Characterization of Ovarian Surface Epithelial Cells from the Hen: A Unique Model for Ovarian Cancer

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To further develop the hen as a model of ovarian adenocarcinoma, we have studied normal and neoplastic ovaries as well as cultured cells from the ovarian surface epithelium (OSE). We characterized the OSE layer of the hen for specific histologic markers and evaluated these markers on tumor tissue. We also isolated and characterized the epithelial cells that are the likely source of the ovarian tumors of the hen. The surface epithelium of normal ovaries demonstrated positive staining for cytokeratin, proliferating cell nuclear antigen (PCNA), progesterone receptor (PR), and negative staining for vimentin. Ovarian tumors demonstrated positive cytokeratin, PCNA, PR, and weak vimentin staining in the gland-like areas. Epithelial cell cultures were obtained by an explant method utilizing small and large yellow follicles. These cells were positive for cytokeratin and negative for vimentin on Days 1 and 3. By Day 10, cytokeratin protein expression was less for some cells, and vimentin expression was weakly present in some cells. Expression of PCNA was observed at Days 1 and 3, but was rarely seen in cells cultured for 10 days. Expression of PR was observed on Day 10 after 24-hr estrogen treatment. Epithelial cells grew slowly in culture, and were susceptible to trypsin or other dissociation treatments. Exp Biol Med 231:1718–1725, 2006

Key words: ovarian cancer; epithelial cells; OSE; ovary; hen

Introduction

Although the lifetime risk of ovarian adenocarcinoma is approximately 1.5%, it is the most lethal of the gynecological malignancies. Despite the fact that many ovarian tumors are associated with abdominal symptoms (1), they are often diagnosed late, after they have metastasized to other organs. At this stage of advancement, the tumors often respond poorly to treatment. Although the origin and early events in the disease are poorly understood, the best predictor is a family history of ovarian cancer, with 5%–10% of patients with ovarian cancer presenting this genetic association (2). Reproductive history is the next-best indicator, with an increased risk of ovarian cancer correlated with ovulation rate. In this regard, Fathalla (3) proposed that the repeated rupture and repair of the ovarian surface epithelial (OSE) layer during the ovulatory process may encourage the malignant transformation of these cells. Subsequently, researchers focused on the isolation, culture, and characterization of the OSE cells. Many of these studies were hampered by the fact that the OSE cells are a relatively minor proportion of the ovarian mass and are easily damaged. Contaminating cell types have also made characterization studies difficult. Procedures have been improved, and normal OSE cells, as well as lines of ovarian tumor cells, are currently the most commonly utilized model systems to study this disease. It is generally believed that inclusion cysts/glands are the sites of origin of human epithelial ovarian cancer, and that these lesions result from the entrapment of surface epithelial cells in the stroma of the ovary (2, 4).

Few cases of ovarian cancer occur spontaneously in species other than the human, with one exception: the domestic hen (5–8). It has been shown by several investigators that the chicken spontaneously develops ovarian cancer (6–8). Interestingly, the hen is a persistent ovulator, laying almost daily, and therefore models the reproductive history of modern-day women, who often have 10–20 years of monthly ovulations before 1 or 2 pregnancies, followed by 10–20 years of ovulations prior to menopause. Fredrickson (8) conducted a 3.5-year study, in which he evaluated the incidence of reproductive tract neoplasia in white leghorn hens ranging from 2 to 7 years of age. He found that 24% of all hens developed age-dependent malignant ovarian adenocarcinoma. In evaluating the origin of ovarian tumors in the hen, Fredrickson (8)

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considered both the germinal (surface) epithelial layer and the thecal layer. The epithelial origin of the tumors was supported by the lack of detectable steroidogenic activity and lack of ultrastructural similarity between the tumors and theca layers. Although he did not observe any ingrowth of epithelial layer cells into the cortex of the ovary, Fredrickson (8) reported that there was moderate ultrastructural similarity between the germinal epithelium and ovarian adenocarcinoma cells. In addition, the malignant phenotype of the adenocarcinoma cells was consistent with epithelial cell origin (8).

More recently, Rodriguez-Burford *et al.* (9) further validated the occurrence of ovarian cancer in the hen by demonstrating that ovarian tumors were immunopositive for a panel of antibodies frequently used to characterize human ovarian tumors. In addition, Barnes *et al.* (10) showed that the incidence of ovarian cancer in hens could be manipulated by reproductive hormones/factors. These authors reported that treatment of hens with a progestin (medroxyprogesterone acetate) decreased the incidence of tumors in 4-year-old hens. We have recently reported (11) that apoptosis occurs in the OSE cells in the area of the ovulatory stigma of hens, and aberrations in this process could underlie malignant transformation. In addition, Murdoch *et al.* (12) have shown that epithelial cells near the stigma show evidence of oxidative DNA damage.

