

Stromal-epithelial interactions in the progression of ovarian cancer: influence and source of tumor stromal cells

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Abstract

Stromal cells are essential for the progression of many cancers including ovarian tumors. Stromal cell-epithelial cell interactions are important for tumor development, growth, angiogenesis, and metastasis. In the current study, the effects of normal ovarian bovine stromal cells on ovarian tumor progression was investigated. The hypothesis tested is that ovarian stromal cells will alter the onset and progression of ovarian tumors. Conditioned medium from normal bovine ovarian surface stromal cells was found to stimulate the growth of normal ovarian surface epithelium and had no effect on the growth of human tumor cell lines SKOV3 and OCC1. Human ovarian cancer cell lines, SKOV3 and OCC1, were injected subcutaneously into nude mice to examine tumor progression. Tumor growth in the nude mice was dramatically reduced when normal ovarian surface stromal cells were co-injected with SKOV3 or OCC1 cells. Similar results were obtained with normal bovine or human ovarian stromal cells. In contrast, irrelevant testicular stromal cells and epithelial cells had no effect on tumor growth in the nude mouse. Histological examination of these tumors revealed a characteristic stromal cell component adjacent to epithelial cell colonies. Sections of these tumors were hybridized with species specific genomic probes using fluorescence in situ hybridization to identify cell populations. Epithelial cells were shown to be of human origin (i.e. SKOV3 or OCC1), but stromal cells were found to be primarily murine in origin (i.e. host tissue). No detectable bovine cells were observed in the tumors after one week post-injection. Results suggest that stromal cells are an essential component of ovarian tumors. Interestingly, normal ovarian stromal cells had the ability to inhibit tumor growth, but were not able to survive long-term incubation at the tumor site. The developing tumor appears to recruit host (i.e. murine) stromal cells to invade the tumor and support its growth. In summary, normal ovarian stromal cells can inhibit ovarian tumor progression and the developing tumors recruit adjacent host stroma to become “tumor stroma”. The tumor stroma likely develop an altered phenotype that cooperates with the tumorigenic epithelial cells to help promote the progression of ovarian cancer. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Ovarian cancer ranks fifth as a cause of cancer related death among women. It is estimated that there will be $\approx 20\,000$ deaths per year from ovarian cancer in the United States (ACS, 1998). Greater than 95% of ovarian cancers originate in the epithelial cells on the

surface of the ovary (Weiss et al., 1977; Piver et al., 1991) referred to as the ovarian surface epithelium (OSE). The OSE is a modified mesothelium covering the surface of the ovary. It is a simple epithelium separated from underlying ovarian stromal tissue by a basal lamina of collagenous connective tissue (Nicosia and Nicosia, 1988). Both the OSE and stroma contribute to the extracellular matrix (ECM) that separates the two cell types (Auersperg et al., 1991). The cellular associations between OSE and stroma have been shown to influence the intermediate filaments in the OSE that may be compared with the early stages of neoplastic

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progression (Hornby et al., 1992). Tumorigenic tissue derived from the OSE also have close associations with stromal tissue (van den Hooff, 1988). Tumor invasion often requires an association with host stromal tissue and most ovarian tumors have a stromal-like component (Scully, 1979; Kurman, 1987; van den Hooff, 1988). Therefore, stromal-epithelial cell interactions appear to have a critical role in the function and growth of tumorigenic OSE. A useful model to study the role of stromal cells in tumor development and growth is the congenitally-athymic nude mouse (nu/nu) (Flanagan, 1966; Pelleitier and Montplaisir, 1975). These severely immune-deficient animals accept transplants of foreign tissue. Many human tumor cells have been injected directly into the subcutaneous space in nude mice and these cells form tumors (Povlsen, 1980). Growth rate and histology of transplanted human tumor cells are relatively constant and predictable (Povlsen, 1980; Mills et al., 1990; Pizer et al., 1996). Human ovarian cancer cell lines (i.e. SKOV3 and OCC1) readily form tumors in nude mice that resemble the ovarian tumors from which they originated. Currently, no other useful *in vivo* ovarian tumor models exist that are of OSE origin. Bovine ovaries recently have been used as a source of ovarian stroma (Parrott and Skinner, 2000, 2000a,b,c). The ovarian surface stromal cells that are adjacent to the OSE produce the mesenchymal factors keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) that can both influence OSE growth. Kit ligand/stem cell factor production by the OSE can act on the ovarian surface stromal cells to promote KGF and HGF production (Parrott and Skinner, 2000, 2000a,b,c). Therefore, ovarian stromal cells and OSE can interact through the local production and action of these growth factors. The current study utilized these cells to examine the effect of normal bovine ovarian stromal cells on human SKOV3 and OCC1 tumor progression in nude mice. The hypothesis tested is that normal ovarian stromal cells will alter the onset and progression of ovarian tumors. Results demonstrate that co-injected normal stromal cells (SCM) can inhibit ovarian tumor progression. Developing tumors were found to recruit host stromal cells (i.e. mouse) suggesting a critical need for stromal cells in the progression of ovarian tumors.

