Secretion of basic fibroblast growth factor in corpora lutea of pregnant sows

by

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LIST OF ABBREVIATIONS

Ab	Antibody
ABC	Avidin biotin complex
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
BSA	Bovine serum albumin
CL	Corpus luteum
DAB	3, 3'- diaminobenzidine tetrahydrochloride
ddH_2O	double distilled water
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
HCG	Human chorionic gonadotropin
ICC	Immunocytochemistry
Kb	Kilobase
KDa	Kilodalton
LH	Luteinizing hormone
NGS	Normal goat serum
NRS	Normal rabbit serum
PBS	Phosphate buffer saline
RIA	Radioimmunoassay
SDS	Sodium dodecylsulfate
TNF	Tumor necrosis factor
TGFα	Transforming growth factor α
TGFß	Transforming frowth factor ß

GENERAL INTRODUCTION

The ovary is an important organ of the female reproductive system. It is an endocrine gland which is capable of secreting several hormones and growth factors which are essential for the functions, growth and development of female reproductive system.

The most important function of ovary is to produce oocytes. Oocyte maturation results from a series of events. During the initial phase (hormone-independent) of follicle development, the oocyte increases in both size and activity with increased production of RNA and ribosomes. Granulosa cells, also called follicular cells, begin to grow and divide during this period (Stabenfeldt *et al.*, 1984). As the ovarian follicle grows in size under hormone stimulation, a large amount of fluid is produced to exert pressure on the *tunica albuginea* of the ovary. This results in a definite bulging and consequent thinning of the surface of the follicle and it eventually ruptures. At this time, the ovum is expelled out of ovary together with follicular fluid (Frandson, 1981). In swine, ovulation occurs about 36-42 hours after the onset of estrus or about 6 hours prior to the end of estrus. It always occurs in both ovaries (Stabenfeldt *et al.*, 1984).

The rupture of follicles after ovulation leads to formation of corpus luteum (CL). The formation of the CL, or luteinization of the granulosa cells, begins with the conversion of the granulosa cell secreting capacity from estrogen secretion to progesterone secretion which is stimulated by the luteinizing hormone (LH) surge (Stabenfeldt *et al.*, 1984). There is a rapid proliferation of primarily granulosa cells and a few theca cells lining the follicle wall (Anderson, 1974). During luteinization, the epithelial cells lining the empty follicular cavity

also begin to multiply (Frandson, 1981). Vascular development of the ovarian follicles becomes even more impressive after ovulation, in association with development of the CL (Reynolds *et al.*, 1992). Generally, the CL consists of granulosa cells, some theca cells, and a fibrin clot which forms in the cavity of the ruptured follicle. The fibrin serves as a framework for granulosa cells and blood vessels to develop. The blood vessels will eventually grow into the interior of the follicle allowing vascularization of granulosa cells and migration of theca cells. The luteal activity lasts about 14 days in the sow.

The corpus luteum is a unique tissue. Its presence is essential for the maintenance of pregnancy, especially in the early stages as it produces progesterone. Progesterone can also be produced by the placenta and adrenal cortex. However, the amount of progesterone produced by the placenta or adrenal cortex is not sufficient to maintain pregnancy in sows (Stabenfeldt *et al.*, 1984). Thus, the CL is the primary source of progesterone in pregnancy and lasts in swine throughout the gestation period as a CL of pregnancy (Frandson, 1981). However, if the ovum is not fertilized, the CL regresses and disappears within another 9 days.

It is obvious that the CL plays an important role in the pregnancy. But how does the CL function and how is its function regulated? It has been demonstrated that not only hormones, but also growth factors, have regulatory effects on CL function. In many animals, the preovulatory surge (LH surge) is the driving force for both formation and initial maintenance of the CL (Stabenfeldt *et al.*, 1984). Usually, the basal circulating levels of LH are sufficient for continued maintenance of the CL. Prolactin, together with LH, may be important for the maintenance of the CL in a few animals such as sheep, mice, and rats (Stabenfelt *et al.*,

1984). Past studies have demonstrated that growth factors, including epidermal growth factor (EGF) and fibroblast growth factors (FGF), can induce proliferation of granulosa cells *in vitro* (Tapanainen, *et al.*, 1987). Additionally, the presence of several other growth factors has been established in ovarian tissues (Hsueh *et al.*, 1984; Bellve and Feig, 1984). One of them, bFGF, is a potent angiogenic, growth and differentiation factor. Basic FGF was first isolated from bovine brain and pituitary as a mitogen for fibroblast. In addition, its presence has been identified in various other tissues, including kidney, adrenal, retina, CL and liver (Baird *et al.*, 1986). Moreover, the effects caused by each growth factor and their interactions with each other on the cellular responsiveness in gonadal tissues have been studied.

The most dramatic effect of bFGF in the CL is angiogenesis. Gospodarowicz *et al.* in 1985, established that the angiogenic factor in the CL is related to bFGF. The effect of bFGF on granulosa cells and endothelial cells in the ovary has also been extensively studied (Gospodarowicz *et al.*, 1986; Subhasis *et al.*, 1987). In 1987, Tapanainen *et al.* found that bFGF could stimulate granulosa-luteal cell proliferation, but did not affect their production of progesterone. However, it was found that in some instances, bFGF has almost no effect on cell proliferation, except for the differentiation function (Baird *et al.*, 1985). In addition, bFGF is proposed to have an effect on the modulation of other hormone or growth factor secretions. For example, bFGF inhibits basal relaxin secretion by large luteal cells *in vitro* (Taylor and Clark, 1992).

Taken together, these reports suggest that bFGF could play a role in the regulation of the growth, development and possible function of the CL. It functions in cell proliferation,

regulation of other hormones and growth factors, and on angiogenesis. Since the CL during pregnancy maintains its function until postpartum, one can postulate a potential role for bFGF in this tissue throughout pregnancy. However, this role has not been established. If a functional role of bFGF in CL of pregnancy exists, there may be an alteration in the amount of bFGF secretion, a change in its receptor expression, or certain steps changed in the process of signal transduction. In fact, there is a possibility that the concentration of bFGF may change during pregnancy. Since relaxin secretion by luteal cells increases during pregnancy and peaks just before parturition (Huan *et al.*, 1991), and the work previously done in our lab (Taylor *et al.*, 1992) suggests that there is an effect of bFGF on relaxin secretion in the CL during pregnancy, it is possible and worthy of investigation that changes in the concentration of bFGF in the pregnant CL might occur which would relate to the changes in relaxin secretion. Therefore, the first objective of this research was to examine the amount of bFGF in the CL throughout pregnancy in sows. In the first part of the experiment, the existence of bFGF in different stages of CLs was examined by Western Blot analysis. The resulting immunoreactive bands on the blots were then analyzed for their intensities. Using the data obtained from above analysis, together with the known concentration of the purified bFGF, we calculated the concentrations of bFGF in every stage of CL sampled. In this way, we were able to tell if there was a difference in levels of bFGF in each stage of pregnancy.

Many types of cell are known to produce bFGF. Potential sources in the CL include endothelial cells, granulosa-luteal cells and macrophages. In the CL, endothelial cells and epithelial cells of vascular tissue comprise almost 50%, while most of parenchyme are small

and large luteal cells. However, the cell source of bFGF in the CL of pregnant swine and whether the same cell remains the primary source for bFGF secretion during the whole stage of pregnancy is uncertain.

Therefore, the second objective of the present study was to determine the cell source of this growth factor in luteal tissue during pregnancy.

In the second section, the cellular source for bFGF secretion was studied by using immunocytochemistry. The avidin-biotin technique was used for localization of bFGF and the cell source of this growth factor was examined.

From the present research, we hoped to find if there was a pattern of bFGF secretion in the whole life span of corpora lutea during pregnancy which would help to elucidate the possible physiological relevance of bFGF in luteal tissue. In addition, we hoped to establish the cell source for bFGF production in luteal tissue, which would provide an insight into its role in the local function in luteal tissues.

This thesis consists of two sections. Section I is about the study of the level of bFGF in the CL by Western Blot analysis, and section II is to describes the cellular location of bFGF in the luteal tissue using immunocytochemistry. Both sections begin with the introductions followed by materials and methods. The results and discussion are then presented. Finally, a general discussion is provided that summarize the whole project.

SECTION I WESTERN BLOT ANALYSIS OF BASIC FIBROBLAST GROWTH FACTOR IN CORPORA LUTEA OF PREGNANT SOWS

INTRODUCTION AND LITERATURE REVIEW

General history and biological properties of FGF

During the 1970s, several laboratories found that pituitary extracts exhibited mitogenic activity. It was in 1974 that Gospodarowicz *et al.* established the basic properties of a FGF from the studies on both brain and pituitary (Gospodarowicz *et al.*, 1974). In the following years, the biological activities of FGF were established, and it became clear that FGF could stimulate not only fibroblast growth but also the growth of other cell types including chondrocytes, adrenocortical cells, vascular smooth muscle cells and vascular endothelial cells (Baird *et al.*, 1986). Therefore, while the name FGF implies its activity on fibroblasts, it became obvious that its activity could be expanded to include several cell types closely connected by their derivation from the primary and secondary mesenchyme (Baird *et al.*, 1986).

Recently, there has been progress made in delineating the biochemical and biological properties of FGF. In brief, the FGFs are a family of polypeptides consisting of seven structurally related polypeptides among which bFGF (bFGF) and acidic FGF (aFGF) are the best characterized. Basic FGF is a 146-amino acid polypeptide. Lobb found in 1988 that tissue and species-specific forms are found to originate from N-terminal truncation due to proteolysis that results in molecular masses between 16 and 18 kDa. Basic FGF's high affinity for heparin or heparin-like molecules is a unique property and has been applied in the purification procedure by many laboratories (Makris *et al.*, 1989). Basic FGF is an angiogenic factor and a mitogen for many mesoderm-derived cells. In addition, bFGF also

influences proliferation and differentiation of glial cells (Grothe *et al.*, 1990). It plays an important role in normal physiological processes *in vivo* such as embryonic development, angiogenesis, nervous cell system differentiation, and wound repair (Baird *et al.*, 1991).

Receptor diversity of FGF

FGF appears to act through plasma membrane receptors to exert its effects on target cells. The cell surface heparin sulfate proteoglycans were revealed first as low-affinity receptors that modulate FGF activity (Baird *et al.*, 1991). So far, at least four distinct genes encoding FGF receptors have been cloned. The receptors for FGFs comprise a super family. The diversity of FGF receptor forms probably arises in part from the transcription of four different receptor genes, each of which may produce alternatively spliced transcripts to yield alternative protein forms. There are at least 7 FGFs and 4 tyrosine kinase-linked FGF receptors. Many studies on the ligand/receptor specificity imply a very high level of redundancy in FGF receptor/ligand interactions in that multiple ligands can bind multiple receptors with essentially equal affinity (Dionne *et al.*, 1991).

Action mechanism of bFGF

Basic FGF is produced by cell types and released into the extracellular matrix. It exerts its effect in a paracrine or autocrine manner. Like protein hormones and growth factors, bFGF binds to a plasma membrane receptor, and the resulting complex initiates the activity of a tyrosine kinase which is associated with the receptor (Dionne *et al.*, 1991). The tyrosine kinase may act through a G protein to elicit activity of other enzymes or regulatory protein

in the cytosol, resulting in an immediate cellular response or a long-term response produced by transcription in the nucleus. The exact signal transduction pathway for bFGF is not fully understood.

Multiple forms of bFGF

Although various forms of bFGF have been identified in many tissues, not all the forms are present in each tissue. Basic FGF isolated from bovine pituitary and brain has a molecular weight of 16.5 kDa, and the same size of bFGF has been purified from human brain. Later, another form of bFGF with a molecular weight of 25 kDa was identified from guinea pig brain (Moscatelli *et al.*, 1987). Truncated forms of bFGF lacking the first 15 amino acids have been isolated from bovine kidney, adrenal gland, and CL (Gospodarowicz *et al.*, 1978, 1982). In a human cell line, it was found that four molecular forms of bFGF were present: 17.8 kDa, 22.5 kDa, 23.1 kDa and 24.2 kDa (Florkiewicz *et al.*, 1989). In bovine ovary extracts, however, the immunoreactive bands of bFGF were about 24 kDa, 30-33 kDa, and 46 kDa (Grothe *et al.*, 1989). In porcine corpora lutea, the common immunoreactive bands found were 20 kDa and 33 kDa, identified by using three kinds of antibodies against internal, C-terminal and N-terminal sequences, respectively (Makris *et al.*, 1989).

Due to the existence of multiple forms of bFGF, it is necessary to find out whether the forms observed come from multiple RNA transcripts or whether they are products of alternative splicing of mRNA. In 1991, Stirling found a major mRNA species of 7.3 Kb and a second species of 3.7 Kb in bovine CL of all stages by Northern analysis using a cRNA

probe. The 7.3 Kb species was more abundant than the 3.7 kb species. Some other studies have suggested that all forms of bFGF were derived from a single mRNA (Quarto *et al.*, 1991).

As mentioned earlier, various forms of bFGF may arise from alternative splicing. It has been reported that translation of the 18 kDa form appears to initiate from an AUG codon, while translation of the higher molecular weight forms of bFGF are initiated at CUG codons 5' to the AUG codon (Quarto *et al.*, 1991). Interestingly, none of the forms of bFGF contains a classic signal sequence of hydrophobic amino acids, which are used by most secretory proteins for vectorial translation into the endoplasmic reticulum preceding eventual release into the extracellular space (Abraham *et al.*, 1986; Sommer *et al.*, 1987). These growth factor molecules are believed to function without containing the usual sorting sequences required for secretion (Quarto *et al.*, 1991).

Presence of bFGF in various tissue cells

Through two decades of studies, it was established that the biological effect of bFGF was not restricted to nervous tissue, but various kinds of other tissues. It has been found that bFGF can induce proliferation of granulosa cells, endothelial cells, epithelial cells, smooth muscle cells, etc. It has also been established that numerous cell types produce bFGF *in vitro*, including fibroblasts, smooth muscle cells, granulosa cells, endothelial cells, glial cells, and breast carcinoma cells (Gospodarowizc *et al.*, 1988; Moscatelli *et al.* 1986; Neufeld *et al.* 1987; Hatten *et al.*, 1988).

Bioactivities of bFGF in ovarian tissue

Effect of bFGF on cell growth

As mentioned before, the activity of bFGF in ovarian tissue is multi-functional. Its function is related to cell proliferation and differentiation, angiogenesis, and regulation of hormonal or growth factor secretion by luteal cells. Although the physiologic relevance of bFGF to ovarian function remains under investigation, several lines of evidence suggest that bFGF may play a central role in supporting the growth and development of the granulosa-luteal cell (Gospodarowizc and Bialecki, 1978, 1979; Savion *et al.*, 1980; Gospodarowizc *et al.*, 1985). Indeed, bFGF constitutes the main mitogenic factor isolated from crude luteal extracts (Gospodarowizc *et al.*, 1985) and has previously been shown to stimulate the replicative life span of cultured granulosa cells of bovine (Gospodarowizc and Bialecki, 1978), porcine, rabbit, guinea pig, and human origin (Gospodarowizc and Bialecki, 1979; Tapaninen *et al.*, in 1987).

