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Expression and action of transforming growth factor beta (TGFβ1, TGFβ2, TGFβ3) in normal bovine ovarian surface epithelium and implications for human ovarian cancer

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Abstract

The majority of ovarian tumors are derived from the single layer of epithelial cells on the surface of the ovary termed the ovarian surface epithelium (OSE). Stromal cell–OSE interactions are postulated to be an important aspect of normal OSE biology and the biology of ovarian cancer. Transforming growth factor beta (TGFβ) has been shown to often be a mesenchymal cell-derived growth factor that mediates stromal cell–epithelial cell interactions in a variety of different tissues. The current study investigates the expression and action of TGFβ isoforms (TGFβ1, TGFβ2, and TGFβ3) in OSE and the underlying stroma in both normal bovine and human tumor tissues. Normal bovine ovaries are similar to human ovaries and are used as a model system to investigate normal OSE and stromal cell functions. All three TGFβ isoforms and their receptor, transforming growth factor beta receptor type II (TGFβRII), proteins were found to be detected in the OSE from normal bovine ovaries using immunohistochemistry. Ovarian stromal tissue also contained positive immunostaining for TGFβ isoforms and TGFβRII. RNA was collected from normal bovine OSE and ovarian stromal cells to examine TGFβ gene expression. TGFβ1, TGFβ2, and TGFβ3 transcripts were detected in both freshly isolated and cultured bovine OSE and stromal cells by a sensitive quantitative polymerase chain reaction assay. TGFβ1 and TGFβ2 mRNA levels were found to be present at similar levels in freshly isolated OSE and stroma. Interestingly, TGFβ3 mRNA levels were significantly higher in freshly isolated OSE than stromal cells. All but TGFβ3 mRNA in OSE increased when the cells were cultured. Observations indicate that normal bovine OSE and stroma cells express the three TGFβ isoforms in vivo and in vitro. Human ovarian tumors from stage II, stage III and stage IV cases were found to express TGFβ1, TGFβ2, TGFβ3 and TGFβRII protein primarily in the epithelial cell component by immunohistochemistry analysis. The stromal cell component of the human ovarian tumors contained little or no TGFβ or TGFβRII immunostaining. TGFβ actions on bovine OSE and stromal cells were also investigated. TGFβ was found to inhibit the growth of OSE, but not stromal cells. To further examine the actions of TGFβ on OSE, the expression of two growth factors previously shown to be expressed by OSE were analyzed. TGFβ1 was found to stimulate the expression of both keratinocyte growth factor (KGF) and kit ligand/stem cell factor (KL) by bovine OSE. Therefore, TGFβ actions on OSE will likely promote a cascade of cell–cell interactions and cellular responses involving multiple growth factors. The effects of regulatory agents on TGFβ expression by the bovine OSE were examined. Transforming growth factor alpha (TGFα) stimulated TGFβ1 expression, TGFβ1 stimulated TGFβ2 expression, and follicle stimulating hormone (FSH) stimulated TGFβ3 expression. These results demonstrate that TGFβ isoforms are regulated differently by the regulatory agents tested. In summary, all the TGFβ isoforms are differentially expressed by the OSE and TGFβ appears to have an important role in regulating OSE and possibly stromal–OSE interactions. A complex network of endocrine and paracrine interactions appears to influence the expression and actions of TGFβ on OSE. Abnormal expression and/or action of TGFβ is postulated to in part be involved in the onset and progression of ovarian cancer.

Keywords: Transforming growth factor beta; Mesenchymal–epithelial; Ovary; Ovarian cancer; Ovarian surface epithelium

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1. Introduction

Ovarian cancer ranks fifth as a cause of cancer deaths among women and results in more deaths than any other cancer of the female reproductive system. It is estimated that there will be about 14000 deaths this year from ovarian cancer in the United States. Greater than 95% of these ovarian cancers originate in the epithelial cells on the surface of the ovary (ACS, 1998; Piver et al., 1991).

The ovarian surface epithelium (OSE) is a single layer of simple epithelial cells that are separated from underlying ovarian stromal tissue by an extracellular matrix (Auersperg et al., 1991). During normal ovarian cycles the OSE undergoes periodic changes and has the capacity to remodel the ovarian cortex through synthetic and proteolytic functions (Auersperg et al., 1991; Woessner et al., 1989). Similarly, the stroma produces growth factors and cytokines that may act on the OSE (Vigne et al., 1994). The OSE can release enzymes that contribute to the breakdown of the underlying stroma which is adjacent to the preovulatory follicle and thus has been suggested to be involved in the process of ovulation (Murdoch, 1996). After ovulation, the OSE proliferates and covers the site of follicular rupture. The hypothesis has developed that the altered cellular activity of the OSE at ovulation and aberrations in the proliferative wound repair process following ovulation may lead to neoplasia (Murdoch, 1996; Godwin et al., 1992a,b).