2. Materials and methods

2.1. Tissue isolation and cell culture

Bovine ovaries were obtained from young non-pregnant cycling heifers less than 10 min after slaughter and delivered on ice. Human ovarian tissue was obtained from the UCSF Cancer Center Pathology Core Laboratory directed by Dr. Bethan Powell. Ovaries were

washed twice in Hanks' balanced salt solution (without Ca^{++} , Mg^{++} , or phenol red) containing penicillin, streptomycin, and fungizone. OSE was scraped from the surface of the ovary with a rubber policeman as previously described (Kruk et al., 1990; Vigne et al., 1994). Sheets of epithelial cells were suspended in Hanks' balanced salt solution, pelleted and washed prior to suspension for plating. After the removal of OSE cells, the ovarian surface stromal cells were microdissected from areas of the ovary devoid of follicles. A section of surface stromal cells 1–2 mm wide by 5–8 mm long and 1 mm deep was collected. This contains the layers of stromal cells adjacent to the OSE and was selected from areas of the ovary devoid of developing follicles. The stromal tissue piece was minced and digested with 1 mg/ml collagenase for 2 h at 37°C or 18 h at 4°C. Cells were plated with an initial density of $\approx 10^6$ cells/2 cm², and were maintained at 37°C in 5% CO₂ atmosphere in Ham's F-12 (Gibco, Grand Island, NY) supplemented with 10% calf serum. Medium was changed every 48–72 h. Once the cells had grown to confluence, the cells were trypsinized and split into appropriate plates. The same procedure was used for both bovine and human ovarian stromal cells and the purity of the cell populations were greater than 98% as previously described (Vigne et al., 1994). For injection into nude mice, cells were split into 100 mm large culture plates (Nunc) and maintained in Ham's F-12 supplemented with 10% calf serum. For collection of stromal conditioned media, ovarian surface stromal cells were washed in Ham's F-12 without calf serum for 24 h. Stromal conditioned media were collected, centrifuged to remove debris, and stored at –80°C. For growth assays, cells were plated in 24-well plates in DMEM (Gibco) supplemented with 10% calf serum. When cells achieved 50–70% confluency, cells were washed in DMEM containing 0.1% calf serum for growth assays. The purity of bovine OSE and stromal cells isolated by this procedure is greater than 98% by keratin staining with no detectable cross contamination (Vigne et al., 1994). Human ovarian cancer cell lines, SKOV3 and OCC1, were obtained from American Type Tissue Culture Collection (Rockville, MD). SKOV3 and OCC1 cells were maintained in Ham's F-12 supplemented with 10% calf serum.

2.2. Growth assays

Cell growth was analyzed by quantifying (³H) thymidine incorporation into newly synthesized DNA. OSE, SKOV3, and OCC1 cells were plated at $\approx 50\%$ confluency in 0.5 ml DMEM medium containing 0.1% calf serum. After 48 h, cells were treated with no growth factor (control), 50 μ l (i.e. 10%) stromal conditioned medium (SCM), or 40 ng/ml epidermal growth factor (EGF). Cells were treated for 20 h with growth

factor. This treatment duration corresponds to the appropriate length of a cell cycle. After treatment, 0.5 ml DMEM containing 2.5 μCi (^3H) thymidine was added to each well, and the cells were incubated for 4 h at 37°C and then sonicated. The quantity of (^3H) thymidine incorporated into DNA was determined, as previously described (Vigne et al., 1994). Data were normalized to total DNA per well using an ethidium bromide procedure previously described (Vigne et al., 1994).

2.3. DNA assays

DNA was measured fluorometrically with ethidium bromide as previously described (Vigne et al., 1994). An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide, 100 U/ml heparin in ethidium bromide buffer (EBB:20 mM sodium chloride, 5 mM ethylene diamine tetracetate, 10 mM Tris, pH 7.8); Sigma), was diluted 1:2 with EBB, and was allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantify DNA levels in the culture wells. This assay has a sensitivity of ≈ 0.1 μg DNA.

2.4. Nude mouse injections

Female 4–6 week old athymic nude mice (nu/nu) on a Balb/c background were obtained from Charles River, and used within 2 weeks of arrival. The care and manipulation of mice were in accordance with the approved guidelines of Washington State University for the ethical treatment of animals. Mice were given injections of SKOV3 or OCC1 cells alone or in combination with ovarian surface stromal cells. Cells were injected subcutaneously (i.e. directly into the subcutaneous space) on either side of the back of the mouse. Experiments were designed so that cancer cells (i.e. SKOV3 or OCC1) alone were injected on one side of the mouse, and the same number of cancer cells plus ovarian stromal cells were injected together on the other side of a different mouse. In some experiments an individual injection used an individual mouse. In order to be prepared for injections, cells in culture were trypsinized in STV (trypsin, EDTA; Gibco) for 5 min at 37°C. After gentle removal using a glass pipette, cells were centrifuged and resuspended in Hanks' balanced salt solution. A small aliquot was placed on a hemocytometer and the cells were counted. Cell suspensions were diluted in Hanks' and placed into a syringe for injection. Initially, 1–4 million SKOV3 or OCC1 cells were injected, but this number resulted in unpredictable tumor occurrence. Therefore, ≈ 10 million SKOV3 or OCC1 cells were injected in the current study. When

indicated, ≈ 10 million ovarian surface stromal cells were co-injected. All injected mice contained an observable mass at the site of injection that steadily decreased in size until approximately day 15–20 post-injection. After day 20, an observable tumor mass developed and grew in size. Mice were monitored daily and tumor area was determined every other day for a total of 70–90 days. A minimum of nine mice for each tumor condition were used for each experiment ($n = 9$).