Similar studies have shown that bFGF is a mitogen for bovine vascular endothelial cells derived from large vessels. CL-derived capillary endothelial cell were almost completely dependent on bFGF for growth *in vitro* (Gospodarowicz *et al.*, 1986).

Taken together, these observations suggest that bFGF may play an autocrine or paracrine regulatory role at, or adjacent to, the sites of its synthesis.

Effect of bFGF on angiogenesis

Basic FGF has been identified in a wide range of highly vascularized tissues. This observation has led to the suggestion that bFGF is a major angiogenic factor in luteal tissue.

Angiogenesis refers to the formation of new blood vessels, or neovascularization. It is an essential component of tissue growth and development. The angiogenic process begins with capillary proliferation and culminates in the formation of a new microcirculatory bed composed of arterioles, capillaries, and venules. The initial component of angiogenesis, capillary proliferation, has been shown to consist of at least three processes: 1) fragmentation of the basal lamina of the existing vessel, 2) migration of endothelial cells from the existing vessel toward the angiogenic stimulus, and 3) proliferation of endothelial cells (Reynold *et al.*, 1992).

The female reproductive organs contain some of the few tissues in the adult that exhibit periodic growth and regression. In addition, growth and regression of these tissues are extremely rapid. When studying the vascular growth in ovarian tissues, it was observed that vascular development of the ovarian follicle becomes even more impressive after ovulation, in association with development of the CL. In fact, approximately 50% of the cells of the mature CL are endothelial cells, and the majority of parenchymal cells are adjacent to one or more capillaries (Reynold *et al.*, 1992).

Angiogenesis plays an extremely important role in the development of the functional CL, mainly because of the vascular changes that take place in the capillary wreath surrounding the follicle at the time of ovulation. Capillary sprouts grow into the granulosa cell layer and develop into a complex network of sinusoidal vessels which invade avascular cell layers, inducing luteinization, and later nourish the parenchyma of the CL (Baird *et al.*, 1986). Gospodarowicz and Thakral in 1977 have previously demonstrated that granulosa and luteal cells produced a diffusible substance that could trigger the early vascular changes that occur

during the development of the CL. Additional observation noted that crude exacts of both porcine and bovine CL are mitogenic for vascular endothelial cells (Gospodarowicz *et al.*, 1985). Similar activities were described by Jakob *et al.* in 1977, who demonstrated that bovine corpora lutea grafted on the chick chorioallantoic membrane could elicit a strong angiogenic response.

Effect of bFGF on other hormones and growth factors

Various hormones and growth factors are known to play important roles in ovarian function. Basic FGF was found to suppress expression of both LH and hCG receptors which were induced by FSH and FSH-mediated induction of progesterone secretion (Biswas *et al.*, 1988). Similarly, bFGF was shown to regulate ovarian steroidogenesis in experimental animals. Basic FGF together with EGF, suppress FSH-dependent LH receptor induction (Tapaninen *et al.*, 1987; Stirling *et al.*, 1991). Also, bFGF was found to be a potent inhibitor of FSH-stimulated aromatase activity. Doses that were capable of eliciting a half-maximal mitogenic response in several cell types were capable of inhibiting the conversion of androstenedione to estradiol (Baird *et al.*, 1986; Stirling *et al.*, 1991). Previous work in our lab (Taylor and Clark, 1992) showed that bFGF serves as a local inhibitory mechanism that regulates relaxin secretion. Also in 1987, Larson and Koos found that bFGF exerts its effect on the secretion of prolactin by rat lactotropes. It is clear that bFGF can play a regulatory role for hormones which are important in ovarian function.

Secretion of hormones and growth factors in CL of pregnancy

When the oocyte is fertilized, the corpus luteum enters into the luteal phase of pregnancy. As mentioned before, the CL is essential for maintaining pregnancy as it is the major source of progesterone. Pregnancy lasts about 114 days in pigs. Corpora lutea of the swine produce not only progesterone, but also relaxin, a peptide hormone that plays a critical role in suppressing uterine motility during pregnancy and in remodeling connective tissues in preparation for imminent parturition (Taylor *et al.*, 1987; Li *et al.*, 1991). In addition, the CL is also a source of oxytocin, another polypeptide hormone. On the contrary, corpora lutea can produce substances that prevent progesterone secretion, such as prostaglandin $F_{2\alpha}$ and 20 α-hydroxysteroid dehydrogenase (Constance *et al.*, 1991).

The release of relaxin and progesterone by corpora lutea have been widely studied. It was well established that soon after mating, porcine progesterone secretion peaks by day 8 and remains elevated until it decreases just before parturition (Adair *et al.*, 1989). Levels of relaxin in blood and luteal tissue increase during pregnancy and peak just before parturition followed finally by a fall to undetectable levels after parturition (Anderson *et al.*, 1983; Changjian *et al.*, 1991).

The CL is a source of not only peptide hormones, but also peptide growth factors. Lines of evidence have identified the existence of several growth factors inside the CL, including bFGF, EGF, tumor necrosis factor (TNF), and transforming growth factor ß (TGFß)(Baird *et al.*, 1986). Thus, the CL is unique in its ability to produce multiple hormones and growth factors.

Statement of existing problems

The changes in progesterone and relaxin concentrations during pregnancy put forward a question: are similar changes seen for bFGF during pregnancy? Since bFGF has been reported to have effects on relaxin and prolactin secretion, it is possible that the concentration of bFGF is also changed in the tissue so as to modulate its local function. On the other hand, the angiogenesis of CL is most rapid after ovulation, and bFGF is the major angiogenic factor in the developing CL. After maturation of the CL, no further change in structure occur until regression in late pregnancy, suggesting that bFGF may not change after the initial development of the CL. It is reasonable to speculate that levels of bFGF are highest during post-ovulation period.

The objectives of present research

The objectives of this study were to confirm the existence and size of bFGF in CL tissues collected from pregnant swine, and to explore the secretion level of bFGF in the whole life-span of the pregnant CL. Thus, we were able to investigate the possible involvement of bFGF in CL during pregnancy.

Introduction of methodology

In order to address this issue, we adopted Western Blot procedures to detect bFGF in the CL. Western Blot is a technique that is useful for the identification and quantitation of a single protein that exists in a mixture of proteins. The basic procedure for Western blotting involves solubilizing the sample to be assayed with detergents and reducing agents,

separating sample proteins by SDS-polyacrylamide gel electrophoresis, and transfering the proteins from the gel to a solid support such as a nitrocellulose membrane. The nitrocellulose membrane is stained to mark the location of molecular weight standards and then exposed to primary antibody (Ab). Bound Ab is then detected by using a secondary Ab. There are several choices of secondary Abs which are classified into two groups: radiolabeled and non-radiolabeled Abs. The non-radioactive techniques for the detection of primary Ab are very simple and safe to perform without sacrificing sensitivity. The location of the protein can be visualized by chromogen development, or after autoradiography if using radiolabeld secondary Ab. In this study, we used biotin conjugated anti-IgG as secondary Ab, and an avidin/biotin system was used subsequently. The location of the protein was detected after adding a chromogenic substrate.

Basic FGF is a peptide growth factor, and has a variety of molecular forms. These multiple forms of bFGF have similar biological function but differ in molecular weights. Based on these properties, Western Blot is an ideal method to detect bFGF, and has been used in many studies to examine the molecular weights of bFGF (Makris *et al.*, 1989).

Advantages of the avidin/biotin system

The strengths of the avidin/biotin system are: 1) It can improve the sensitivity because it provides more potential for amplification due to multiple site binding. Also, avidin conjugates are very stable. 2) It overcomes the problem of high background caused in other techniques. 3) Biotinylation has minimal effects on the biological activities of proteins, and

permits more accurate studies. 4) High affinity between avidin and biotin assures a rapidly formed and stable complex between avidin and the biotinylated protein. This affinity is over one million times higher than that of antibody for most antigens. The binding of avidin to biotin is essentially irreversible. Also of importance is that avidin/biotin system is more safe to use because no isotope is involved. The relation between antigenic portion on the blot, antibodies, and avidin/biotin system will be shown in detail in the next section.

After finishing Western blotting, the intensities of the immunoreactive bands were quantitatively analyzed. After comparing the intensity of the purified bFGF to the unknowns, the concentration of bFGF in CL from each stage of pregnancy was calculated. A graph of bFGF concentrations was plotted and compared with the ages of CL. This was used to evaluate the possible physiological role of bFGF in luteal tissue during the time of pregnancy, so as to further understand the local regulatory function of bFGF in this tissue.

MATERIALS AND METHODS

Animals

Porcine corpora lutea from ovaries of pregnant sows were collected from the Iowa Packing Plant and the Meat Laboratory, ISU. Tissues were kept in cold phosphate buffer saline (PBS) immediately after removal. The gestational age of the CL was determined by measuring the length of fetus from crown to rump (Evans and Sack, 1973). CL were collected from pigs at 9 stages of pregnancy: Day 8, 15, 37, 47, 54, 70, 77, 82, and 112 (term is day 114+/-2 days).

Materials

The reagents for protein assay were bought from Bio-Rad Company (Richmond, CA.). The Western Blot reagents were purchased from Sigma Chemical Co. (St Louis, MO.). All reagents were of analytic grade. The system used for electrophoresis and electrotransfer was from Schleicher & Schuell (S&S) (Keene, NH). Purified bFGF (human recombinant bFGF(1-154) generated from *E. coli*) and anti-human bFGF was obtained from Biomedical Technological Inc.(Stoughton, MA). The secondary Ab anti-rabbit IgG, and Vectastain kit were purchased from Vector Company (Burlingame, CA). Normal rabbit serum (NRS) was from Accurate Chemical & Scientific Corp. (Westbury, NY).

Methods

Sample protein preparation

The corpora lutea were obtained by using a sterile scalpel to cut the CL membranes and excise the CL. In this way, only CL tissues were used. Nine stages of CL were obtained in the same way. Corpora lutea for each stage were weighed first, and then minced in a glass petri dish containing 5 ml cold phosphate-buffer saline (PBS). All CLs of the same stage were used as a single pool. The suspended samples were transferred to a Wheaton homogenizer, and homogenized thoroughly for 5 minutes with a pestle in order to break up cell membranes and release cellular substances. The homogenate was then poured into a 15 ml polypropylene centrifuge tube. It was then centrifuged at a speed of 1500 xg in a clinical centrifuge for ten minutes. The pellet was discarded. The resulting supernatant was further centrifuged at 5^0C, 21,000rpm, no brake condition for 90 minutes to sediment cellular organelles, like mitochondria, lysosomes, and cellular debris. Finally, the supernatant was divided into 50 μl aliquots in Eppendorf tubes and stored at -20^0C.

Protein assay

The Bio-Rad protein assay was used to determine the protein concentration in each sample. The Bio-Rad protein assay is a dye-binding assay based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The absorbance differs with the dye color change in response to various protein concentrations. We used bovine serum albumin (BSA) as a standard with a stock concentration of 14.8 mg/ml. Six dilutions of standards were made

with sample buffer(62.5mM tris-HCl, 10% glycerol(v/v)) ranging from 14.8 μg/μl to 88.8 μg/μl. The absorbance of the standard solutions was read at 595 nm by using a spectrophotometer. A standard curve of the absorbance against their concentrations was graphed. Meanwhile, the unknown samples of CL were also made in dilution with sample buffer, and the absorbance measured as described above. The concentration of total proteins in CL samples was calculated from the standard curve. The volume of each protein sample loaded in one well of the gel was adjusted to contain the same amount of total proteins.

Western blot

Electrophoresis

According to the published report, the molecular weight of bFGF in porcine corpora lutea ranged from 20 kDa to 55 kDa (Markris *et al.*, 1989). Our gel should be able to separate the samples with these molecular weights. A 16% tris-glycine polyacrylamide gel was used in the experiments and molecular weight markers ranging from 3kDa to 43kDa were chosen. Two kinds of molecular weight markers (GIBCO BRL, Gaithersburg, MD) were used. One was pre-stained molecular weight markers and the other was non-stained molecular weight marker. The pre-stained served as an indicator for the location of dye front on the gel in the electrophoresis process. The non-stained was used to calculate the molecular weights of the unknown bands. The pre-stained molecular weight markers cannot be used to calculate the molecular weights of the unknown since it contains dye which affects its actual migration rate in the electric field.

The apparatus for gel electrophoresis was set up and running buffer was filled in both

upper and lower cells. The composition of running buffer was as follows: 192mM glycine, 25mM tris-base, and 10% SDS (w/v). Sample proteins or molecular weight markers were prepared with 10 μl sample buffer which contained 62.5mM tris-HCl, 10% glycerol(v/v), 2% sodium dodecyl sulfate (SDS)(v/v), 0.05% 2-mercaptoethanol(v/v), and 0.00125% bromophenol blue(w/v), and deionized water to adjust the volume up to 50 μl for each sample. The volumes for protein samples were determined by the protein assay. The volume for molecular weight markers was 10 μl. All protein samples were heated in water at 70^0C (John Wiley & Sons, Current Protocols in Molecular Biology vol. 2, published by Green Publishing Association and Willey-Interscience) for 3 minutes, and were applied onto the wells with 20 μl each sample per well which contained 132 μg total protein in CL tissues. The gel was run at a constant current of 30 mA for 40-50 minutes. It was stopped when the pre-stained markers were observed to move close to the bottom of the gel.

When the sample protein was treated with sample buffer, proteins were fully denatured by reduction with ß-mercaptoethanol, and filled with SDS in sample buffer. ß-mercaptoethanol was used to break down disulfide linkages between polypeptide chains so as to dissociate the protein into its smallest subunits. SDS binds to most proteins, with the non-polar hydrophobic portion buried in the non-polar regions of the protein and the negatively-charged sulfate portion exposed to the solvent. Binding of SDS interfered with native hydrophobic and ionic interaction, causing the dissociation of most oligomeric proteins into their monomer subunits and the disruption of their secondary structure. SDS eliminated charge variability between polypeptides, giving them all the same charge-to-mass ratio. So, the protein migration depended exclusively on the molecular weight. SDS in the running

buffer overtook the SDS protein complex formed during sample preparation and maintained the form of SDS protein complex during electrophoresis. SDS in the sample and running buffers was to keep the proteins binding to SDS throughout the electrophoresis.

