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Effects of bTP-1 and oxytocin on prostaglandin and protein production by the bovine endometrium

Roger D. Johnson

To the Graduate Council:

I am submitting herewith a dissertation written by Roger D. Johnson entitled "Effects of bTP-1 and oxytocin on prostaglandin and protein production by the bovine endometrium." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

James D. Godkin, Major Professor

We have read this dissertation and recommend its acceptance:

Hank Kattesh, John Erby Wilkensen, Bert Erickson, Mary Ann Handel

Accepted for the Council:

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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

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Dr. James D. Godkin,
Major Professor

We have read this dissertation
and recommend its acceptance:

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EFFECTS OF bTP-1 AND OXYTOCIN ON PROSTAGLANDIN AND PROTEIN PRODUCTION BY THE BOVINE ENDOMETRIUM

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Roger D. Johnson

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ABSTRACT

There is evidence that conceptal proteins in general, and bovine trophoblast protein-1 (bTP-1) specifically, alter biochemical mechanisms which are responsible for returning cattle to estrus. Endometrial explants, gland fragments, glandular epithelial cells, and stromal cells from uteri of cyclic cows (N=4) were cultured in medium containing 1 ug/ml bovine serum albumen (BSA), 1 ug bovine trophoblast protein-1 (bTP-1), 50 mU oxytocin after 8 h, or 1 ug/ml bTP-1 followed by 50 mU oxytocin at 8 h and examined their effects on prostaglandin and protein secretion. Explants treated with bTP-1 secreted less Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) than explants receiving BSA ($P<0.05$) or oxytocin alone ($P<0.001$). Oxytocin treated tissue or cells secreted sinificantly more $PGF_2\alpha$ than bTP-1 treatments ($P<0.05$) and BSA treatments ($P<0.05$) in all cultures. Glandular epithelial cell cultures treated with oxytocin secreted more prostaglandin E_2 (P,0.05) than cultures treated with BSA or bTP-1. Glandular epithelial cells produced more $PGF_2\alpha$ than any other group when normalized to total cellular protein, while stromal cells produced more PGE_2. There was no effect of treatment on incorporation of radiolabeled methionine and cysteine into macromolecules although stromal cells incorporated more radiolabel than other cell or tissue type. Two-dimensional-SDS PAGE and fluorography revealed that similar proteins were secreted by explants, glandular epithelial cells, and intact gland fragments

but no differences were observed due to treatment. These results indicate bTP-1 and/or oxytocin affect prostaglandin secretion in *in vitro* models of the bovine endometrium in a manner that helps explain *in vivo* observations.

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PART 1: REVIEW OF LITERATURE

CHAPTER 1

INTRODUCTION

Embryonic mortality results in inefficient production of meat, milk, and wool and is a major problem in all food and fiber producing animals. Estimates in cattle and sheep reveal that between 16 to 42% of all embryos are lost during the first 30 days of gestation (Ayalon, 1978). In the cow the sequence of events leading to luteolysis must be blocked or attenuated to maintain the corpus luteum (CL), which sustains progesterone levels required in early pregnancy (Hansel et al., 1973; Thatcher et al., 1984). Timely interactions between the uterus, developing conceptus and corpus luteum are essential to maintain pregnancy. Signals released from the blastocyst attenuate uterine function to prevent luteolysis. The major secretory protein released by the conceptus at this time, bovine Trophoblast Protein-1 (bTP-1), is considered to be this antiluteolytic agent. A breakdown in communication or absence of bTP-1 by Day 15-17 of the cycle results in uterine secretion of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and subsequent luteal regression. It is not known, however, if the effects of bTP-1 on the uterus are due to its influence on a specific endometrial cell or complex paracrine interactions.

The bovine uterus plays a central role in regulating the lifespan of the CL in cycling and pregnant animals. Episodic release of $PGF_2\alpha$ by the nonpregnant uterine endometrium

travels by a counter-current mechanism via the utero-ovarian vasculature to the ovary where it promotes rapid regression of the CL during the late luteal phase of the estrous cycle (See Review, Silvia et al., 1991). Although plasma levels of $PGF_2\alpha$ are reduced during early pregnancy (Kindahl et al., 1976; Betteridge et al., 1984), the amount of $PGF_2\alpha$ secreted by the endometrium appears to be less important than the pulsatile manner in which $PGF_2\alpha$ is released. (Schramm et al., 1983). A complex hormonal system including progesterone, estrogen, and oxytocin regulates $PGF_2\alpha$ secretion. Exposure to progesterone for several days is necessary to facilitate $PGF_2\alpha$ production by the uterus, while increasing levels of estrogen at this time promote the formation of receptors for estrogen and oxytocin. It appears that oxytocin from the posterior pituitary and/or CL is directly involved in the timing and magnitude of $PGF_2\alpha$ secretion. During luteolysis in ruminants, pulsatile secretion of $PGF_2\alpha$ is evoked by oxytocin after binding to specific endometrial receptors and initiating phosphoinositide hydrolysis (Bazer, 1991). While exogenous oxytocin can induce luteolysis, its effect is probably mediated through $PGF_2\alpha$ as hysterectomized animals fail to respond accordingly. Animals immunized against oxytocin exhibit delayed luteal regression, which also suggests that oxytocin is involved in the mechanism that regulates luteolysis. During early pregnancy there is a reduced responsiveness to oxytocin, which is attributed to an

inhibition of the increase in oxytocin receptors observed in cyclic animals (Mirando, et al., 1990a,b).

Prostaglandin synthesis requires several steps, each of which can be limiting depending on study conditions (Fitzpatrick, 1991). Initiation of prostanoid synthesis often is rate limited by release of arachidonic acid, a major component of the plasma membrane. Phospholipase C and Phospholipase A_2 are two enzymes involved in the liberation of arachidonic acid, which may involve a membrane bound G protein (Sternweis, 1992). For example, binding of a signaling molecule to a G-protein linked receptor in the plasma membrane, activates a phosphoinositide-specific phospholipase C which cleaves phosphatidylinositol-bisphosphate to generate inositol triphosphate and diacylglycerol (Bansal, 1990). Arachidonate is released during the rapid metabolism of diacylglyceride. Liberated arachidonic acid is the substrate for prostaglandin endoperoxide synthase (PGS) also called prostaglandin H synthase (PGHS) or cyclooxygenase, a membrane bound glycoprotein concentrated in the endoplasmic reticulum. Details of the chemistry, action and regulation of this enzyme have recently been reviewed (Fitzpatrick, 1991; Smith and Marnett, 1991; DeWitt, 1991). PGS exhibits two enzyme activities: a bis-oxygenase (cyclooxygenase) activity catalyzing PGG_2 formation and peroxidase activity catalyzing the reduction O PGG_2 to PGH_2. Both enzyme activities are present in the same molecule that exists as a homodimer. After

formation of the PGH_2 by PGS, these are specific synthases or isomerases for the prostanoid end-products, including $PGF_2\alpha$ and PGE_2. In addition, there are multiple genes that control PGS expression, a constitutively expressed PGS-1 gene and an inducible PGS-2 gene (Rosen et al. 1989; Masferrer et al. 1992).

Progesterone, a hormone required to maintain pregnancy in the cow, is produced primarily by the CL for the first one-third of gestation. It is generally accepted that the developing conceptus (embryo and associated membranes) is responsible for preventing luteal regression, thereby ensuring continued progesterone production. This phenomenon constitutes an endocrinological component of "maternal recognition of pregnancy" (MRP). MRP, as described above, occurs around Day 12-13 in the ewe and Day 16-17 in the cow (Moor, 1969; Northy and French, 1980). Removal of the conceptus after these times results in an extended estrous cycle, while prior removal has no effect (Rowson and Moor, 1967; Ellinwood et al., 1979a). Intrauterine infusion of Day 12-14 homogenates (Rowson and Moor, 1967) or extracts (Martel et al., 1967) prolonged luteal function, while intrauterine administration of Day 25 ovine conceptus homogenates or heat treated Day 12-14 conceptus ovine conceptuses had no effect on the ewe's estrous cycle. The latter observation indicated that the signal was most likely a protein.

In both cattle and sheep the large episodic pulses of

$PGF_2\alpha$ associated with luteolysis are diminished during pregnancy, suggesting the presence of an antiluteolysin. Since implantation occurs relatively late in development, the conceptus is able to communicate with the mother before the trophoblast has undergoes definitive attachment to the endometrium. Evidence indicates that a low molecular weight, acidic protein produced by the trophoblast of the conceptus during MRP is the probable candidate (Godkin et al., 1982; Bartol et al., 1985). These trophoblast proteins, ovine trophoblast-1 (oTP-1) in the ewe, and bovine trophoblast protein-1 (bTP-1) in the cow, are the major secretory proteins secreted at this time (Godkin et al., 1984). The complex of proteins that constitute bTP-1 has isoelectric points from 5.3 - 5.7, and molecular weights ranging from 22,000 to 24,000, which is somewhat larger than oTP-1 (mol. wt. = 18,000) apparently due to glycosylation of the bovine TP-1. Secretion of bTP-1 is maximal between Day 16-19 of pregnancy (Bartol et al.; Lifsey et al., 1989), although mRNA for bTP-1 is present as early as Day 12 (Roberts 1992).

It is now clear that oTP-1 and bTP-1 are Type 1 τ-interferons (Roberts, 1992). Significant homology in amino acid sequence between oTP-1, bTP-1, and human and bovine $INF\alpha_2$ has recently been demonstrated by N-terminal analysis and cDNA sequencing (Imakawa et al., 1987; Stewart et al., 1987). The greatest similarity of the bovine trophoblast gene products (boIFNτ), however, is to interferon tau (IFNτ) (Roberts,

1992). Recently it has been demonstrated that recombinant human INFα and oTP-1 had similar effects on prostaglandin and protein synthesis by ovine endometrial cells in vitro (Salamonsen et al., 1988). Both INFα and oTP-1 significantly attenuated $PGF_2\alpha$ and PGE_2 release. Treatment of ovine endometrial cells with INFα or oTP-1 revealed the proteins to have identical effects on uterine cell protein synthesis (Salamonsen et al., 1988). These studies, however, do not discriminate between luminal and glandular epithelium. It has been our experience that luminal epithelial cells during the late luteal phase of the estrous cycle are very fragile and do not survive enzymatic treatment. Intramuscular injection of rboIFNα into nonpregnant ewes induced the synthesis of the pregnancy-specific protein (p70)in the endometrium (Francis, 1991). Rueda et al. (1993) identified two secretory protein that are synthesized by the endometrium of pregnant cows in response to rboIFN-τ.

OTP-1 binds to the endometrial epithelium cells of Day 13 non -pregnant ewes, demonstrating that the uterus is a target organ (Salamonsen et al., 1988). In addition, high affinity binding sites for oTP-1 were identified in uterine membrane preparations by competitive binding studies (Godkin et al., 1984a). Comparatively high specific binding of labelled IFN was demonstrated in tissue derived from ovariectomized ewes and increased significantly by estrogen treatment of ewes, but not by progesterone treatment (Russell,

et al., 1993). Earlier Niswender et al. (1989) demonstrated that binding sites on endometrium from cyclic ewes were maximal on Day 4 and progressively decreased to Day 12, and increased again to Day 16. Intrauterine administration of the trophoblast protein-1 extends luteal function in cyclic cattle (Vallet et al., 1987) and ewes (Fincher et al., 1986). Another study also reported that intrauterine infusion of bTP-1 extends luteal function in cycling cattle (26.5 days) while other bovine conceptal secretory proteins released at this time had no effect (21.5 days) when compared to control animals (19.5) (Helmer et al., 1989). Both oTP-1 and bTP-1 alter the production of prostaglandins, and have been shown to modify the production of specific endometrial proteins in vitro (Godkin et al., 1984a; Helmer et al., 1989). Godkin, et al. (1984) demonstrated that purified oTP-1 selectively stimulated synthesis and release of at least six uterine proteins when incubated with uterine endometrial explants. Explants from the gravid uterine horn produced two specific proteins, p70 and p15 (Sharif et al., 1987). These proteins produced to a lesser extent by the nongravid horn, and were not present in the nonpregnant ewe (Sharif et al., 1989). Similarly, oTP-1 was shown to stimulate synthesis and release of specific proteins by primary cultures of Day 13 uterine epithelial cells (Salamonsen et al., 1988). Finally, bovine conceptal secretory proteins and bTP-1 specifically have been shown to decrease protein synthesis and secretion as well as

reduce $PGF_2\alpha$ secretion by bovine endometrial explants (Helmer et al., 1989). Cellular mechanisms, however, which mediate the actions of bTP-1 and oTP-1 are not clearly defined at present.

Interferons (Type I and II) are pleiotropic cytokines named originally for their ability to confer resistance to subsequent viral infection (DeMaeyer, 1988). They have since been implicated for their involvement in cellular differentiation, proliferation, immune function, and reproduction (Rogerts, 1992). Type II IFN, also known as IFN-gamma is a product of T cells and functions to activate macrophages. Recently, La Bonnardierre et al., (1991) reported that IFN-gamma was a product of the preimplantation pig blastocyst. Although IFN-gamma has wide reaching effects, there is no evidence of its role as an antiluteolysin in this species.

