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Expression and Action of Kit Ligand/Stem Cell Factor in Normal Human and Bovine Ovarian Surface Epithelium and Ovarian Cancer[1]

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ABSTRACT

Greater than 95% of ovarian cancers originate from the epithelial cells on the surface of the ovary termed ovarian surface epithelium (OSE). A normal aspect of OSE function is repeated proliferation after ovulation, and this is postulated to be involved in part in the onset of ovarian cancer. The hypothesis tested is that locally produced growth factors have an important role in controlling OSE proliferation. The current study investigates the potential role of the growth factor kit ligand (KL)/stem cell growth factor and its receptor c-*kit* in normal OSE biology and ovarian cancer. Human tumors from borderline, stage I, and stage III cases of ovarian cancer were found to express KL and c-*kit* protein in the epithelial cell component by ICC analysis. The stromal cell component of human ovarian tumors contained little immunostaining. Bovine ovarian physiology and endocrinology are similar to the human such that cow ovaries were used as a model system to investigate normal OSE functions. KL and c-*kit* proteins were detected in the OSE from both normal human and bovine ovaries. Adjacent ovarian stromal tissue contained less intense but positive KL and c-*kit* immunostaining. To extend the ICC results, RNA was collected from normal bovine OSE and ovarian stromal cells to examine KL gene expression. KL transcripts were detected in cultured OSE and stromal cells by Northern blot analysis. KL gene expression was found to be high in freshly isolated OSE but low in freshly isolated stroma using a quantitative polymerase chain reaction procedure. Levels of KL gene expression in cultured OSE and stroma increased to high levels. Observations indicate that normal OSE expresses high levels of KL in vivo and in vitro. The actions of KL on the growth of both normal OSE cells and ovarian cancer cells was investigated. KL was found to stimulate the growth of normal OSE cells in a similar manner to epidermal growth factor. Observations demonstrate the production and action of KL by normal OSE cells and ovarian cancer cells. Coexpression of KL and c-*kit* by normal OSE suggests that KL can act as an autocrine factor for OSE. The local production and action of KL on OSE provides insight into normal OSE biology, and a factor that may be involved in the onset and progression of ovarian cancer.

INTRODUCTION

In the late 1800s Spencer Wells first recognized the role of the ovarian surface epithelium (OSE) in the disease referred to as ovarian cancer [1]. Greater than 95% of ovarian cancers originate in the epithelial cells on the surface of the ovary [2]. These OSE cells are modified peritoneal mesothelial cells that undergo a mesenchymal-to-epithelial cell transition during development [3]. Although extensive research has focused on established ovarian tumors, relatively little is known about the normal biology of the OSE that gives rise to ovarian cancer. The local expression of growth factors and their receptors is postulated to be required for ovarian cancer cells and normal OSE cells. The kit ligand (KL, also named stem cell factor, mast cell factor, or steel factor) and its tyrosine kinase receptor c-*kit* are encoded at the steel (Sl) and white spotting (W) loci of the mouse, respectively [4–7]. In adult ovaries, KL is expressed in granulosa cells and has been shown to be important for oocytes [8–11] and theca cells [12] during follicular development. Because OSE cells and granulosa cells are thought to arise from a common cellular lineage, KL may also be important for OSE cells. This study examined the potential role of KL and c-*kit* in normal OSE biology and ovarian cancer.

Cell–cell interactions between OSE cells and ovarian surface stromal cells are proposed to be important for OSE biology [13]. The OSE is a simple epithelium separated from underlying ovarian stromal tissue by a basal lamina [14]. Both the OSE and stroma appear to contribute to the formation of various extracellular matrix components that separate the two cell types [15]. During normal ovarian function the OSE undergoes cyclic changes including the release of enzymes that contribute to the breakdown of the underlying stroma that overlies the preovulatory follicle [16, 17]. After ovulation, the OSE proliferates and covers the area affected by follicular rupture [18]. The alteration in OSE function and growth at ovulation implies that cellular association with the underlying stroma may influence the OSE. When OSE is not in its normal stromal environment, the intermediate filaments in the OSE may be compared with the early stages of neoplastic progression [19]. Tumorigenic tissue derived from the OSE also has close associations with stromal tissue. Ovarian tumors often require an association with host stromal tissue, and most ovarian tumors have a stromal-like component [20, 21]. Therefore, stromal cell–epithelial cell interactions may have a role in the function and growth of normal and tumorigenic OSE.

Kit ligand can have a wide range of activities on germ cells, melanocytes, mast cells, and primitive hematopoietic cells of the myeloid, erythroid, and lymphoid cell lineages [22]. Many of these cell types initiate their developmental program and differentiate in response to KL. It also appears that KL can cause many of these cell types to proliferate. Kit ligand and c-*kit* also play a role in several human cancers such as lung [23], thyroid [24], breast [25], and testicular [26] malignancies [27]. Although the tumor stage information was provided, coexpression of KL and c-*kit* has been reported in gynecological tumors including serous adenocarcinomas and germ cell tumors of the ovary [28]. The

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potential role of KL and c-*kit* in normal OSE biology and in the onset of ovarian cancer remains to be elucidated.

Normal OSE of the rat, rabbit, and human have been isolated and cultured [29–31], but the size and availability of these tissues often limit the use of these models. Bovine ovaries present a useful model for OSE and ovarian stromal interactions. The bovine ovary has essentially the same physiology and size as the human ovary. The cow is a mono-ovulator that ovulates regularly and has an ovarian cycle similar to that of the human. Bovine ovarian cancer of epithelial origin has been reported [32], suggesting that bovine OSE have similar tumorigenic potential as human OSE. Large amounts of fresh bovine ovaries can be obtained and large numbers of fresh OSE and stromal tissue can be isolated from them. Therefore, the bovine ovary provides a useful model for examining the specific cell–cell interactions involving normal OSE. Once established these cellular interactions can be compared with those of human OSE and ovarian tumor cells.

The current study was designed to examine the local production and action of a specific KL and c-*kit* in human and bovine ovarian surface epithelium. The hypothesis is tested that KL and c-*kit* may have an important role in normal OSE and stromal cell functions as well as in ovarian cancer. Information regarding the expression and actions of KL and c-*kit* will be useful in understanding the cell–cell interactions that regulate OSE.