Transferring

Because the gel is so fragile and the proteins on it diffuse rapidly, it is difficult to study the property of proteins on gel. This problem is overcome by transferring the fractionated proteins to a solid support matrix which can immobilize the protein and thus can be easily manipulated. Nitrocellulose membranes are the standard medium for protein transfer techniques. The mechanism of protein binding to the membrane is most likely a hydrophobic interaction.

After the protein mixture was separated, the gel was removed from the gel plates to a big plastic vessel with approximately 500 ml transfer buffer of following composition: 96mM glycine, 20% methanol(v/v), and 12mM tris-base. The gel was soaked in transfer buffer for 30 minutes. The nitrocellulose membrane (BA83; S&S Inc., with pore size of 0.2μm), five foam pads and two filters (S&S; GB003 papers) were soaked for 10 minutes prior to transferring. Sufficient soaking of the gel was required to make sure that the gel was free of SDS. Also, soaking ensures that the nitrocellulose membrane, filters and pads are filled with transfer buffer and contain no air bubbles. The blotting sandwich was then assembled in the following way. One pad was first placed inside the cathode plate. Above the pad, there was one filter, one gel, one nitrocellulose membrane, again one filter, and 4 pads in that order from bottom to top. Then the anode plate was placed on the top of the 4 pads to close

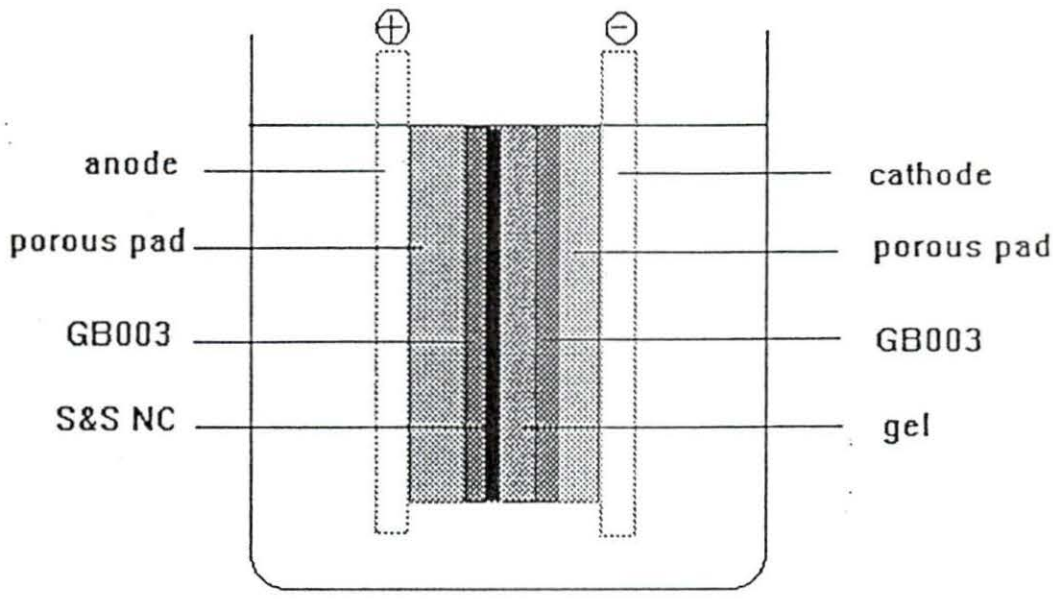
the blotting assembly. The diagram showing the assembly of electroblot was in Figure 1. The blotting sandwich was set in this manner so that the transfer direction was from the gel to the membrane. Therefore, the protein on the gel can be transferred to the nitrocellulose membrane. While transferring the gel, the contact should be good and care should be taken to avoid air bubbles between the filter, gel and nitrocellulose membrane.

The electrotransfer was carried out at 200 mA at room temperature for one hour. The nitrocellulose membrane containing the protein of interest (which is called the blot), was taken from the assembly and stained with 0.5%(w/v) Ponceau S (0.5g Ponceau S was dissolved in 1ml pure glacial acetic acid and brought to 100 ml with deionized water). Staining was done to check if the proteins were transferred properly. At the same time, the non-stained molecular weight marker locations were marked with a pencil. The bottom of well was also marked for measuring the migration distance of each band. Then the molecular weight lanes were cut off and the remaining blot was cut into strips and used for the immunoblotting.

Immunoblotting

In order to detect the presence of bFGF in the sample, the blot was incubated with primary, secondary antibodies, avidin-biotin complex, and developed with chromogen.

After transfer, all active sites remaining on the blot not occupied by the immobilized proteins were occupied to prevent high background staining caused by reagents in the subsequent steps. Blotto was a protein blocking agent made of 1% dry milk in TBST buffer (150mM NaCl, 0.05% tween 20(v/v), 50mM tris-HCl, pH7.2). It filled the unoccupied sites



GB003 filter

S&S NC nitrocellulose membrane

Figure 1. Assembly for transferring (Schleicher & Schuell Inc.)

on the blot. Tween 20, a detergent was used to reduce the affinity of the binding reagents for the surface of the blot.

Blot strips were put into a disposable dish with about 25ml Blotto, and blocked for 70-80 minutes on a platform rocking machine at speed 3 (Research Products International Corp., Mount Prospect, IL). After blocking, the Blotto was poured off and the primary Ab at a 1:400 dilution in 5ml TBST was added. The primary Ab was anti-bFGF raised in the rabbit against human recombinant bFGF. This Ab has been shown to detect human bFGF, bovine and mouse bFGF (Biomedical Technological Inc.). The blot was incubated with primary Ab over night at room temperature with a continuous gentle shaking. On next day, the Ab solution was poured away and the blot was washed in TBST at room temperature for four times, 10 minutes each. The washing buffer was poured away before proceeding to the next step. The next step the secondary Ab incubation. The secondary Ab used was anti-rabbit IgG raised in the goat. It is also a biotinylated Ab which can directly recognize the primary Ab attached on the blot. The secondary Ab, 1:400 dilution in 5ml TBST, was added to the blot for one hour on a shaker. Then, the blot was washed in TBST in the same way of four times, 10 minutes each. While washing the blot, the reagent for next step was prepared which was avidin-biotin complex (ABC). ABC is the complex of avidin and biotinylated peroxidase. Avidin has a high affinity for biotin or biotinylated molecules. Since the secondary Ab was biotinylated, it can be bound by avidin which is associated to biotinylated peroxidase. Thus, ABC was used as a linkage between the enzyme and secondary Ab. ABC formation needs at least 30 minutes, and should be made 30 min in advance before of the incubation. The best time to prepare ABC was during the washing procedure, so that it can

be used right after the washing step was done. The ABC was made of 1:100 dilution in 5ml TBST, and the blot was incubated with ABC for 40 minutes at room temperature with continuous shaking. The blot was washed with TBST again in the same way and an additional wash with PBS. The location of antigenic portion on the blot was finally visualized with chromogenic substrates: 3, 3'-diaminobenzidine tetrahydrochloride (DAB). DAB is the substrate for peroxidase which is attached to biotin. The DAB solution, which contained 50% DAB(w/v) and 0.1% H_2O_2, was prepared fresh daily in PBS. The reaction was accelerated by H_2O_2 and resulted in a brown color. The blot was transferred to a new disposable dish before adding the substrate. Then, the blot was developed in DAB for 5 or 10 minutes, and several bands appeared. The location of the immunoreactive band on the blot indicates the place where the antigen exists. The relation between the protein, primary Ab, secondary Ab, ABC and the substrate is shown in Figure 2.

Controls

In order to be sure that the bands on the blot are the specific immunoreactive bands we are interested in, several controls were run. Positive controls are to test the reactivity of the primary Ab. The common sample for positive control is the purified antigen or certain tissue which is rich in the antigen. The antigen is loaded as usual and the running procedure is the same. If the resulting band detects the antigen, then it indicates the primary Ab can detect its antigen. Negative controls are to detect the non-specific bands on the blot. It could be achieved by omission of primary Ab, or secondary Ab, or ABC. Since the final result is dependent on the interaction of primary Ab, secondary Ab and ABC, missing any of them

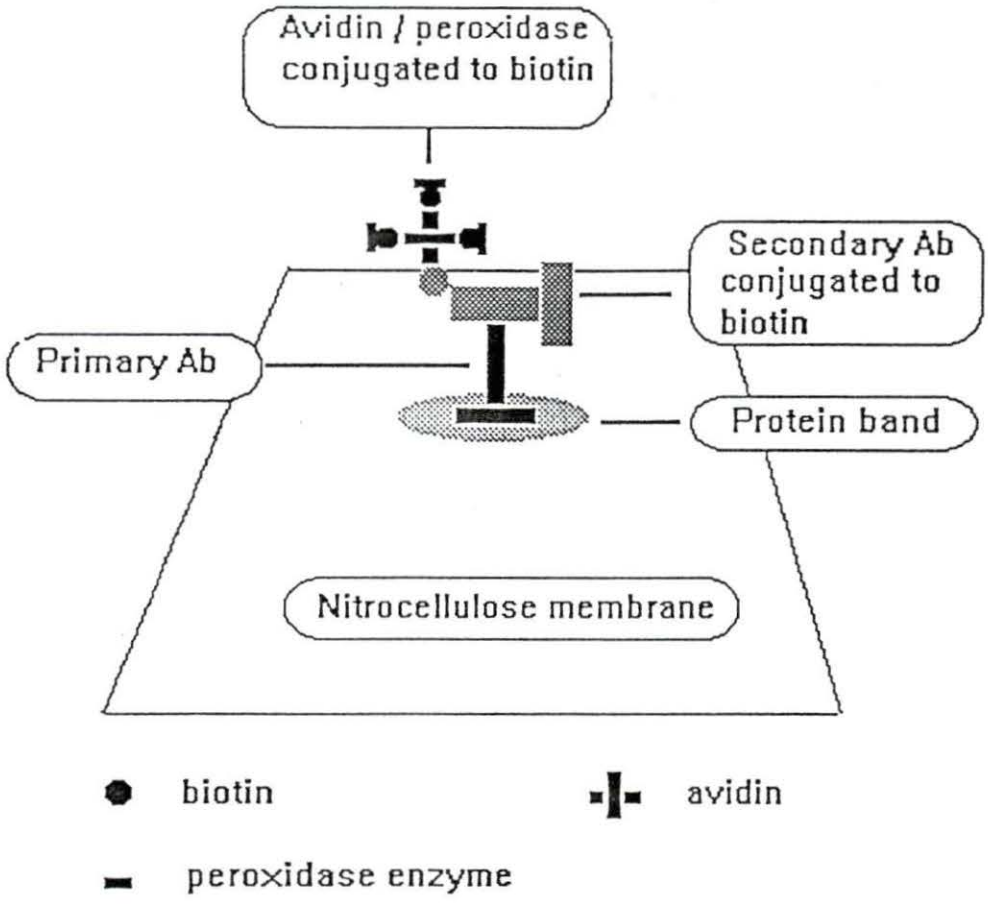


Figure 2. Diagram of relationship between antigen on the blot, primary Ab, secondary Ab, ABC and peroxidase enzyme.

would result in losing the specific immunoreactive band except for the non-specific bands. It also could be achieved by using pre-immune serum or pre-absorbed Ab which is made by incubating excess purified antigen with primary Ab so that all the binding sites on the Ab are filled by the antigen. In both cases, the running procedure is the same except that the primary Ab is replaced by pre-immune serum or pre-absorbed Ab. No specific band for antigen is detected except the non-specific bands which are due to the reaction caused by other serum components to protein.

Two positive controls and one negative control were studied here. Since it has been established that brain tissue is one of the tissues producing abundant bFGF, porcine brain tissue was used as one of the positive controls. Another positive control was the purified bFGF purchased from Biomedical Technological Inc. Controls were studied along with the reference CL sample in the same way and probed with anti-bFGF at the same dilution of 1:400. Finally, the bands in the control lanes indicated bFGF. Therefore, we can identify the specific bFGF in the sample lane by comparing to the control lane.

Because our primary Ab was raised in the rabbit, we used normal rabbit serum (NRS) as our negative control. NRS control was conducted along with each CL sample and porcine brain tissue. The running procedure was the same. Each sample of its stage was run on two adjacent lanes. One was probed with primary Ab and the other with NRS at a 1:45000 dilution (which contains same amount of immunoglobin as 1:400 primary Ab) in 5ml TBST. Therefore, the resulting bands on the blot were non-specific bands.

While doing Western Blot, each experiment was run twice. Nine stages of CL were examined for bFGF. Day 47 CL was the reference sample used in each blot.

Data analysis

The distances from the bottom of a well to the localization of each band were measured for both the molecular weight standards and the unknown proteins. A graph of the molecular weights vs. their migration distances of logarithm was made as a standard curve. The molecular weights of the unknowns were then calculated from the plot.

In order to determine whether the concentration of bFGF varies with different stages of pregnancy, the intensity of each band of its stage was determined by image analysis of the photographs. First, a computer program was written for detecting the intensities of bands. The intensity was evaluated by a number from 0 to 255, which indicates the greyness of bands. The smaller the value, the stronger the intensity. The analysis was done by drawing a line in the middle of a band which had the most dense staining on the computer, and then grading each point on the line to measure its intensity. Finally, the intensity of the band was obtained from the average value of those points on the band. Considering that the background color of each pictures was different, the values of the bFGF band from different blots could not be compared. In addition, a Day 47 CL sample was used in each blot as a reference, and it can be used to eliminate the variance caused by different blots. By setting the value of all Day47 CL on blots at 1, each band of its age had a relative value simply by taking the ratio of the absolute value of Day 47 to that of itself. In this way, all the values of bands are normalized and can be compared with each another.

Since the total amount of purified bFGF loaded on the well, and its relative intensity was known, the concentration of bFGF in Day 47 CL sample and other stages of CLs

can be calculated.

As the CL concentration of each stage was calculated, a graph of bFGF concentration vs. CL age in pregnancy can be made.

RESULTS

Protein assay

The sample of Day 47 CL was used as a reference throughout the experiment. Day 47 CL sample protein was made in serial volumes of 10μl, 15μl, 20μl, 25μl and 30μl. They were loaded on the wells and probed with antibodies. The suitable volume of sample which gave a final clear band with lower background was chosen for the experiment. The proper volume was 15 μl for Day 47 CL in this study. Then, the total protein concentration in 15 μl Day 47 sample was measured by Bio-Rad protein assay. This concentration was set as the suitable amount of total sample protein to be loaded on each well in Western Blot. Subsequently, each CL sample was examined for its protein concentration. The volume of CL which contained all equal amount of total protein was determined for each stage sample. Each CL sample was loaded on the gel with a determined volume to examine bFGF. Therefore, the total amount of sample protein loaded per well was the same. The result of the protein assay for the concentration of Day 47 sample was 22 μg/μl. Thus, the total protein loaded on each well was 132 μg.