The cellular associations between OSE and stroma also have been shown to influence the intermediate filaments in the OSE similar to that observed in early stages of neoplastic progression (Hornby et al., 1992). Tumor invasion often requires an association with host stromal tissue and most ovarian tumors have a stromal-like component (Kurman, 1987; Scully, 1995). Therefore, stromal–epithelial cell interactions appear to have a critical role in the function and growth of normal and tumorigenic OSE.

Transforming growth factor beta (TGFβ) belongs to a super family of structurally related multifunctional proteins that regulate many aspects of cellular function and have diverse functions in a variety of cell types (Massague, 1983). The isoforms TGFβ1, TGFβ2, and TGFβ3 have been isolated from mammalian tissues (Derynck et al., 1988), while TGFβ4 and TGFβ5 were identified in chick and *Xenopus*, respectively (Jakowlew et al., 1988; Kondaiah et al., 1990). When the sequences for TGFβ isoforms are compared between mammalian species there is $>97\%$ identity (Derynck et al., 1987; Massague, 1990). The presence of multiple forms of TGFβs and the conserved sequences of individual isoforms suggests that these isoforms mediate distinct and evolutionarily conserved processes (Massague, 1990). In culture all three isoforms show similar function and behave as inhibitors of normal and transformed epithelial cell growth (Roberts et al., 1985; Tucker et al., 1984; Coffey et al., 1988).

All three isoforms, TGFβ1, TGFβ2, and TGFβ3, are expressed by the normal and malignant OSE (Jakowlew et al., 1997; Henriksen et al., 1995; Bartlett et al., 1997; Bristow et al., 1999). Previous reports on normal ovaries and ovarian tumors indicate that TGFβs are important growth factors in these tissues (Jakowlew et al., 1997; Lafon et al., 1996; Hurteau et al., 1994, 1999). TGFβ elevates the expression of several extracellular matrix proteins including fibronectin and collagen types (Ignotz and Massague, 1986) and has been shown in a variety of tissues (Lin et al., 1992; Marchant, 1980; Kruk et al., 1990; Doraiswamy et al., 1998). Thus TGFβs not only affect growth of epithelial and mesenchymal cells but also regulate extracellular matrix deposition which influences tumor invasion and metastasis.

The TGFβ ligands bind three cell surface receptors (type I, II and betaglycan) with high affinity (Massague, 1990). Type I and II receptors bind TGFβ with greater affinity than betaglycan and are necessary for biological responses to TGFβ (Massague et al., 1987). The type I receptor is a 50–60 kDa protein that possesses conserved regions resembling serine–threonine kinases, but unlike the type II receptor does not bind ligand independently. The type II receptor is a 75–85 kDa glycoprotein that has been shown to associate with type I receptor on binding to TGFβ (Hu et al., 1998; Lin et al., 1992). The increased cell surface expression or complete loss of TGFβ receptors has been observed in a number of tumors and cell lines (Arteaga et al., 1988; Kimura et al., 1999; Li et al., 1998).

The bovine ovary physiology and size are similar to 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 has been reported in super-ovulated and aged cows (Marchant, 1980) suggesting the bovine OSE may have similar tumorigenic potential to human OSE. The availability of normal bovine ovaries is also an advantage compared to the limited supply of normal human tissue. Therefore, the bovine ovary provides a useful model for examining the specific cell–cell interactions involving normal OSE (Vigne et al., 1994; Parrott and Skinner, 1998; Parrott et al., 2000a,b).

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 or about the adjacent layer of stromal cells that has been shown to influence the OSE. The current study was designed to examine the local production and action of TGFβ in bovine OSE and underlying stroma.

2. Materials and methods

2.1. Tissues

Bovine ovaries were obtained from young non-pregnant cycling heifers less than 10 min after slaughter and transported on ice by the WSU/UI Center for Reproductive Biology, Animal Reproduction Core Laboratory (Vigne et al., 1994). Human cancer tissues were surgically collected from women with serous type stage II, stage III and stage IV ovarian tumors and were obtained from the ovarian cancer bank at the University of California, San Francisco under the direction of Dr Bethan Powell. When required, ovaries were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5 μm sections. All tissues were collected with appropriate university approvals.

2.2. OSE and stromal cell isolation and cell culture

OSE cells and ovarian stromal cells were prepared fresh for RNA isolation or cultured. OSE cells were scraped from the surface of the ovary with a rubber policeman as previously described (Vigne et al., 1994; Kruk et al., 1990). Sheets of purified epithelial cells adhered to the rubber policeman were suspended in Hanks' buffered salt solution and then pelleted and washed prior to suspension for plating. After the removal of the OSE cells, the ovarian surface stromal cells were microdissected from areas of the ovary devoid of follicles (Vigne et al., 1994). A section of surface stromal cells 1–2 mm wide by 5–8 mm long and 1-mm deep was 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 and maintained at 37 °C in 5% CO_2 atmosphere in Ham's F-12 (Gibco Labs., 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. For RNA isolation from cultured cells, OSE and stromal cells were plated in 100 mm large culture plates and maintained in Ham's F-12 supplemented with 10% calf serum.