MATERIALS AND METHODS

Tissues

Fresh human adult ovarian tissues were obtained from Dr. Bethan Powell in the Department of Obstetrics and Gynecology at the University of California, San Francisco. The normal human tissues were collected from salpingo-oophorectomy specimens removed for benign diseases from women of child-bearing age. Human cancer tissues were surgically collected from women with borderline, stage I and stage III ovarian cancer. Bovine ovaries were obtained from young nonpregnant cycling heifers less than 10 min after slaughter. When required, ovaries were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Sections were stored at 4°C until immunocytochemistry (ICC) was performed.

*Embedding, Histology, and ICC for KL and c-*kit

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin according to standard procedures. Immunocytochemistry for KL was performed according to standard procedures. Briefly, 5-μm sections were deparaffinized and rehydrated, quenched in 20% methanol:3% hydrogen peroxide, and blocked in 5% serum for several hours at room temperature before incubation with primary antibody polyclonal goat antihuman KL (R&D Systems, Minneapolis, MN) or monoclonal rat antimouse c-*kit* (ACK-2; Gibco, Grand Island, NY) overnight at 4°C. Secondary antibody (biotinylated rabbit anti-goat IgG or goat anti-mouse IgG from Vector Laboratories (Burlingame, CA) was detected by using the Vectastain kit (Vector) and diaminobenzadine (DAB). Slides were counterstained lightly with hematoxylin to visualize the tissue. The KL-positive and c-*kit*-positive cells are stained brown. Antibodies have previously been shown to react with bovine tissue [12].

Stromal Cell and OSE Isolation and Cell Culture

The OSE cells and ovarian stromal cells were isolated fresh (for quantitative reverse transcriptase–polymerase chain reaction [QRT-PCR] studies) or cultured (for Northern and growth studies). The OSE cells were scraped from the surface of the ovary with a rubber policeman as previously described [13, 33]. Sheets of epithelial cells were suspended in Hanks' buffered salt solution and then 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. Sections of surface stromal cells 1–2-mm wide by 5–8-mm long and 1-mm deep were collected. The tissue piece was minced and digested with 1 mg/ml collagenase and 1 mg/ml hyaluronidase for 2 h at 37°C or 18 h at 4°C. Cells were plated with an initial density of approximately 10^6 cells/2 cm^2 and were maintained at 37°C in a 5% CO_2 atmosphere in Ham's F-12 (Gibco) 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. For isolation of RNA from cultured cells, OSE and stromal cells were plated in 100-mm large culture plates (Nunc, Naperville, IL) and maintained in Ham's F-12 supplemented with 10% calf serum. For growth assays, OSE cells were plated in 24-well plates in Dulbecco's minimum essential medium (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 OSE isolated by this procedure is greater than 98% by keratin staining with no detectable stromal contamination [13]. The purity of the freshly isolated and cultured ovarian stromal cells is also greater than 98% with negligible epithelial cells [13]. Human ovarian cancer cell lines, SKOV3 and OCC1, were obtained from the American Type Culture Collection (Rockville, MD).

RNA Preparation

Total RNA was prepared from freshly isolated or cultured cells using Trizol reagent (Gibco). Trizol was added directly to freshly isolated cells or to the culture plate to prevent RNA degradation. Total RNA was used to purify mRNA using the FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA). The RNA was stored at −70°C until use.

Northern Blot Analysis

Total RNA and mRNA from OSE cells and ovarian stromal cells were isolated as described above. Approximately 6 μg of total RNA and 6 μg mRNA were fractionated on a 1.2% formaldehyde-agarose gel. Following fractionation, the RNA in the gel was transferred into Nylon membrane (Hybond N+; Amersham, Arlington Heights, IL) in single-strength MOPS buffer and UV cross-linked. The membranes were then prehybridized (500 mM phosphate buffer, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS) for 2 h at 60°C. The hybridization was carried out at 60°C overnight with 32P-labeled KL probe obtained by random primer extension (Prime-It II; Stratagene, La Jolla, CA) of a bovine KL partial cDNA [12]. The membrane was washed in buffer (0.2 strength SSC, 0.1% SDS) at room temperature for 10 min then 60°C for 20 min. Membranes were exposed to x-ray film (X-OMAT; Kodak, Rochester, NY) overnight at −70°C using an intensifying screen. The membrane was

subsequently stripped and rehybridized with bovine cyclophilin using a similar procedure.