Antibody dilutions

In order to determine the proper Ab dilution for the experiment, a serial dilution of primary Ab was used to probe the blot strips containing Day 47 CL sample. As a consequence, the Ab dilution which showed the best result was selected as the suitable dilution of primary Ab. The appropriate dilutions for the secondary Ab and ABC complex

were tested in the same manner. After the optimal dilution for all reagents was obtained, the nine stages of luteal tissue were examined for the existence of bFGF. The ages of these tissues were Day 8, 15, 37, 47, 54, 70, 77, 82, and 112 of pregnancy. The sample of each age was run on the two adjacent lanes. One lane was probed with primary Ab and the other with NRS in place of the primary Ab. The primary Ab was anti-human bFGF raised in the rabbit. It was used at a dilution of 1:400 in all stages of samples in this experiment. The secondary Ab was anti-rabbit IgG raised in goat and used at 1:400 dilution to each blot. The ABC dilution used was 1:100 throughout the experiment.

Control group

In order to test if the bands on the blot were specific immunoreactive bands, control lanes were run. There were one negative control and two positive controls. NRS was used as a negative control to identify the non-specific binding on the blot. The NRS lane was run along with each stage sample. The non-specific band was easily identified in the NRS lane, and the specific immunoreactive bFGF band of each stage was determined by eliminating the non-specific band found in NRS lane. With the help of molecular weight standards, the molecular size of the immunoreactive band was calculated on a logarithm graph. The result turned out that there was one bFGF band on the blot for each stage. The molecular weight of bFGF was 31kDa and the same molecular weight was found in every sample.

There were two positive controls in this part of the experiment. They were used to test the specificity of the primary Ab. The first one was the human recombinant bFGF(1-154). One μg and 0.5 μg purified bFGF were run and probed as usual. As a result, one band of

17 kDa appeared on the blot as expected (Figure 3). This indicates that our primary Ab can detect bFGF specifically. Lane 1 and 6 were molecular weight markers. The bFGF band in lane 3 has stronger intensity than that in lane 2, because bFGF in Lane 2 was in half amount of that in lane 3. Lane 4 and 5 were Day 47 CL sample. Lane 5 was incubated with NRS, and represented the non-specific bands. Lane 4 was probed with anti-bFGF at 1:400 dilution. The immunoreactive bFGF band in Day 47 CL sample was 31 kDa.

Because the purification process of our CL samples differs from that of the bFGF bought commercially, it may cause the difference in molecular weight of bFGF between our sample and the commercial bFGF. Thus, it is necessary for us to find a positive control which was treated in the same manner as our samples. Therefore, the second control used was porcine brain. The reason for using porcine brain tissue is that brain was found to be one of the tissues abundant in bFGF. The brain tissue was homogenized and run in duplicate along with Day 47 CL (Figure 4). Lane 2 and 3 were probed with NRS, and lane 4 and 5 with primary Ab. The result showed two bFGF bands in the positive control lanes. One bFGF band in brain tissue was consistent with the bFGF band found in Day 47 CL sample which were on lane 6 and 7. The molecular weight of this bFGF band was also 31 kDa. The other bFGF in brain tissue was 28 kDa which may be the nonspecific band. The specificity of our primary Ab was tested and the immunoreactive bFGF band was further verified.

Detection of bFGF in CL samples with reference Day 47 CL

Nine stages of CL samples were examined for the bFGF, which were Day 8 (Figure

Figure 3. Identification of the specificity of primary Ab. Lane 1 and 6 were molecular weight markers. Lane 2 and 3 were human recombinant bFGF. Sample in lane 2 was in half amount sample in lane 3. The immunoreactive band in both lanes were of 17 kDa molecular mass, resulting from probing with primary Ab: anti-human basic FGF. Lane 4 and 5 were Day 47 CL. Lane 4 was resulted from probing with NRS instead of primary Ab. Lane 5 was the result of probing with anti-human bFGF.

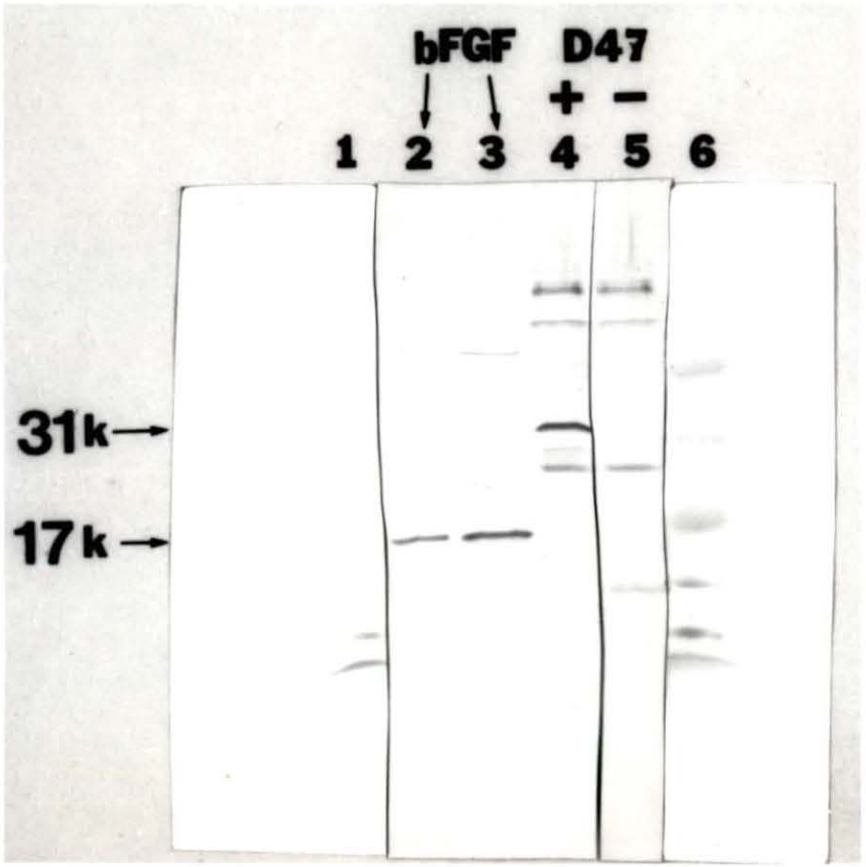
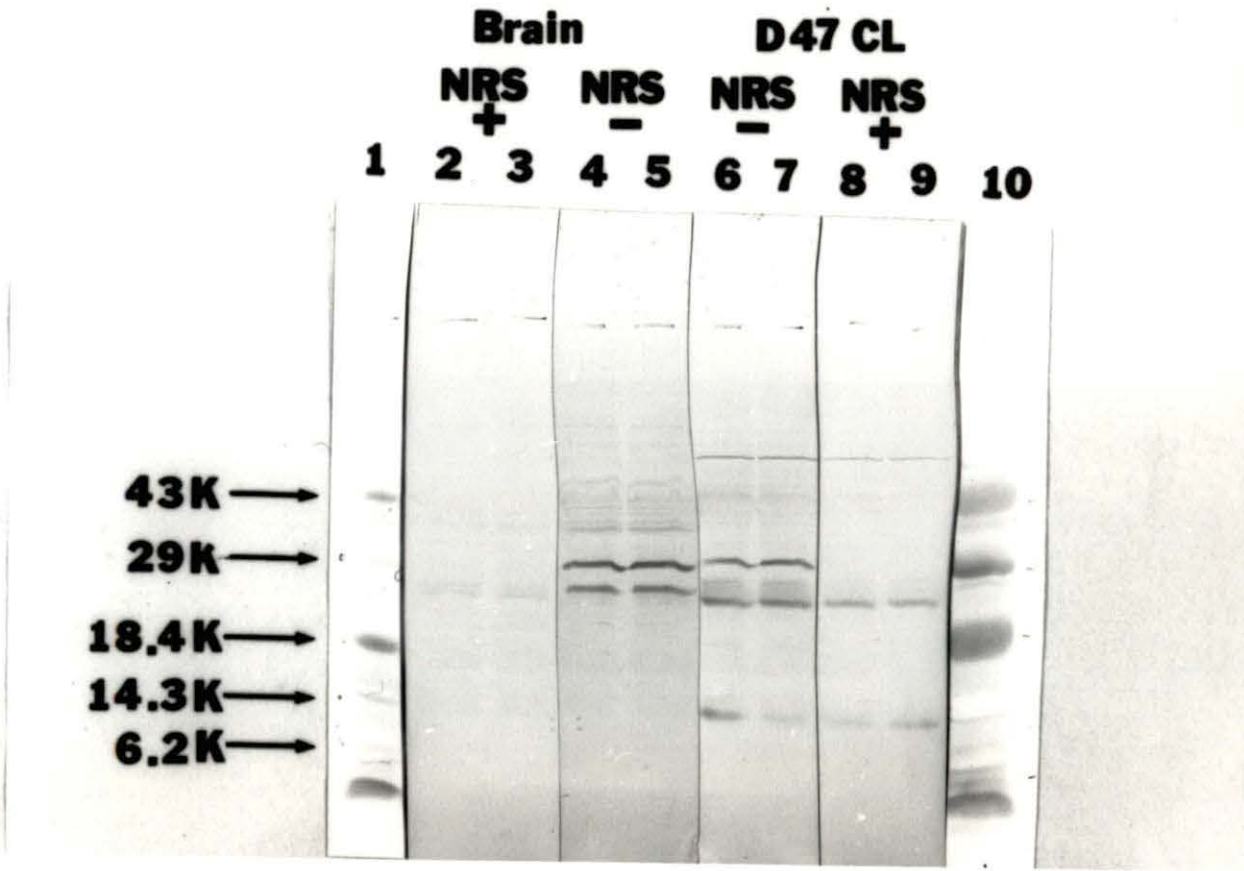

bFGF
D47
+
−
1
2
3
4
5
6
31k
17k

Figure 4. Validation of positive control using porcine brain treated with same conditions as CL samples. Lane 1 and 10 were molecular weight markers. Lane 2, 3, 4 and 5 were brain tissues. Lane 6, 7, 8, and 9 were Day 47 CL. Lane 2, 3, 8, and 9 were treated with NRS as negative control lanes. Lane 4, 5, 6, and 7 were treated with anti-bFGF. The immunoreactive bands in brain tissue were 28 kDa and 31 kDa. The band of 31 kDa was consistent with the band in CL.


Brain
D47 CL
NRS +
NRS −
NRS −
NRS +
1 2 3 4 5 6 7 8 9 10
43K
29K
18.4K
14.3K
6.2K

5), Day 15 (Figure 6), Day 37 (Figure 6), Day47 (Figure 6), Day 54 (Figure 7), Day 70 (Figure 8), Day 77 (Figure 8), Day 82 (Figure 7), Day 112 (Figure 8). Only one immunoreactive bFGF band found in all CL samples which were studied. The molecular weight of the bFGF band was found to be 31 kDa in nine samples.

Image analysis of bFGF band intensity

The immunoreactive bFGF band in each age of CL sample was measured for its intensity by image analysis. The intensity was graded by a number from 0 to 255, where 255 stands for white and 0 stands for black. Thus, the smaller the number, the stronger the intensity. Each point on the middle line of bFGF band was graded for the intensity by such a number, and the final value for the intensity resulted from an average value of every number graded for the intensity of each point on the band. The intensities of nine stages of CL and controls were all analyzed in this way. The results of these intensities were listed in the Table 1.

The intensities of the bands can not be compared with each other if they were not on the same blot. This is because of the small differences caused by experiments. To correct for this difference, a reference sample Day 47 was run in every blot. In order to diminish the variance made on Day 47 sample in each blot, the intensity value for the bFGF band in Day 47 sample was set at 1.0000. Thus, a set of relative values for the intensities of bFGF in all samples were obtained by dividing the value for reference bFGF band by the value for bFGF in each sample on the same blot. The relative values for bFGF band in all samples were shown in Table 1.

Figure 5. Detection of bFGF in Day 8 CL sample. Lane 1 and 6 were molecular weight markers. Lane 2 and 3 were Day 8 CL. Lane 4 and 5 were Day 47 CL which serves as a reference. Lane 2 and 4 were NRS lanes whereas lane 4 and 5 were incubated with anti-human bFGF. The immunoreactive band in Day 8 CL was of 31 kDa.

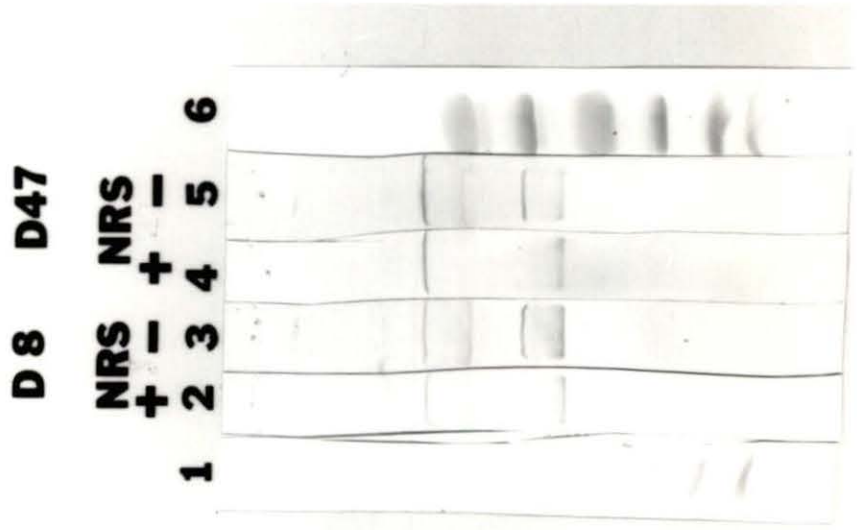

D8
D47
NRS
NRS
+
−
+
−
1
2
3
4
5
6

Figure 6. Detection of bFGF in Day 15 and Day 37 CL samples. Lane 2 and 9 were molecular weight markers. Lane 3 and 4 were Day 15 CL; lane 5 and 6 were Day 37 Sample; lane 7 and 8 were Day 47 samples which serve as a reference. Lane 3, 6 and 7 were incubated with anti-human bFGF; lane 4, 5, and 8 were NRS lanes. The immunoreactive bands in both Day 15 and Day 37 CL samples were of 31 kDa.