To study the effect of growth factors and gonadotropins on TGFβ gene expression, OSE or stromal cells were plated in six-well plates in Ham's F-12 supplemented with 10% calf serum. When cells achieved confluency, cells were washed in Ham's F-12 and maintained in Ham's F-12 in the absence of calf serum for 24 h and then an additional 48 h for treatments. Cells were treated with no growth factor (control), transforming growth factor alpha (TGFα) (25 ng/ml), TGFβ (10 ng/ml), epidermal growth factor (EGF) (50 ng/ml), follicle stimulating hormone (FSH; 100 ng/ml), human chorionic gonadotropin (hCG; 100 ng/ml) or kit ligand (KL; 50 ng/ml). Treated cells were cultured for 48 h and harvested for total RNA isolation. The purity of OSE and stromal cells isolated by this procedure are greater than 98% as determined by specific cytoskeletal component staining with no detectable cross contamination: only non-staining cells of stromel phenotype (Vigne et al., 1994).

2.3. Growth assays

Cell growth was analyzed by determining (^{3}H) thymidine incorporation into newly synthesized DNA. OSE or stroma cells were plated (approximately 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 TGFα, 10 ng/ml TGFβ, combination of TGFα and TGFβ, 50 ng/ml EGF, or 10% bovine calf serum (Gibco). Cells were treated with growth factors for 20 h. After treatment, 0.5 ml DMEM containing 2.5 μCi (^{3}H) thymidine (25 Ci/mmol) 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 (Vigne et al., 1994).

2.4. Immunohistochemistry

Tissues were fixed in 10% formalin and embedded in paraffin according to standard procedures. Immunohistochemistry was performed according to a standard protocol (Pelton et al., 1991) as previously modified (Doraiswamy et al., 1998; Parrott and Skinner, 1998; Parrott et al., 2000a,b). Briefly, 5-μm sections were deparaffinized and rehydrated, quenched in 3% hydrogen peroxide in 20% methanol, and non-specific protein binding sites were saturated by incubation with 10% serum (normal goat serum, Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Slides were incubated with polyclonal rabbit anti-human TGFβ isoform specific antibody or TGFβRII antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) at 1:50 to 1:200 dilutions overnight at 4 °C. Secondary antibody (biotinylated goat anti-rabbit IgG, Vector) was detected by using the Vectastain kit (Vector) and diaminobenzadine (Vector). Negative controls of non-immune antibody were used on adjacent tissue sections. Competition with excess antigen was also performed as controls (Doraiswamy et al., 1998; Parrott and Skinner, 1998; Parrott et al., 2000a,b).

2.5. 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. RNA was stored at −70 °C until use.

2.6. Quantitative PCR assays

Steady state levels of TGFβ and cyclophilin (i.e. 1B15) mRNAs were analyzed using a specific quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay for each gene. These quantitative RT-PCR assays have previously been described in detail (Parrott and Skinner, 1998). The primers used in this quantitative analysis of TGFβ, TGFβRII, keratinocyte growth factor (KGF), kit-ligand (KL) and 1B15 were: TGFβ1, 5′-GGA CCT GGG CTG GAA GTG-3′ (5′ primer, 18-mer) and 5′-CTG CTC CAC CTT GGG CTT-3′ (3′ primer, 18-mer) which generated a specific 205-bp TGFβ1 PCR product; TGFβ2, 5′-TTC GCA GGT ATC GAT GGC AC-3′ (5′ primer, 18-mer) and 5′-GCA ATT ATG CTG CAC ATT CC-3′ (3′ primer, 18-mer) which generated a specific 204-bp TGFβ2 PCR product; TGFβ3, 5′-TGC CCA ACC CCA GCT CCA AGC G-3′(5′ primer, 22-mer) and 5′-CCT TTG AAT TTG ATT TCC A-3′(3′ primer, 19-mer) which generated a specific 289-bp TGFβ3 PCR product; KGF, 5′-ATA CTG ACA TGG ATC CTG CCA AGT TTG CTC TAC AGA TGC TTC-3′ (5′-primer; 45-mer) and 5′-TCC AAC TGC CAC GGT CCT GAT-3′ (3′-primer; 45-mer), which generated a specific 306-bp KGF PCR product; and 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), which generated a specific 452-bp KL PCR product. 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) which 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 five different primers including 1B15), 0.1 mM dNTPs, 10 mM DTT, 40 Units RNAse inhibitor (Promega, Madison, WI), and 200 Units M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) in 40 μl RT buffer (50 mM Tris–HCl pH 8.3, 75 mM KC1, 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 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 TGFβ1, TGFβ2, TGFβ3, KGF, KL, or1B15 subclones were used to generate standard curves from 1 attogram/μl (10^{-15} g/μl) to 10 pg/μl (10×10^{-9} g/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 simultaneously assay five 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 Units AmpliTaq polymerase in 50 μl GeneAmp PCR buffer (containing 1.5 mM $MgCl_2$, Perkin–Elmer). Each PCR amplification consisted of an initial denaturing reaction (5 min, 95 °C); 25–31 cycles of denaturing (30 s, 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 Phosphor Imager (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 TGFβ data were normalized for 1B15.