Quantitative RT-PCR Assays

Steady-state levels of KL and cyclophilin (i.e., 1B15) mRNAs were analyzed using a specific QRT-PCR assay for each gene. These quantitative RT-PCR assays have previously been described in detail [12]. The primers used in this quantitative analysis of KL and 1B15 were: KL, 5′-GGA CAA GTT TTC GAA TAT TTC TGA AGG CTT GAG TAA TTA TTG-3′ (5′ primer, 42-mer) and 5′-AGG CCC CAA AAG CAA ACC CGA TCA CAA GAG-3′ (3′ primer, 30-mer) that generated a specific 452-bp KL PCR product; and 1B15, 5′-ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC-3′ (5′ primer, 33-mer) and 5′-ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG-3′ (3′ primer, 33-mer) that generated a specific 105-bp product from all cell types, demonstrating the integrity of the RNA samples. Before reverse transcription, tubes containing total RNA and specific 3′ primers were heated to 65°C for 10 min to facilitate denaturing and cooled to room temperature to facilitate annealing. Total RNA (1 μg) was reverse transcribed for 1 h at 37°C using the following conditions: 1 μg total RNA, 1 μM specific 3′ primers of interest (up to four different primers including 1B15), 0.1 mM dNTPs, 10 mM dithiothreitol, 40 U RNase inhibitor (Promega, Madison, WI), and 200 U M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) in 40 μl RT buffer (50 mM Tris-HCl ph 8.3, 75 mM KCI, 3 mM $MgCl_2$). After 1 h, samples were heated to 95°C for 5 min to inactivate the reverse transcriptase enzyme. Samples were immediately diluted 2.5-fold and carrier DNA (Bluescript plasmid; Stratagene) was added to a final concentration of 10 ng/μl. This concentration of Bluescript carrier DNA (10 ng/μl) was included in all subsequent dilutions of samples and standards. Immediately before amplification, each unknown sample was further diluted 1:10 in order to improve the fidelity of the PCR reaction. Plasmid DNAs (i.e., Bluescript) containing bovine KL or 1B15 subclones were used to generate standard curves from 1 autogram/μl (10^{-15} μg/μl) to 10 pg/μl (10×10^{-9} μg/μl), each containing 10 ng/μl Bluescript carrier DNA. Identical 10-μl aliquots of each sample and standard were pipetted in duplicate into a 96-well reaction plate (Marsh Biomedical Products, Rochester, NY) and sealed with adhesive film (Marsh Biomedical Products) for PCR amplification. By this design it was possible to assay simultaneously 5 known standard concentrations and 40 unknown samples for each gene. Amplification was performed in a Perkin-Elmer 9600 equipped with a heated lid using the following conditions: 0.4 μM each primer, 16 μM dNTPs, and 1.25 U AmpliTaq polymerase in 50 μl GeneAmp PCR buffer (containing 1.5 mM $MgCl_2$; Perkin-Elmer, Irvine, CA). Each PCR amplification consisted of an initial denaturing reaction (5 min, 95°C); 25–31 cycles of denaturing (30 sec, 95°C), annealing (1 min, 60°C), and elongation (2 min, 72°C) reactions; and a final elongation reaction (10 min, 72°C). At least 0.25 μCi of ^{32}P-labeled dCTP (Redivue; Amersham Life Sciences, Arlington Heights, IL) was included in each sample during amplification for detection purposes. Specific PCR products were quantitated by electrophoresing all samples on 4–5% polyacrylamide gels, simultaneously exposing the gels to a phosphor screen for 8–24 h, followed by quantitating the specific bands on a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT samples. Equivalent steady-state mRNA levels for each gene were determined by comparing each sample to the appropriate standard curve. All KL data were normalized for 1B15.

Optimal cycle number for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e., logarithmic phase of PCR reactions). Kit ligand quantitative PCR products were amplified for 33 cycles and 1B15 PCR products were amplified for 25 cycles. The sensitivity of each quantitative PCR assay was below 1 fg, which corresponds to less than 125 fg target mRNA/μg total RNA. For each assay, all samples were simultaneously measured in duplicate resulting in intra-assay variabilities of 8.9% (KL) and 6.5% (1B15).

Growth Assays

Cell growth was analyzed by quantifying [^{3}H]thymidine incorporation into newly synthesized DNA. The OSE cells were plated (approximately 1 million cells/cm^2 providing 50% confluence) in 0.5 ml DMEM medium containing 0.1% calf serum. After 48 h, cells were treated with no growth factor (control), 50 ng/ml KL (R and D, Chicago, IL), or 40 ng/ml epidermal growth factor (EGF) (Sigma, St. Louis, MO). Cells were plated for 48 h and then treated for 20 h. After treatment, 0.5 ml DMEM containing 2.5 μCi [^{3}H]thymidine was added to each well, and the cells were incubated for 4 h at 37°C and then sonicated. The quantity of [^{3}H]thymidine incorporated into DNA was determined, as previously described [13]. Data were normalized to total DNA per well using an ethidium bromide procedure previously described.

DNA Assays

The DNA was measured fluorometrically with ethidium bromide as previously described [13]. 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 approximately 0.1 μg DNA.

Statistical Analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of growth factors on [^{3}H]thymidine incorporation into DNA and differences among fresh versus cultured OSE and stromal cell hepatocyte growth factor (HGF) expression were analyzed by a one-way analysis of variance (ANOVA). Observed significance probabilities of 0.05 (Prob F) or less were considered evidence that an ANOVA model fits the data. Significant differences between treated cells and control (untreated) cells were determined using the Dunnett's test that guards against the high alpha-size (type I) error rate across the hypothesis [34]. Significant differences among fresh versus cultured OSE and stromal cell HGF expression was determined using the Tukey-Kramer HSD (honestly significant difference) test that protects the significance tests of all combinations of pairs [34–36].