Although there has been abundant research on human OSE cells and tumor cell lines, and there has been significant recent interest in the hen as an animal model for the disease, no one has reported the isolation or culture of OSE cells from the ovary of the hen. To further develop this spontaneous model of ovarian adenocarcinoma, we studied the OSE of the hen. A primary aim of this study was to characterize this cell layer with conventional cell markers. In addition, we evaluated these markers on tumor tissue. Ultimately, we characterized cultured OSE cells, which are the likely source of the ovarian tumors of the hen, utilizing these same markers. The ability to isolate and culture these cells provides a tool to examine neoplastic alterations involved in ovarian carcinogenesis. The spontaneous occurrence of ovarian cancer in the hen means that questions related to etiology can be addressed in this model.

Materials and Methods

Animals. Single-comb white leghorn hens (Babcock B300) were individually caged, and were maintained on a lighting schedule of 15:9-hr light:dark cycle (lights on at 0600 hrs). They were provided with a commercial layer diet and had access to water. Animal care and use was in accordance with the institutional animal care and use committee guidelines.

General Design. *Study I—Characterization of Surface Epithelium of Normal Ovary and Ovarian Tumors.* Immunocytochemical and histologic markers were evaluated on paraffin-embedded ovarian sections to determine if these markers were specifically reactive with the chicken epithelial cell layer. Both normal ovarian tissues free of any neoplasms, as well as ovarian tumors that had been diagnosed as epithelial ovarian cancer, were examined with these markers. We evaluated tissues for cytokeratin, vimentin, proliferating cell nuclear antigen (PCNA), and progesterone receptor (PR). The procedure was performed at least three separate times with each marker, and we used ovarian sections from hens of 2–5 years of age. In an effort to identify appropriate tissue to recover proliferating OSE cells, we counted the number of PCNA-positive cells in histologic sections derived from the ovary proper (devoid of all yellow follicles; $n = 6$), small yellow follicles (SYF; $n = 4$), and the fifth largest follicle in the large yellow follicle (LYF) hierarchy (F5 follicles; $n = 4$). Positive cells were expressed as a proportion of the total OSE cells in a field of view. Five random fields of view were counted for each tissue.

Normal ovaries and tumors were fixed in buffered formalin, and paraffin-embedded blocks were prepared and cut by the Cornell Histology Laboratory. Sections were deparaffinized in xylene and rehydrated in reducing concentrations of ethanol, washed in phosphate-buffered saline (PBS), and boiled in 0.01 *M* citrate buffer for 10 min. Tissue sections were blocked with 10% goat serum in PBS for 30 min at 37°C and incubated with primary antibody overnight at 5°C. Primary antibodies were anti-cytokeratin peptide 18 (1:50; clone KS-B17.2; Sigma Chemical Co., St. Louis, MO), anti-vimentin (1:100; clone VIM 3B4; Research Diagnostics, Inc., Concord, MA), anti-PCNA (20 μg/ml; clone PC10; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-PR (20 μg/ml; Clone hPRa 6; Affinity BioReagents, Inc., Golden, CO). Slides were washed in PBS three times and incubated with secondary antibody for 1 hr at 37°C. Secondary antibody was Alexa Fluor 488 goat anti-mouse IgG conjugate (1.0 μg/ml; Invitrogen Molecular Probes, Carlsbad, CA). Following washing, coverslips were applied and tissue sections examined with a Nikon E600 microscope under epifluorescence using fluorescein isothiocyanate excitation and barrier filter sets (Nikon Instruments Inc., Melville, NY). Photographs were taken with an RT slider Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Negative controls were incubated with blocking solution and showed no staining.

*Study II—Characterization of Cultured OSE Cells. **Culture of OSE cells.*** Initial attempts to isolate OSE cells from the ovary of the hen were modeled after those done in humans (13, 14). Scraping the ovarian surface in an area devoid of follicles was difficult to do in the hen, because the mature hen's ovary is normally covered with follicles. We therefore scraped the surface of large yellow follicles and SYFs to isolate the OSE cells. Although the OSE cells were removed from the follicles, they appeared to roll up in balls and failed to attach to the dish, whereas fibroblast and endothelial-like cells attached very readily and grew well. Subsequently, we utilized the explant method (15) modified for the chicken. Initially, we used

small (3–5 mm diameter) explants of ovarian tissue devoid of any macroscopic follicles. Contaminating cells prompted us to culture whole-follicle explants, which had fewer nonepithelial cells. Hens laying regular sequences were selected and euthanized by carbon dioxide asphyxiation at 1.5–3 hrs after oviposition for collection of tissue. SYF (8–12 mm in diameter), which are pedunculated in the hen and completely covered by the surface epithelial cells, were removed aseptically, washed and placed in culture dishes with media (Day 0) and incubated at 37°C under a humid atmosphere of 5% CO_2 in air. Medium was RPMI (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; Gibco), penicillin G sodium (100 IU/ml; Gibco) and streptomycin sulfate (100 μg/ml; Gibco). Follicles were removed on Day 1 and fresh medium was applied. Cultures were maintained for 1, 3 or 10 days and examined for viability (trypan blue exclusion) or characterized with markers as given below.