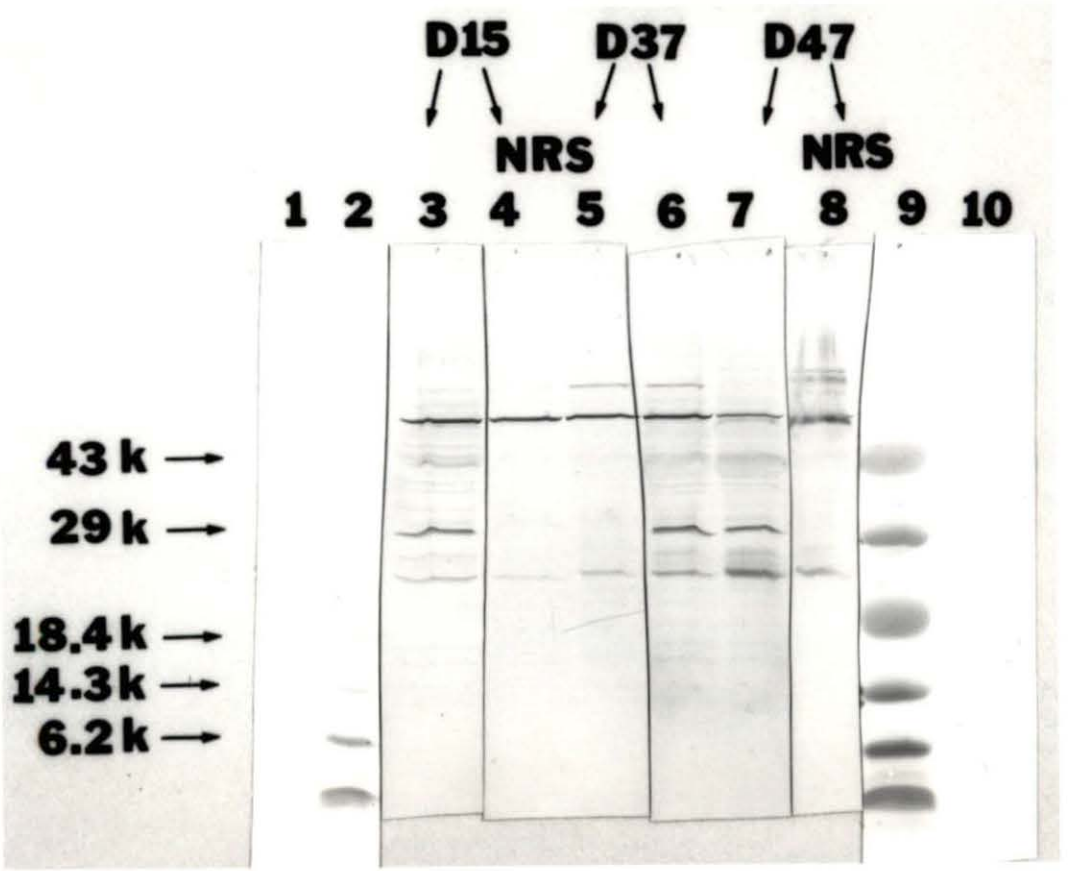

D15
D37
D47
NRS
NRS
1 2 3 4 5 6 7 8 9 10
43 k
29 k
18.4 k
14.3 k
6.2 k

Figure 7. Detection of bFGF in Day 54 and Day 82 CL samples. Lane 1 and 8 were molecular weight markers. Lane 2 and 3 were Day 82 samples. Lane 4 and 5 were Day 54 CL samples. Lane 6 and 7 were Day 47 reference lanes. Lane 2, 4, and 6 were NRS lanes. Lane 3, 5, and 7 were probed with anti-human bFGF. The immunoreactive bands in Day 82 and Day 54 samples were of 31 kDa in molecular weight.

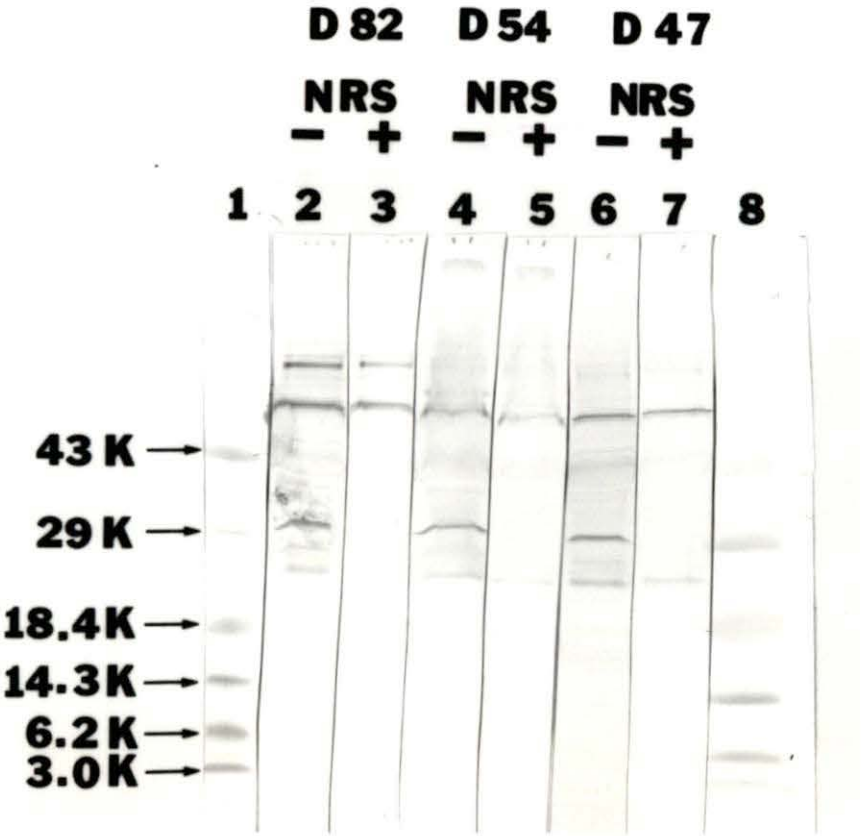

D 82
D 54
D 47
NRS
NRS
NRS
− + − + − +
1 2 3 4 5 6 7 8
43 K
29 K
18.4K
14.3K
6.2K
3.0K

Figure 8. Detection of bFGF in Day 70, Day 77, and Day 112 CL samples. Lane 1 and 10 were molecular weight markers. Lane 2 and 3, lane 4 and 5, lane 6 and 7 were Day 112, Day 77 and Day 70 CL sample respectively. Day 47 CL were the reference in lane 8 and 9. Lane 2, 4, 6 and 8 were incubated with anti-human bFGF. Lane 3, 5, 7, and 9 were NRS lanes. The immunoreactive bands in three ages of CL samples were of 31 kDa in molecular weight.

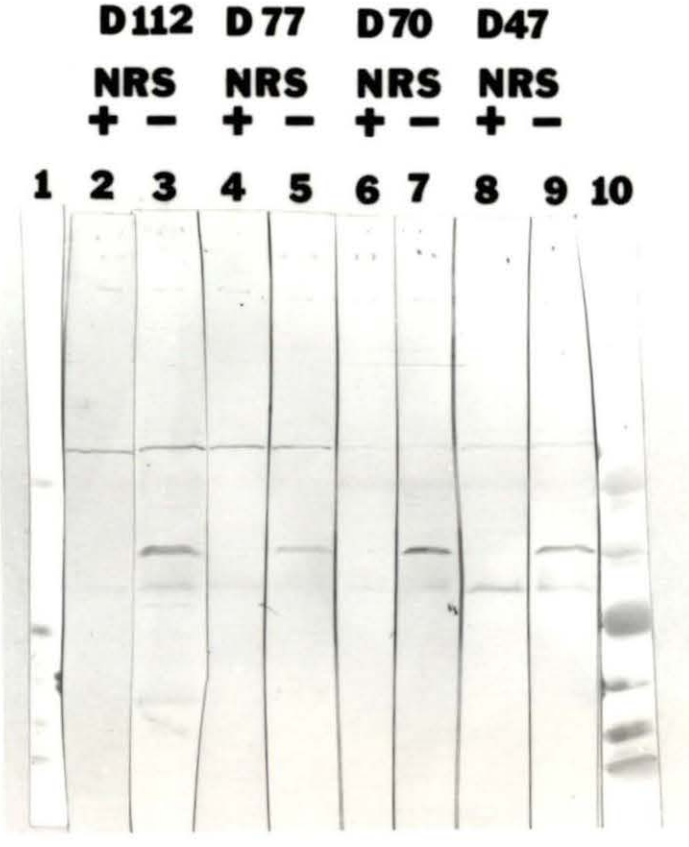

D112
D77
D70
D47
NRS
NRS
NRS
NRS
+
−
+
−
+
−
+
−
1
2
3
4
5
6
7
8
9
10

Table 1. Immunoreactive bFGF produced in luteal tissues of different stages.

	Sample	Absolute greyness	Relative greyness	Concen. of bFGF (ng/ug)
blot 1	Day 8	183.85	0.9991	10.45
	[a] Day 47	183.68	1.0000	10.53
blot 2	Day 15	146.33	0.9151	9.62
	Day 37	127.62	1.0492	10.98
	[a] Day 47	133.90	1.0000	10.53
blot 3	Day 54	169.26	1.0609	11.14
	Day 82	194.76	0.9220	9.70
	[a] Day 47	179.57	1.0000	10.53
blot 4	Day 70	149.79	1.1230	11.82
	Day 77	176.65	0.9522	10.0
	Day 112	145.23	1.1580	12.23
	[a] Day 47	168.22	1.0000	10.53
Control	Purified bFGF	29.230	0.7174	7.58
	[a] Day 47	20.970	1.0000	10.53

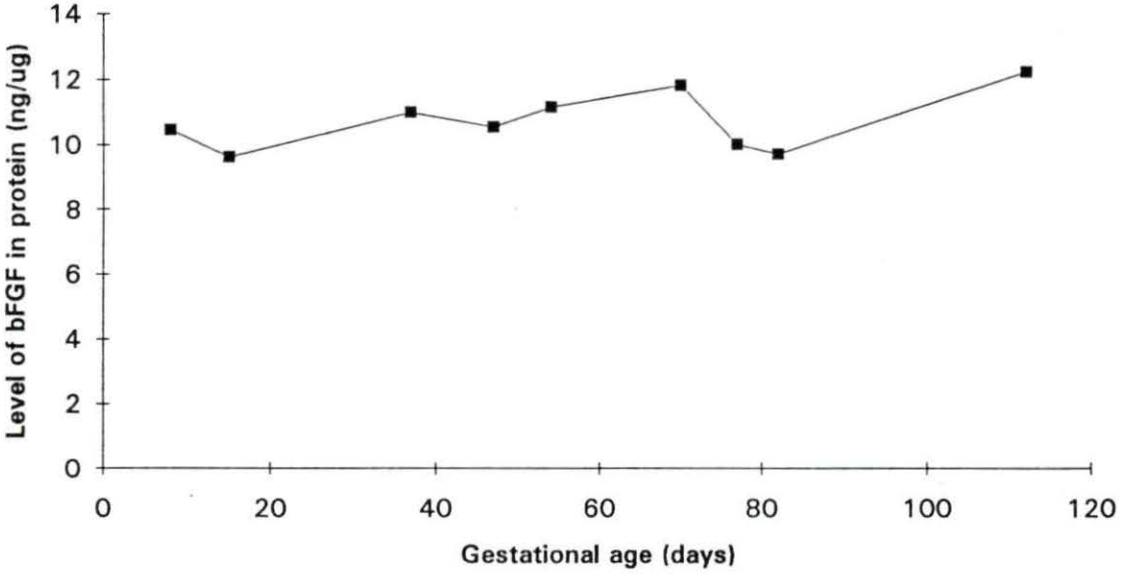
[a] Day 47 is the reference in each blot and used to normalize the values from different blot. The relative greyness for bFGF band in Day 47 sample was set at 1.0000. Its concentration was calculated from control blot with 10.53 ng/ug in the sample. n=1.

Effect of pregnancy on the secretion of bFGF

Since the amount of purified bFGF loaded on the well was known, the amount of bFGF in each sample was calculated by taking the ratio of relative value of the band to that of bFGF band, and multiplying with the known amount of bFGF in the control. In this way, the content of bFGF in each age of CL was obtained. These results were also listed in Table 1.

Since the concentration of bFGF in each stage of CL was known, the relationship of bFGF concentrations with different stages in pregnancy was plotted on a Figure 9. The effect of pregnancy on the concentration of bFGF in CL was estimated from the graph. The bFGF content of CL tissue derived from donors at different stages of pregnancy was not different.

Figure 9. Concentration of bFGF in sample of CL vs the stages of pregnancy. The gestational ages are expressed in days on X-axis, while the concentration of bFGF in CL sample are expressed in a unit of ng/ug on Y-axis. The levels of bFGF fluctuate during the period of pregnancy. The highest levels appear in Day 112 and Day 70, whereas the lowest was found in Day 15 and D 82 CL samples.


Level of bFGF in protein (ng/ug)
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
Gestational age (days)

DISCUSSION

The present study not only has demonstrated the presence of the 31 kDa bFGF in porcine pregnant luteal tissues, but has also examined the relationship between the concentrations of bFGF in CL samples and the stages of pregnancy. Since relaxin secretion has been found to increase as gestation advances (Changjian *et al.*, 1991) and bFGF has some regulatory effects on its secretion (Taylor *et al.*, 1992), it is reasonable to speculate a level change for bFGF associated with its function. Our results showed that the concentrations of bFGF in luteal tissue fluctuated during pregnancy, but there was no specific pattern. This result implies following possibilities:

First, there may not be a pattern for bFGF secretion in the pregnancy. While luteal tissue is in pregnant phase, the biological responses to bFGF might be decreased during this quiescent period, or bFGF may not have dramatic functions in pregnancy. It has been demonstrated that luteal cells do not proliferate after formation of CL. Previous study had examined the effect of pregnancy on luteal weight, composition and mitosis in the ewe, and found that luteal weight remained constant until Day 120, and the quantitative aspects of the composition of the luteal tissue showed no significant change (O'Shea *et al.*, 1988). In addition, mitosis was seen just in a few cells (O'Shea *et al.*, 1988).

Angiogenesis is manifested during the time of ovulation and CL development. Basic FGF has been an established angiogenic factor in the CL (Gospodarowicz *et al.*, 1985). The angiogenic process may not be active during pregnancy or other hormones and growth factors may be sufficient as angiogenic factors during pregnancy. Recently, a vascular

endothelial growth factor and its mRNA were identified in rat luteal cells which suggested its involvement in the process of CL angiogenesis (Phillips *et al.*, 1990). Moreover, EGF, TGFα, and TGFß were found to possess the capacity to stimulate neovascular tissues (Baird *et al.*, 1989). On the basis of these observations, we can speculate that angiogenesis might be dependent on several factors in local ovarian tissue, and resulting in a combined function of several factors which interact each other to exert a synergistic effect. Thus, bFGF may not be essential in angiogenesis during pregnancy. Some studies have demonstrated that bFGF has no effect on progesterone secretion *in vitro* and progesterone content was not significantly changed during the pregnancy (Tapanainen *et al.*, 1987; O'Shea *et al.*, 1988). This suggests that bFGF is probably not involved in the production of progesterone.