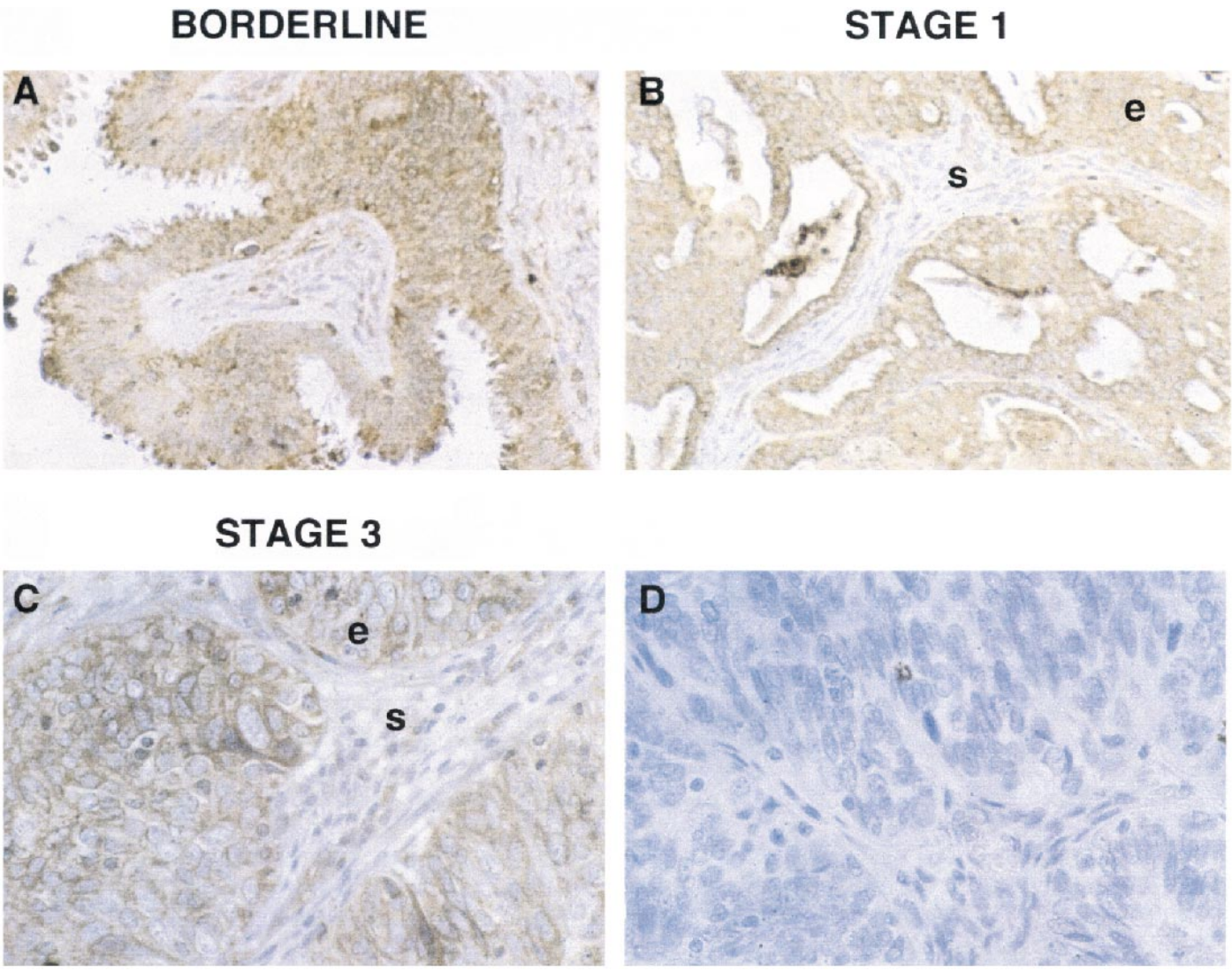


FIG. 1. Kit ligand protein expression in human ovarian tumors. Human ovarian tumors were surgically removed and immediately fixed in 4% paraformaldehyde followed by paraffin embedding. Ovaries were obtained from patients diagnosed with borderline, stage I, and stage III ovarian cancer. Sections were cut at 5 μm thickness and stored on glass slides at 4°C. Immunocytochemistry was performed using a KL antibody as described in the *Materials and Methods.* Sections were visualized and photographed at approximately ×400 magnification. The same results were obtained in four separate experiments using four different ovarian tumors at each stage. (**A**) Borderline ovarian cancer; (**B**) stage I ovarian cancer; (**C**) stage III ovarian cancer; (**D**) control section stained with nonimmune IgG. The brown staining identifies the glandular and colonies of epithelial cells. Epithelial cells are designated (e) and stromal cells (s).

RESULTS

Expression of KL and receptor c-*kit* protein was examined at different stages of ovarian cancer by ICC. Tissues were examined from cases of borderline, stage I, and stage III ovarian cancers. At all stages, KL and c-*kit* protein were detected in the epithelial cell component of the tumor (Figs. 1, 2). Data are representative of four different tumors at each stage. Light staining could also be detected in the stromal cells that border the epithelial cells. Control slides were analyzed using nonimmune IgG and showed no staining. Although ICC is not quantitative, the staining intensity for both KL and c-*kit* decreased in the later stage tumors. Observations support the hypothesis that expression of KL and its receptor c-*kit* proteins may be important during the progression of ovarian cancer. Experiments were next designed to characterize KL and c-*kit* expression in normal OSE and ovarian stromal cells.

Expression of KL and c-*kit* protein were examined in normal human and bovine ovaries by ICC. The surface morphology of the bovine ovary is very similar to that of the human as previously described [13]. A single layer of OSE is present on the outer surface of the ovary adjacent to multiple layers of ovarian stromal cells. In both human and bovine ovaries KL and c-*kit* proteins were detected in the OSE (Fig. 3). Light staining could also be detected in the stromal cells that border the epithelial cells. Some cells of stromal morphology distant from the OSE had slightly more intense staining in both the human and bovine ovaries (Fig. 3). No staining was detected in control slides using nonimmune IgG. Observations suggest that KL and c-*kit* protein are expressed at high levels by normal OSE and to a lesser extent by ovarian stromal cells.

In order to examine KL gene expression further, steady-state levels of KL mRNA were examined in bovine OSE and ovarian stromal cells by Northern blot analysis. Total RNA and poly(A) RNA from cultured OSE and ovarian