Type I IFN bind to a common receptor despite the presence of three distinct subtypes (α,β, and), each with a different amino acid sequence. Bovine IFNτ most closely resembles boIFN , although oIFNτ has greater sequence homology than any bovine IFN. It has been well documented that trophoblast protein-1 (oIFNτ and boIFNτ) play a major role in MRP. The mechanism by which these Type I IFN prevent luteolysin, however is not completely understood. Endometrial IFN receptor are present, and trophoblast IFN decreases the expression of oxytocin receptors. It has also been reported that low concentration of IFNα stimulated progesterone secretion by cultured luteal

cells (Luck, 1992).

Another characteristic of all IFNs is their ability stimulate 2',5', oligo-adenylate synthetase, which is involved in the antiviral and antiproliferative affects of IFN (Johnson, 1984). Short et al., (1993) indicated that bovine endometrial 2', 5', oligo-adenylate synthetase content increases significantly on Day 18 of pregnancy compared to cyclic animals. In addition, Schmitt et al. (1993) reported that 2', 5', oligo adenylate synthetase was higher in surface epithelium, glandular epithelium, and stromal cells of pregnant animals when compared to endometrial cells of animals at a similar point in their estrous cycle. Finally, ovine endometrium infused with oTP-1 caused an increase in the actiity of the 2',5' oligo adenylate synthetase enzyme (Mirando et al., 1991). A major problem in studying fetal-maternal interactions at the cellular level has been the relative inaccessibility of the system. However, utilization of <u>in vitro</u> technologies has allowed researchers to study physiological parameters not previously possible. Three uterine endometrial cell types; 1) luminal epithelium; 2) glandular epithelium; and 3) stromal cells have been isolated and cultured in our laboratory from Day 15-17 cycling cows. It is possible, therefore, to study conceptus protein-uterine interactions using <u>in vitro</u> cell culture systems and isolated conceptus proteins. BTP-1 has been well characterized (Lifsey et al., 1988) and demonstrated to interact with uterine

endometrium to elicit changes in maternal physiology. The development of *in vitro* models are crucial to determine the mechanisms involved with these interactions. Conceptal proteins in general and bTP-1 specifically attenuate the oxytocin stimulation of $PGF_2\alpha$ release, while conflicting data exist concerning the degree of change in protein secretion due to bTP-1. The specific cells in the bovine endometrium that respond to bTP-1 are not known. It has been reported that $PGF_2\alpha$ secretion from ovine uterine epithelial cells treated with oTP-1 is reduced (Salamonson et al., 1988).. However, they fail to discriminate between epithelial cell type that have recently isolated. By comparing the morphological and biochemical characteristics of each cell type isolated, we believe that the mechanisms resulting from endometrial/conceptal protein interactions will be further elucidated.

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PART 2; A SIMPLE METHOD FOR ISOLATING BOVINE ENDOMETRIAL CELLS

CHAPTER 1

ABSTRACT

Cultures of three cell populations were established from late luteal phase bovine endometrium. Luminal cells were obtained by gentle agitation of dissected endometrial tissue and could be maintained on Matrigel™ but not uncoated plastic. These cells exhibited a low columnar morphology, but did not divide in culture. Stromal and epithelial cultures were isolated from the digestion of the dissected endometrium with a collagenase/Dispase solution using differential centrifugation and filtration through a nylon mesh. Stromal cells exhibited a fibroblast-like morphology and divided rapidly in culture. Immunocytochemical localization of vimentin microfilaments in these cells confirmed their mesenchymal origin, and demonstrated that the purity of cultures was >90%. Epithelial cell monolayers began as outgrowths from small glandular fragments obtained from the digestion solution. These cells divided in culture, took on a flattened, spreadout morphology, and stained positive for cytokeratin, a marker for epithelial cells. Both stromal and glandular epithelial cell types survived subculturing for several passages. Ultrastructural evaluation of the endometrium revealed that the gland fragments used to initiate epithelial cultures were tightly adherent cells that rested on basal lamina in which stromal cells were embedded. The

epithelial cells contained prominent nuclei, endoplasmic reticulum, and golgi.

CHAPTER 2

INTRODUCTION

The bovine endometrium is composed of glandular epithelium, luminal or surface epithelium, stromal cells, endothelium, and blood cells (Marinov and Lovell, 1968). Epithelial cell layers are separated from other cellular components by a basement membrane made up of collagen, laminin, fibronectin, and proteoglycans which is generally a product of the epithelium and underlying stroma (Freshney, 1992). Together with soluble factors from the stroma, the basement membrane serves to regulate the differentiated function of the epithelium. Surface (luminal) epithelial cells have been classified pseudostratified columnar throughout the estrous cycle, with variation in the size of the cells occurring at different phases of the estrous cycle (Asdell et al., 1949; Borell et al., 1959). Hypertrophy of uterine glands has been associated with development of the corpus luteum (CL), while regression of the CL leads to atrophy of the glands (Marinov and Lowell, 1968). Epithelial cells are constantly regenerated from stem cells in many tissues such as the basal layer of the epidermis and in the crypts of the intestine. This process may also serve an important role as to the physiological state in the endometrial epithelium of the cow.

Fortier et al. (1988) reported a technique to isolate

luminal epithelial cells and stromal cells from cows during early to mid-luteal phase of the estrous cycle (Day 2-12), indicating optimal recovery and viability of epithelial cells during this period. However, the endometrium tissue in general, and the individual cell types specifically, change morphology as well as their physiological responsiveness over the course of the estrous cycle (Marinov and Lovell, 1968). Late luteal phase ovine endometrial cells have also been isolated (Salamonsen et al., 1988) and were reported to alter their prostaglandin and protein synthesis in response to ovine interferon tau (oIFNτ), the anti-luteolysin in sheep.

The purpose of this study was to develop methods to isolate and characterize cells from the bovine endometrium to be used as an *in vitro* model for feto-maternal interactions. The use of *in vitro* techniques presents alternative methods of studying these interactions which facilitates a more controlled experimental environment from *in vivo* methods. Endometrial epithelial cell lines have been reported for humans (Centola, et al., 1984; Kirk, et al., 1986), mice (Sengupta, et al., 1986), rats (Lieberherr, et al., 1984) guinea pigs (Sumida et al., 1988), and cattle (Munson, et al., 1988). However, primary cells may represent a more accurate physiological state of the *in vitro* situation due to different endocrinological affects of the ovarian steroids on the uterus during the estrous cycle.

In the present study, bovine endometrial glandular and

luminal epithelial, and stromal cells were isolated from cattle on Day 17 of the estrous cycle. Bovine interferon tau (bIFNτ; formerly bTP-1), the major protein secreted by the trophoblast between Day 16-23 is regarded as the signal for recognition of pregnancy in the cow (See Roberts, 1992 for Review). In the cow paracrine interactions between the uterus, developing conceptus, and CL are essential for a successful pregnancy. For the study of feto-maternal interactions, it may be necessary to isolate endometrial cells during this critical time period to obtain a more accurate delineation of their response to this signal from the developing conceptus.

CHAPTER 3

MATERIALS AND METHODS

Reagents.

Dulbecco's modified Eagles's medium (low glucose) (DMEM), DMEM methionine free, minimal essential media (MEM), fetal calf serum (charcoal treated; 5 g/100 ml), Hank's balanced salt solution (HBSS), HBSS without calcium and magnesium, amino acids and basal medium Eagle vitamin solution were purchased from Gibco Laboratories, Grand Island, NY. Collagenase was obtained from Boehringer Mannheim, Indianapolis, IN. Matrigel$_{tm}$ and dispase were purchased from Collaborative Research Inc., Bedford, MA. Insulin was acquired from Eli Lilly and Co., Indianapolis, IN. Oxytocin and glucose were purchased from Sigma Chemical Co., St. Louis, MO. Antibodies (vimentin and cytokeratin) and peroxidase anti-peroxidase kits were obtained from BioGenex, San Ramon, CA. [^{35}S]-methionine/cysteine trans ^{35}S-label (specific activity 1071 ci/mmole) was obtained from ICN Biochemicals, Inc., Irving, CA. Supplies for electrophoresis were from BioRad Laboratories, Richmond, CA.

Animals.

Angus and Hereford crossbred cattle were checked twice daily for standing estrous. On day 17 of the estrous cycle (estrus = day 0), cows were slaughtered and the reproductive

tracts removed and placed on ice until processed further in the laboratory. Uteri from 4 animals were successfully used in this investigation.

Isolation of endometrial cells.

Reproductive tracts were trimmed, rinsed with Hank's Balanced Salt Solution (HBSS) and placed in a laminar flow hood. The following procedures were performed aseptically. Each uterine horn was cut longitudinally along the antimesometrial border. The exposed endometrium was dissected away from the myometrium and washed in two changes of HBSS without calcium and magnesium, ($-Ca^{+2}$, $-Mg^{+2}$) containing penicillin (200 units/ml), streptomycin (200 ug/ml), and fungizone (0.5mg/ml) (ABAM, Gibco Laboratories, Grand Island, NY). Luminal cells were observed under a dissection microscope to detach from the endometrium and these were collected. The sheets of cells were allowed to settle in a 50 ml conical tube containing HBSS and subsequently cultured in Dulbeco's Minimum Essential Medium (DMEM) containing 10 % Fetal Bovine Serum (FBS). The remaining endometrial strips were minced into small fragments (2-4 mm^2) using sterile scalpel blades and placed into a sterile 500 ml Erlenmeyer flask containing a digestion medium of 0.1 % collagenase and 0.5 U/ml dispase in HBSS -Ca-Mg. After 2 hours in a 37° C shaking water bath digestion was terminated. The cell suspension was diluted to twice the volume with HBSS -Ca,-Mg

and passed through a 200 μm sterile nylon mesh (Spectrum Medical Industries, Inc. Los Angeles, CA.) into a large petri dish (150 mm). Visible glandular fragments too large to pass through the mesh were repeatedly dispersed through a sterile pipette and again passed through a 200 μm sterile mesh. This cell suspension was centrifuged (50 x g) for 3 minutes. Uniform size was obtained by further disruption of the fragments through repeated pipeting. The fragments containing between approximately 50-500 cells were cultured in DMEM containing 10% FBS, and ABAM. Cell fragments could not be disrupted further without loss of viability. The supernatant contained primarily stromal cells was centrifuged at 400 x g for 10 minutes and the pellet was resuspended in culture medium.

Cell viability was estimated at > 95 % using the trypan blue exclusion method (Kuypers and Hulsman, 1984).

Immunocytochemistry.

Immunocytochemical localization of cytokeratin microfilaments, a marker for epithelial cells, and vimentin microfilaments, a marker for mesenchymal cells was performed using the peroxidase-anti-peroxidase (PAP) system (BioGenex, San Ramon, CA). All procedures were performed at room temperature. Cells cultured to 80% confluence were washed with phosphate buffered (PBS), fixed in Bouin's, and washed 3 times in PBS. After blocking for nonspecific proteins,

cytokeratin or vimentin antisera, (diluted 1:100) were added to the appropriate cells and incubated for one hour. Samples were rinsed twice with PBS, incubated for 20 min. with the link antibody, rinsed again with PBS and finally incubated with PAP for 30 min. Controls were treated identically except non-immune sura were used in place of the primary antibody. Cells were counterstained with hematoxylin and examined under light microscope.

Ultrastructure

Samples of each cell type was fixed in 3% glutaraldehyde and postfixed in 1%OsO4. After deydration and embedment, cells were stained with lead citrate and examined in a Phillips 201 transmission electron microscope.

CHAPTER 4

RESULTS

The cells which line the uterine lumen constitute a low to medium columnar epithelium (Marinov and Lovell, 1968; Smith et al, 1990) with lateral separations between cells (Smith et al., 1990). Isolated endometrial luminal epithelial cells were found to be very fragile and fragmented when subjected to enzymatic digestion. Structurally intact sheets of cells were obtained, however, by mechanically agitating the endometrial tissue in HBSS $-Ca^{2+}$,$-Mg^{2+}$ on ice. These cells did not attach to uncoated polystyrene petri dishes and became fragmented within 48 h in culture. However, cells plated on a Matrigel™ substrate bound tightly and were maintained in culture for several weeks. Typical luminal cells retained their columnar morphology throughout the culture period. Luminal cells did not divide in culture but remained in groups and appeared as polarized clumps, while single cells without the support of other cells toppled over and adhered to the substrate by their lateral sides (Fig. 1).

Contrary to luminal cell cultures, endometrial glandular epithelial cells obtained through enzymatic (collagenase/dispase) digestion of bovine endometrium attached and grew well in culture on uncoated plastic. These cells assumed a flattened, cuboidal, sheet-like morphology typical of epithelial cells (Fig. 2). At the time of plating the

viability of the epithelial cells, estimated by trypan blue dye exclusion, was greater than 95%. Originally, these cells were plated as cell clumps of different sizes (Fig. 3) with colonies extending from them over a period of 3-5 days in culture. (Fig. 4). They exhibited contact inhibition at confluence which generally occurred within 1 week depending on seeding density (Fig. 5). Cells growing in the center of the confluent sheet were regular, polygonal, and demonstrated a clearly defined edge. At the outer edge of a patch cells became more irregular and distended.