2.5. Tumor histology and fluorescent *in situ* hybridization

Tumors formed in nude mice were excised and placed in Histochoice tissue fixative (Amresco, Solon, OH). Fixed tumors were embedded in paraffin and cut into 7 μm sections. Tumor sections were stained with eosin and hemotoxylin as previously described (Vigne et al., 1994). Tumor sections were assayed for the presence of mouse, bovine and human cells using fluorescence *in situ* hybridization (FISH) with species-specific genomic probes. In this process, paraffin sections fixed on slides were treated in the with: Xylene 2×10 min; 100% ethanol 2×10 min, air dry; 0.1 M NaSCN at 80°C for 1 min; H₂O 2×5 min; 1 mg/ml RNAase at 37°C for 20 min; H₂O 2×5 min; 0.04 mg/ml pepsin in 0.2 N HCl at 37°C for 2 min; H₂O 2×5 min; dehydrated in 70%, 85%, 100% ethanol series, air dried; denatured in 70% formamide in $2 \times \text{SSC}$ (pH 7) at 72°C for 5 min; dehydrated in 70%, 85%, 100% ethanol series and air dried. Probes used for FISH were mouse Cot 1 DNA purchased from Gibco, human genomic DNA extracted from lymphocytes from a female donor, and bovine genomic DNA extracted from calf thymus tissue (Current Protocols in Human Genetics, Appendix 3B, John Wiley & Sons, Inc., 1995). Each DNA sample was labeled by nick translation as previously described (Current Protocols in Human Genetics, 4.6) with Fluorescein-12-d-UTP (NEN) or Alexa 568-5-d-UTP (Molecular Probes). Probe size ranged between 300 and 2000 bp when electrophoresed on a nondenaturing 1% agarose gel. Twenty nanograms of each labeled probe in 10 μl of hybridization mix (final concentration of 50% formamide, 10% dextran sulfate, and $2 \times \text{SSC}$ at pH 7) was denatured at 72°C for 5–10 min. The mix was applied to the denatured tissue section, covered with a 22 mm² cover slip, and allowed to hybridize overnight at 37°C. Following hybridization, the cover slip was gently floated off in $2 \times \text{SSC}$ and the slide was washed 3 times in wash solution at 45°C (50% formamide, $2 \times \text{SSC}$, pH 7), once in $2 \times \text{SSC}$ at 45°C, once in PN at room temperature (0.1 M phosphate buffer, 0.05% NP-40 at pH 8, made by mixing 0.1 M Na₂HPO₄ and 0.1 M NaH₂PO₄ to achieve pH 8), followed by a final rinse in water before air drying. All washes were 10 min each. The sample was counter-

stained with 1 μM 4,6-diamino-2-phenylindole (DAPI) in antifade.

2.6. Statistical analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of treatments on (3H) thymidine incorporation into DNA were analyzed by a one-way analysis of variance (ANOVA). Observed significance probabilities of 0.05 ($\text{Prob} > F$) or less were considered evidence that ANOVA model fits the data. Significant differences between treated cells and control (untreated) cells were determined using the Dunnett's test which guards against the high alpha-size (Type I) error rate across the hypothesis (Dunnett, 1955). This multiple comparison test is recommended for multiple comparisons with control (Hsu, 1996).

3. Results

In order to investigate the potential mechanisms involved in cell–cell interactions between ovarian stromal cells and ovarian tumor cells, the effect of stromal cell secreted proteins on cell growth was examined. Ovarian stromal cells were cultured under serum-free conditions and SCM collected. SCM was then used to treat normal bovine OSE and human SKOV3 and OCC1 cells in culture. As has been previously shown (Vigne et al., 1994), SCM stimulated (^3H) thymidine incorporation into DNA in normal bovine OSE (Fig. 1(A)). SCM had no significant effect on SKOV3 (Fig. 1(B)) or OCC1 (Fig. 1(C)) growth. EGF was used as a positive control and stimulated the growth of all three cell types (Fig. 1). Previously SCM has been shown to reduce the growth promoting ability of EGF on OSE (Vigne et al., 1994). In contrast, SCM at 5 and 50 μg SCM protein/ml did not influence EGF actions on SKOV3 or OCC1 ($P < 0.01$) in three different experiments. These results suggest that normal OSE are influenced by ovarian stromal cells but transformed ovarian carcinoma cells may respond differently than their normal counterpart. The potential effects of ovarian stromal cells on in vivo tumor formation and growth were examined next.

Two human ovarian carcinoma cell lines, SKOV3 and OCC1 (Wong et al., 1990), were injected subcutaneously into athymic nude mice to examine tumor formation and growth. When injected into nude mice these cell-lines develop tumors with a similar morphology as the original tumors. Therefore, SKOV3 and OCC1 cells injected into nude mice can be used as a model to study ovarian tumor progression. Ovarian stromal cells were co-injected (i.e. mixed together) with SKOV3 and OCC1 to examine the effects of normal ovarian stroma on tumor progression. Mice were in-