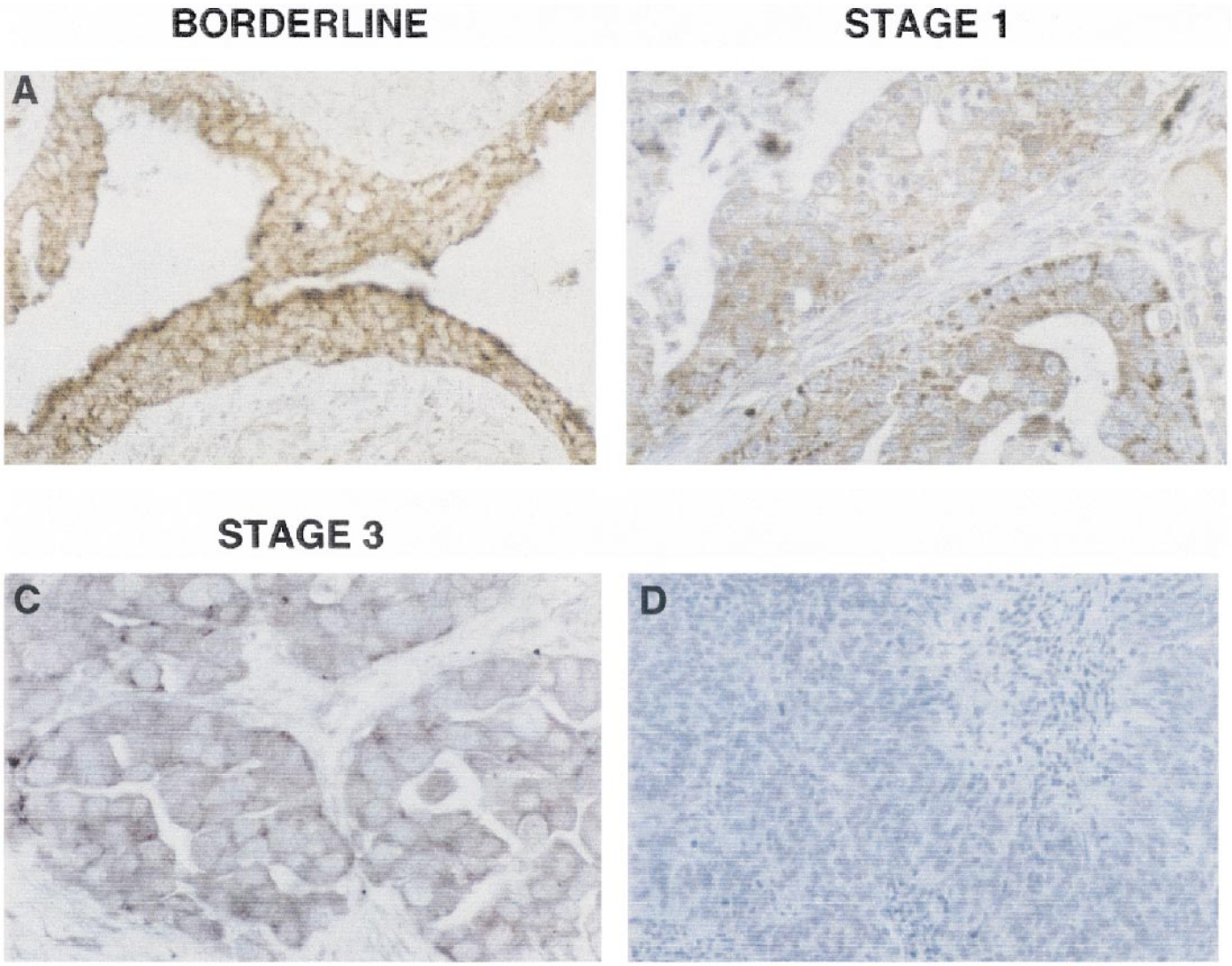
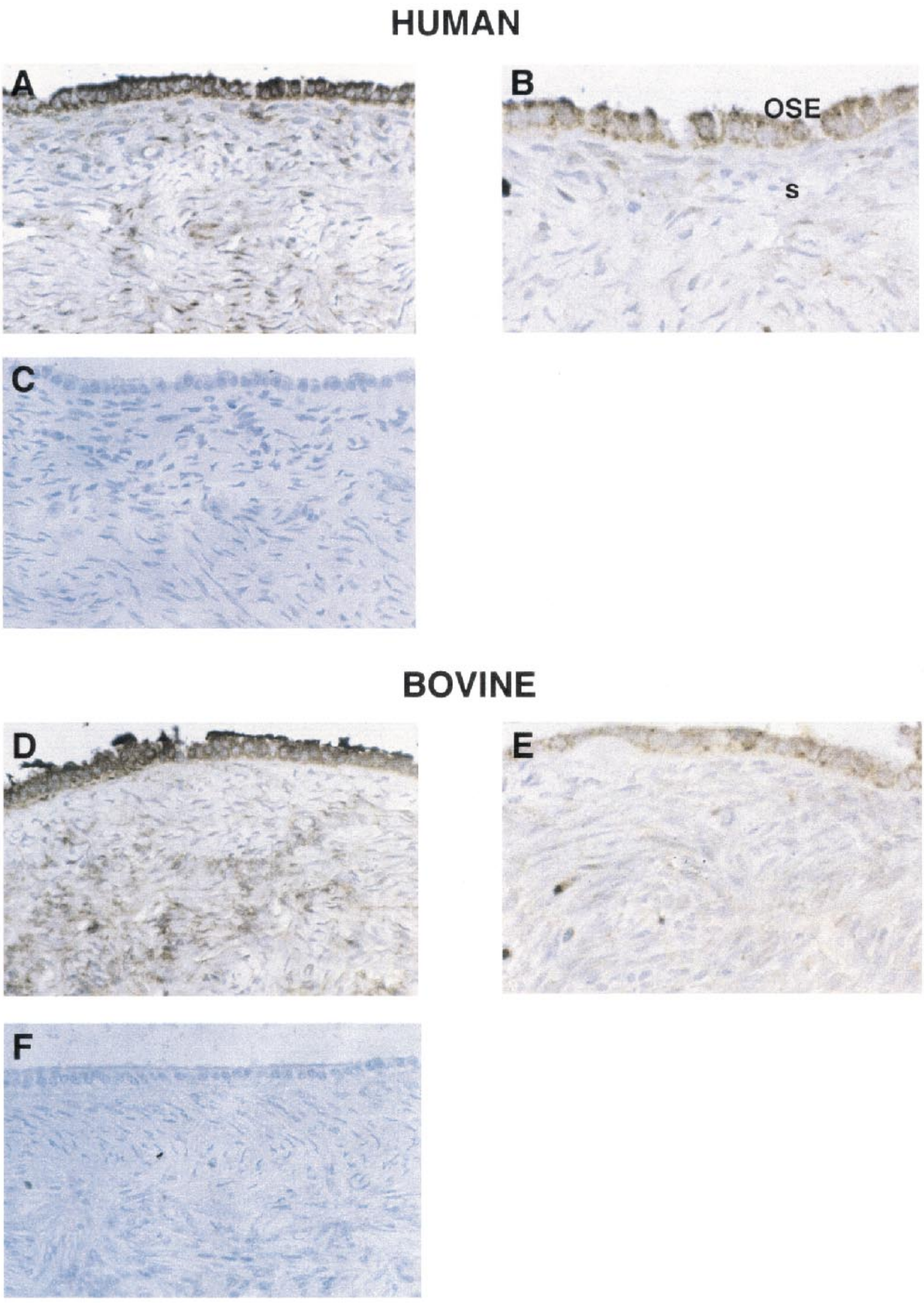


FIG. 2. Protein expression of c-*kit* in human ovarian tumors. Human ovarian tumors were surgically removed and immediately fixed in 4% paraformaldehyde followed by paraffin embedding. Ovaries were obtained from patients diagnosed with borderline, stage I, and stage III ovarian cancer. Immunocytochemistry was performed using a c-*kit* antibody as described in the *Materials and Methods.* Sections were visualized and photographed at approximately 400× magnification. The same results were obtained in four separate experiments using four different ovarian tumors at each stage. (**A**) Borderline ovarian cancer; (**B**) stage I ovarian cancer; (**C**) stage III ovarian cancer; (**D**) control section stained with nonimmune IgG. The brown/gray staining identifies the glandular and colonies of epithelial cells.