All the above evidence indicates that bFGF might not have much of an effect in angiogenesis or hormone regulation during pregnancy in the swine. However, a wealth of information suggests that bFGF plays an important role in gonadal tissue (Baird *et al.*, 1986). It is possible that the role of bFGF during pregnancy is merely to maintain the normal luteal tissue function and its structure, not to modulate an essential function required in a certain stage of pregnancy. Thus, the concentration of bFGF required in this process would not be altered considerably throughout pregnancy. However, the exact physiological role of this growth factor in luteal tissue remains to be further investigated.

Secondly, it is possible that bFGF has a functional role in some aspects of pregnancy. However, the biological responses of bFGF are not dependent on the concentration of the ligand, but on the amount of its receptors expressed during the pregnancy. So far, little is known about the receptor expression in ovarian tissue during pregnancy. But it is possible

that the expression of the bFGF receptor is under tight regulation. Changes in receptor concentration will alter the responses to bFGF. Another reason might be that no changes in bFGF concentrations or the levels of its receptors occur during pregnancy. The extent of response is controlled by certain unknown regulatory proteins in the cytosol or associated with certain transcriptional factors. Therefore, the biological responses to bFGF may not be totally ligand-dependent. Thus, the concentration of bFGF around its action sites could be always sufficient, and the level of basic FGF does not need to change for the response.

Finally, a pattern for bFGF secretion could exist, yet we failed to detect it is due to insufficient CL samples from the sows. Since the CL sample from each stage of pregnancy we used in the research came from different sows, the amount of bFGF contained in each sample may be confounded by different individual variation even when they were at the same stage of pregnancy. To test this we would have to obtain a CL sample from each stage of pregnancy in the same pig (which is impossible), or we could collect more CL samples of every stage from more individual pigs to eliminate variation due to individual difference. With increased number of samples, trends of the concentration change of bFGF would be more accurate. Due to the difficulty in the tissue source and the limitation of time, we were unable to obtain more samples which may somehow affect the result presented here.

Other methods can be used to improve the accuracy of our results. Although northern blots on each stage of CL sample to examine the mRNA levels of bFGF is an indicator of the level of bFGF during pregnancy, radioimmunoassays (RIA) are a more accurate method for quantification. By the use of RIA on samples of each stage to obtain the relationship of bFGF concentration and the time course of pregnancy would have obtained with higher

accuracy. However, RIA for bFGF are not widely available.

Although we are not sure at this point whether there is a pattern for bFGF secretion, the presence of this growth factor in the luteal tissue is confirmed. Although we established that the amounts of bFGF in the CL throughout pregnancy appeared to be the same, the function of bFGF during gestation is not clear.

Basic FGF has been found to have four forms of molecular weights. They are 18 kDa, 22 kDa, 24kDa, and 25 kDa. The 18 kDa form is the most common one which can be found in many tissues. Past studies have shown that bFGF found in the bovine CL is a truncated form with biological activity (Baird *et al.*,). We found only one bFGF in this study, and the molecular weight of bFGF in luteal tissues in all stages of pregnancy was determined as 31 kDa. The reason for the difference between our molecular weight form of bFGF and the published molecular weight of bFGF could be following possibilities. Basic FGF may bind to other protein molecules which have high affinity with bFGF and are not dissociated during sample preparation and electrophoresis (Makris *et al.*, 1989). A similar molecular weight of bFGF was observed in porcine brain tissue which underwent the same process as CL samples. The other band found in brain tissue was proven to be also bFGF. This may be the higher form of bFGF in brain. Our results were consistent with previous finding that 25 kDa was the higher molecular weight form of bFGF from the guinea pig (Moscatelli *et al.*, 1987). The result of two molecular weight forms of bFGF found in brain tissue also supported the early speculation that brain FGF consists of multiple forms (Gospodarowicz *et al.*, 1984).

Another reason for the high molecular form of bFGF in our study is that 31kDa bFGF

may be a non-truncated form existing in luteal tissue. Up to the present date, few studies have reported this form of bFGF except one, which found a 33 kDa bFGF in the porcine luteal tissue using the purified sample protein (Makris *et al.*, 1989). So far, it is not clear whether this 31 kDa bFGF is a large molecular form present in luteal tissue, or is the result of the dimer of the low molecular weight bFGF.

It is also possible that the 31 kDa protein is an oncogene product rather than bFGF. Oncogene products have high structure similarity to FGFs, and have 66% of homology in structure in its internal sequence (Makris *et al.*, 1989). Thus, these oncogene products may react with bFGF Ab and become visible on the blot. Two oncogenes were identified, to date, *int-2* gene and *hst-1* gene. Immunoblot of *int-2* from transfected cell lysates with an *int-2* specific antiserum result in four major *int-2* species. The large pair of protein were found at 30.5 kDa and 31.5 kDa which is similar to the size of our bFGF (Dickson *et al.*, 1991).

Our primary Ab was able to detect the porcine bFGF in CL tissue. This was verified by the fact that the purified bFGF was detected using our primary Ab, although the bFGF band in the purified sample was at 17 kDa. In fact, different antibodies against bFGF detected different forms of bFGF (Makris *et al.*, 1989). It is possible that our bFGF antibody could only detect 31 kDa form of bFGF.

The fact that the molecular weight of bFGF is 31 kDa suggested it may be the dimer of the 17 kDa molecule. The reason for the existing of the dimer might be that the temperature used to heat the CL sample proteins was not high enough to break down the dimer molecule.

Other reasons for the difference in molecular weight might be that different clones encode different forms of bFGF. It is also possible that the bFGF form varies with different

species and tissues. The biggest molecular form of bFGF has been reported as 70 kDa (Makris *et al.*, 1989).

SECTION II IMMUNOCYTOCHEMICAL LOCALIZATION OF BASIC FIBROBLAST GROWTH FACTOR IN CORPORA LUTEA OF PREGNANT SOWS

INTRODUCTION AND LITERATURE REVIEW

Background knowledge of bFGF in ovarian tissues

In the early 1970's, several laboratories found the existence of an angiogenic factor in the CL. It was also established that the presence of an angiogenic factor was associated with the perifollicular neovascularization during folliculogenesis using human follicular fluid (Frederick *et al.*, 1984). It has been suggested that granulosa and luteal cells could produce an angiogenic factor that triggers capillary invasion in the avascular granulosa cell layer. It seemed logical, therefore, that the activity present in this tissue was due to the local presence of an FGF-like growth factor. This was proved in Gospodarowcizs's work that angiogenesis in CL was related to FGF (Gospodarowicz *et al.*, 1985). This work was supported by Andrew Baird *et al.* in 1986, who demonstrated the presence of an immunoreactive FGF in the ovary using RIA. FGF has also been tested for its ability to induce angiogenesis on the chick chorioallantoic membrane, where it is a potent inducer of neovascularization (Gospodarowicz *et al.*, 1985). Shortly after, many groups established that the angiogenic activity could be due to the local presence of FGF in the ovary (Jakob *et al.*, 1977; Koos and Le Maire, 1983; Makris *et al.*, 1983, 1984). Basic FGF is a single-chain, non-glycosylated, cationic polypeptide with a molecular mass of about 18 kDa and PI 9.6 (Shing *et al.*, 1984). Basic FGF has 146 amino acids. The structure analysis suggested that the sequence of bFGF responsible for receptor activation may be in the core sequence of bFGF. It has a high affinity for heparin, and has been identified in various tissues, including brain, pituitary, kidney, ovary, and uterus.

Statement of purpose of this study

There is still little information concerning the cellular location of bFGF in tissues that contain bFGF. A study of pituitary tissue indicated that an immunoreactive bFGF may be localized in a subpopulation of corticotropes (Baird *et al.*, 1986). The cellular localization of bFGF in pituitary was reported using the immunoperoxidase technique (Baird *et al.*, 1986). Recently, a report provided the information of the specific localization of bFGF within mouse uterus by an immunohistochemical technique (Wordinger *et al.*, 1992). In this study, it was found that the connective tissue stroma and the basal lamina associated with uterine glandular and surface epithelial layers were a source of bFGF. The basal lamina and endothelial cells associated with blood vessels within the uterus and the smooth muscle cells of the myometrium were stained positively.

However, the precise cell source of bFGF in luteal tissue remains to be elucidated. Since the biological activities of bFGF suggest it may potentially be a critical local regulatory factor to modulate various physiological responses within the luteal tissue, it is necessary to find out the exact luteal cell source of bFGF.

The objective of this part of the study was to use the immunoperoxidase staining method to localize bFGF specifically within luteal tissues and to determine whether the localization of bFGF within the CL was influenced by different stages of pregnancy in sows.

Explanation of immunoperoxidase staining technique

Methods of immunoperoxidase staining can be used to localize cellular antigens. This method involves the use of antibodies and the enzyme, peroxidase. Immunoperoxidase allows

visualization of cell components in a variety of specimens. For a specific antigen it can be determined what type of cells produce this substance in normal and neoplastic tissue, levels of the substance produced, the identification of cells of unknown origin, and the determination of tumor cell differentiation (Bourne, 1983). Here we are interested in the cell type for bFGF secretion during the different stages of pregnancy.

There are four main methods of immunoperoxidase staining. The direct, indirect, PAP, and avidin-biotin methods. In the direct method, the specific Ab is chemically linked to peroxidase. A substrate is then applied to produce a color precipitating at the site. It can be quickly performed. The main drawback is that for every antigen to be localized, a different conjugated Ab is needed. The most common application of this method is for the detection of immunoglobulin, complement and immune complex. In the indirect method, a primary Ab is added first, then the conjugated secondary Ab is added followed by adding the substrate. PAP is comprised of the peroxidase and an Ab against peroxidase. PAP method utilizes three reagents: primary, secondary Ab, and PAP complex. We used the avidin-biotin method. The strong affinity of avidin for biotin gives this method greater sensitivity than other conjugated Ab techniques. This method is based on the ability of the egg-white glycoprotein avidin to non-immunologically bind four molecules of the vitamin biotin. Three reagents are used. The first is a primary Ab specific for the antigen to be localized. The secondary Ab which is conjugated to biotin, is capable of binding to the first Ab. The third reagent is a complex of peroxidase conjugated biotin and avidin. The free sites of the avidin molecule allow binding to the biotin on the secondary Ab. The peroxidase enzyme, and therefore the original antigen, are visualized with an appropriate chromogen, DAB (Bourne,

1983).

By using this technique, we were able to identify the cellular source of bFGF in the CL, and to observe the level of the growth factor at each age by the intensity of positive staining inside the cells.

MATERIALS AND METHODS

Animals

The fresh corpora lutea were collected from ovaries of pregnant sows from Iowa Packing Plant and Meat Laboratory, ISU. Five stages of CL were obtained as Day 15, 45, 54, 82, and 112. Each CL was dissected from ovary by a sterile scalpel. All CLs were frozen in liquid nitrogen immediately until the liquid nitrogen around the tissue stopped bubbling. Each age of CLs were wrapped in a separate aluminum foil. All tissue sampels were stored in freezer at -70^{0}C.

Materials

All the antibodies used were bought commercially. The primary Ab, anti-bFGF, was purchased from R&D System (Minneapolis, MN). Normal goat serum (NGS), secondary Ab and Vectastain kit were obtained from Vector Laboratories, Inc. NRS was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY). The substrate, DAB, and Tris base, were purchased from Sigma Chemical CO.(St.Louis, MO). Hydrogen peroxide, and PBS were purchased from Fisher Scientific Co. These reagents were all of analytic grade.

Methods

Tissue preparation

CL tissues were sectioned at 8 μm on a rotary microtome at a temperature of -20°C. Tissue sections were placed onto one side of a glass slide which was previously treated with

Vectabond (Vector Labs.), a non-protein tissue section adhesive to prevent the sections from detaching from the slide. Slides were placed in acetone for 5 minutes and then air dried. All slides were kept in a slide box and put into a freezer at -20^{0}C.

Immunocytochemistry

Tissue sections were circled by a pap pen (Daido Sangyo CO., LTd. Japan), and placed into a long plastic vessel with three wet paper towels in it. All procedures were carried out in this moisture chamber at room temperature, unless otherwise specified. The section was first washed with Tris buffer (0.05M tris-base, pH7.6) for 5 minutes. After the buffer was aspirated, 4 or 5 drops of 0.3% hydrogen peroxide were added to the tissue and incubated for one hour in order to quench endogenous peroxidase activity. The slide was gently rinsed with Tris buffer and incubated in buffer for 5 minutes.

In order to remove non-specific binding, normal goat serum (NGS) was used at a dilution of 1:20. NGS filled the charged sites, leaving no room for absorption of the primary Ab. Addition of 5% bovine serum albumin (BSA) in NGS increased the protein concentration and further reduced the non-specific staining. The 5% BSA (v/v) containing NGS was added to tissue section and incubated for a period of 20 minutes. The excess serum was tapped off and sucked by air vacuum without washing with Tris buffer. Then, the tissues were treated with 5 drops of anti-bFGF at a 1:100 dilution made in Tris-PBS buffer, and incubated over night at 4°C. This primary Ab can recognize the antigenic portion in the CL tissue and bind it. In order to eliminate the background staining, a high salt buffer Tris-PBS (10ml 0.05M tris buffer, pH 7.6; 90 ml 0.01M PBS, pH 7.2) was used in the following washing steps. After

the primary Ab was removed, Tris-PBS buffer was added to rinse the slide and allowed to stay for 20 minutes in order to elute the unbound Ab.

Five drops of secondary Ab (anti-rabbit IgG; 1:200 dilution in Tris-PBS buffer) was then added to the tissue and incubated for 30 minutes at room temperature. Since the primary Ab is raised in rabbit, the secondary Ab which was raised in goat against rabbit, can bind to the first Ab in the tissue. Also, the secondary Ab is a biotinylated Ab, and it provides binding sites for ABC subsequently. So, the secondary Ab serves as a linking bridge between the primary Ab and ABC. Again the Ab solution was sucked off and tissue was washed in Tris-PBS for 10 minutes.

While doing the above steps, the ABC complex needs to be made at least 30 minutes in advance before use. The tissue sections were then incubated with ABC (1:200 in Tris-PBS) for 30 minutes, followed by a buffer bath in Tris-PBS for 10 minutes. The relationship of tissue antigen, primary Ab, secondary Ab, and ABC is shown in Figure 10. The DAB solution (50% DAB (w/v), 0.01% H_2O_2 in Tris buffer) was made by putting 1.5 mg DAB powder in 2.97 ml Tris buffer until fully dissolved, and 30 μl 1% hydrogen peroxide was added. The solution should be made immediately before use. The slides were developed in Ab for 5 minutes before being rinsed with double distilled H_2O (ddH_2O). Finally, the slides were counter-stained with hematoxylin (Shandon, Pittsburgh, PA) for 10 seconds, and decolorized in ammonium water (1ml of 17M ammonia hydroxide, 500ml tap water) for eight seconds. Lastly, the slides were washed in ddH_2O for 5 minutes and mounted with aqua-mount (Learner Laboratory, Pittsburgh, PA) to cover the tissue section.