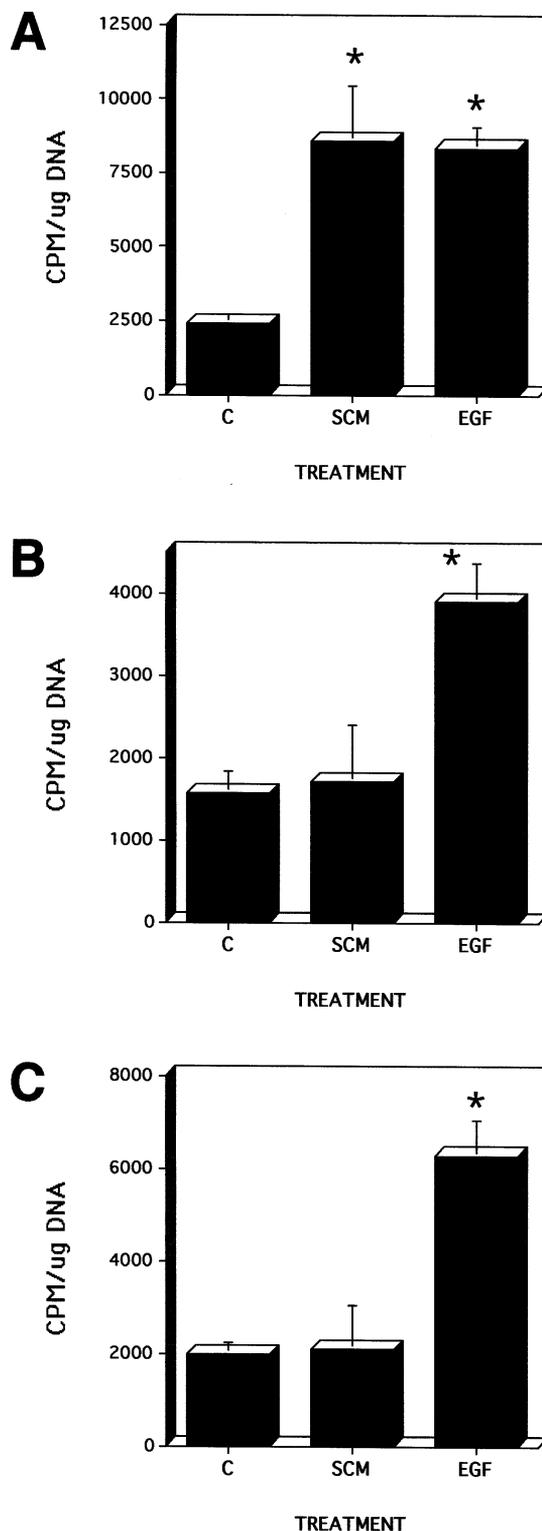


Fig. 1. SCM regulation of DNA synthesis in normal OSE and human ovarian cancer cells: Ovarian surface stromal cells were cultured under serum-free conditions to collect SCM. The effects of SCM on cell growth was determined with a thymidine growth assay. The mean \pm SEM from three different experiments done in replicate is presented. An ANOVA was performed and significant differences from control were determined using the Dunnett's test. Bars with asterisks differ from control ($P < 0.05$). (A) results using normal bovine OSE; (B) results using SKOV3 cells; and (C) results using OCC1 cells.

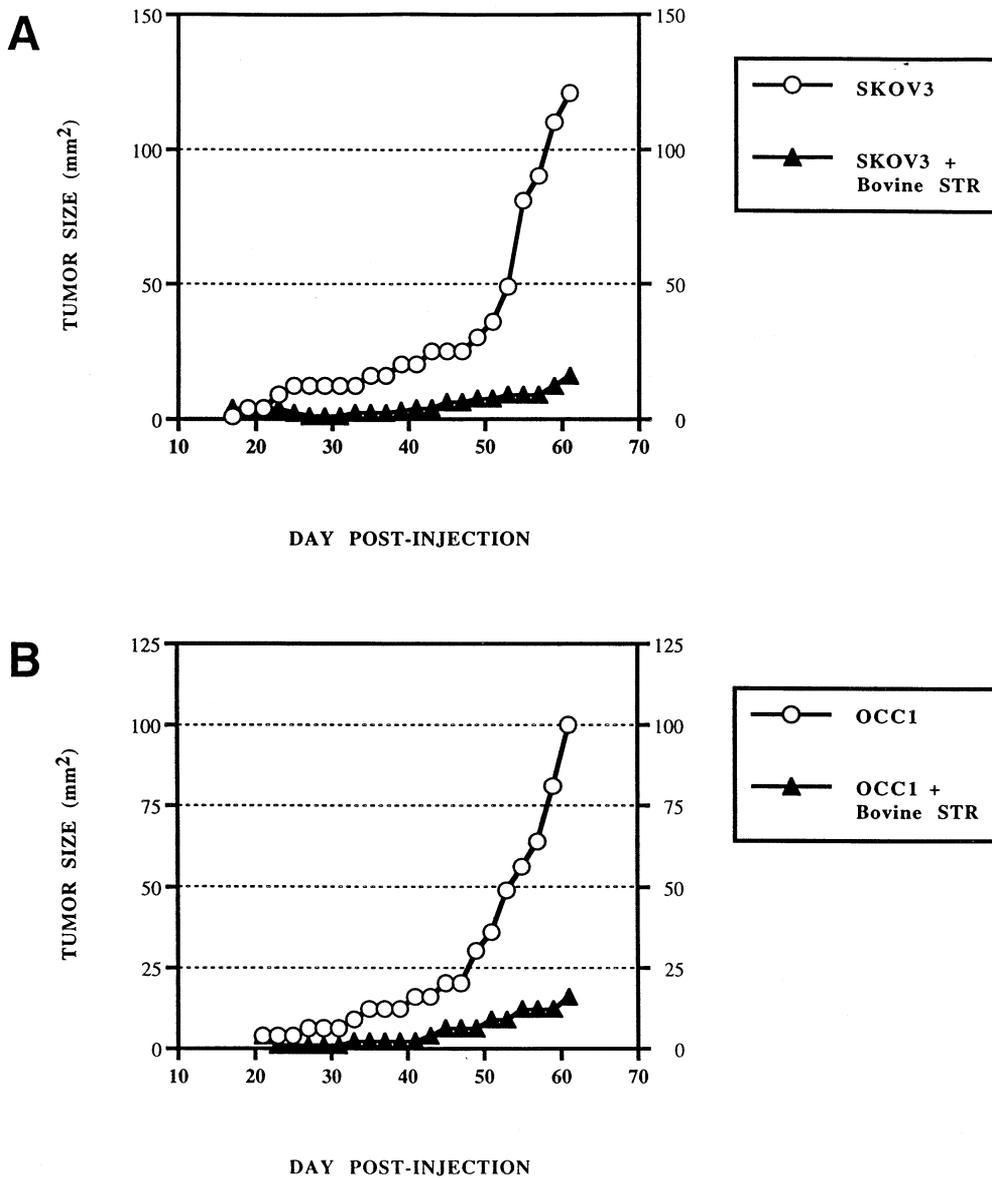


Fig. 2. Effects of ovarian stromal cells on tumor growth in nude mice: Athymic nude mice (nu/nu) were given injections of SKOV3 cells (A) or OCC1 cells, (B) alone or in combination with bovine ovarian surface stromal cells, (C) Injections of SKOV3 cells alone or in combination with human ovarian stromal cells and (D) Injections of OCC1 cells alone or in combination with rat testicular peritubular (stromal) cells (PC) or Sertoli (epithelial) cells (SC). Cells were injected subcutaneously (i.e. directly into the subcutaneous space). Cancer cells (i.e. SKOV3 or OCC1) alone were injected (i.e. 10 million) in the absence or presence of ovarian stromal cells or testicular cells (i.e. 10 million), therefore mixed and co-injected. All injected mice contained an observable mass at the site of injection that steadily decreased in size until approximately day 15–20 post-injection. After day 20, an observable tumor mass developed and grew in size. Mice were monitored daily and tumor area was determined every other day for a total of 70 days. Data are representative for each condition of at least nine experiments for ovarian stromal cells and three experiments for testicular cells. (A and C) results after injecting SKOV3 cells; (B and D) results after injecting OCC1 cells.