Stromal cells were also acquired through enzymatic treatment of endometrial tissue. A relatively homogeneous population of stromal cells could be obtained through differential centrifugation of the heterogeneous population of cells. Stromal cells rapidly adhered to uncoated plastic dishes and exhibited a fibroblast-like appearance, with numerous cytoplasmic processes (Fig. 6). Confluence was dependent on time and seeding density. Upon reaching confluence these cells could be subcultured for several passages.

Coculture of epithelial gland fragments with stromal cells resulted in colonies of epithelial cells with clearly defined borders (Fig. 7) or epithelial islands surrounded by stromal cells (fig. 8). Most co-cultures eventually resulted in cultures primarily composed of stromal cells due to their higher rate of proliferation.

Immunocytochemistry.

Luminal cell cultures could not be stained as they fragmented during fixation due to their fragile nature. Alternatively, we attempted to affix these cells to slides using a cytospin procedure. However, the cells did not withstand even low speed centrifugation and fragmented during the procedure.

Immunocytochemical staining of stromal and attached epithelial cells confirmed the microscopic morphological observations as to the identity of each cell type. Stromal cells stained positive for vimentin (Fig. 9), and negative for cytokeratin, while epithelial cells stained negative for vimentin and positive for cytokeratin (Fig.10).

Ultrastructural Morphology.

Gland fragments from which the epithelial cell cultures were initiated were tightly adherent and rested on a basal lamina embedded with stromal cells (Fig. 11) Further examination of the individual epithelial cells revealed that the apical membrane had irregular, blunt microvilli (Fig. 12). Oval-shaped nuclei were usually located in the middle-to-basal region of the epithelial cells and were somewhat irregular in shape with indentations and lobes (fig. 12). Scattered strands of rough endoplasmic reticula were present around the nucleus and the basal region of the cell. Apical regions often contained many vesicles of variable size and

shapes. Mitochondria that were round or elongated in shape were plentiful in the basal region of the cell and in the apical region beneath the area of vesicles. Junctional complexes typical of epithelial cells were present as were numerous bundles of intermediate filaments. Cilia was present on some of the epithelial cells and high magnification revealed the 9 + 2 bundle arrangement typical of cilia (not shown).

CHAPTER 5

DISCUSSION

This study describes a simple method for isolation of epithelial and stromal cells from the bovine uterine endometrium. The cell isolation procedure used resulted in highly enriched (>90%) primary cultures of epithelial or stromal cells as demonstrated with the phase-contrast microscope and by immunocytochemistry. By carefully monitoring estrus, we established cell cultures from animals in the late luteal phase of estrus. In the non-pregnant state $PGF_2\alpha$ is released by the endometrium at this time and travels by a counter-current mechanism via the utero-ovarian vasculature to the ovary where it eventually causes rapid luteal regression (Bazer et al., 1991). During pregnancy the trophoblast of the developing conceptus secretes bTP-1, now recognized as bovine interferon tau (bIFN-τ), the major secretory protein released between Day 16-22. Interferon tau is considered to be the signal responsible for maternal recognition of pregnancy, which implies communication between the conceptus and bovine uterus. Isolation of endometrial cells during the critical period of maternal recognition of pregnancy, therefore, may be useful for performing studies involving fetal/maternal interactions prior to attachment.

Failure of the luminal epithelial cells to divide and migrate in culture suggests that these cells may be terminally

differentiated by Day 17. This agrees with the observation of Fortier et al. (1988), who suggested that recovery and viability of endometrial cells were optimum between Days 2-12. It is not clear whether the luminal epithelial cells develop from stem cells separately from glandular epithelium, or evolve from the glands as they migrate into the lumen of the endometrium.

Stromal and glandular epithelial cells were separated readily after enzymatic digestion using differential centrifugation. As indicated, the epithelial cells isolated from enzymatic digestion were cultured initially as small fragments of between 50-500 cells, while the stromal cell population emerged as single cells. Electron micrographs illustrated that glands within the endometrium were composed of tightly adhered cells that remained attached even after digestion with our collagenase/Dispase solution. Stromal cells embedded in a matrix *in situ* appeared as single cells after isolation. Glandular epithelial cells emerging as single cells after digestion exhibited poor viability and growth characteristics. However, the fragments formed outgrowths, colonies, and eventually monolayers in culture. As epithelial layers are closely associated *in vivo* and are strongly self-adherent, it was not surprising to find that they tended to survive better *in vitro* as clusters or sheets of cells. Perhaps the effect of the enzymatic treatment was much more harsh on individual cells due to disturbances on their cell

membranes. In addition, communication between adherent cells may affect their initial subsistence (Freshney, 1992).

Morphologically, the isolated cells varied little from endometrial cells isolated from other sources, such as human (Liu & Tseng, 1979) rat (McCormack % Glasser, 1980) and rabbit (Gerschenson et al., 1981; Fortier et al., 1987). Epithelial cells were polygonal in shape and formed multicellular colonies, while stromal cells appeared fibroblast-like and were scattered randomly immediately after seeding. Both cell populations reached confluence within 5-7 days in culture. Also, these cells were morphologically similar under the light microscope to cells isolated from cattle by Fortier et al. (1988). The current method, however, does not utilize the extra step of layering the digested cell suspension on a Ficoll hypaque gradient while maintaining similar purity.

While cultured endometrial cells may appear morphologically similar, the physiological function of these cells may differ dramatically. Trophoblast proteins secreted between 16-23 days after conception alter prostaglandin secretion from the endometrium of sheep (Salamonsen et al., 1988) and in cattle (Helmer et al., 1989). The pulsatile manner in which prostaglandin $F_2\alpha$ is secreted by the endometrium in cycling animals is modified by bIFN-τ, during this period. The bovine uterus, therefore, must play a critical role in regulating the lifespan of the corpus luteum, the major source of progesterone at this time. Exposure to

progesterone for several days is necessary to facilitate $PGF_2\alpha$ production by the uterus, while increasing levels of estrogen at this time promote the formation of receptors for estrogen and oxytocin. It also appears that luteal oxytocin is directly involved in the timing and magnitude of $PGF_2\alpha$ secretion. Therefore, for studies involving conceptal protein/endometrial interactions, cells may need to be isolated from animals after these complex hormonal events have taken place. Indeed, the effects of bIFN-τ are manifested only during a critical period designated as "maternal recognition of pregnancy".

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Fig. 1 Luminal epithelial cells in culture (560x). Notice columnar morphology. Cells in groups remain polarized, while single cells appear as they have "toppled" on their sides.

Fig. 2 Glandular epithelial cells after 1 week in culture (140x). These cells have a flattened, "cobblestone" morphology typical of epithelium.

Fig. 3 Fragments of glands from the endometrium immediately after enzyme digestion (75x). Notice the different sizes of cell aggregates.

Fig. 4 Glandular epithelial cells extending from one of the cell aggregates after 4 days in culture(168x).

Fig. 5 Glandular epithelial cells after 1 week in culture (155x). At 100 % confluence cells become vacuolated and begin to die shortly thereafter.

Fig. 6 Stromal cells in culture after 2 days in culture (140x). Notice appendages extending from the cell typical of mesenchymal cells.

Fig. 7 Stromal and glandular epithelial cells cultured together (255x). Notice the distinct barrier formed by the two cell types.

Fig. 8 Epithelial cells forming an "island" surrounded by stromal cells (140x).

Fig. 9 Stromal cells stained positive for vimentin microfilaments, a marker for mesenchymal cells (175x).

Fig. 10 Glandular epithelial cells indicating the presence of cytokeratin, a marker for epithelial cells (175x).

Fig. 11 Endometrial tissue illustrating glandular epithelial cells resting on a matrix. Notice stromal cell within the matrix.

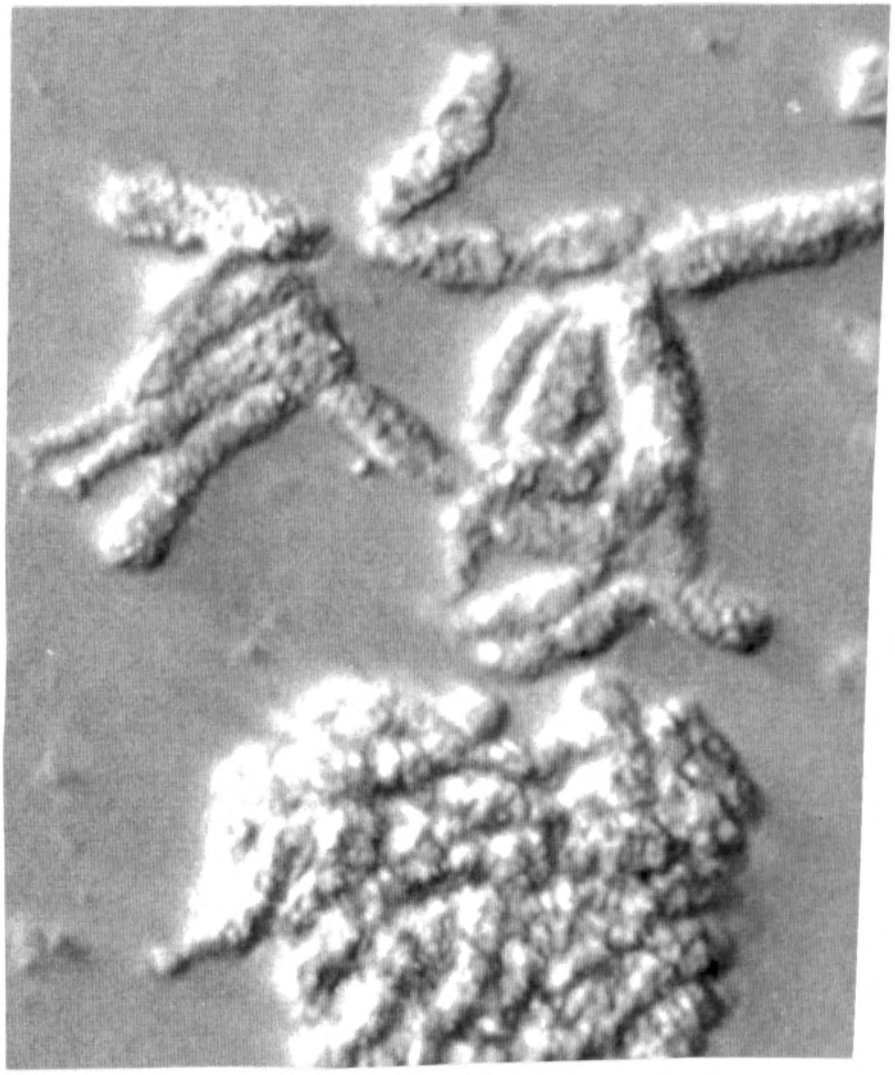
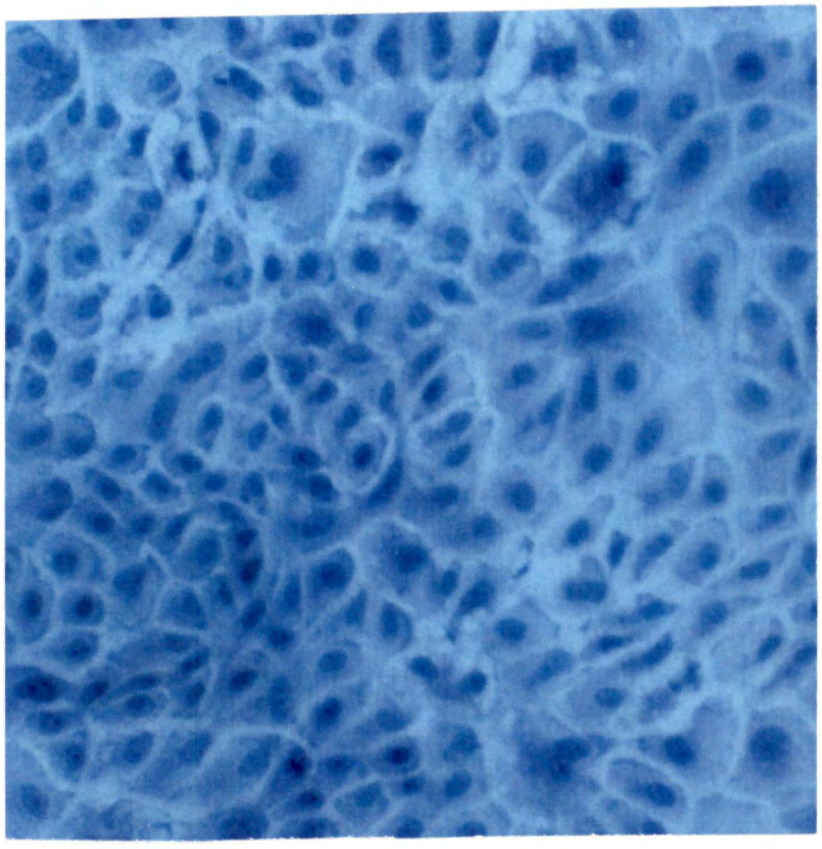