In a separate study we examined the effect of follicle size on the relative size of cultured OSE cells. We also observed cultures for mitotic nuclei. Hens ($n = 3$) were euthanized and the five LYFs as well as the SYFs were removed from the ovary and cultured as described above. After 24 hrs, the follicles were removed, and the attached cells were washed and fixed. Cells were stained with propidium iodide (PI; 1 μg/ml in PBS) for 15 min at room temperature, washed, and coverslips mounted. Using epifluorescence, the number of nuclei was determined in five randomly chosen fields (2.5×10^{-3} mm^2) for each follicle size. The study was replicated three times.

Characterization of cultured OSE cells. Cultured cells were examined for the protein expression of cytokeratin, vimentin, and PCNA on Days 1, 3, and 10, and for PR on Day 10. Cells were fixed in acetone and milliQ water (80:20) for 3 min on ice, blocked, and treated with primary and secondary antisera, as described above. Negative controls were incubated with blocking solution instead of the primary antibody.

In addition, cultured cells were examined on Days 1, 3, and 10 for the uptake of DiI acetylated low-density lipoprotein (DiI AcLDL; Molecular Probes), a fluorochrome-labeled complex that is taken up by macrophage and endothelial cells *via* scavenger receptors specific for the modified LDL (16). Cells were incubated in media containing 15 μg/ml DiI AcLDL for 1 hr at 37°C, washed, and examined under epifluorescence. Cells taking up the DiI AcLDL showed cytoplasmic staining and were considered to be endothelial or macrophage in origin (16). Negative controls were fibroblast cells, which showed no staining, whereas positive controls were bovine arterial endothelial cells (data not shown). The study was replicated three times using follicles from three hens in each replicate.

Statistical Analysis. Data were analyzed with SAS using the GLM procedure (SAS Institute, Cary, NC). Differences among means were determined by Duncan's multiple range test, with $P < 0.05$ considered significant.

Results

A representative example of an excised normal ovary and one with cancer is shown in Figures 1A and B, respectively. The normal ovary is characterized by a hierarchy of LYF with the largest (F1) destined to ovulate next, followed by the second largest follicle (F2), which will ovulate approximately 26 hrs later, and so on (Fig. 1A). In addition, there are SYF and large white follicles (LWF) that will grow in size and replace the LYF. The ovarian tumors are characterized by firm, white, cauliflower-like nodules, often with fluid-filled cysts (Fig. 1B). Ascites is frequently found in the abdomen of hens with more advanced cases, and metastases are often observed on the serosa of the intestines and oviduct. Photomicrographs of a paraffin section of normal ovary and an ovarian tumor stained with hematoxylin and eosin (H&E) are shown in Figures 1C and D, respectively. The micrograph of the ovarian tumor (Fig. 1D) shows a glandular growth pattern lined predominantly by simple columnar or high cuboidal epithelial cells. In contrast, the age-matched normal ovary (Fig. 1C) is devoid of these gland-like structures.

Study I—Characterization of Normal Ovary and Ovarian Tumors. *Normal Ovary.* The hen's ovary has a layer of flat to cuboidal epithelial cells on the surface (Fig. 2A). These surface cells demonstrated positive staining in the cytoplasm after incubation with a monoclonal antibody to the intermediate filament cytokeratin (marker for epithelial cells; Fig. 2B), and showed no staining when probed with an antibody to vimentin (marker for fibroblast cells; Fig. 2C), although underlying stromal-like cells, as well as follicular granulosa cells, were positive. In addition, many of the OSE cells showed nuclear staining for PCNA and PR (Figs. 2D and E). Analysis of PCNA expression in paraffin sections showed that the percentage of PCNA-positive cells was greater in the OSE on the ovary proper as compared with the OSE on either SYF or F5 follicles (Fig. 3).

Ovarian Tumor. Paraffin-embedded sections of epithelial ovarian cancer demonstrated strong cytokeratin and weak vimentin staining in the gland-like areas (Figs. 4A and B). In addition, the gland-like areas showed abundant PCNA- and PR-positive cells (Figs. 4C and D).

Study II—Characterization of Cultured OSE Cells. *Culture of OSE Cells.* Ovarian tissue explants resulted in colonies of epithelial-like cells, but contaminating cells quickly overgrew the colonies within 3 days. Our efforts to eliminate the contaminating cells were unsuccessful. We therefore used whole-follicle explants in subsequent work. Culture of SYF for 1 day prior to removal from the dish resulted in colonies of OSE cells (Fig. 5A). Cultures often had at least some contaminating cells on the border of the OSE cell colonies initially. By Day 10, contaminating cells had grown more than the OSE cells, although pure patches of epithelial cells were present. Although proliferation of the OSE cells was limited compared with