jected with either SKOV3 or OCC1 cells alone or co-injected together with normal ovarian stromal cells. As shown, the tumors derived from tumor cells co-injected with normal bovine ovarian stromal cells were smaller than tumors derived from injected tumor cells alone (Fig. 2). The number of tumor cells injected for a specific experiment was equivalent and was ≈ 10 million tumor cells. When Stromal cells were co-injected together with tumor cells, the same number of stromal cells (i.e.

≈ 10 million) were used as tumor cells such that all mice were injected with the same number of tumor cells. After injection, a small but observable mass of cells was present at the site of injection. This mass of cells was similar between injection sites for tumor cells alone or tumor cells combined with stromal cells. Mice were observed daily and the area of each tumor mass was measured every other day. Tumors were measured with calipers on opposing poles to calculate an approximate tumor area.

After injection, the mass of cells continuously regressed down to an area of $\approx 2 \text{ mm}^2$. Prior to 7 days post-injection the mass of cells was not solid and histological analysis not possible. At 14–20 days post-injection, all sites contained small masses that appeared similar with or without co-injected stroma. The approximate tumor cell number and tumor histology was similar for tumor cells injected alone or in combination with ovarian stromal cells (Fig. 3). Tissue collected at day 7, 14, 21, 30 and 60 all had similar morphology (Fig. 3), but the larger tumors had a higher degree of vascularization. After approximately day 20 post-injection, tumor development and growth started to occur at

the injection sites. Both SKOV3 and OCC1 cell lines developed into tumors with predictable growth characteristics. Tumor masses steadily increased in size from $\approx 2 \text{ mm}^2$ up to 100–125 mm^2 (Fig. 2). However, at sites where ovarian stromal cells had been co-injected, tumor growth was inhibited. Tumor growth was negatively affected by both bovine and human ovarian stromal cells (Fig. 2). Therefore, the species appears not to be a factor. By post-injection day 61 there was a remarkable difference in the size of tumors with or without initial ovarian stroma injection (Fig. 2). The data are representative of 9 mice for each tumor condition in a minimum of at least three different experi-