stromal cells were probed with a previously obtained bovine KL probe [12]. It was necessary to isolate RNA from cultured cells due to the relatively large amount of RNA required for Northern blots. A specific KL transcript was observed in both OSE and stromal cells with an apparent size of approximately 3 kb (Fig. 4). Bovine granulosa cells were found to have a similar size transcript and rat ovarian tissue had multiple transcripts (data not shown). The transcript size detected in bovine OSE and stromal cells is smaller than the primary transcript detected in mice [8, 37, 38] by Northern analysis. However, several KL and c-*kit* transcript sizes have been detected in rat testis [39] and mouse granulosa cells [8]. Up to five alternatively spliced transcripts have been observed in human cells [40]. It was necessary to examine poly(A) RNA from OSE cells and ovarian stroma in order to obtain a detectable KL band by Northern analysis. The level of expression was apparently similar in cultured ovarian surface stromal cells and cultured OSE cells. Blots were reprobed with a cyclophilin probe (1B15) to demonstrate integrity of the RNA. These results suggest that both normal OSE and ovarian surface stromal cells express the KL gene when placed in cell culture. Experiments were further designed to quantitate KL gene expression in freshly isolated OSE and ovarian stromal cells by quantitative PCR analysis.

FIG. 3. Kit ligand and c-*kit* protein expression in normal human and bovine ovarian tissue. Normal human ovaries were surgically removed and bovine ovaries were obtained from a slaughterhouse. Ovaries were immediately fixed in 4% paraformaldehyde followed by paraffin embedding. Immunocytochemistry was performed using a KL or c-*kit* antibody as described in the *Materials and Methods.* Sections were visualized and photographed at approximately 400× magnification. The same results were obtained in four separate experiments using four different human ovaries. (**A**) Human ovary stained with KL antibody; (**B**) human ovary stained with c-*kit* antibody; (**C**) human ovary stained with nonimmune IgG; (**D**) bovine ovary stained with KL antibody; (**E**) bovine ovary stained with c-*kit* antibody; (**F**) bovine ovary stained with nonimmune IgG. The brown staining identifies the ovarian surface epithelium labeled (OSE) and stromal cells are labeled (s).



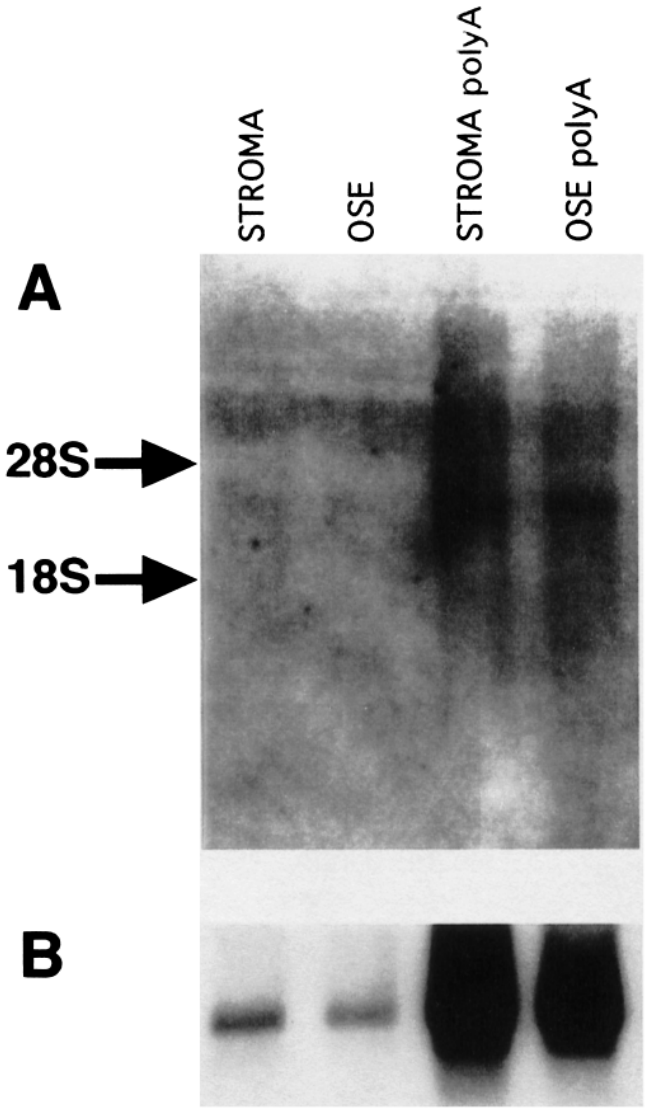


FIG. 4. Kit ligand mRNA expression in OSE and ovarian stromal cells. Bovine OSE cells and ovarian stromal cells were isolated and grown to confluence as described in the *Materials and Methods.* Total RNA was isolated from cultured cells, and mRNA was purified from total RNA. The RNAs were separated on 1.5% agarose gels, blotted to nylon membranes, and probed for KL mRNA expression. (**A**) Probes were prepared using random primer extension of a previously isolated KL subclone isolated from bovine ovarian cells [12]. (**B**) Membranes were reprobed with constitutively expressed cyclophilin (1B15) to control for differences in loading and transfer.