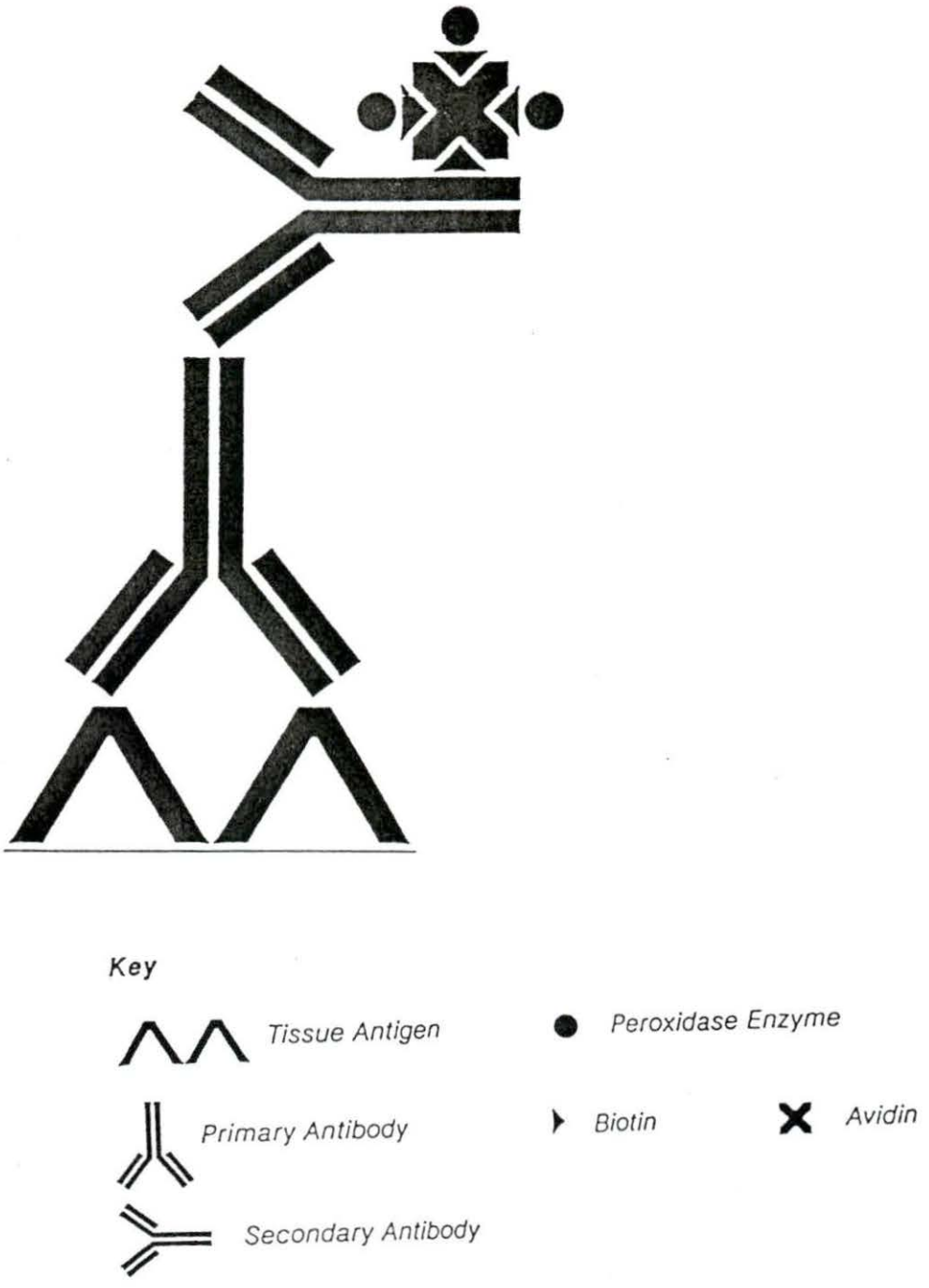


Figure 3. Diagram of the relationship between tissue, primary Ab, secondary Ab, ABC and peroxidase enzyme (Handbook of Immunoperoxidase Staining Methods, Dako Corp.)

Controls

While doing immunocytochemistry (ICC), the following slides were run to determine whether the staining we obtained in the tissue sections was a result of non-specific or background staining. Three control slides were run regardless of the age of CL: 1) Omission of primary Ab: the slide was processed as usual, but was probed only with several drops of Tris buffer instead of primary Ab. There should be no staining observed in the section because the antigen was not recognized; 2) NRS: measure the amount of protein in 1:100 dilution Ab solution, then determine how much NRS is equivalent to the Ab. NRS was made at 1:3000 in Tris-PBS and added to replace primary Ab in the over night-incubation. Other steps were the same. 3) Pre-absorbed Ab: the pre-absorbed Ab needed to be prepared first. The amount of primary Ab added was the same as for probing at a 1:100 dilution. Anti-bFGF (4.0 μg) was mixed with 4.2 μg purified human bFGF, and then diluted to a final volume of 200 μl with Tris-PBS buffer. Incubate for 2 hours at room temperature. Gently tap the tube every half an hour. Pre-absorbed Ab was added at the step usually add primary Ab. Other steps are all the same.

RESULTS

Control group

To determine if the positive staining was the result of specific binding to the antigen in question, three control slides were run. The first one was the NRS slide in which the NRS was added to the tissue for incubation instead of primary Ab. This substitution control serves as a blank since any staining observed is due not to Ab localization of the antigen, but to non-specific protein binding, endogenous peroxidase activity, or non-specific binding of the other Ab reagents. The second one was pre-absorbed anti-bFGF which was used instead of primary Ab. In theory, all Abs would be absorbed by the antigen and no staining would be observed. In the third control, the specimen was processed identically to the CL tissue section, but the primary Ab incubation was omitted. This was to eliminate the possibility that the staining was the result of the binding of secondary Ab to antigen.

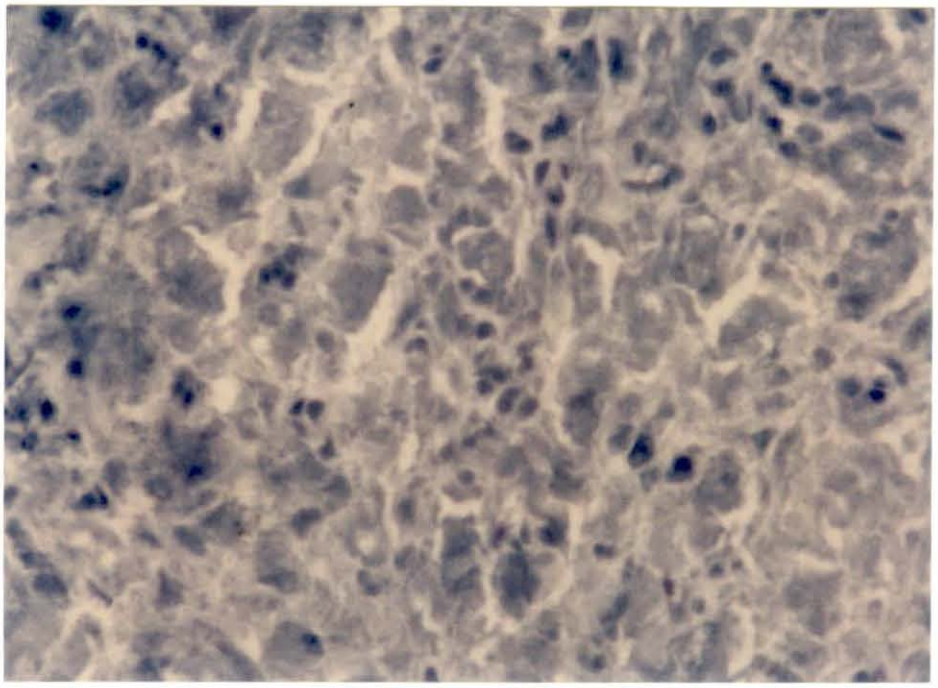
The results of the three controls were presented in Figure 11, Figure 12, and Figure 13, respectively. It is obvious to see that the slides incubated with NRS and without primary Ab showed no staining at all. The pre-absorption slide showed very little positive staining in the luteal tissue, and a slight staining in the connective tissue. However, compare the staining to that found in sample slides, and the staining in the pre-absorption slide was negligible.

Immunoperoxidase staining of bFGF in CL tissue sections

Five stages of frozen CL tissue were studied, which were Day 15 (Figure

Figure 11. Negative control with omission of primary Ab. Day 54 CL sample was run without incubation with primary Ab. No any positive staining was found in the specimen.

Figure 12. Negative control with 1:3000 NRS. Day 112 CL sample was run and incubated with 1:3000 of NRS instead of primary Ab. No positive staining was observed in the specimen.



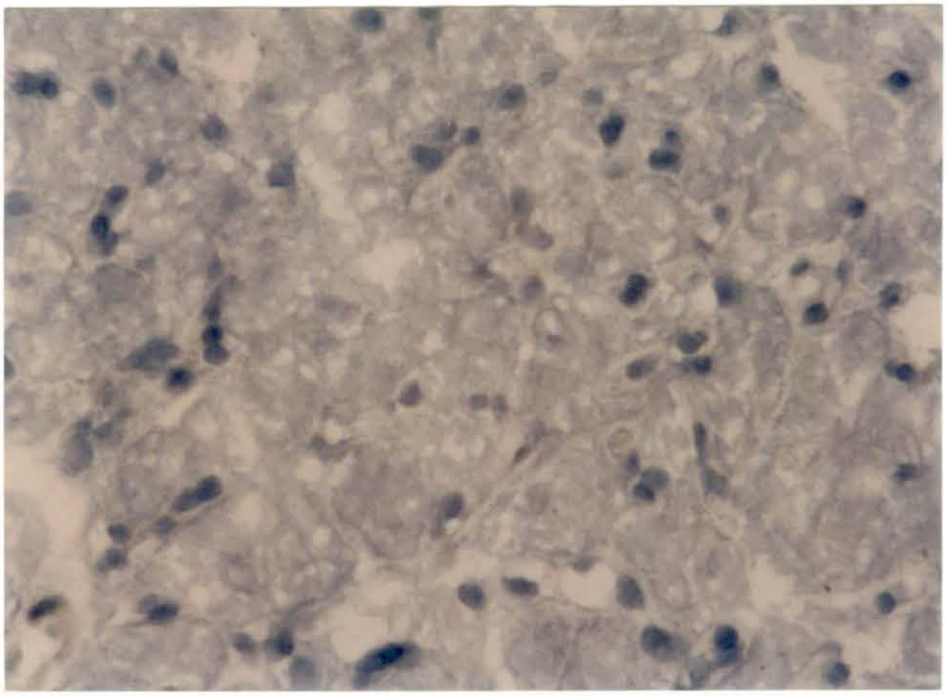
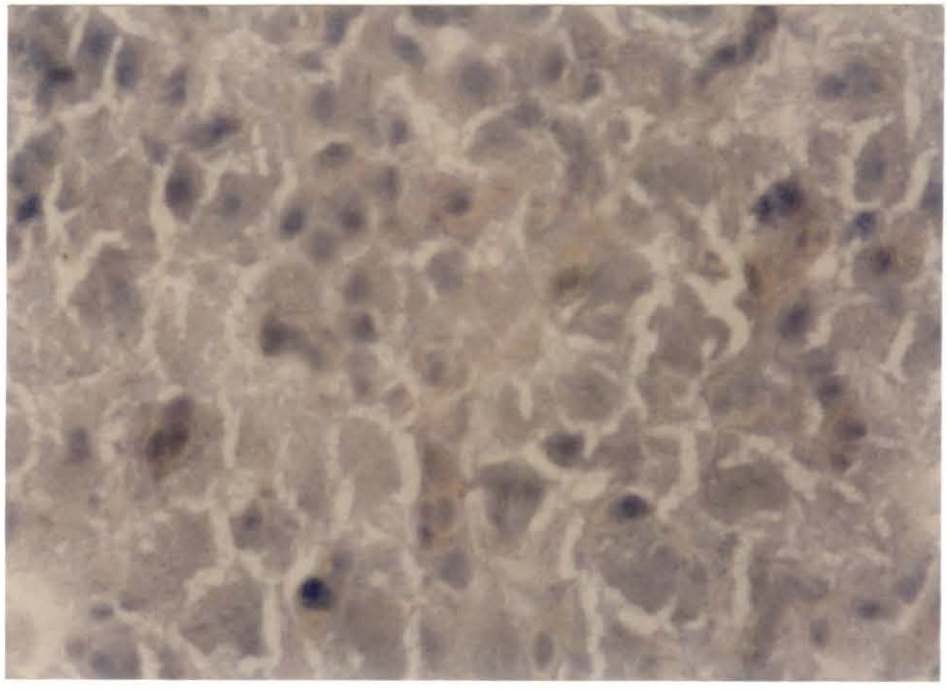
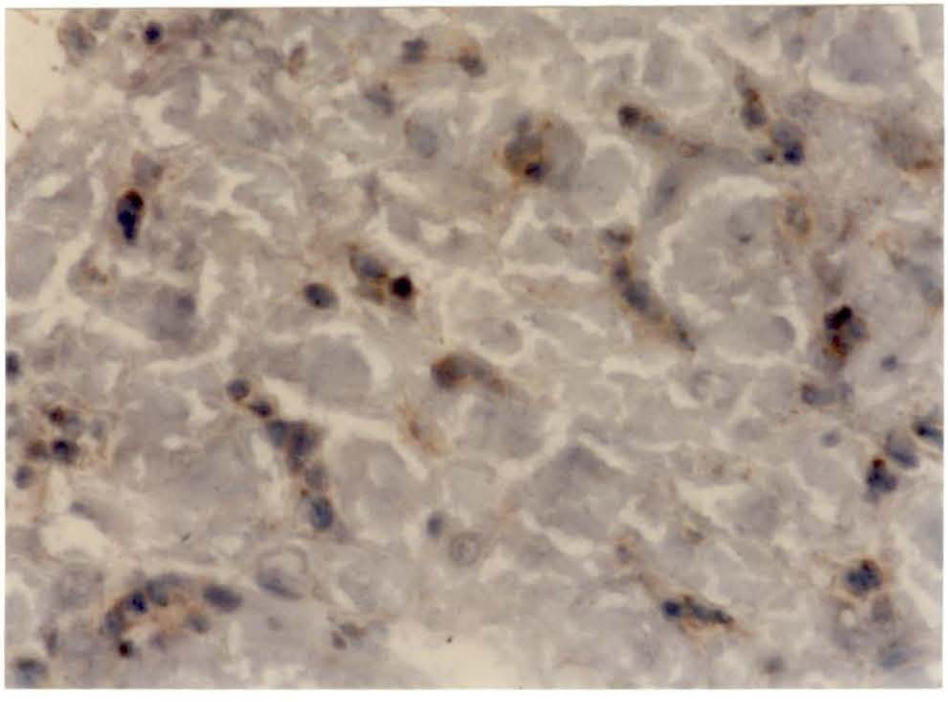


Figure 13. Negative control with 1:100 pre-absorption Ab. Day 112 CL sample was run and incubated with 1:100 pre-absorbed Ab to replace the primary Ab. A light staining was observed in some luteal cells and in connective tissue.