The optimal number of cycles for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e. logarithmic phase of PCR reactions). TGFβ1 quantitative PCR products were amplified for 31 cycles, TGFβ2 PCR products were amplified for 30 cycles, TGFβ3 PCR products were amplified for 27 cycles, KGF PCR products were amplified for 28 cycles, KL PCR products were amplified 31, and 1B15 PCR products were amplified for 26 cycles. The sensitivity of each quantitative PCR assay was below 1 fg which corresponds to less than 125 fg target mRNA/μg total RNA.

2.7. Statistical analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of treatment were analyzed by a one-way analysis of variance (ANOVA). Observed significance probabilities of 0.05 ($P > F$) or less were considered evidence that ANOVA model fits the data. Significant differ-

ences 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. This multiple comparison test is recommended for multiple comparisons with control.

3. Results

The ability of TGFβ1 to influence the growth of bovine OSE and stromal cells was investigated. TGFβ1 inhibited transforming growth factor-alpha (TGFα) stimulated DNA synthesis in bovine OSE (Fig. 1A). This inhibition of TGFα stimulated growth was not observed with bovine stroma treated with TGFβ1 (Fig. 1B). Therefore, TGFβ can act as a growth inhibitory factor for OSE, but not stroma cells under the conditions utilized. The ability of TGFβ to regulate the growth of OSE suggests that TGFβ may be involved in the normal growth functions of OSE.

Expression of TGFβ and TGFβRII protein was examined in normal bovine ovaries by immunohistochemistry. In bovine ovaries TGFβ1, TGFβ2, TGFβ3, and TGFβRII protein were detected in the OSE and underlying stroma (Fig. 2A–D). While TGFβ staining was intense in the OSE, staining could also be detected in the stromal cells that border the epithelial cells. Staining for TGFβRII was present in both the bovine OSE and stroma at similar intensities. No detectable staining was seen in control slides (Fig. 2F). Negative controls with competing peptide antigens also were negative on bovine tissue (data not shown). Similar results were obtained for TGFβ localization in normal human ovaries (data not shown), as previously described (Jakowlew et al., 1997; Henriksen et al., 1995; Bartlett et al., 1997; Bristow et al., 1999; Lafon et al., 1996; Hurteau et al., 1994, 1999). Observations suggest that TGFβ protein is expressed by both normal bovine and human OSE and stroma.

Expression of TGFβ protein was also examined at different stages of human ovarian cancer. Tissues were examined from cases of serous type stage II, stage III, and stage IV ovarian cancers. TGFβ1, TGFβ2, and TGFβ3 protein were detected in the epithelial cell component of the tumor tissue at all stages examined (Fig. 3A–C). Less intense staining could also be detected in the stromal cells that border the epithelial cells. TGFβRII staining was detected at all stages of tumors and was observed in both epithelial and stromal cells (Fig. 3D). Tumor stromal staining was less intense than that seen in normal ovaries. The expression was primarily present in the epithelial component and to a lesser extent in stromal tissue. These results demonstrate the expression of TGFβ isoforms and receptor in all these different stages of ovarian tumors in humans.

TGFβ gene expression in normal OSE and ovarian surface stromal cells was examined using a sensitive quantitative PCR assay (Parrott and Skinner, 1998; Parrott et al., 2000a,b). Steady state levels of TGFβ1, TGFβ2 and TGFβ3 mRNA expression were determined and normalized for the constitutively expressed gene cyclophilin, termed 1B15. Normalization for 1B15 expression corrected for changes in cell number, amount of RNA, the integrity of initial mRNA and for small differences in the efficiency of reverse transcription reaction between samples. TGFβ gene expression was observed in both freshly isolated and cultured bovine OSE and ovarian surface stromal cells (Fig. 4). TGFβ1 and TGFβ2 mRNA levels were found to be similar in freshly isolated OSE and stromal cells. Interestingly, TGFβ3 mRNA levels were significantly higher in freshly isolated OSE than stromal cells. TGFβ1 and TGFβ2 mRNA levels were higher in cultured cells than in freshly isolated samples. In contrast, TGFβ3 was higher in freshly isolated OSE than cultured cells. Therefore, all isoforms were expressed by OSE and stroma at similar levels except for the high levels of

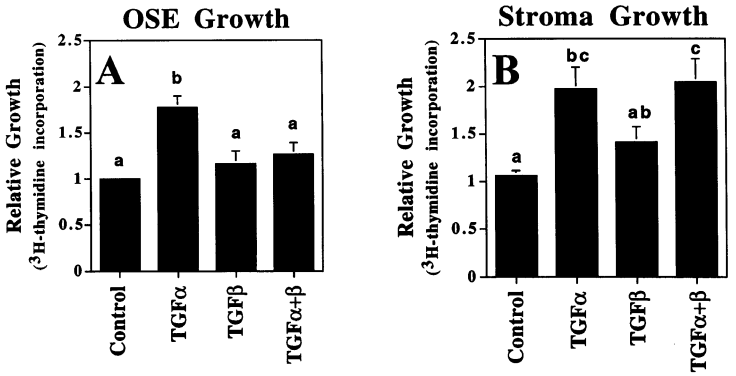

Fig. 1. Relative cell growth as determined by ^{3}H-Thymidine incorporation into DNA. (A) Bovine OSE cell growth in response to treatment with TGFα and TGFβ1. (B) Bovine Stroma cell growth in response to treatment with TGFα and TGFβ1. ^{3}H-Thymidine incorporation values are expressed as counts per minute (CPM) and normalized to μg DNA and expressed relative to control groups set equal to one. Data is presented as the mean ± S.E.M. from three different experiments performed in replicate. Different letters at the top of columns indicate a significant difference at $P \leq 0.05$ using ANOVA.