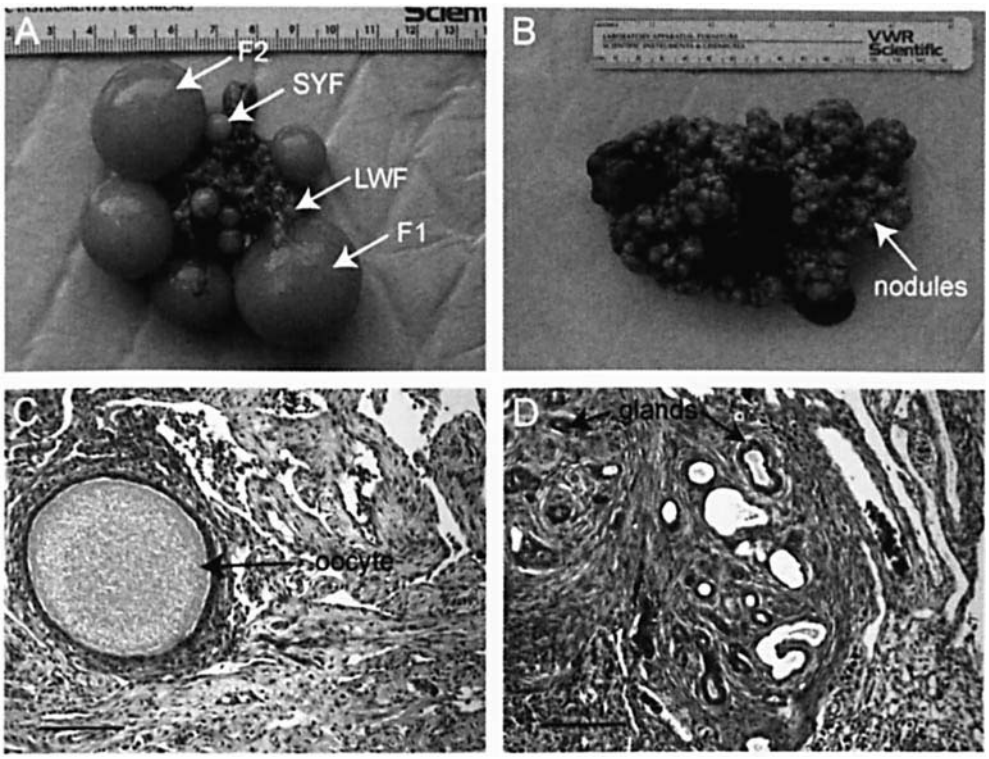

Figure 1. Normal (A) and cancerous ovary (B) from the hen. H&E-stained sections of normal (C) and cancerous (D) ovary. Scale bar, 50 μm. Color figure available in the online version.

contaminating cells, vital dye exclusion confirmed viability of the cells up to at least Day 10.

Characterization of Cultured OSE Cells. OSE cells were positive for cytokeratin and negative for vimentin protein expression on Day 1. Furthermore, the OSE cells did not take up DiI AcLDL (data not shown). Fibroblast cells positive for vimentin, and endothelial cells demonstrating DiI AcLDL staining, were also observed initially, but were minor contributions to the cultures, and were always on the border of the OSE cell colonies. By Day 10, however, these contaminating cells were often crowding the OSE cells, and cytokeratin staining was less for some OSE cells relative to Day 1 (Fig. 5B). Furthermore, although vimentin was absent initially, it was weakly expressed in some cells by Day 10 (Fig. 5C). Examination of cells for expression of PCNA protein showed that, although a few OSE cells were positive on Days 1 and 3 (Fig. 5D), they were rarely positive on Day 10. PR was not initially observed in the OSE cells, although abundant and strong staining for PR was seen after 10 ng/ml of estradiol benzoate was added to the cultures for 24 hr on Day 9 (Fig. 5E).

Examination of cell colonies derived from either SYF or larger follicles and stained with PI showed no differences in morphology (data not shown). In addition, the mean number of cell nuclei counted within defined confluent areas of OSE colonies was not different among the follicle types (data not shown). Mitotic nuclei (Fig. 6) were present on Day 1 in cultures derived from SYF and F5, but were rarely observed in cultures from larger follicles.

Discussion

Similar to the human, the hen spontaneously develops ovarian cancer with an age-related incidence (8). Indeed, few cases of epithelial ovarian cancer have been documented in any species other than the human and the chicken (5). Although OSE cells have been isolated and cultured from a variety of species (2), we are unaware of any reports on the isolation of normal or neoplastic ovarian epithelial cells from an avian species. The identification and characterization of cultured OSE cells from the hen is critical, because these cells are the putative source of ovarian tumors. Although contaminating cells were often observed in our cultures, they were relatively minor contributions initially. Improvements in the culture system could possibly result in pure populations of these cells, which would be useful for studies examining the expression of mRNAs and/or protein that may be associated with pathologic changes in the cells occurring during ovarian carcinogenesis.