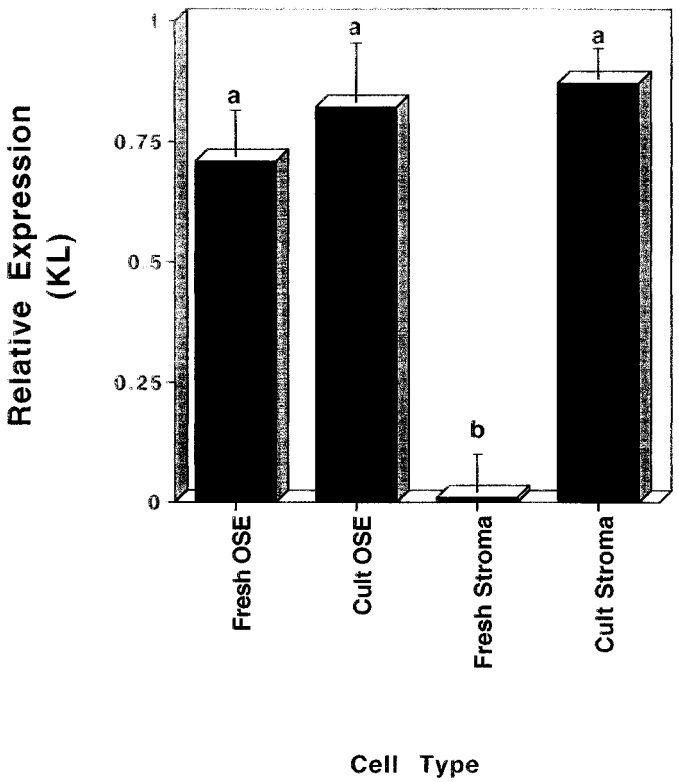


FIG. 5. Quantitation of KL mRNA expression in OSE and ovarian stromal cells. Bovine OSE cells and ovarian stromal cells were isolated and grown to confluence as described in the *Materials and Methods.* Total RNA was extracted from freshly isolated cells or cultured cells. Steady-state levels of KL mRNA were determined in replicate using quantitative RT-PCR as described in the *Materials and Methods.* Levels of KL mRNA were determined and normalized to levels of cyclophilin (1B15) mRNA (fg KL mRNA/fg 1B15 mRNA). Data are presented as the mean ± SEM of duplicate determinations from 8 to 12 different sets of RNA from each cell type. An analysis of variance was performed and significant differences among cell types were determined using the Tukey-Kramer HSD test. Bars with different superscript letters differ from each other ($P < 0.05$).

The KL gene expression in normal OSE and ovarian stromal cells was examined using a sensitive quantitative RT-PCR assay [12]. Total RNA was isolated from 8 to 12 different preparations of freshly isolated normal OSE and ovarian surface stromal cells. Samples were reverse transcribed using the specific 3′ primers of the KL and cyclophilin (i.e., 1B15) genes. Next, samples were simultaneously amplified by PCR along with known KL or 1B15 standard plasmids to quantify gene expression. Steady-state levels of KL mRNA expression were determined and normalized for the constitutively expressed gene cyclophilin, termed 1B15. Normalization for 1B15 expression corrected for changes in cell number, for the amount and integrity of initial mRNA, and for the efficiency of reverse transcription between samples. The small error in the data shown in Fig. 5 between different experiments suggests a high level of reproducibility of the data and minimal variability in the procedure. Consistent with the results from Northern analysis, KL gene expression was observed in cultured OSE and ovarian surface stromal cells (Fig. 5). Kit ligand expression was higher in freshly isolated OSE cells than in freshly isolated stromal cells. Fresh stromal cells had low but detectable levels of KL gene expression. These results demonstrate that KL gene expression is higher in freshly isolated OSE cells, but ovarian stromal cells have the capacity to express KL in cell culture. The potential physiological significance of these observations is discussed.

The ability of KL to influence the growth of bovine OSE cells was investigated. The OSE were plated at 50% confluence and maintained in 0.1% calf serum for 48 h. Cells were then treated with 50 ng/ml KL for 20 h followed by a 4-h incubation with [^{3}H]thymidine. Cells treated with 40 ng/ml EGF or 10% calf serum were used as positive controls. Higher doses of growth factor (100 ng/ml) gave similar results (data not shown). The KL and EGF have previously been shown to react on bovine cells [13]. Kit ligand was found to stimulate DNA synthesis in bovine OSE (Fig. 6) similar to EGF. The combined treatment of KL and EGF did not consistently exceed that of EGF alone (data not shown). The high level of stimulation with calf serum was likely due to the presence of multiple growth factors such

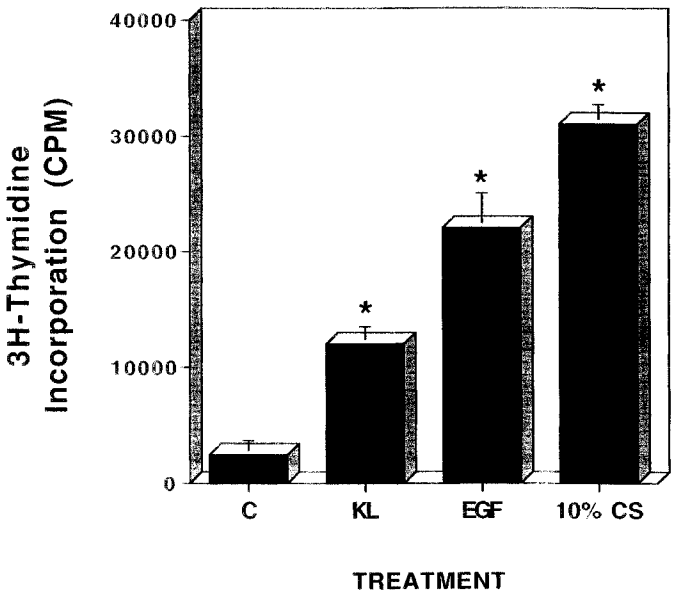


FIG. 6. Kit ligand regulation of DNA synthesis in bovine OSE cells. Bovine OSE cells were isolated as described in the *Materials and Methods.* Cells were deprived of serum when approximately 70% confluent, and [3]H-thymidine incorporation into DNA was determined after 20 h of treatment with 50 ng/ml KL, 40 ng/ml EGF, or 10% bovine calf serum. Cells were incubated with [3]H-thymidine for 4 h. Data are representative of four different experiments done in replicate. An analysis of variance was performed, and significant differences from control were determined using Dunnett's test. Bars with asterisks differ from control ($P < 0.05$).

as insulin-like growth factor. The ability of KL to promote the growth of OSE suggests that KL may be involved in the normal growth functions of OSE. Two ovarian cancer cell lines, SKOV3 and OCC1, did not appear to grow in response to KL under the conditions tested (data not shown). These observations show that KL may be involved in regulating growth in normal OSE biology, and its role in regulating the growth of ovarian tumor cells remains to be elucidated.