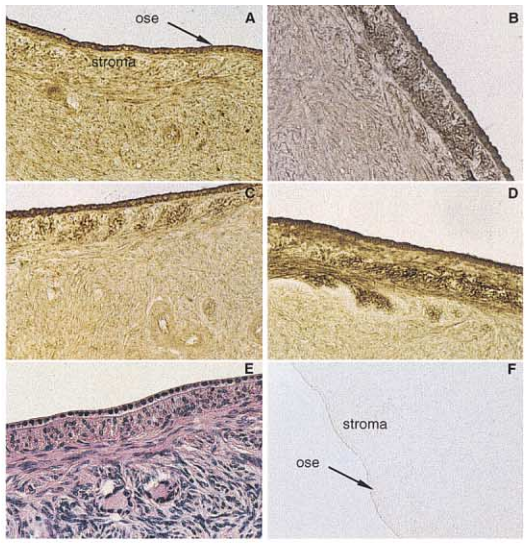

Fig. 2

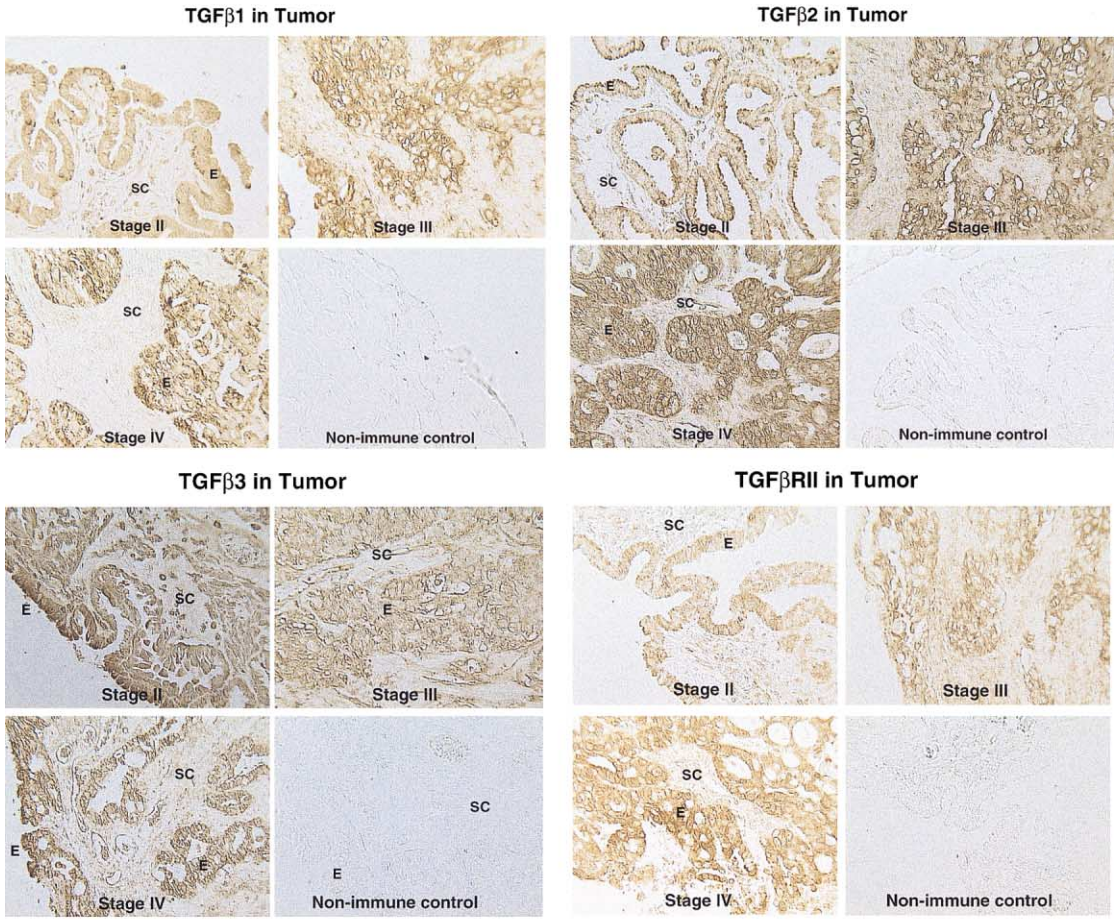

Fig. 3

Fig. 2. Immunohistochemical localization of TGFβ isoforms and their receptor in bovine ovary sections showing OSE and stromal cells: (A) TGFβ1; (B) TGFβ2; (C) TGFβ3; (D) TGFβRII; (E) hematoxilin and eosin morphological stain and (F) non-immune serum negative control. The brown/gray stain indicates positive protein staining. Microscope magnification = 400 × . Data are representative of three different experiments. Fig. 3. (A) Immunohistochemical localization of TGFβ1 in human stage II, III and IV ovarian tumors. The control refers to a negative control which used non-immune serum. (B) Immunohistochemical localization of TGFβ2 in human stage II, III and IV ovarian tumors. The control refers to a negative control which used non-immune serum. (C) Immunohistochemical localization of TGFβ3 in human stage II, III and IV ovarian tumors. The control refers to a negative control which used non-immune serum. (D) Immunohistochemical localization of TGFβRII in human stage II, III and IV ovarian tumors. The control refers to a negative control which used non-immune serum. E = OSE derived tumor epithelium. SC = stromal cell-derived tumor tissue. Microscope magnification = 200 × . Data are representative of three different experiments.

TGFβ3 by OSE. Absolute levels of TGFβ3 mRNA expression in OSE and stroma are over three orders of magnitude higher than TGFβ2 which is 10-fold higher than TGFβ1 (Fig. 4).

The effect of regulatory factors on bovine OSE and stroma cell TGFβ gene expression was investigated. Gene expression for TGFβ isoforms was determined using the quantitative RT-PCR as described in Section 2. No treatments of stroma cells resulted in significant changes in TGFβ1, TGFβ2, or TGFβ3 mRNA expression (data not shown). Bovine OSE cells in culture treated with TGFα showed a significant increase ($P < 0.05$) in TGFβ1 expression compared to control cultures (Fig. 5A). TGFα had no effect on TGFβ2 or TGFβ3 mRNA levels. TGFβ1 was found to stimulate ($P < 0.1$) TGFβ2 mRNA levels, but had no effect on TGFβ1 or TGFβ3 expression (Fig. 5B). Interestingly, FSH significantly stimulated TGFβ3 mRNA levels over control ($P < 0.05$), but had no effect on TGFβ1 or TGFβ2 expression. Neither human chorionic gonadotropin (hCG) nor kit ligand (KL) had an effect on TGFβ expression (Fig. 5). Therefore, the TGFβ isoforms are differentially regulated by different regulatory factors of bovine OSE.