We first evaluated our histologic markers on normal ovarian sections to determine their appropriateness for use on cultured OSE cells. Similar to the human (2), the hen has a single layer of flat to cuboidal surface epithelial cells covering the ovarian surface (Fig. 2A). These surface cells demonstrate the cytoplasmic staining pattern of cytokeratin (Fig. 2B), indicative of epithelial cells, and showed no staining when probed with an antibody to vimentin (Fig. 2C). Furthermore, some surface epithelial cells were

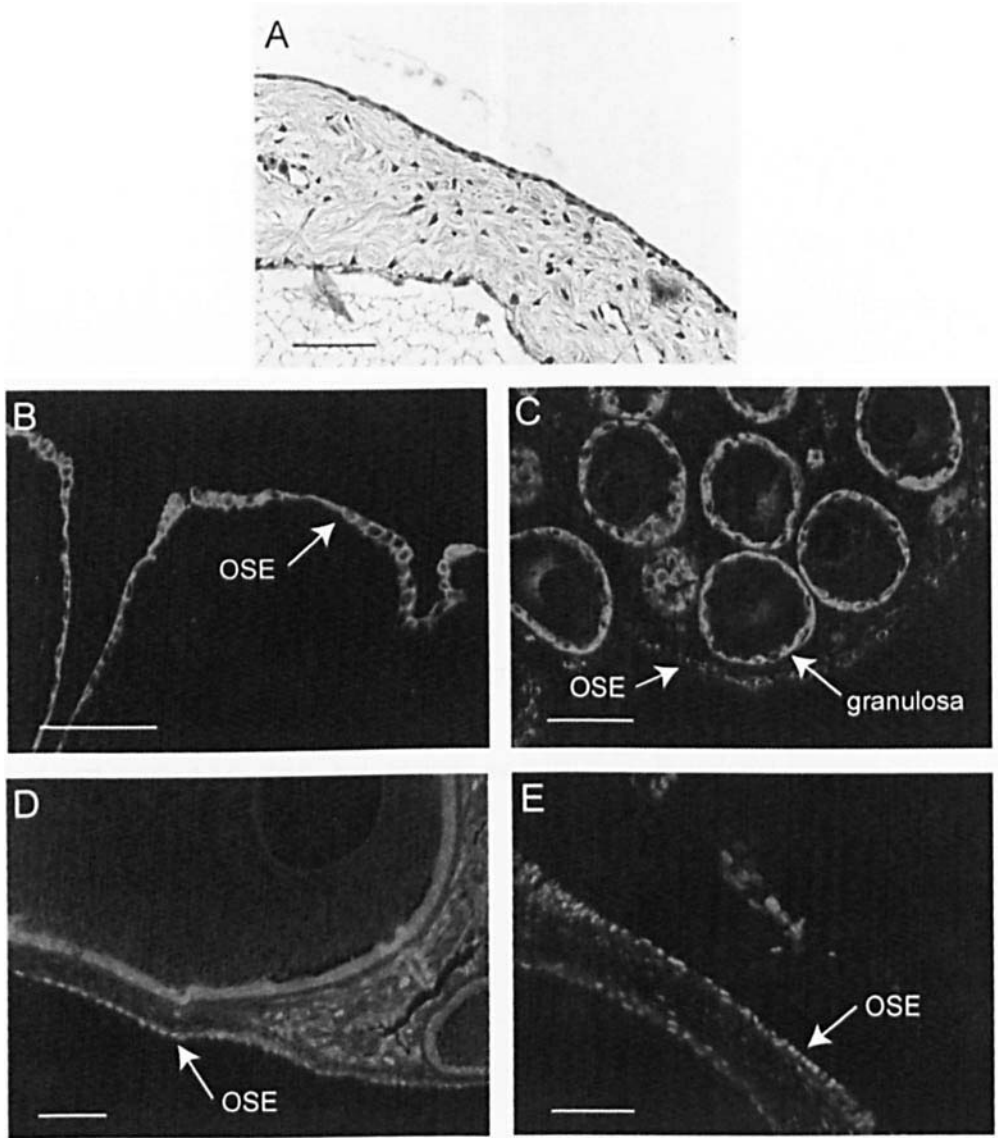

Figure 2. Histologic and immunostaining of normal ovary in the hen (A–E; scale bar, 50 μm). H&E-stained section of normal OSE cells (A); immunohistologic detection of cytoplasmic cytokeratin in OSE cells (B); absence of vimentin staining in OSE cells but strong staining in underlying cells as well as in granulosa cells of the follicle (C); PCNA staining observed in the nuclei of OSE cells as well as in many granulosa cells (D); strong staining for PR observed in the OSE cells (E). Color figure available in the online version.