Figure 1



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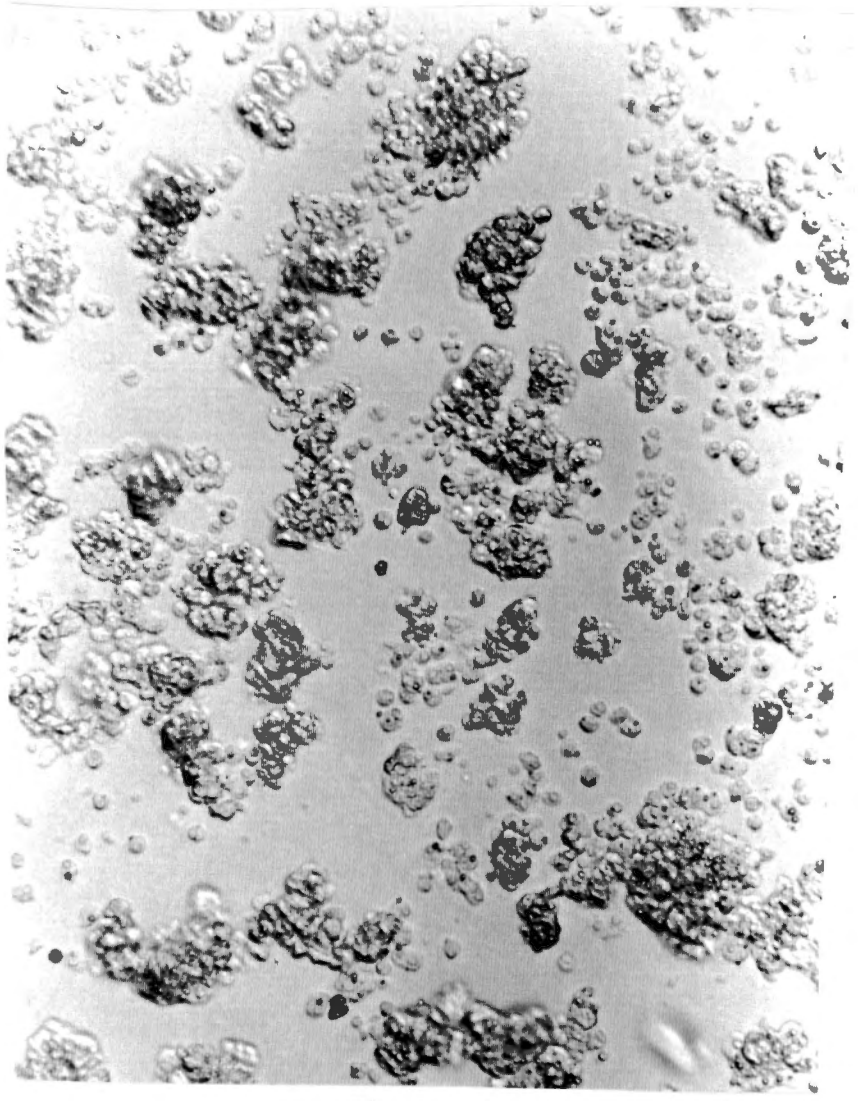

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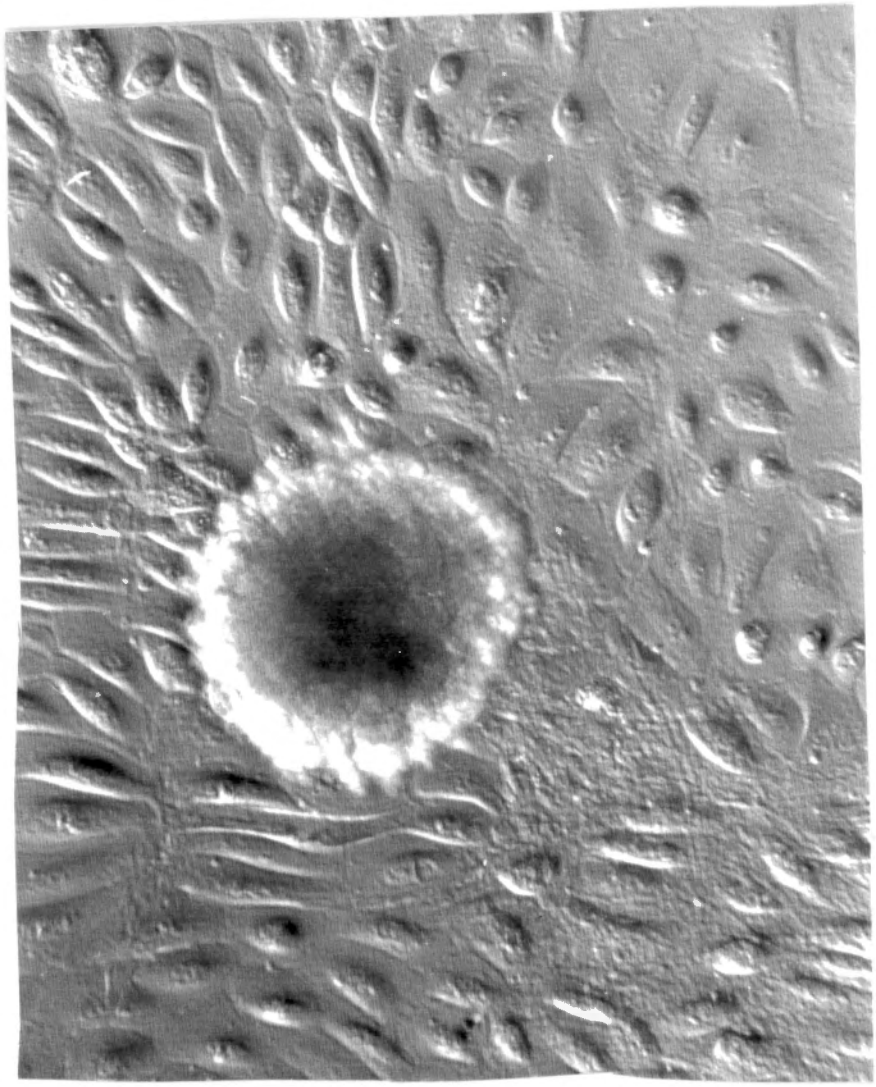

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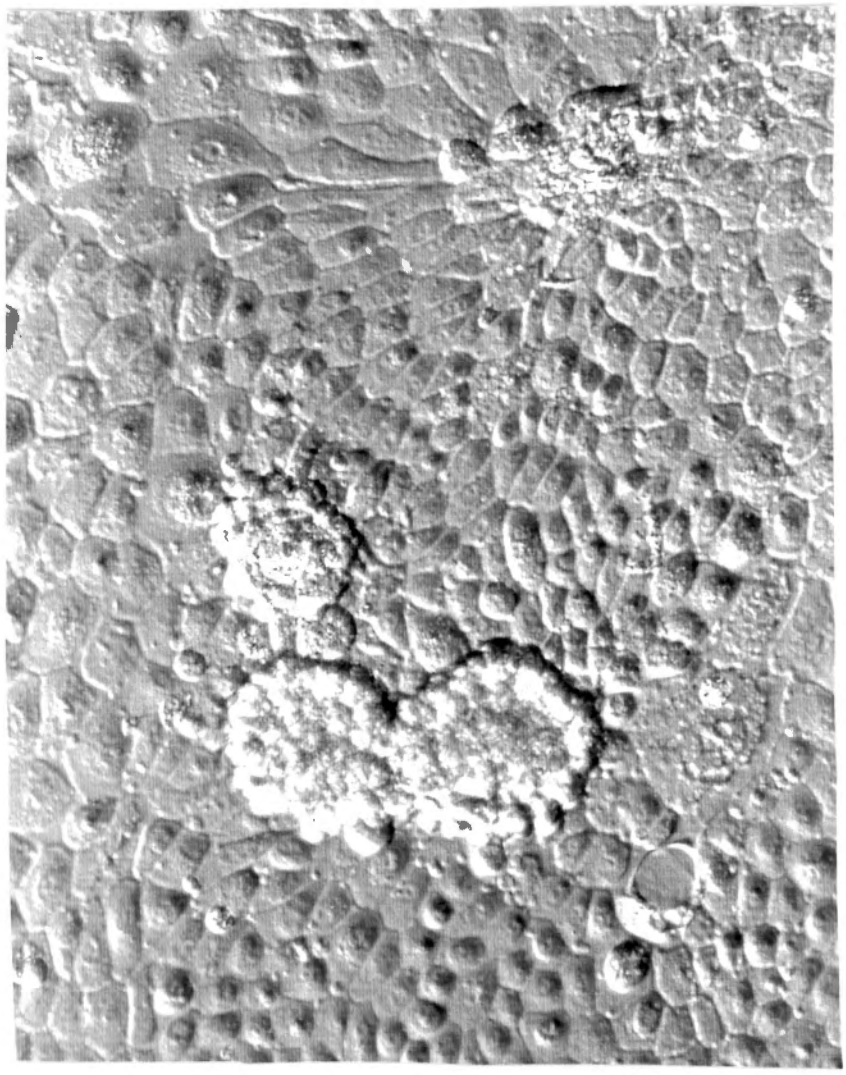

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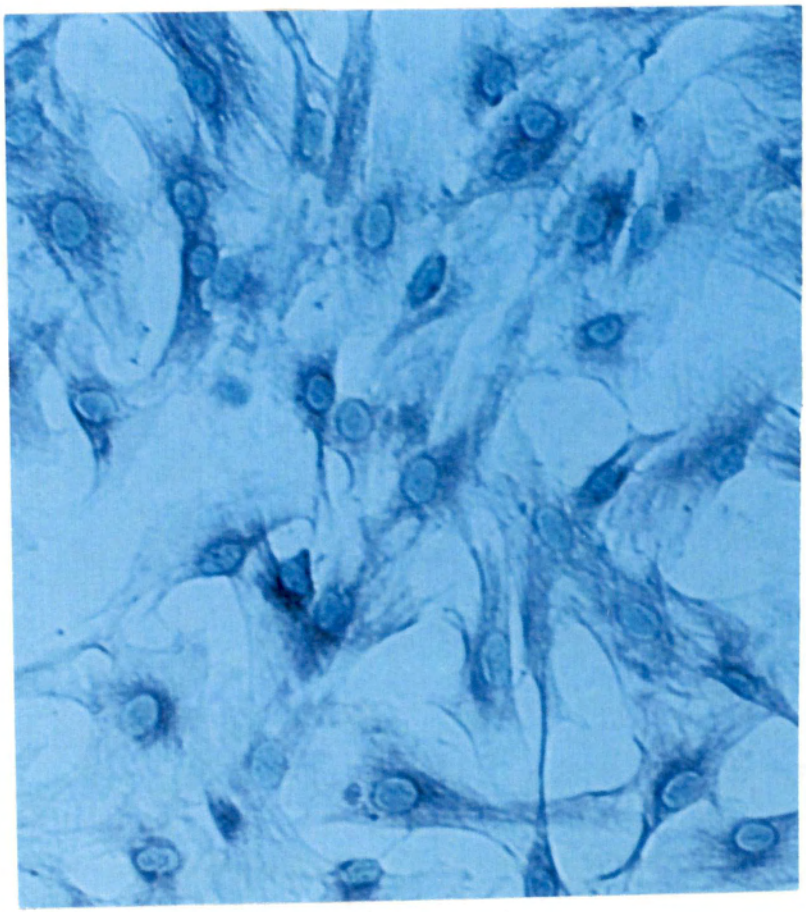

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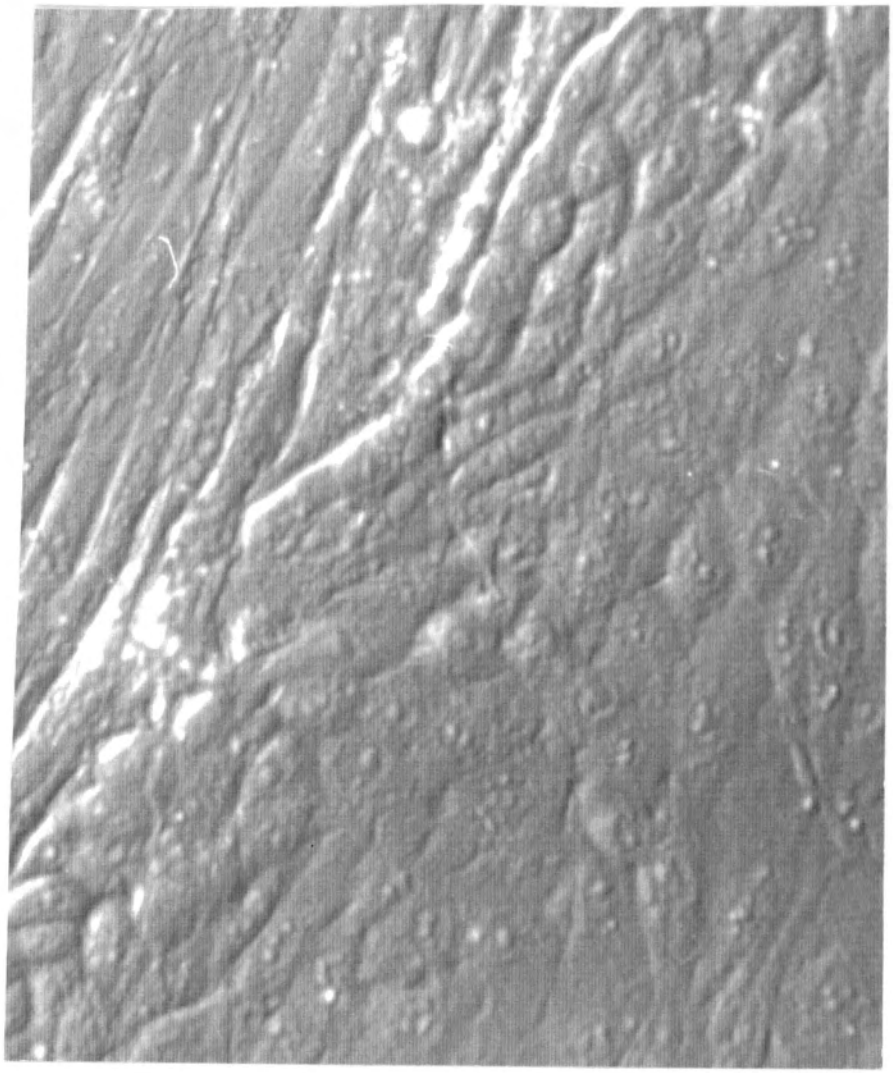