The ability of TGFβ to influence the expression of other growth factors produced by bovine OSE was also investigated. KGF and KL have previously been shown to be expressed by OSE and stimulate OSE growth (Parrott et al., 2000a,b). Interestingly, TGFβ1 treatment of OSE cells stimulated the expression of both KGF and KL (Fig. 6). Therefore, TGFβ can influence OSE expression of growth factors that can subsequently influence OSE function and growth.

4. Discussion

The current study examines the local expression and action of TGFβ in normal and tumorigenic OSE and stroma. Immunohistochemistry was used to localize all isoforms of TGFβ protein to both bovine OSE and stroma cells. The mRNA for TGFβ isoforms were also found to be expressed in both OSE and stroma cells. This confirms previous work showing TGFβ in human OSE (Henriksen et al., 1995) and extends this observation to ovarian surface stroma. Therefore, TGFβ may act in an autocrine and/or paracrine manner in normal OSE and stroma cells. When human ovarian tumors were examined all TGFβ isoforms were shown to be at high levels in the epithelial component of the tumors, compared to the stroma. This is consistent with TGFβ acting in a more autocrine manner to influence tumorigenic OSE. Loss of TGFβ receptors has been observed in a number of tumor cell lines (Shipley et al., 1986; Kimchi et al., 1988; Arteaga et al., 1988). In the current study, TGFβ RII was detected in the tumors analyzed. Further analysis of ovarian tumors is required to elucidate the role TGFβ may have in tumor progression. Tumorigenic OSE can lose TGFβRII receptors or receptor responsiveness (e.g. Smad action) resulting in a decrease in the ability of TGFβ to suppress proliferation. The inability of the tumorigenic OSE to be inhibited by TGFβ may facilitate tumor progression of ovarian cancer. A limitation to the current study is the lack of correlation with bovine ovarian tumors. The infrequent detection of bovine ovarian tumors is largely due to the fact that domestic cows do not generally live long enough to develop tumors.