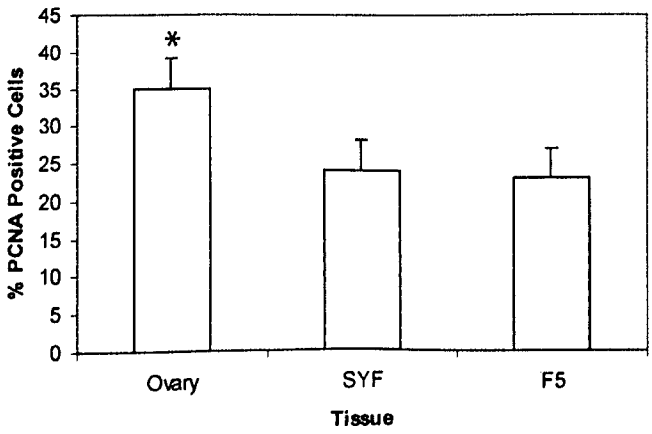

Figure 3. The percentage of OSE cells staining positive for PCNA in paraffin sections of ovary proper, SYF, and F5 follicles ($P < 0.05$).

positive for PCNA, indicating mitotic activity in this layer (Fig. 2D). Surface epithelial cells also demonstrated nuclear staining for PR (Fig. 2E) similar to the human (17). These data agree with those of Isola *et al.* (18), who observed the most intense PR staining in the germinal epithelium of the ovary of the laying hen. Epidemiologic data suggest that progesterone may confer some protection from ovarian cancer in the human (19). Additionally, treatment of hens with a progestin was correlated with reduced incidence of the disease (10).

Evaluation of ovarian sections from hens diagnosed as having epithelial ovarian adenocarcinoma (Fig. 4) indicated strong cytokeratin and weak vimentin staining in the gland-like areas. Furthermore, PCNA and PR were strongly expressed in these areas as well. The abundance of PCNA

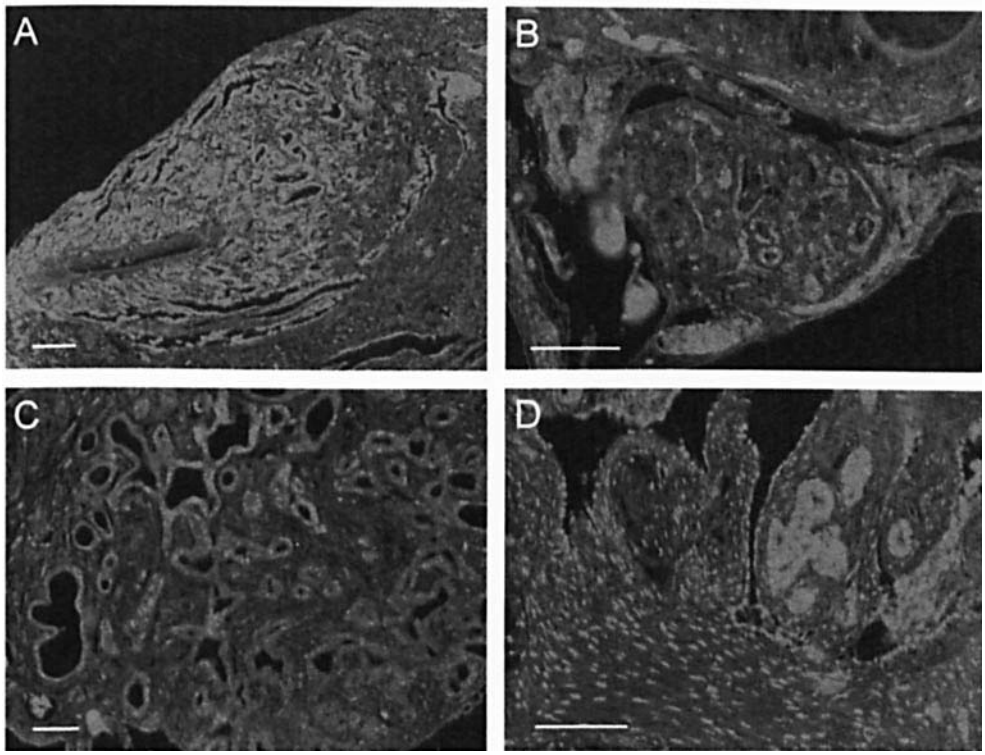

Figure 4. Immunostaining of ovarian cancer in the hen (A–D; scale bar, 50 μm). Cytokeratin staining throughout the glandular-like areas (A); weak vimentin staining also present in these areas, and autofluorescence of red blood cells observed surrounding the nodule (B); nuclear staining for PCNA (C) and PR (D) observed in the glandular areas. Color figure available in the online version.

expression is suggestive of cellular proliferation, and the expression of PR may suggest hormone dependence/regulation of the tumor in the hen. Rodriguez-Burford *et al.* (9) examined ovarian adenocarcinomas from 2-year-old hens with a panel of antibodies that are frequently expressed in human ovarian cancer, and also observed strong cytokeratin and PCNA staining in the ovarian neoplasms.

Cultured OSE cells from a variety of species and human ovarian cancer cell lines have provided insight into the genetics and biology of epithelial ovarian cancer (20). Although scraping of the ovary is commonly used to initiate cultures of OSE cells in many mammalian species (13), this procedure has not been successful in the hen. Observation under a dissection microscope while scraping the follicle showed that cells often came off the surface of the ovary but failed to attach to the culture dish. We tried the explant method after numerous attempts with various substrates and media.