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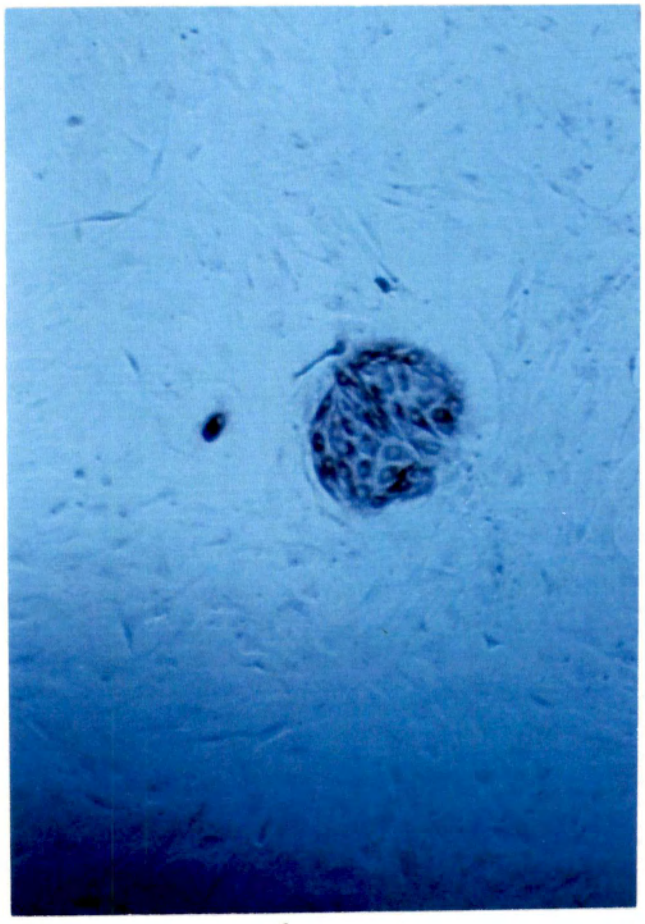

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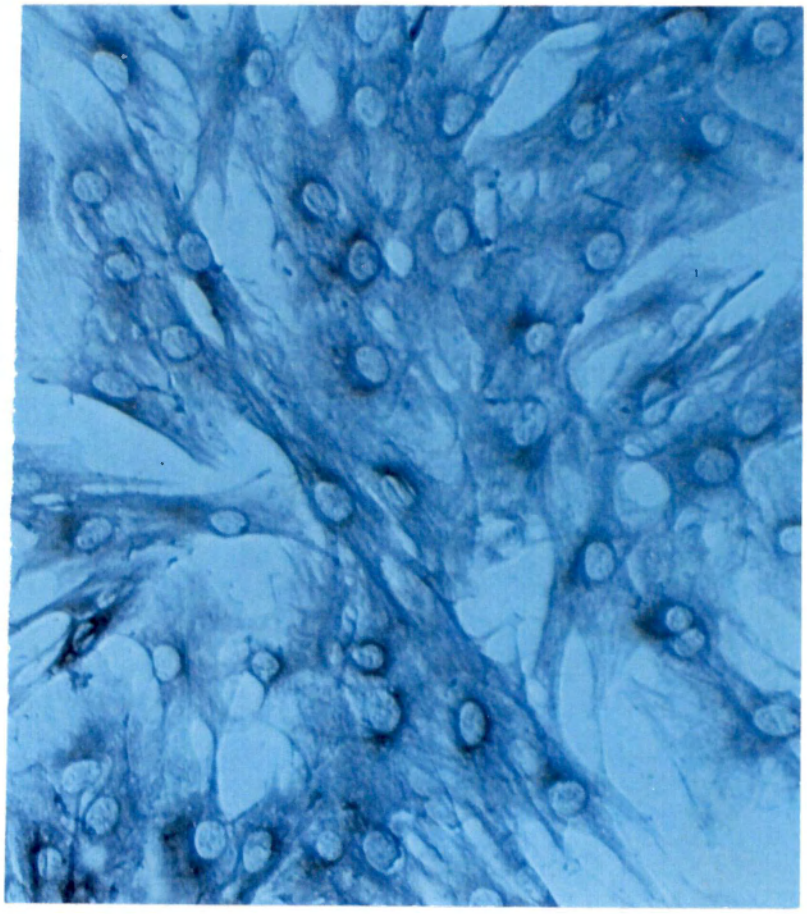

Figure 9

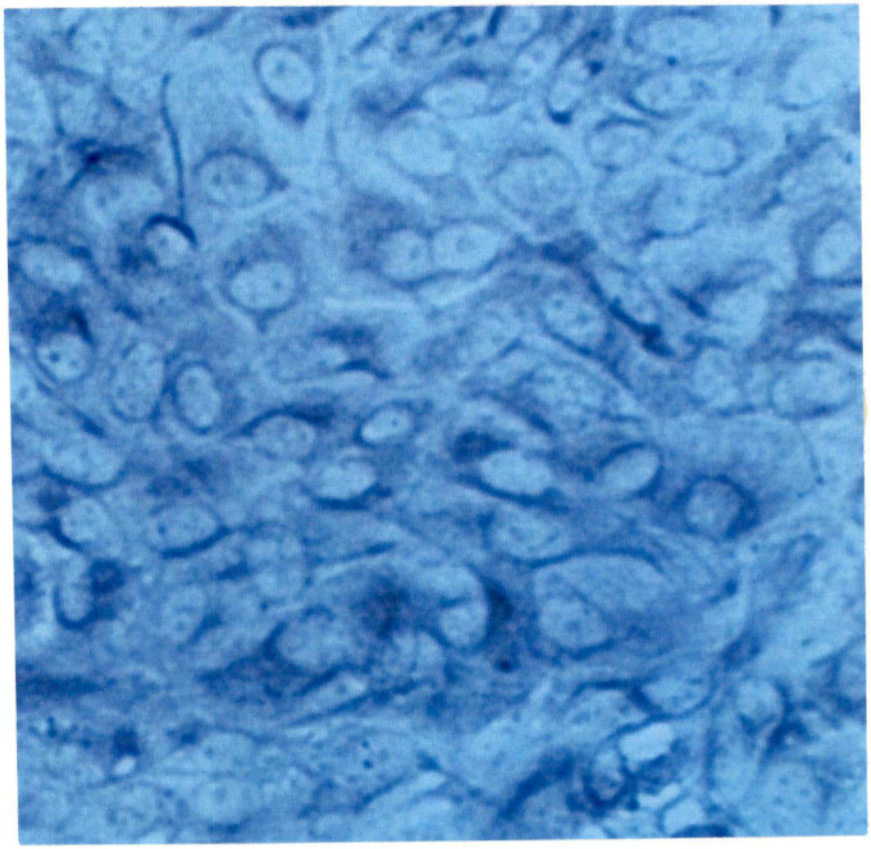

Figure 10

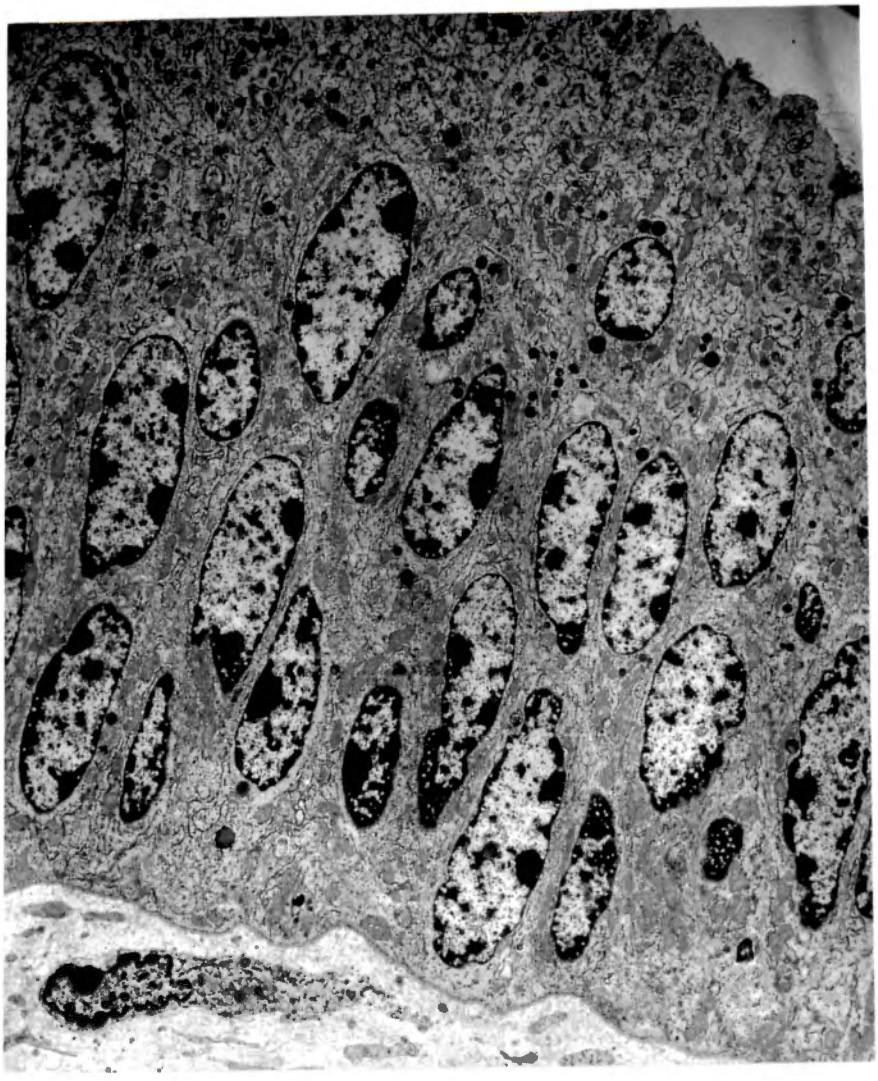

Figure 11

PART 3: BOVINE TROPHOBLAST PROTEIN-1 AND OXYTOCIN ALTER PROSTAGLANDIN PRODUCTION BUT NOT PROTEIN SYNTHESIS

CHAPTER 1

ABSTRACT

There is evidence that conceptal proteins in general, and bovine trophoblast protein-1 (bTP-1) specifically, alter biochemical mechanisms which are responsible for returning cattle to estrus. Endometrial explants, gland fragments, glandular epithelial cells, and stromal cells from uteri of cyclic cows (N=4) were cultured in medium containing 1 ug/ml bovine serum albumen (BSA), 1 ug bovine trophoblast protein-1 (bTP-1), 50 mU oxytocin after 8 h, or 1 ug/ml bTP-1 followed by 50 mU oxytocin at 8 h and examined their effects on prostaglandin and protein secretion. Explants treated with bTP-1 secreted less Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) than explants receiving BSA ($P<0.05$) or oxytocin alone ($P<0.001$). Oxytocin treated tissue or cells secreted significantly more $PGF_2\alpha$ than bTP-1 treatments ($P<0.05$) and BSA treatments ($P<0.05$) in all cultures. Glandular epithelial cell cultures treated with oxytocin secreted more prostaglandin E_2 (P,0.05) than cultures treated with BSA or bTP-1. Glandular epithelial cells produced more $PGF_2\alpha$ than any other group when normalized to total cellular protein, while stromal cells produced more PGE_2. There was no effect of treatment on incorporation of radiolabeled methionine and cysteine into macromolecules although stromal cells incorporated more radiolabel than other cell or tissue type. Two-dimensional-SDS PAGE and fluorography

revealed that similar proteins were secreted by explants, glandular epithelial cells, and intact gland fragments but no differences were observed due to treatment. These results indicate bTP-1 and/or oxytocin affect prostaglandin secretion in *in vitro* models of the bovine endometrium in a manner that helps explain *in vivo* observations.