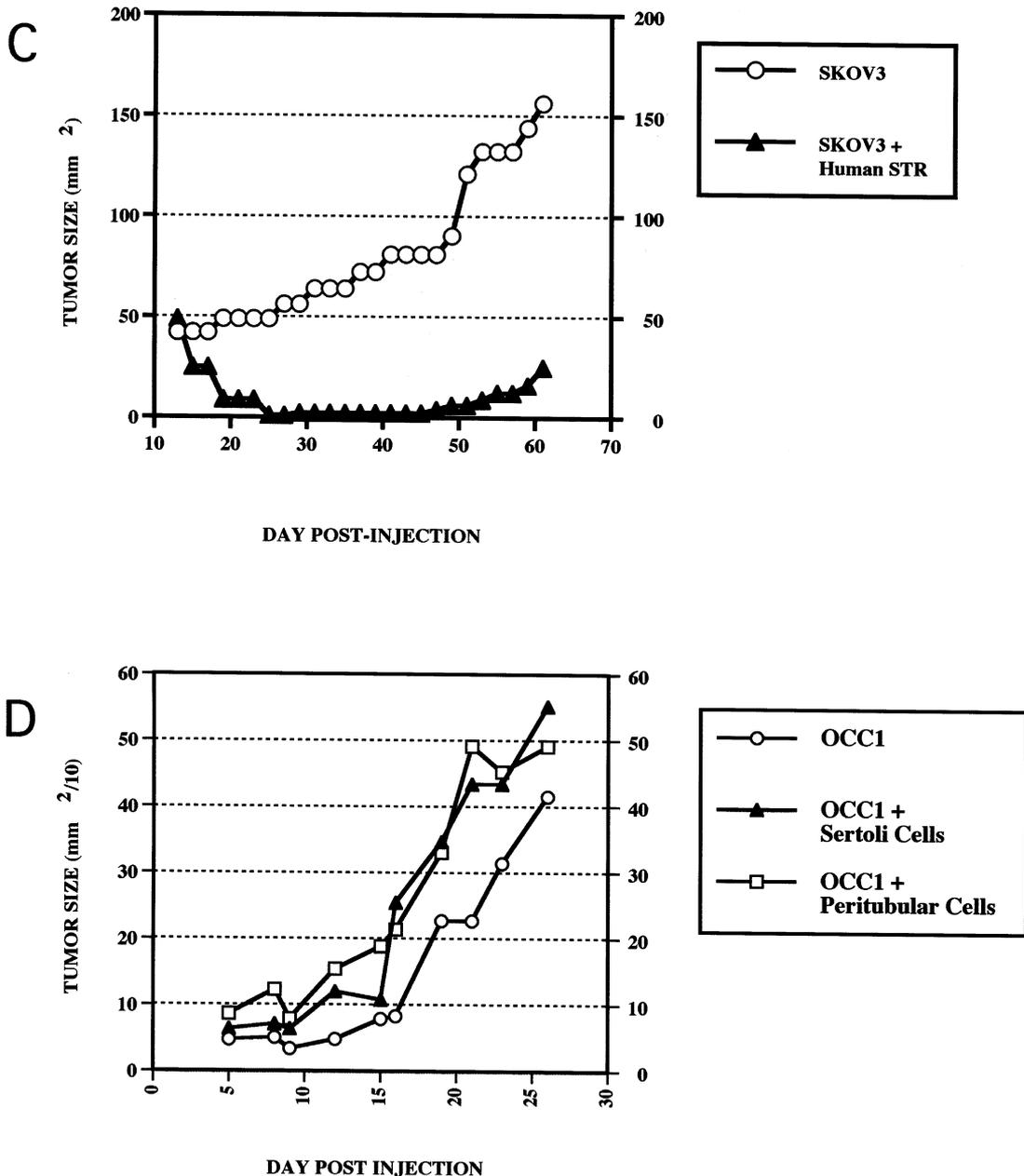


Fig. 2. (Continued)

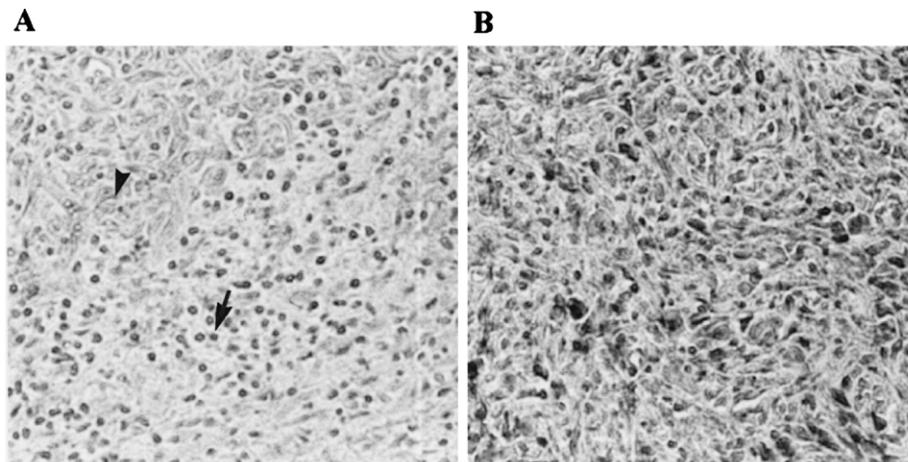


Fig. 3. Histology of SKOV3 nude mouse tumors co-injected with bovine stromal cells. Tumors were isolated after 7 days (A) and 21 days (B) of development. Histology is presented at 200X magnification. The arrow denotes tumor epithelium and arrow head stromal. Representative of at least three different experiments.

ments ($n = 9$). The average percent inhibition of tumor development observed for all the experiments was $80 \pm 15\%$ inhibition when ovarian stromal cells were present. Two mice with stromal suppressed tumors were allowed to develop up to 90 days post-injection. The tumors continued to increase in size, but did not reach the size of tumor cells done at 60 days. The presence of the stromal cells appeared to delay the onset of the tumor and delay the rate of tumor growth. Observations suggest that ovarian stromal cells inhibit early tumor development or growth, but does not completely block tumor formation.

To investigate the specificity of the effects of ovarian stroma cells on tumor progression, several irrelevant cells of non-ovarian lineage were used in the nude mouse tumor model. Testicular peritubular cells and Sertoli cells from 20 day old rats were isolated and purified as previously described (Chaudhary and Skinner, 1999). These cells were used to co-inject with the OCC1 and SKOV3 human ovarian tumor cell-lines. Neither the testicular stromal derived peritubular cells nor the epithelial derived Sertoli cells influenced OCC1 or SKOV3 tumor progression (Fig. 2(D)). Of the three different experiments performed, only a slight enhancement of tumor development was observed with the co-injected testicular cells. Therefore, the procedural aspects of the co-injection of doubling of the cell number does not appear to be the causal factor in the tumor suppression observed with the ovarian stromal cells (Fig. 2(A–C)).

Tumors were excised from the nude mice and characterized for histology and to identify cell populations. The histology of the tumors suggests a defined stromal component that is adjacent to colonies or islands of epithelial tumor cells (Fig. 3). The morphology of the tumor was similar at day 7, 14, 21, and 60 of tumor

development (data not shown). Tumors were potentially composed of cells from three different species. Human tumor cell lines were injected in the absence or presence of bovine stromal cells in a host mouse. Species-specific chromosomal markers were used to distinguish human, bovine, and murine cells. Species-specific probes were fluorescently labeled and hybridized to tumor sections as described in Section 2. After washing, sections were visualized by fluorescence microscopy. To confirm species specific hybridization, each probe was hybridized individually to human lymphocytes, bovine tissue sections, and mouse tumor tissue sections. Each probe displayed species specific hybridization without cross species hybridization (data not shown). As an example, the bovine probe used on a bovine section is shown in Fig. 4(B). FISH results are shown in Fig. 4. The DNA DAPI stain is shown to identify the nuclei of viable cells (Fig. 4(D and F)). As expected, tumors were composed primarily of human epithelial cells derived from SKOV3 or OCC1 cells (i.e. green/yellow staining) (Fig. 4(C)). However, no detectable bovine cells (i.e. green/yellow staining) was observed in the sections suggesting that the bovine ovarian stromal cells were absent (Fig. 4(E)). Similar results were obtained at day 7 post-injection and at day 20 post-injection. As stated above, histological sections could not be obtained less than 7 days post-injection. Therefore, the bovine cells appear to be lost from the tumors within the first week of post-injection. Tumors contained characteristic regions of stromal tissue that was primarily composed of murine cells (i.e. reddish/orange staining) (Fig. 4(C and E)). The presence of murine stromal cells in these tumors suggests that the host (i.e. mouse) tissue was recruited by the tumor. These results suggest that SKOV3 and OCC1 human tumors require a stromal cell component to grow and that these stromal cells appear to be recruited from the host site.