The ability of TGFβ to regulate cell growth was examined. Bovine OSE and stromal cell growth in culture was shown to be stimulated by treatment with TGFα. This increase in OSE growth was inhibited by treatment with TGFβ1. TGFβ has previously been shown to inhibit the proliferation of numerous cell types including mesenchymally derived cell lines (Tucker et al., 1984), fibroblasts (Roberts et al., 1985), epithelial cells, human melanoma, lung carcinoma, and breast carcinoma cells (Roberts et al., 1985; Coffey et

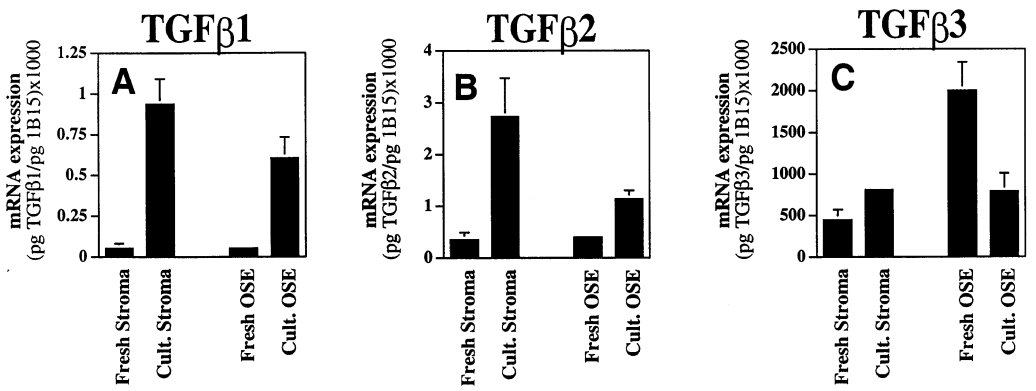

Fig. 4. Relative TGFβ mRNA levels in freshly isolated and cultured bovine OSE and stromal cells. TGFβ mRNA expression is normalized to cyclophilin (1B15) mRNA expression: (A) TGFβ1 expression; (B) TGFβ2 expression and (C) TGFβ3 expression. Data are the mean ± S.E.M. from three different experiments performed in replicate.

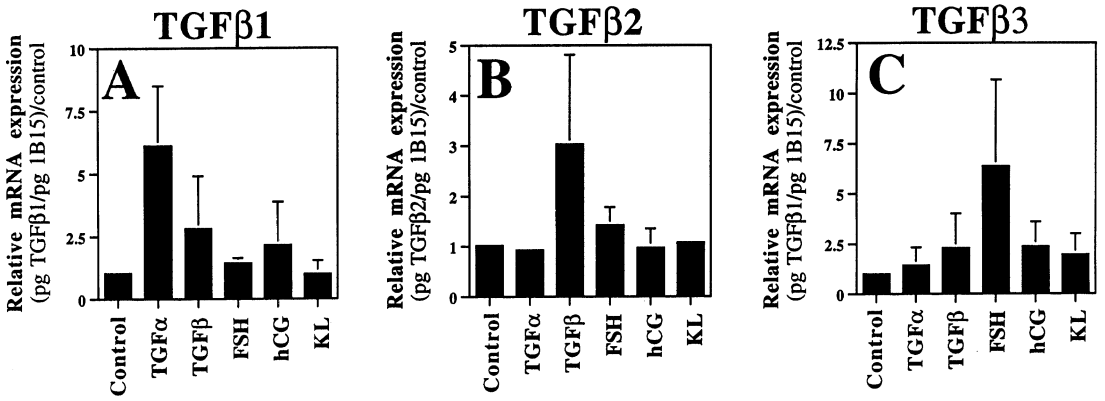

Fig. 5. Relative TGFβ mRNA levels in bovine OSE treated with regulatory factors: (A) TGFβ1; (B) TGFβ2 and (C) TGFβ3. TGFβ mRNA expression is normalized to cyclophilin (1B15) mRNA expression and adjusted so that the value of control groups equals 1. OSE cells were treated with TGFα, TGFβ, FSH, hCG, KL or untreated (control). Data are presented as the mean ± S.E.M. from three different experiments done in replicate.

al., 1988). In the course of a normal ovulatory cycle the OSE is stimulated to proliferate and cover the ruptured follicle site after ovulation occurs. Production of TGFβ isoforms by OSE or stroma could act in an autocrine or paracrine manner to prevent over-proliferation of the OSE. In support of this model, it was found that TGFβ1 expression in bovine OSE cells was increased in response to treatment with TGFα. Stromal TGFβ mRNA expression was not influenced by treatment with TGFα. This suggests that stromal cells may be responsive to other regulatory agents than those which influence OSE cells. Previous investigations have found levels of TGFβ isoforms to be abnormal in cancer tissues. TGFβ isoforms have been found to be high in thyroid carcinoma, breast cancer and urethral tumors (Kimura et al., 1999; Li et al., 1998; Shibata et al., 1998). Conversely, TGFβ3 was found to be abnormally low in prostate carcinoma (Djonov et al., 1997). None of these studies correlated TGFβRII expression to TGFβ levels.

TGFβ1, TGFβ2 and TGFβ3 expression are differentially regulated in the bovine OSE. TGFβ1 and TGFβ2 mRNA levels were similar in OSE and surface ovarian stroma. However, TGFβ3 levels were higher in OSE than in stroma. In addition, TGFβ3 levels were significantly higher than TGFβ1 and TGFβ2 levels in freshly isolated OSE. Differential levels of TGFβ isoform expression have been demonstrated in other tissues. TGFβ1, TGFβ2 and/or TGFβ3 expression levels were found to change independently of each other in rat testis (Cupp et al., 1999), rat prostate (Chang et al., 1999; Itoh et al., 1998), pig embryonic ectoderm (Gupta et al., 1998) and ventricular tissue of hypertrophic rat heart (Li and Brooks, 1997). The fact that TGFβ3 levels are high in OSE raises the possibility that TGFβ3 plays a unique role in regulating the physiology of the OSE. The specific function of TGFβ3 in the OSE requires further investigation to elucidate.