CHAPTER 2

INTRODUCTION

Events that lead to a successful pregnancy must be coordinated between the embryo and its associated membranes (the conceptus) and maternal tissue. Maternal recognition of pregnancy is a term used to describe the response of the mother due to the presence of the developing conceptus. The conceptus must signal its presence prior to luteal regression which implies communication exists before implantation. In nonpregnant animals pulsatile episodic secretion of uterine $PGF_2\alpha$ during the late luteal phase of the estrous cycle (Day 17-18) causes luteolysis followed by a decrease in progesterone production and eventual return to estrus (See Bazer, 1992). Ovarian oxytocin is associated with these $PGF_2\alpha$ pulses and stimulates secretion of $PGF_2\alpha$ from the endometrium of ruminants, including the ewe (Sheldrick and Flint, 1986) and cow (Bazer et al., 1991). In addition, oxytocin receptors in the endometrium reach peak levels during luteolysis in sheep (Flint, 1986) and in the cow (Jenner, 1991). The conceptus must signal its presence to extend the functional lifespan of the corpus luteum (CL) and ensure a continued source of progesterone, an essential hormone for maintenance of early pregnancy in all eutherian mammals. During early pregnancy the pattern of $PGF_2\alpha$ secretion is attenuated, CL function is maintained and the number of oxytocin receptors

are reduced. It has become evident that the substance responsible for CL maintenance originates from the conceptus, is proteinaceous, and elicits its effect locally.

Preimplantation development of the conceptus in domestic ruminants is more extensive than in many other eutherian mammals. Communication must exist between the conceptus and mother prior to implantation in order to prevent luteolysis. In the cow, for example, the spherical blastocyst with a diameter of several millimeters on day 14-15 elongates to approximately 15 centimeters within a three day period. This elongation phase is primarily due to extensive growth of the outer cell layer, the trophectoderm, and as a result it lies in direct apposition to the uterine lumen where it eventually attaches at specific sites. Thus, vascularization between the developing conceptus and uterine wall does not occur until after at least 3 weeks of pregnancy. Secretory conceptus protein production has been characterized in the ewe (Godkin et al., 1984b) cow (Bartol et al., 1985; Lifsey et al., 1989) and goat (Gnatek et al., 1989). Godkin et al. (1982) identified a protein secreted by the conceptus during the critical period in which the CL must be maintained for a successful pregnancy to continue. Initially called ovine trophoblast protein-1 (oTP-1) and currently referred to as interferon tau (Roberts, 1992), it is the major secretory protein produced by the sheep conceptus during maternal recognition of pregnancy. Ovine trophoblast protein-1 binds

specifically to uterine epithelium (Godkin et al., 1984a; Stewart et al., 1987) and has been reported to alter uterine prostaglandin production (Salamonsen et al., 1988). Other studies have indicated that oTP-1 prompts the synthesis and secretion of pregnancy specific proteins by ovine epithelial endometrial cells (Salamonsen, 1991; Vallet, 1991). Godkin et al. (1984a) and Vallet et al., (1988) have demonstrated that oTP-1 extends the lifespan of the CL when injected into the uterine lumen of nonpregnant ewes between Days 12 and 18.

A comparable bovine conceptus protein which crossreacts with antiserum to oTP-1 has been designated bovine trophoblast protein-1 (bTP-1), (Helmer et al., 1987). It is the major secretory protein produced by the bovine conceptus between Days 17 and 22 and extends the interestrous interval when infused into the uterine lumen. Many isotypes of bTP-1 exist (pI 5.8-6.7) with molecular weights existing at 22000 and 24000, slightly higher than oTP-1 due to glycosylation. Initial cDNA cloning and protein sequencing identified oTP-1 and bTP-1 as type 1 interferons (IFN). Bovine TP-1 is know referred as interferon tau (IFNτ), which has the greatest similarity to bovine trophoblast interferon gene products (boIFNτ). Bovine trophoblast protein-1 has been shown to inhibit the secretion of PGF and PGE from bovine endometrial explants (Barros, et al., 1991), while a recombinant bovine interferon-alpha$_2$ inhibited PGF secretion and caused luteal maintenance in cyclic ewes (Parkinson, et al., 1992). In

addition, Helmer (1989) reported the induction of an endometrial protein secreted from explants receiving bTP-1. Rueda et al. (1993) identified two secretory proteins synthesized by the endometrium in response to rboIFNτ.

The purpose of this study was to determine the effects of bTP-1 and oxytocin on bovine endometrial explants and cells (epithelial and stromal) that comprise this tissue. Their effects on isolated endometrial cells will ultimately lead to identification of appropriate target cells and receptors to understand cellular mechanisms involved in maternal recognition.

CHAPTER 3

MATERIALS AND METHODS

Reagents.

Unless specified otherwise, tissue culture products were obtained from GIBCO Laboratories, Grand Island, NY. Collagenase and Dispase were obtained from Collaborative Research Incorporated, Bedford, MA; Trans-^{35}S-Label from ICN Radiochemicals, Irvine, CA; oxytocin, albumen, beta-mercaptoethanol, dithiothreitol, molecular weight standards, NP-40, TEMED, Trizma base and urea were purchased from Sigma Chemical Company, St. Louis, MO; acrylamide, Coomassie blue R-250, N,N-diallyltartardiamide, N, N -methylene-bis-acrylamide, and SDS from BioRad Laboratories, Richmond, DE; radioimmunoassay kits for $PGF_2\alpha$ and PGE_2 were purchased from Advanced Magnetics, Inc., Cambridge, MA; ampholines from LKB, Uppsala, Sweden and X-OMat /x-ray film were purchased from Eastman-Kodak, Rochester, NY. The bTP-1 was isolated as described in Lifsey et al. (1989).

Animals.

Angus and Hereford crossbred cattle were checked twice daily for signs of standing heat. On day 17 of the estrous cycle (estrus = day 0), cows were slaughtered and the reproductive tracts removed and placed on ice until further processed in the laboratory. The physiological status of the

ovary was confirmed by examination of ovarian morphology (Ireland, et al., 1979).

Explants Culture.

The uteri were trimmed, rinsed with sterile HBSS and placed in a laminar flow hood. Strips of endometrium were carefully dissected from the underlying myometrium and included both caruncular and intercaruncular tissue. The endometrial strips were placed in a 100mm dish containing Hank's Balanced Salt Solution (HBSS) and diced with sterile scalpel blades into small pieces (30 mg each). Approximately 250 mg of tissue were washed in HBSS and placed in 35 mm petri dishes containing 5 ml Minimum Essential Media (MEM) containing the following treatments in triplicate: **1)** 1 ug/ ml BSA, **2)** 1 ug/ml bTP-1, **3)** 50 mU/ml oxytocin (after 8 h in culture, and **4)** 1 ug/ml bTP-1 + 50 mU oxytocin after 8 h of culture. Cultures were maintained at 37° C in a 5% CO_2 incubator on a rocking platform. Medium was collected at 24 h, centrifuged (1000 x g for 15 min.) to remove debris and stored in aliquots at -80 degrees C.

Isolation of endometrial cells.

Reproductive tracts were trimmed, rinsed with HBSS and placed in a laminar flow hood. The following procedures were performed aseptically. Each uterine horn was cut longitudinally. The exposed endometrium was dissected away

from the myometrium and washed in two changes of Hank's Balanced Salt Solution without calcium and magnesium (HBSS - Ca, -Mg), containing penicillin (200 units/ml), streptomycin (200μg/ml and fungizone (0.5 mg.ml)(ABAM, Gibco Laboratories, Grand Island, NY). Luminal cells were observed under a dissection microscope and when detached from the endometrium, these were collected from the final wash after physically agitating the tissue with forceps. These sheets of cells were allowed to settle in a 50 ml conical tube containing HBSS and subsequently cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS). The remaining endometrial strips were minced into small fragments (2-4 mm^2) and placed into a 500 ml flask containing a digestion medium of 0.1 % collagenase and 0.5 U/ml dispase in HBSS -Ca-Mg. After 2 hours in a 37 degree shaking water bath digestion was terminated . The cell suspension was diluted in HBSS and passed through sterile nylon mesh into a large petri dish (150 mm). Visible glandular fragments too large to pass through the mesh were removed and placed in DMEM containing 10 % FBS. The remaining cell suspension was centrifuged (50 x g) for 3 minutes and the pellet containing smaller fragments was combined with the larger fragments. A more uniform fragment size consisting of between 50 to 250 cells was obtained by disrupting the larger fragments through repeated pipeting. Single cells could not be obtained due to the inability of the collagenase to disrupt the tight association between the cells

within a gland. The fragments were cultured in DMEM containing 10% FBS, and antibiotics. The supernatant of the above procedure containing primarily stromal cells was centrifuged at 400 x g for 10 minutes. The pellet was resuspended in culture medium.

Radioimmunoassay.

Concentrations of prostaglandin E_2 (PGE_2) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) were determined by radioimmunoassay (RIA) using kits obtained from Advanced Magnetics Inc., (Cambridge, MA). Interassay and intra-assay coefficients of variation were 7.6% and 12.8% for $PGF_2\alpha$, and 9.4% and 13.2% for PGE_2, respectively.

Radioactive labelling of tissue and cells.

Endometrial explants and isolated cell cultures (glandular epithelial, luminal epithelial, and stromal) were cultured with treatments as described except methionine was reduced 90% and 5 μCI/ml ^{35}S-Tran label was added. After 24 h medium was removed, centrifuged (1000 x g for 15 min) and stored at -80 C.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE).

Aliquots of unfractionated, dialyzed medium from tissue and cell cultures were lyophilized and dried samples were dissolved in a volume of 5 mM K_2CO^3 containing 9.4 M urea, 2%

(v/v), Nonidet P-40, and 0.5% (w/v) dithiothreitol, sufficient to allow 100,000 cpm of nondialyzable radioactivity or 150 ug total protein (Lowry et al., 1951) to be loaded onto each gel in a total volume of not more than 100 ul. Methods of sample preparation and electrophoretic separations of polypeptides by 2D-PAGE were described by Roberts (1984). After electrophoresis, Coomassie Brilliant Blue R-250-stained, 12% (w/v) polyacrylamide slab gels were photographed, impregnated with sodium salicylate (Chanberlain, 1979), dried, and exposed to Kodak XAR film (Kodak, Rochester, NY) as described previously (Roberts et al., 1984).

Incorporation of ^{35}S-Trans Met/Cys into macromolecules.

The amount of radiolabel incorporated into macromolecules was determined by trichloroacetic acid (TCA) precipitation. Cells from incubations with ^{35}S-Trans Met/Cys were solubilized by sonication in 50mM Tris buffer. Aliquots of medium and solubilized cells were each placed and dried onto Whatman 3MM paper that previously had been saturated with 20% TCA (wt/vol). Precipitation of proteins onto the filter paper and removal of nonproteinaceous compounds was accomplished by serial washing of filter paper with 20% and 5% TCA followed by 95% ethanol as described by Mans and Novelli (1961). Radioactivity of precipitated protein was quantified by scintillation counting.

Protein Determination.

Total protein was determined by the method of Lowry (Lowry, 1951), using bovine serum albumen (BSA) as a standard.

Statistical Analysis.

Means of detectable prostaglandins from the medium of triplicate dishes were log transformed and statistical analysis was performed using analysis of variance (ANOVA), followed by Tukey-Kramer Multiple Comparisons Test. (InStat, GraphPad Softwares, Inc.).

CHAPTER 4

RESULTS

Prostaglandin secretion by endometrial explants.

Figure 1 illustrates the effect of treatment on $PGF_2\alpha$ and PGE_2 secretion by endometrial explants. Comparison of treatment means revealed that explants treated with bTP-1 produced significantly less $PGF_2\alpha$ than either oxytocin treated tissue ($p<0.001$), or tissue receiving BSA alone ($P<0.05$). Other treatment means were not different from one another. Secretion of Prostaglandin E (PGE) indicated no difference due to treatment. As illustrated the amount of $PGF_2\alpha$ was approximately 5-7 times greater than PGE_2.

Prostaglandin secretion by isolated endometrial cells.

Figures 2-4 illustrate the prostaglandin production by the different isolated cells due to treatment.

Glandular Epithelial Cells. $PGF_2\alpha$ secretion by oxytocin treated cells was significantly higher than cells treated with bTP-1 ($P<0.01$) or BSA ($P<0.001$), while cells receiving bTP-1 plus oxytocin produced more $PGF_2\alpha$ than cells given BSA alone.

Oxytocin treated epithelial cells also produced more PGE_2 than cells receiving either BSA ($P<0.001$) or bTP-1 ($P<0.01$). Cells given both bTP-1 and oxytocin produced more PGE_2 than BSA treated cells ($P<0.05$) or bTP-1 ($P<0.01$).

Glandular epithelial cells produced more $PGF_2\alpha$ than PGE_2

regardless of treatment (Figure 2).

Stromal Cells. Stromal cells were the only cell type that produced more PGE_2. Although comparatively low levels of $PGF_2\alpha$ were detected in stromal cell culture medium all treatment means were significantly different except cells treated with both bTP-1 plus oxytocin compared to cells receiving BSA alone. No treatment differences in PGE_2 production were detected (Figure 3).

Gland Fragments. Prostaglandin production by gland fragments was determined during the first 24 h after isolation. More $PGF_2\alpha$ was produced than PGE_2 in these cultures. Treatment had no effect on PGE_2 production. However, oxytocin treated glands produced more $PGF_2\alpha$ than cultures given BSA ($P<0.001$) or bTP-1 ($P<0.001$), while gland cultures receiving both bTP-1 and oxytocin produced more $PGF_2\alpha$ than BSA treated cultures ($P<0.01$) or cultures given bTP-1 alone ($P<0.05$). (Figure 4)

Luminal Cells. Luminal cells did not secrete prostaglandins above the limits of detection.

Comparison of prostaglandin production by different cells.