We based our explant method on procedures used in humans (15). Small excised explants of ovary proper, devoid of any macroscopic follicles, were placed into culture. The OSE from this tissue was shown to have a relatively high proportion of PCNA-positive cells, based on observations made on paraffin sections of normal ovary (Fig. 3). Epithelial-like colonies were observed following removal of the explanted tissue, but contaminating cells were also present. Efforts to control the growth of the contaminating cells were unsuccessful and, therefore, we used whole-follicle explants. The culture of SYF for 1 day prior to removal resulted in epithelial-like colonies. Although some contaminating cells were initially observed surrounding the colonies, fibroblasts and endothelial cells often crowded the OSE cultures by Day 10. In subsequent work (data not shown), we reduced this problem by leaving the follicle in culture for less than 1 day and by scraping the dish to remove these contaminating cells early in the culture. Uptake of DiI AcLDL, a marker for endothelial and macrophage cells (16), was not observed for the OSE cells at any time. Endothelial and/or macrophage cells were often present on the margins of the colonies, and were positive for this marker. The OSE cells were positive for cytokeratin throughout the study, but expression had decreased in some cells by Day 10 (Fig. 5). Auersperg *et al.* (13) found primary cultures of human OSE cells to be characteristically, but not invariably, positive for cytokeratin. In the present study, vimentin appeared to be absent initially, but was weakly expressed in some epithelial cells by Day 10 (Fig. 5). In the human, vimentin was expressed in cultured OSE cells (13) and was independent of passage. Furthermore, human OSE cells are known to coexpress cytokeratin and vimentin filaments *in vivo* (21). The cultured cells can undergo epithelial–mesenchymal conversion (2) and lose the epithelial marker, keratin, over time. The reason for this conversion has not been defined, but can be influenced by many factors, including substrates, epithelial growth factor, and sera (2).

