cancers (Scully, 1995) and in grossly normal ovaries from patients with genetic risk for ovarian cancer (Salazar et al., 1996).

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