The various cells isolated from the bovine endometrium secreted prostaglandins differently when controls (BSA) of each cell type were compared (Table 1). Glandular cells produced significantly more PGF than either the fragments or stromal cells. Gland fragments were not different than stromal cells. Stromal cells secreted significantly more PGE than

gland fragments ($p<0.001$) and glandular cells ($p<0.05$). Of all the cell groups only stromal cells produced more PGE than PGF.

Incorporation of radiolabel into macromolecules.

There was no effect of treatment on incorporation of radiolabel into macromolecules in explant cultures or cell cultures. However, stromal cells incorporated more radiolabel into macromolecules than either glandular fragments ($p<0.001$) or glandular cells ($p<0.05$) (Table 2).

Analysis of endometrial secretory proteins by SDS-PAGE.

Treatment had no effect on radiolabeled proteins secreted into the medium. Explant cultures and glandular epithelial cells produced many proteins with similar electrophoretic mobilities (Figure 5). These included a band of acidic proteins (pH=5.3) with molecular weights (mw) of approximately 95kd, 80kd, 43kd. Proteins secreted by explants and gland fragments included a group of 45kd proteins (pH-5.9-6.6), while proteins with the same pH, with mw=78kd were expressed by gland fragments and glandular cells. Fluorographs of proteins secreted by stromal cells did not demonstrate any commonality to explant, fragment, or glandular epithelial cell cultures. Despite relatively high incorporation of radiolabel by the stromal cells, few proteins were evident from the fluorographs. This was probably due to the amount of time before SDS-PAGE was performed. Luminal cells did not exhibit

secreted proteins as predicted from their failure to incorporate radiolabel.

CHAPTER 5

DISCUSSION

Investigators have previously demonstrated that conceptal proteins alter prostaglandin production by the endometrium in the bovine (Gross, et al., 1988; Helmer et. al., 1989; Thatcher et al., 1992) and in the ovine (Vallet, et. al., 1988; Salamonsen, et. al., 1988). Parkinson et al. (1992) reported that bovine IFNα caused a reduction in plasma $PGF_2\alpha$ levels of cyclic ewes while extending the estrous cycle. These data were based on studies utilizing intact animals or explant culture. Results from the present experiment indicates that bovine Trophoblast Protein-1 (bTP-1), recognized as the protein responsible for maternal recognition of pregnancy, significantly modifies prostaglandin production by bovine endometrial explants and isolated endometrial stromal cells. Although bTP-1 reduced the prostaglandin production in glandular fragments and glandular epithelial cells in a monolayer, the effects were not significantly different.

Helmer et al. (1989), reported that the reduction of PGF secretion by bovine endometrial explants treated with bCSP (bovine conceptal secretory proteins) or bTP-1 occurred within the first 12 h and was attributed to an inhibitor, while the subsequent rate of secretion was unchanged. In the present experiment glandular epithelial cells and stromal cells were allowed to reach confluency prior to treatment which may

partially explain their failure to respond to bTP-1 at a significant level. Also, the antiviral activity of bTP-1 isolated in different laboratories is probably different and may reflect a dose response. Helmer (1989) also reported an increase in PGE_2 production and attributed this finding to the combined effects of the conceptal proteins on the distinct cells that comprise the bovine endometrium. We observed a decrease in both $PGF_2\alpha$ and PGE_2 production, suggesting that bTP-1 affects enzymes involved in arachidonic cleavage from the membrane or the cyclooxygenase activity on arachidonic acid prior to the formation of Prostaglandin H_2, an intermediate metabolite common to all prostanoids. Salamonsen et al. (1988) also reported that oTP-1 or human interferon-α2 significantly inhibits both $PGF_2\alpha$ and PGE_2 production by isolated ovine endometrial cells, but could not associate the change in production to cyclooxygenase activity (Salamonsen, 1991). While the affects of bTP-1 indicated a similar response on all cell types, the degree of variability among cows may also affect the results.

While oTP-1 and bTP-1 are similar (Lifsey, 1989; Helmer, 1987), there are apparent differences in their biochemical make-up, binding properties, and possible mode of action. Stewart et al., (1987) demonstrated that unlabelled oTP-1 or IFN can inhibit ^{125}I-labelled human IFN binding to membrane receptors from sheep uteri. In the present study bTP-1 had a significant effect on prostaglandin secretion by endometrial

explants and isolated stromal cells. This suggests that the underlying stromal cells may be involved in a paracrine interaction with other endometrial components to alter biochemical responses. Our results indicate that explant cultures and epithelial cells produce more $PGF_2\alpha$, while stromal cells produce more PGE_2. Salamonsen et al. (1991) has reported similar findings in the ewe.

Oxytocin affected prostaglandin secretion by explant cultures and isolated endometrial cells more than any treatment. Receptors for oxytocin located on the endometrium increase rapidly between Days 17 and 20 in the cow (Bazer, 1992) and Days 14 and 16 in the ewe. Although endometrial oxytocin receptors are present between estrus and Day 4, they are low or undetectable between Days 5 and 16 in the cow (Days 5 and 13 in the ewe). McCracken et al. (1984), postulated that progesterone may inhibit synthesis of oxytocin receptors on the endometrial epithelium by binding to its receptor as oxytocin receptors increase following down regulation of progesterone receptors. Oxytocin from the CL as well as the posterior pituitary acts through its receptors on the endometrial epithelium to stimulate the inositol phospholipid second messenger system which induces the pulsatile release of $PGF_2\alpha$. Soloff and Fields (1989) reported that oxytocin binds to the myometrium of bovine uterus at estrus more than during the early luteal phase. Parkinson et al., (1991), reported that endometrial oxytocin receptor concentrations were higher

in non-pregnant heifers at Day 18 when compared to pregnant animals during this time. In addition, recombinant bovine IFNα caused a decrease in oxytocin receptor in cyclic and progesterone treated ovariectomized ewes (Vallet, 1991). Presumably, oxytocin receptor concentrations are important determinants of physiological changes in uterine sensitivity for oxytocin. There is strong evidence that luteolysis occurring after an injection of oxytocin into cows is linked to the stimulation of uterine prostaglandin synthesis (Armstrong and Hansel, 1959; Anderson et al., 1965; Ginther et al., 1967; Milvae and Hansel, 1980). Our results indicate that exogenous oxytocin (50mU/ml) induces prostaglandin F_2 secretion from endometrial explants, gland fragments, glandular cells, and stromal cells, while its effect on PGE production was limited to glandular cells. Perhaps the PGF pathway is enhanced by oxytocin more than alternative pathways.

The present study also demonstrates that the cells which comprise the bovine endometrium secrete prostaglandins differentially. Glandular epithelial cells, gland fragments and explants produce more $PGF_2\alpha$ than PGE_2, while stromal cells produce more PGE_2. Previous studies have also reported that stromal cells produce PGE_2 predominantly (Fortier et al., 1988; Salamonsen et al., 1991). $PGF_2\alpha$ from the endometrium travels via the utero-ovarian vasculature in a counter current mechanism to the ovary where it interacts specific receptors.

The interaction initiates luteal regression during the late luteal phase of the cycle. Presumably, bTP-1 secreted by the unattached conceptus during the period of maternal recognition of pregnancy alters this process, possibly by altering prostaglandin synthesis by the cells that line the endometrium. While our study did not show a significant reduction in $PGF_2\alpha$ by cells or explants treated with bTP-1, there was a tendency for lower values.

Previous studies have demonstrated that bTP-1 not only alters prostaglandin production by the bovine endometrium, but also affects the incorporation of radiolabel into macromolecules and modifies secretory proteins (Gross et al., 1988; Helmer et al., 1989; Rueda et al., 1991; Geisert et al., 1988). Helmer (1989) reported that explants treated with bTP-1 incorporated less radiolabeled amino acid than controls treated with BSA. However, the control group containing medium alone, bTP-1 (1 μg/ml) treatment, and bCSP treatments were not different from one another. In another study Gross (1988) indicated that bCSP reduced the amount of radiolabel incorporated into proteins from endometrium from cyclic animals, but did not affect pregnant cows. Changes in incorporation of radiolabel due to treatment (BSA, bTP-1 and/or oxytocin) were not different in the present study. However, stromal cells incorporated radiolabel more than the epithelial cells.

There have been several studies investigating the effect

of the conceptus and its secretory products (including bTP-1) on protein synthesis by the bovine endometrium. Helmer (1989) and Gross (1988) both reported enhancement of a secreted protein (mw=14,900) in response to bCSP. However, this protein was not altered by bTP-1 alone (Helmer et al., 1989). Geisert et al. (1988) described two groups of intensified low molecular weight proteins (Mr 14-16 X 10^{-3} and Mr 35 X 1^{-3}) secreted on Day 17 from endometrial explants of pregnant cows compared to cyclic animals. Rueda et al. (1993) reported that bovine endometrial explants from pregnant and nonpregnant animals cultured in the presence of recombinant bovine IFNτ showed a dose dependent increase in synthesis of 12 and 28 kD molecular weight classes of proteins. Our results did not indicate an effect of treatment on proteins secreted by the endometrium (explants or cells) as revealed by 2D-SDS PAGE and fluorography. It is possible that proteins secreted from the conceptus, other than bTP-1, affect maternal tissue in an eclectic manner to alter its environment favorable to the conceptus.

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Figure 1. Comparison of $PGF_2\alpha$ and PGE_2 secretion due to treatment (BSA, bTP-1, oxytocin and bTP-1 plus oxytocin) by cultured bovine endometrial explants. Data expressed as log of the mean of each treatment value.

Figure 2. Comparison of PGF2α and PGE2 secreted by isolated bovine glandular epithelial cells.

Figure 3. Comparison of PGF2α and PGE2 secreted by isolated bovine stromal cells.

Figure 4. Comparison of PGF2α and PGE2 secreted by isolated bovine glandular fragments.

Figure 5. Two-dimensional PAGE analysis of [^{35}S] Tran MET/CYS metabolically labeled proteins secreted by bovine uterine endometrial explants.

Figure 6. Two-dimensional PAGE analysis of [^{35}S] Tran MET/CYS metabolically labeled proteins secreted by endometrial glandular epithelial cells

Prostaglandin Production

Bovine Endometrial Explants

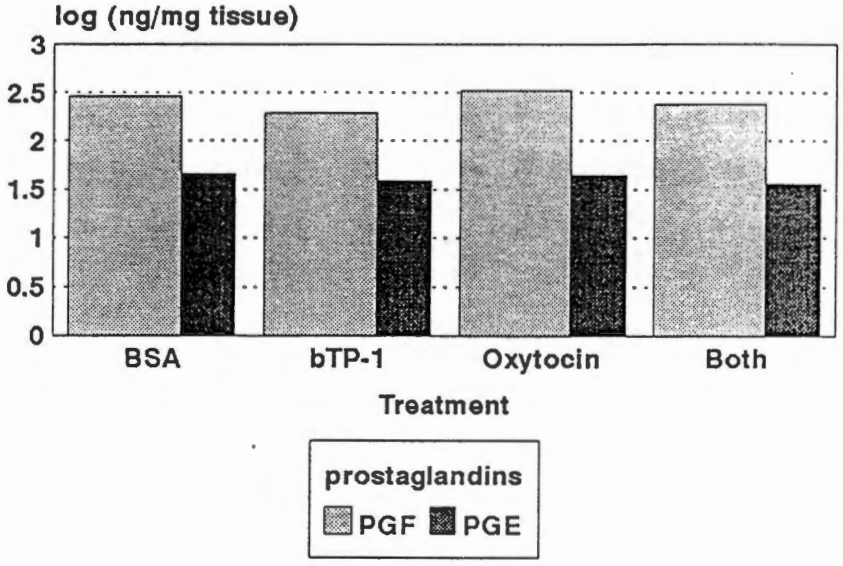

Figure 1.

Prostaglandin Production

Glandular Epithelial Cells

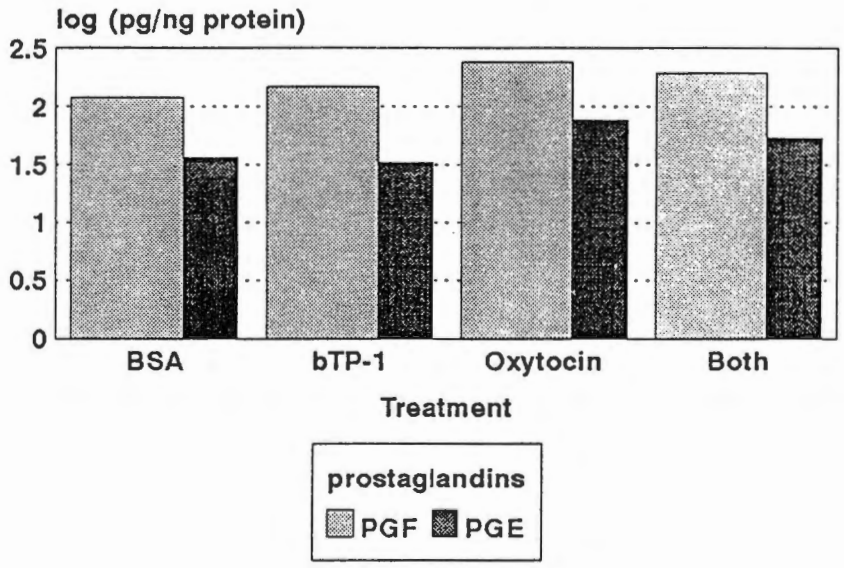

Figure 2.