DISCUSSION

The autocrine actions of growth factors to stimulate OSE proliferation have been proposed to be involved in the onset and progression of ovarian cancer [41]. The local production of these growth factors may also be important for the normal biology of the OSE. The current study demonstrates that KL and its receptor c-*kit* may be important for normal OSE and ovarian cancer. This year some 30 000 new cases of ovarian cancer will be diagnosed in the United States, and it is estimated that there will be about 20 000 deaths from ovarian cancer [1, 2]. Ovarian cancer accounts for 4% of all cancers in women. Ovarian cancer ranks fifth as a cause of cancer deaths among women. In order to understand the factors involved in the onset and progression of ovarian cancer, it is necessary to examine the expression and action of growth factors such as KL in normal OSE and ovarian cancer.

Several stages of human ovarian cancer were found to express KL and c-*kit* protein. Kit ligand and c-*kit* were immunologically observed in the epithelial components of borderline, stage I, and stage III ovarian tumors. Although not quantitative, the intensity of the ICC stain was decreased in the more advanced stage of ovarian tumors. This implies that KL may have a more important role in the earlier stages of ovarian tumors. The decreased staining in advanced tumors suggests that KL expression may be reduced and/or lost with advanced stages of transformed tumor cells. The presence of detectable levels of KL at all these stages of ovarian cancer suggests that expression of KL may have a role in the development and/or progression of the disease.

Expression of KL in normal OSE appears to be similar between human and bovine ovaries. The high level of KL and c-*kit* detected in OSE by ICC suggests KL will likely have a role in regulating normal OSE. The adjacent stromal cells had low but detectable staining. It remains to be determined if the OSE production of KL may act in a paracrine manner to influence adjacent stromal cells.

Gene expression studies of KL in OSE and ovarian stromal cells were consistent with KL protein expression. Steady-state levels of KL mRNA were observed in both OSE and ovarian stromal cells. Using cultured cells, similar levels of KL transcripts were observed in ovarian stromal cells and OSE cells by Northern analysis. When examining KL gene expression in fresh versus cultured cells using a more quantitative RT-PCR procedure, steady-state levels of KL mRNA were found to be high in freshly isolated OSE and low in fresh stroma. Kit ligand expression was elevated in ovarian stromal cells after cell culture, suggesting that these cells have the ability to express high levels of KL. The potential role of KL production by subsets of ovarian surface stromal cells remains to be elucidated.

The hypothesis that OSE-derived growth factors stimulate OSE proliferation in an autocrine manner is supported by the ability of KL to stimulate normal OSE proliferation. The proliferation of OSE is an important aspect of normal OSE biology. Ovulation creates a wound at the ovarian surface. During the repair, OSE cells proliferate from the perimeter of the ruptured follicle and cover the area affected by ovulation [18, 42]. Over time, repeated ovulations are thought to cause the OSE to proliferate abnormally. As a result, there is an increased risk of ovarian cancer in nulliparous women. This concept has been termed the ''incessant ovulation'' hypothesis [43]. Although this concept of repeated OSE proliferation is well accepted, little information is available concerning the factors involved in normal OSE growth. The coexpression of KL and c-*kit* in the normal OSE and the action of KL on normal OSE suggest that KL/c-*kit* are involved in OSE proliferation. Interestingly, KL had no effect on the proliferation of the two ovarian cancer cell lines examined. Whether this is a unique aspect of these transformed tumor lines or if ovarian tumor cells lose responsiveness to KL in vivo and/or in vitro remains to be examined. These lines do form tumors in nude mice that are histologically similar to human tumors.

The potential role of the ovarian surface stromal cells to promote OSE proliferation through the production of KL is less clear. Bovine ovarian stromal cells have the capacity to express high levels of KL in vitro. However, freshly isolated ovarian stromal tissue contains low levels of KL expression. One possibility is that subpopulations of ovarian surface stromal cells normally express KL, and these cells are preferentially selected in short-term cell culture. An alternative explanation is that the culture conditions induce KL expression in ovarian surface stromal cells. Perhaps the production of KL by ovarian stromal cells is an unusual condition that may promote abnormal OSE proliferation.

The current study is the first report of the expression of KL and c-*kit* in normal human and bovine OSE. However, previous studies have examined the expression of KL and c-*kit* in rat OSE and in cell lines derived from ovarian can-

cers. Kit ligand and c-*kit* were found to be expressed in several gynecological tumors including some ovarian cancers [28]. Immortalized cultures of rat OSE cells (ROSE 199) have been reported to express KL but not c-*kit* [44]. In this same study, primary cultures of normal human OSE did not express KL or c-*kit*, but two human ovarian cancer cell lines expressed KL and c-*kit* [44]. In another study, primary cultures of normal and immortalized rat OSE cells expressed KL that could be regulated by dcAMP [45]. These studies suggest that there may be differences in expression of KL and c-*kit* between rat and human OSE. In the current study, expression of KL and c-*kit* was shown to be similar between bovine and human OSE cells from normal ovaries and ovarian tumors. Therefore, this study helps establish the bovine ovary as a useful model for human OSE functions and suggests that studies involving the bovine ovary may provide information concerning normal and abnormal human OSE that cannot be obtained using rat ovaries.

In the current study, the role that KL and c-*kit* may have in mediating cell–cell interactions involving OSE was investigated. Human and bovine OSE cells were shown to express KL and c-*kit*. Expression of KL and c-*kit* was observed in normal ovaries and ovarian tumors. The role and c-*kit* expression in ovarian surface stromal cells is not known. These observations support the hypothesis that expression of KL and c-*kit* may play a role for KL in normal OSE biology and ovarian cancer. The autocrine actions of KL on other tissues have been previously observed [46, 47]. Both OSE cells and ovarian surface stromal cells had the capacity to express high levels of KL in vitro, but fresh OSE cells appeared to express higher levels of KL than fresh stromal cells. Kit ligand stimulated the growth of OSE cells, supporting the role of KL in OSE biology. Further analysis of the actions of KL on OSE is required with particular attention to the combined effects with other growth factors. Coexpression of KL and c-*kit* in normal OSE cells may help explain why this epithelial cell is susceptible to transformation.

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