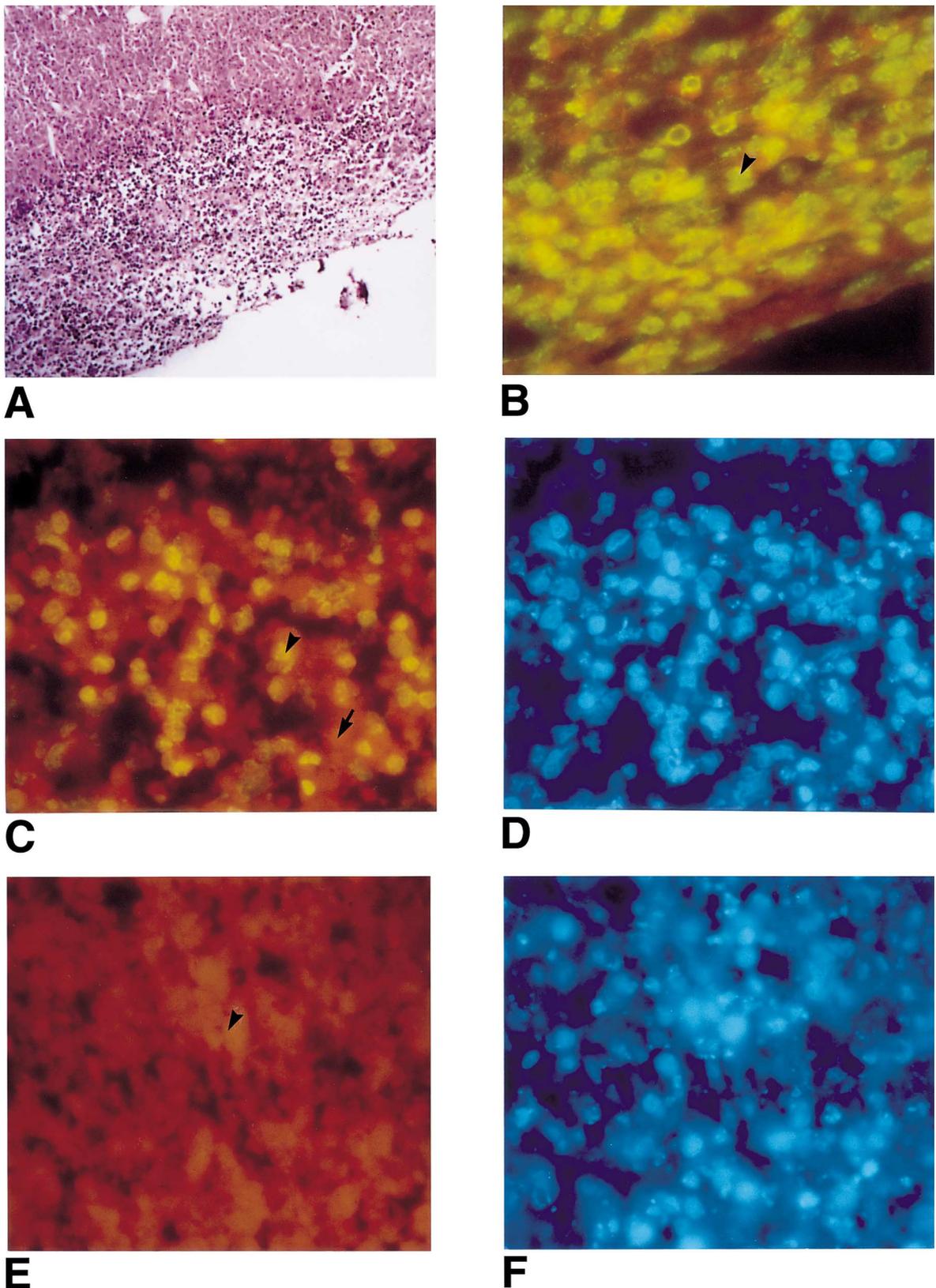


Fig. 4. Species-specific FISH: A tumor from a co-injection of OCC1 cells and bovine stromal cells at 20 days of development was obtained for analysis. Tumor sections were analyzed with species-specific fluorescent DNA probes as described in Section 2 with human (green/yellow), bovine (green/yellow), and murine (reddish/orange) probes. Image A is at 100X magnification. All others are at 600X magnification. (A) Haematoxylin-eosin stained tissue section, (B) positive control: bovine tissue hybridized with FITC labeled calf thymus DNA (arrow denotes positive bovine cell), (C) tumor tissue hybridized with mixture of FITC labeled human DNA (green) and Alexa 568 labeled mouse DNA (red) (arrow denotes human cell and arrow head mouse cell), (D) same region as image C, counterstained with DAPI, (E) tumor tissue hybridized with mixture of FITC labeled bovine DNA (green) and Alexa 568 labeled mouse DNA (red) (arrow head denotes mouse cell) and (F) same region as image E, counterstained with DAPI.

4. Discussion

It has long been recognized that growth and differentiation of epithelial cells is directed by adjacent mesenchymal cells during embryonic development and optimally maintained by adjacent stroma in adult tissues (van den Hooff, 1988; ACS, 1998). Similarly, these types of cell–cell interactions between malignant epithelia and surrounding stroma are postulated to be involved in tumor development and progression (van den Hooff, 1988). Although tumors are primarily derived from epithelial cells, tumors commonly contain a stromal cell component that actively interacts with the tumor epithelia to support tumor progression. The tumor stromal cells are not transformed, but are phenotypically distinct from non-tumor stroma (Vigne et al., 1994). Normal ovarian surface epithelial cells are adjacent to several layers of ovarian surface stromal cells (van den Hooff, 1988) and appear to interact under normal conditions (Parrott and Skinner, 2000, 2000a; Parrott and Skinner 2000b). In the current study, these normal ovarian stromal cells were shown to influence tumor growth and progression in nude mice.