Prostaglandin Production

Stromal Cells

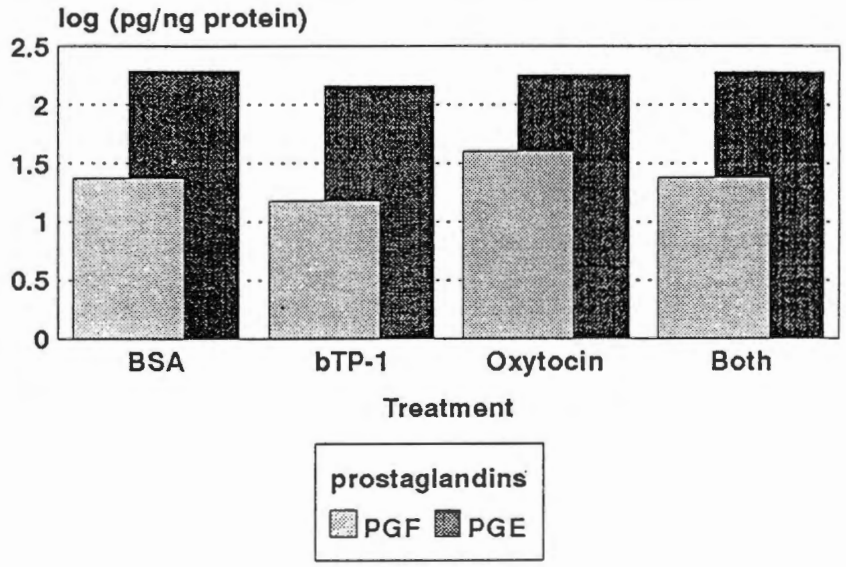

Figure 3

Prostaglandin Production

Glandular Fragments

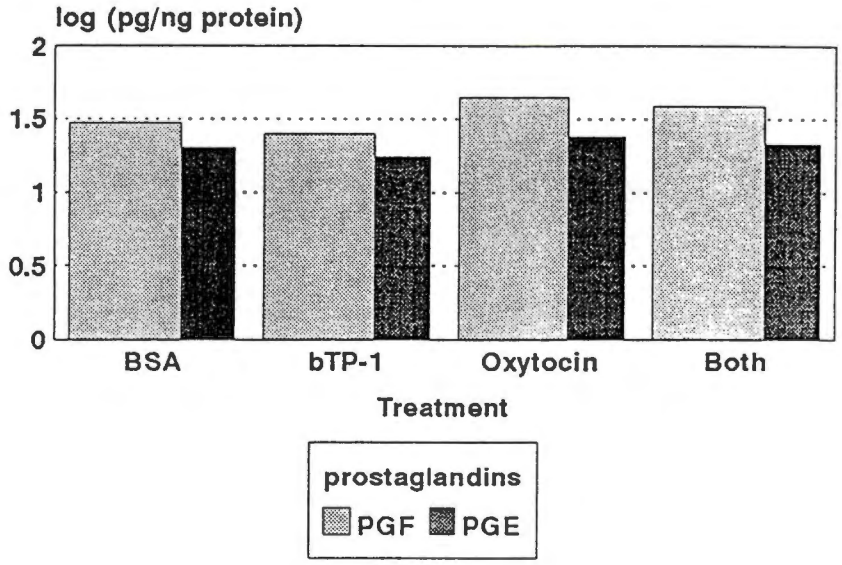

Figure 4.

BOVINE ENDOMETRIUM

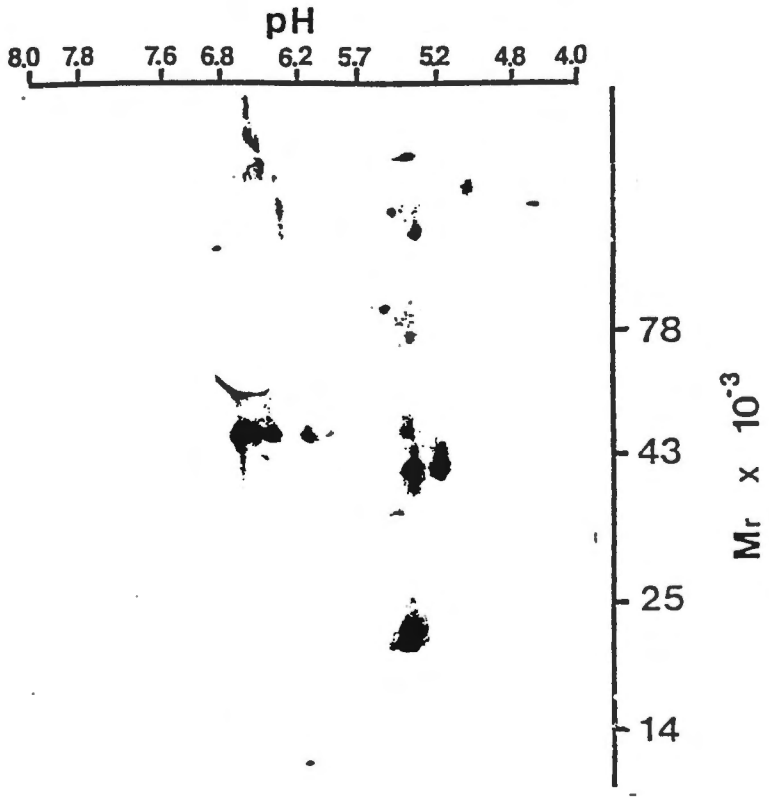

Figure 5.

GLANDULAR CELLS

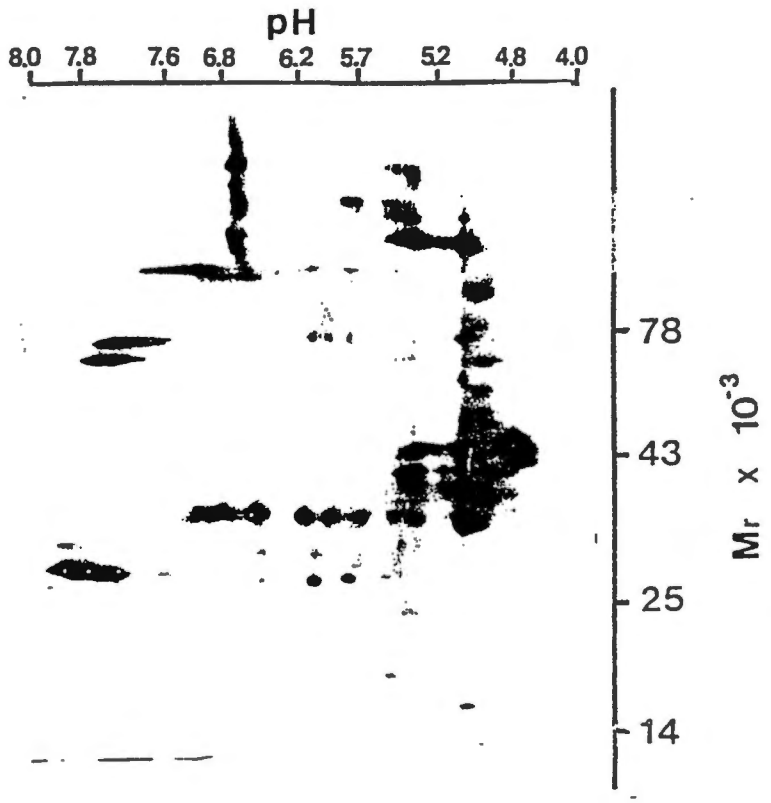

Figure 6.

PART 4: GENERAL SUMMARY

CHAPTER 1

SUMMARY

Maternal recognition of pregnancy is a phrase used to describe the physiological, endocrinological, and immunological response of the mother to the presence of an embryo in the reproductive tract. (Short,1969; Bazer,1992; Roberts,1992). The early pregnancy conceptus product receiving the most attention in the past decade has been interferon-τ, (IFNτ), originally called bovine trophoblast-1, and probably the same protein referred to as trophoblastin by Martal (1979). Within the dynamics surrounding early pregnancy, the affect of IFN-τ on the bovine uterine endometrium in general, and specifically each cell type, seemed an intriguing piece of information lacking from the maternal recognition of pregnancy paradigm. It has long been demonstrated that IFN-τ suppresses the pulses of $PGF_2\alpha$ that causes luteolysis, while the absence of a viable embryo causes the endometrial epithelium to respond to luteal oxytocin, which initiates luteolysis. Therefore, IFN-τ and oxytocin were two diametric cell products used to test our cell model system and to investigate their effects on the bovine uterine endometrium. The data presented in the previous chapters is an effort to add new information to this area of research.

In summary, cells from dissected bovine uterine endometrium were isolated using differential centrifugation

along with a collagenase/Dispase digestion. Eventually, three cell populations emerged as the primary cell types which constitute uterine endometrial tissue. Luminal cells or surface epithelium, isolated from the endometrium without enzymatic treatment, were found to be very fragile and, therefore, difficult to characterize. These cells appeared to terminally differentiated by Day 17 of the estrous cycle as they fragmented after a few days in culture, produced no detectable proteins, and only trace amounts of prostaglandins (PGF and PGE). Endometrial glandular epithelial cells were isolated in clumps using differential centrifugation after exposure to the digestion solution. Cultured glandular fragments formed colonies in culture and eventually generated monolayers of epithelial cells. Morphologically, these cells appeared flattened, cuboidal, and epithelium-like. Immunocytochemical staining of these cells with cytokeratin, a marker for epithelial cells, confirmed the morphologic observation. Ultrastructural examination indicated that the glandular epithelial cells were attached to a basal lamina with an apical membrane composed of irregular microvilli.

Stromal cells were isolated from the endometrium after enzymatic digestion and differential centrifugation. These cells rapidly adhered to uncoated plastic and exhibited a fibroblastic-like morphology exhibiting cytoplasmic processes. Vimentin, a marker for fibroblastic cells, was used to confirm immunocytochemically the morphologic observations.

Isolation and culture of distinct cell types has the apparent advantage of treating only those specific cells and allows scrutiny of their products. One of the disadvantages is that primary cells in culture may not perform as cells *in vivo* without the support of substrate or products from other cells associated with the endometrium. Therefore, bovine uterine explant cultures were used to compare the results obtained from the cell data. Explants containing all three cell types including endothelium and substrate components were cultured in similar culture conditions. Also, the gland fragments used to initiate glandular epithelial cell cultures were subjected to similar treatment. With few exceptions IFN-τ caused a decrease in prostaglandin production (although not all were statistically significant), while oxytocin promoted an increase in the amount of prostaglandin detected. Although IFN-τ and/or oxytocin did not alter the secretory pattern of metabolically labeled protein, several proteins of similar electrophoretic mobility were observed between explant cultures and glandular epithelial cells. Explants, gland fragments, and glandular epithelial cells produced more PGF than PGE, while stromal cells produced much more PGE.

This study provides new information in the area of maternal/fetal interactions during early pregnancy. The description of a relatively simple method for the isolation of epithelial and stromal cells from the bovine uterine endometrium may provide insight for new investigations

involving their mechanism of action. As described in Chapter 3, the time of cell harvest within the estrous cycle is critical. Fortier et al. (1988) suggested that recovery and viability of endometrial cells were optimum between Days 2-12, while luminal cells at Day 17 in this study appeared terminally differentiated. The estrous cycle in the cow is approximately 21 days in length and the hormonal environment changes throughout this period. At Day 17 the bovine uterine endometrium has been exposed to high levels of progesterone for 10-12 days and if a viable embryo is not present, the uterus responds to oxytocin and produces the luteolysin, $PGF_2\alpha$. In addition, this time period coincides with the release of IFN-τ from the trophoblast of the preimplantation conceptus.

The cells used in this study produced differentiated cell products (prostaglandins and proteins) and responded to stimuli in a manner similar to the uterine endometrium *in vivo*. Therefore, they meet the criteria necessary to be used as a viable *in vitro* model system. The contribution of specific uterine cells to the endometrial response and their role in pregnancy recognition may be further elucidated with the use of a workable cell model.

A deficiency of this study is the inability to observe paracrine interactions between the different cell types as well as to examine the contribution of the basal lamina on differentiated cell products. The close relationship between

all endometrial components suggests that all are interrelated not only physically, but also biochemically. Therefore, products from one cell type may influence the response of another. The use of primary cell cultures reduces their reliance on other cell products to a certain extent by obtaining cells that have been "preconditioned" in the animal prior to harvest. For example, uterine epithelial cells from Day 17 cows have a large number of receptors for oxytocin, while cells taken from Day 12 animals have very few.

In addition to describing a method for the isolation of endometrial cells, the data presented in this study differ with information generated by several prior investigations. Helmer (1989) reported an increase in PGE production in response to bTP-1 (IFN-τ) from endometrial explants, while observing a decrease in the production of PGF. In this study it was found that bTP-1 caused a decrease in both PGE and PGF in explants cultures as well as the specific cell types. Several investigators have also reported altered protein production by uterine explants in response to bTP-1 (Helmer, 1989; Geisert et al., 1988; Rueda et al., 1991). However, there is no consensus among investigators on which protein is affected. We report no change in the pattern of metabolically labeled protein production as determined by 2D-SDS-PAGE.

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