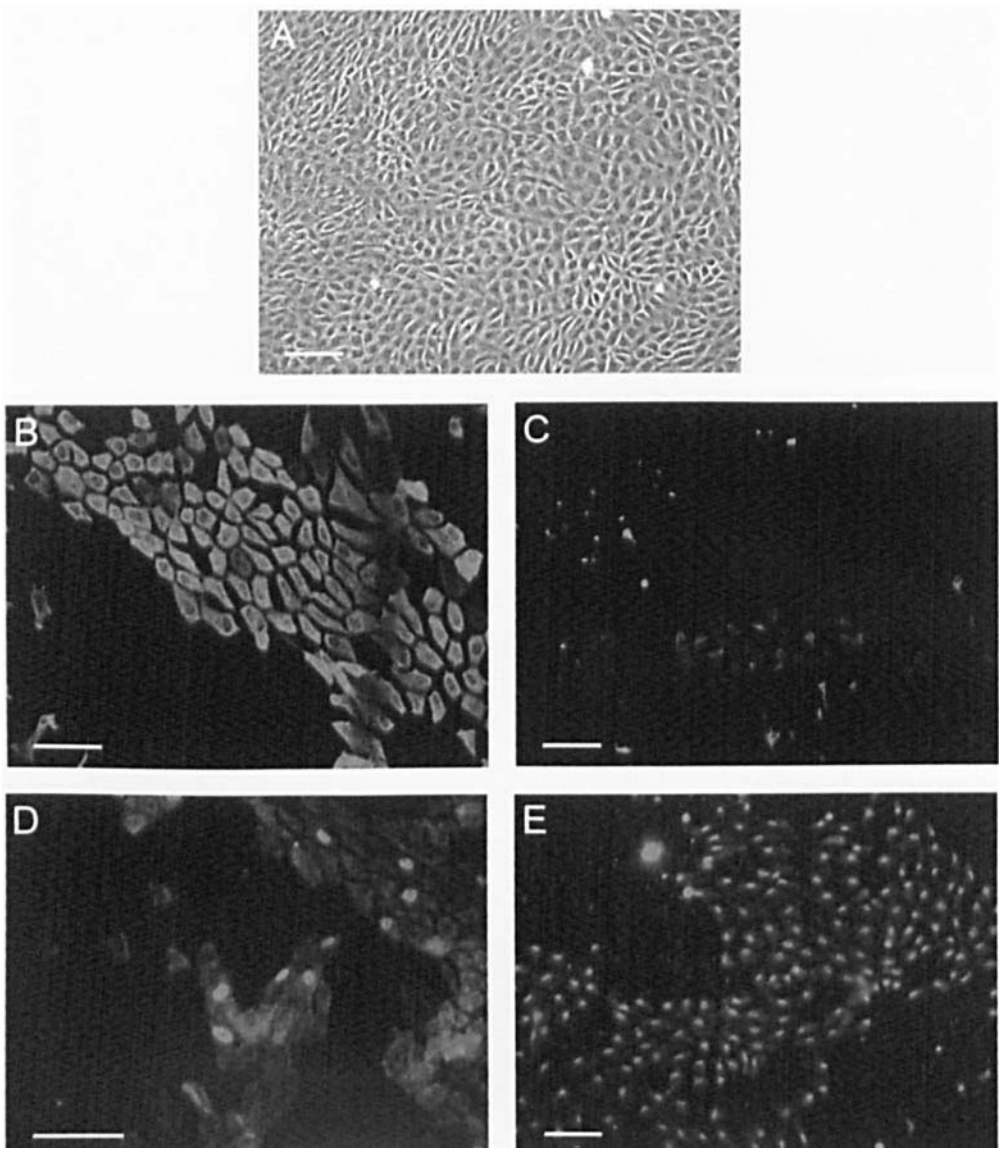

Figure 5. Photomicrographs of cultured OSE cells from the hen (A–E; scale bar, 50 μm). Phase–contrast image of normal OSE cells on Day 1 (A); OSE cells demonstrating positive staining for cytokeratin on Day 10 (B); weak to negative staining for vimentin on Day 10 (C); positive staining for PCNA in a small proportion of OSE cells on Day 1 (D); strong staining for PR on Day 10 in OSE cells cultured in the presence of 10 ng/ml estrogen on Day 10 (E). Color figure available in the online version.

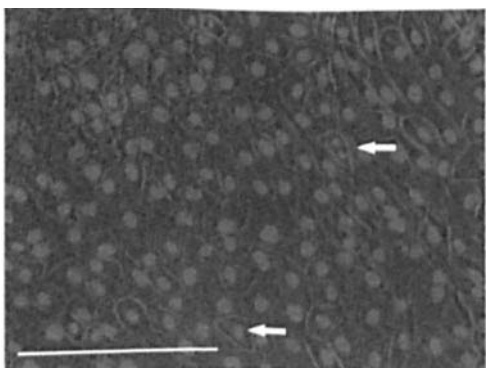

Figure 6. Photomicrograph of OSE cells stained with PI and overlaid with the brightfield image. Arrowheads indicate mitotic nuclei (scale bar, 25 μm). Color figure available in the online version.

Expression of PCNA was observed at Days 1 and 3 (Fig. 5), but was rarely observed in cells cultured for 10 days. PR was evaluated only on Day 10, and was absent in media devoid of estrogen. Addition of estrogen to the media for 24 hrs, however, stimulated expression of PR (Fig. 5). The effect of estrogen on the expression of PR has been previously documented in the hen (18, 22). This normal physiologic response of PR expression after estrogen (18, 22) may be taken as evidence of health of the cultures. However, expression of PR has also been observed in the OSE in the absence of estrogen treatment in the immature chick (23). This finding differs from our observations in the mature laying hen. Although minimal growth was observed in cultures of surface epithelial cells, they were still viable, as determined by viability staining at Day 10.

Our initial studies utilized SYF primarily because this size follicle was more suitable to our culture system. There is considerable variation in follicle size on the hen's ovary and in the rate of growth of these follicles. We hypothesized that there may be a difference in the size or rate of division of the OSE cells related to follicle size. We examined cultures derived from small and larger follicles on Day 1 and observed no differences in the relative size of the cells. Additionally, we observed mitotic nuclei in OSE cells derived from SYF and F5 follicles, suggesting some proliferation was present initially. Although culture conditions may not be optimal for the sustained proliferation of cultured chicken OSE cells, viability was maintained, as well as the expected physiologic response to estrogen.

In related studies, we utilized a variety of media and media combinations, including M199, Dulbecco's modified Eagle's medium (DMEM), DMEM:M199, MEM, MCDB:M199, and Williams' and Waymouth's MB752/1 media (data not shown). In addition, various sera, including fetal calf, cow, and chicken, were used at different times, with levels varying from 0% to 20 % (data not shown). Furthermore, a variety of substrates (plastic, glass, gelatin, laminin, matrigel, fibronectin, lysine, and collagens I and IV) and growth factors (e.g., epidermal growth factor, insulin-transferrin-selenium mixture, estrogen, progesterone, hydrocortisone) have been used. Although contaminating cell types preferred one or another media, no observable difference in growth was seen in the OSE cells. The OSE cells did not attach and/or grow following trypsin or nonenzymatic dissociation procedures, and have not been successfully passaged or frozen.

In summary, we have characterized specific cell markers for the OSE cell layer of the hen and evaluated these cell markers on ovarian tumors. We have also isolated, cultured, and characterized the OSE cells from the hen. The ability to isolate and culture these cells *in vitro* provides the means to examine the potential progenitor cells of ovarian adenocarcinoma and to modify their environment. These studies are important in further developing this unique model of spontaneous ovarian cancer. Although further improvements are needed in the system to prolong and expand these cells *in vitro*, they should prove beneficial in providing insight into the early events associated with ovarian cancer in the hen and ultimately aid in our understanding of the origin of the disease in humans.

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