There are several potential factors involved in stromal cell–epithelial cell interactions that influence tumor development and progression. Tumor stroma and epithelia may produce growth factors that influence cell growth, proteases that degrade basement membranes and ECM, and angiogenic factors that promote new blood vessel formation in the tumor. Conditioned medium from Stromal cells was found to contain growth-promoting activity for normal bovine OSE, but did not stimulate growth of SKOV3 and OCC1 cells. This suggests that normal ovarian stroma may promote the growth of normal OSE, but not transformed ovarian cancer cells. SCM contains a mixture of factors that can both promote and inhibit cell growth (Parrott and Skinner, 2000, 2000a,b). Normal OSE, SKOV3, and OCC1 cells were found to respond differentially to various levels of SCM. This could be a factor of the transformed phenotype of the cells and/or simply that bovine cells and human cells may respond differently to SCM. The identification and characterization of factors produced by ovarian stromal cells that influence OSE and ovarian cancer cells remain to be elucidated.

Both SKOV3 and OCC1 developed tumors in nude mice that were inhibited by normal ovarian stromal cells. However, tumor development was not completely inhibited by ovarian stromal cells. After 61 days, sites co-injected with ovarian stromal cells contained tumors that were $\approx 20\%$ the size of those injected in the absence of stromal cells. The histology of all the tumors was similar. The tumors contained pockets of epithelial cells surrounded by regions of

stromal cells. Using species-specific chromosomal markers it was found that the epithelial components were derived from the human tumor cells and the stromal components were derived primarily from murine cells. Observations suggest that bovine ovarian stromal cells did not survive and/or were lost during tumor progression. Therefore, the inhibitory actions of normal bovine ovarian stromal cells primarily occurred during early tumor development in the nude mice. Ovarian stromal cells may have inhibited tumor development at early stages, but may not have survived as the tumor developed. After these ovarian stromal cells degenerated or migrated, tumor development and progression proceeds as expected involving the stromal cells recruited from the host site.

The specific mechanisms of how the ovarian stromal cell influenced the ovarian tumor progression remain to be elucidated. It is possible that growth control or stromal cell secreted products alters growth regulation of the tumor cells. Equally important will be stromal products that alter the differentiated state of the cells to a more normal phenotype. Since the control experiments of irrelevant testicular cells had no effect on tumor progression, procedural aspects of the study do not appear to be the causal factors in the tumor suppression observed. Possible speculations that will now need to be explored are the ability of the normal ovarian stromal cells to reprogram different tumor cells to an altered state not supporting tumor progression (e.g. increase cell cycle control or reduced growth factor production). Alternatively, the presence of normal ovarian stromal cells may alter the phenotype or function of the recruited host site stromal cells that in turn continue to suppress tumor progression after the loss of the normal bovine cells. These potential mechanistic aspects of the phenomena described now need to be investigated and will provide insight into ovarian tumor biology.

Host (i.e. mouse) stroma was consistently observed in the tumors suggesting that stromal cell–epithelial cell interactions were required for tumor development and progression. Breast cancer cells respond to estrogen by producing several factors which influence their own growth, as well as the growth of surrounding stromal cells. These stromal cells in turn produce factors that influence the growth of the epithelial cells (McGrath, 1983; Haslam and Lively, 1985). Mammary gland epithelial cells, which are responsive to estrogen *in vivo* do not respond to estrogen when isolated and cultured *in vitro*. However, this responsiveness is restored when the epithelial cells are co-cultured with mammary stromal cells (Haslam, 1986). These previous studies suggest that estrogen-induced breast cancer growth may both directly and indirectly involve interactions with stromal cells. Similar conclu-

sions may be made regarding observations in the current study for SKOV3 and OCC1 tumor growth in nude mice. Stromal cells appear to be an essential component of the ovarian tumor.

Previous studies have reported that tumor stroma and normal stroma are phenotypically distinct (van den Hooff, 1988). One study found that conditioned medium from stromal cells cultured from benign and malignant tumors had a stimulatory effect on MCF-7 cells, while conditioned medium from stromal cells cultured from normal tissue had an inhibitory effect on the growth of MCF-7 cells (Adams et al., 1988). Another study found that stromal cells from mammary tissue were stimulatory while stromal cells from other tissues had no effect or were inhibitory on MCF-7 growth (Mukaida et al., 1991). A recent study also demonstrated that spontaneous mammary tumors induce VEGF expression in the adjacent “tumor stroma” (Fukumura et al., 1998). Similar effects were found with stromal cells and prostate tumor progression (Chung, 1995; Hayward et al., 1998). These studies support the postulate that stroma can influence tumor progression. The specific alterations in stromal cells that occur in a tumor environment remain to be elucidated. Since normal ovarian stromal cells produced factors that stimulate growth of normal OSE, but not ovarian cancer cells (Fig. 1), it is possible that epithelial cells in ovarian tumors actively recruit ovarian stroma to become ‘tumor stroma’ and support tumor progression. The SKOV3 and OCC1 cells were shown to recruit local host (i.e. mouse) stroma to become such ‘tumor stroma’ in the nude mouse experiments in the current study. Investigation of the functional changes in the tumor stroma will provide insight into ovarian tumor progression.

The current study supports the concept that stromal cells are required for tumor progression. Tumor stromal cells are not transformed and are not likely to show the genetic plasticity that allows transformed cells to rapidly acquire chemotherapeutic resistance. It may be possible to inhibit the actions of stroma and suppress the overall growth of tumors. A better understanding of the role of stromal tissue in the development, growth, and progression of ovarian tumors will help in the potential design of useful therapeutic agents to treat ovarian cancer.

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