Analysis of TGFβ isoform expression by freshly isolated versus cultured bovine OSE demonstrated an increase in both TGFβ1 and TGFβ2 in culture. Many growth factors have been shown to increase expression during cell culture. This is likely due to the absence of normal regulatory control of gene expression. The use of the cell cultures to examine regulation of gene expression demonstrates the potential for the regulatory agent to influence the expression of the specific growth factor (i.e. TGFβ isoform). The role that this regulatory factor has in vivo requires more direct in vivo experiments.

Treatment of OSE with FSH increased expression of TGFβ3 mRNA. Previous research has shown that the follicle stimulating hormone receptor (FSHR) mRNA appears to be present in normal OSE (Zheng et al., 1996) and FSH can stimulate proliferation of some ovarian cancer lines (Zheng et al., 2000; Feng et al., 1996; Simon et al., 1983; Wimalasena et al., 1992). Recently, we have

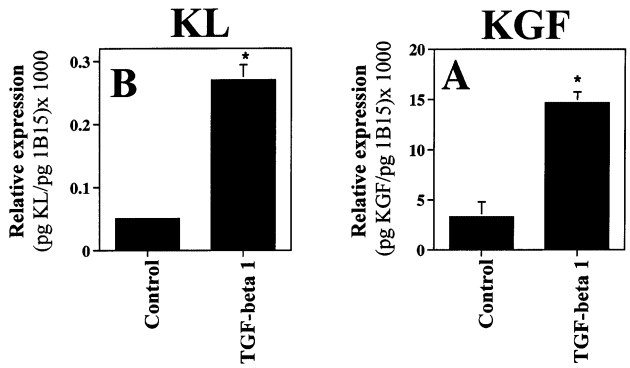

Fig. 6. Relative expression of growth factors in bovine OSE cells stimulated with TGFβ1: (A) KGF expression and (B) KL expression. Growth factor mRNA levels are normalized to cyclophilin (1B15) mRNA expression. Treatment groups marked with (*) are significantly ($P \leq 0.05$) different than control using a student's t-test. Data are presented as the mean ± S.E.M. from three experiments done in replicate.

found that normal bovine OSE express both the FSH receptor and LH receptor (Parrott et al., 2001). FSH and LH were found to stimulate bovine OSE proliferation directly and the expression of growth factors (Parrott et al., 2001). The ability of FSH and LH to influence OSE is critical since the postmenopausal occurrence of ovarian cancer is in an environment of elevated gonadotropins. In the current study FSH was found to influence TGFβ expression. It is possible that FSH stimulation contributes both to cell proliferation and to the timely up-regulation of TGFβ3. TGFβ3 may act as a negative feedback for FSH actions to keep over-proliferation of OSE in check through autocrine actions of TGFβ. Interestingly, it was also demonstrated that treatment with TGFα increased OSE expression of TGFβ1. In a similar manner, TGFβ1 may act as a negative feedback for TGFα to prevent over-proliferation of OSE.

There are a number of growth factors in addition to TGFβ that have been shown to be expressed by OSE. Keratinocyte growth factor (KGF) and kit ligand (KL) are expressed by OSE. Kit ligand (KL) (Parrott et al., 2000b) and KGF (Parrott et al., 2000a) increase OSE cell growth (Parrott et al., 1994; Parrott and Skinner, 2000). It is likely that cell proliferation of the OSE is controlled by the positive and negative influences of a network of locally produced growth factors. Expression and action of one factor may affect the expression levels of other factors. The proliferative response of OSE cells at any one time depends on the combined inputs of the network of growth factors. In the current study the effect of TGFβ1 treatment on levels of KL and KGF was examined. Treatment of bovine OSE with TGFβ1 resulted in an increase in KL and KGF mRNA expression. This demonstrates that the negative regulator of cell proliferation TGFβ1 can up-regulate expression of the positive regulators of cell proliferation KL and KGF. It is speculated this effect of TGFβ1 on KGF and KL expression may be a positive feedback response to maintain the balance of OSE growth required. In this manner TGFβ can act indirectly to affect normal OSE proliferation. This is an example of how a network of growth factors will be needed to control normal OSE biology.

Ovarian tumor development likely in part involves the abnormal expression of a network of growth factors that affect normal OSE proliferation. Abnormal TGFβ regulation or action may directly or indirectly influence ovarian tumor development and progression. While the combined effects of growth factors on OSE and tumor growth are starting to be investigated, the specific growth factors involved remain to be elucidated. The current study supports the potential that TGFβ isoforms and their receptors may be an important part of the control of normal and/or tumorigenic OSE biology. Further studies to directly assess the function of TGFβ in vivo will be required to understand the specific role of TGFβ in OSE biology and ovarian cancer.

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