Figure 14. Immunoperoxidase staining of bFGF in Day 15 CL tissue section. Day 15 CL sample was run and incubated with a 1:100 dilution of anti-bFGF Ab. The positive staining was observed within cytoplasm of luteal cells and associated with cell membranes.

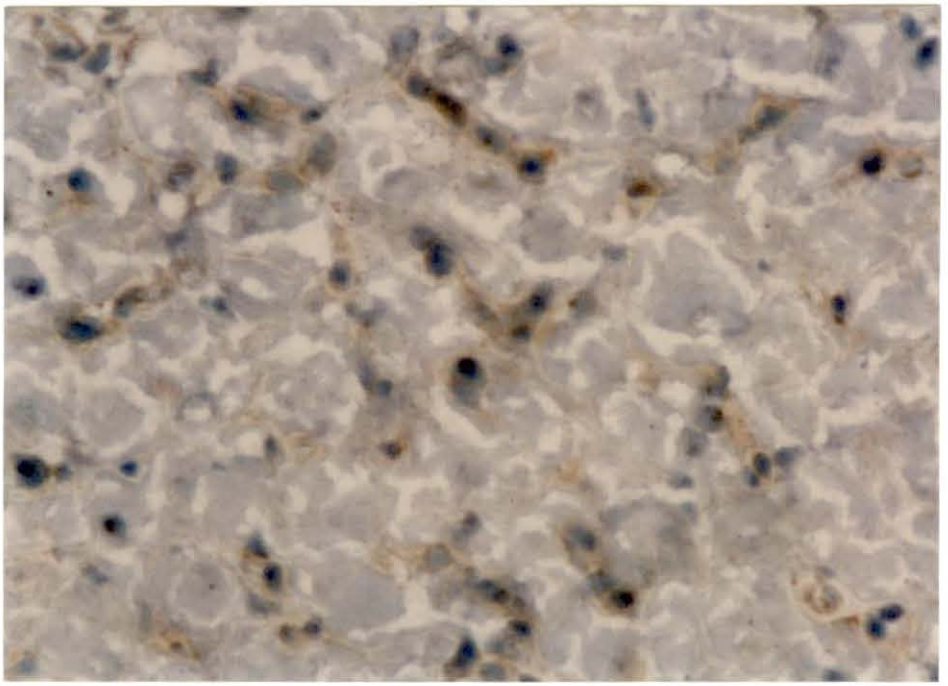




14), Day 45 (Figure 15), Day 54 (Figure 16), Day 82 (Figure 17), Day 112 (Figure 18). The picture showed the blue color which is due to the hemotoxylin reaction, and the brown color which is the reaction of peroxidase, DAB and hydrogen peroxide. All pictures were taken at an amplification of 128.6 X. The positive staining appears in brown color which indicates the locations of antigen in the CL tissue sections. A similar pattern of positive staining is observed in all five tissue sections. The positive staining can be found in the basement membrane of endothelial cells in the vessle wall. Most of the staining is found primarily in the extracellular matrix of the luteal cells and associated with cells membrane. Some of the staining seems to be in the cytosol of the luteal cells. Due to the fact that the morphology of these slides are not good, it is difficult to determine the exact location of the positive staining. In all the slides observed, there is no strong indication of the nuclei staining.

Figure 15. Immunoperoxidase staining of bFGF in Day 47 CL tissue section. Day 47 CL sample was run and incubated with a 1:100 dilution of anti-bFGF Ab. The positive staining was observed within cytoplasm of luteal cells and associated with cell membranes.

Figure 16. Immunoperoxidase staining of bFGF in Day 54 CL tissue section. Day 54 CL sample was run and incubated with a 1:100 dilution of anti-bFGF Ab. The positive staining was observed within cytoplasm of luteal cells and associated with cell membranes.



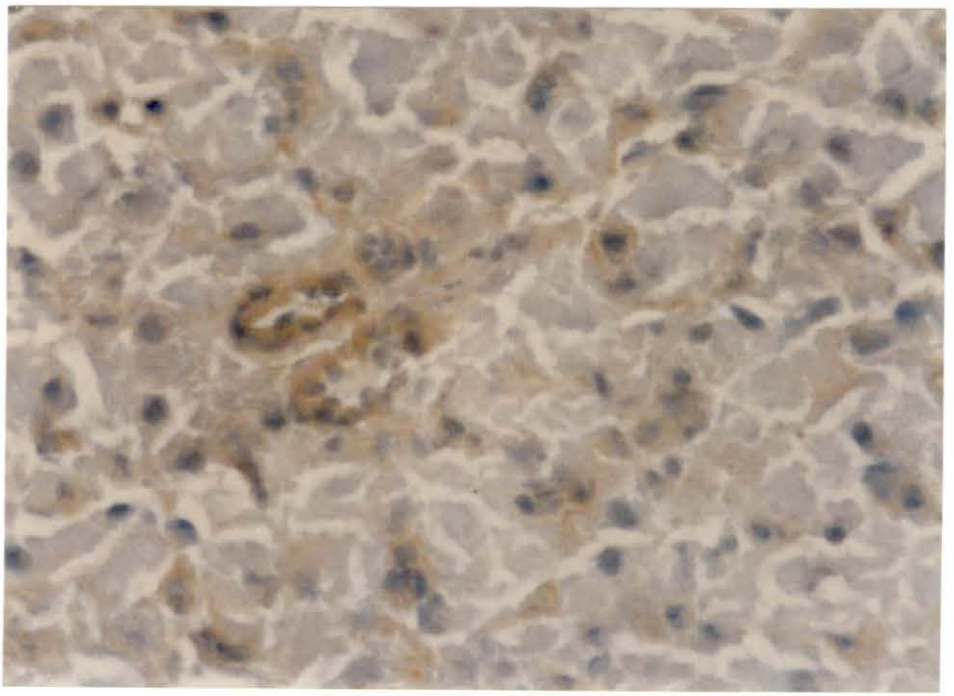
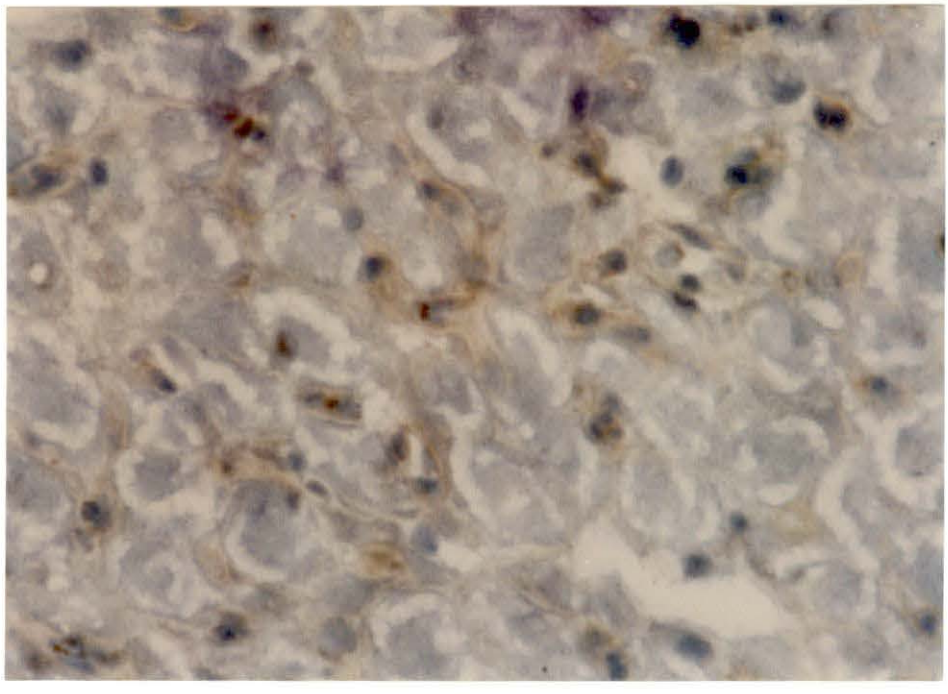
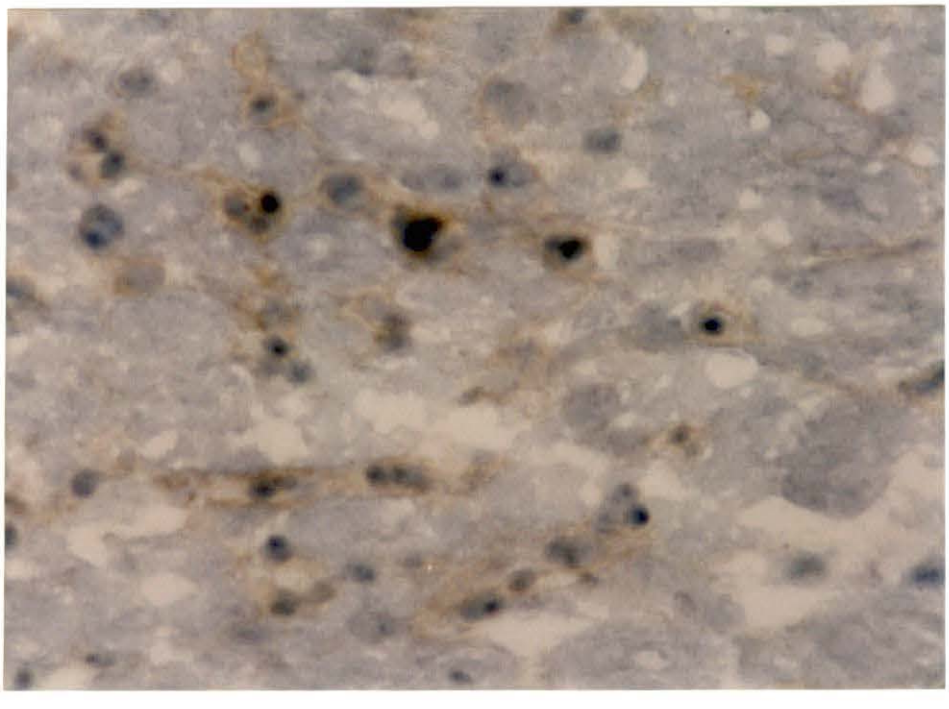


Figure 17. Immunoperoxidase staining of bFGF in Day 82 CL tissue section. Day 82 CL sample was run and incubated with a 1:100 dilution of anti-bFGF Ab. The positive staining was observed within cytoplasm of luteal cells and associated with cell membranes.

Figure 18. Immunoperoxidase staining of bFGF in Day 112 CL tissue section. Day 112 CL sample was run and incubated with a 1:100 dilution of anti-bFGF Ab. The positive staining was observed within cytoplasm of luteal cells and associated with cell membranes.





DISCUSSION

The purpose of the second part of the study was to explore the exact cellular source of bFGF. Our result found that the majority staining was in extracellular matrix around the luteal cells. Staining was observed also in endothelial cells of blood vessel walls and some luteal cells. These observations suggest that the cells for the production of bFGF in luteal tissue may be luteal cells and endothelial cells. Thus, we can postulate that the presence of bFGF in luteal cells may be responsible for the function of luteal cells while the presence of bFGF in endothelial cells may be responsible for the biological activities in the vascular tissues.

It is clear that the staining was associated with the cytoplasm membrane of those cells, and had a diffuse reaction with extracellular matrix in slides. As mentioned in the first section, the immunoreactive band identified was 31 kDa which was higher than the molecular weights reported. If the actual molecular weight of the immunoreactive bFGF is 31 kDa, then its location seems to contrast to the previous finding in which the 18 kDa bFGF was found in cytoplasm and the cell membrane, whereas the 22 kDa and 24 kDa molecules were associated with the nucleus (Quarto *et al.*, 1991). The reason that high molecular forms of bFGF target the nucleus is that these forms of bFGF contain a nuclear localization signal at the NH_2-terminal extension of the molecule. If the 31 kDa bFGF is the high molecular form, then its absence in the nucleus might result from the lack of the nucleus targeting signal or the functional domain for the targeting signal is masked for some unknown reason. If this is not the case, and the higher molecular weight form is a dimer molecule. Then, the actual

molecular weight of the immunoreactive bFGF could be the 18 kDa molecule since the localization of bFGF was observed within the cytosol and associate to cell membrane.

Our result showed the positive staining associated with extracellular matrix in a diffusible manner. This confirms early *in vitro* studies that bFGF is present within the subendothelial extracellular matrix (Burgess *et al.*, 1989). It has been proposed that this diffuse presence of protein may result from the release of growth factors through the activities of hydrolytic enzymes. In addition, studies have shown the extracellular matrix to be a dynamic component capable of modulating gene expression (Burgess *et al.*, 1989). In one recent report, it was found that bFGF has high affinity to heparin sulfate proteoglycans (HSPGs), which was proven to be the component of the extracellular matrix and plasma membrane. Binding to HSPG could protect bFGF from digestion of proteases (Moscatelli *et al.*, 1991). These observations suggest that the extracellular matrix may provide a reservoir of bFGF that can mediate long-term responses required for angiogenesis upon the exposures to bFGF. The existence of soluble HSPG may serve as a carrier to provide a diffusible form of bFGF for function without being digested. Therefore, the interaction between bFGF and the extracellular matrix might be crucial to the biological activities of bFGF.

There was a significant staining in the endothelial cells in the blood vessel walls. The staining was localized in the basal membrane. This phenomenon can be observed in all ages of samples examined by ICC. The result provides evidence that endothelial cells could produce bFGF which accounts for the proliferation of the endothelial cells in vascular tissues. The presence of positive staining of bFGF in the five stages CL samples indicates the physiological requirement for this growth factor. It is possible that some angiogenesis or

vascular development were still going on slowly caused by the bFGF during pregnancy instead of the dramatic effect after ovulation when the vascular development of the ovarian follicle becomes more impressive. At the same time, the growth factor may be stored inside the cells at a certain level and released for function upon receiving signals released by certain physiological changes from the conceptus.

In conclusion, immunoperoxidase staining suggested that the the cell source for bFGF secretion in luteal tissues might be luteal cells and endothelial cells. The immunostaining was localized in the cell membrane and a few in the luteal cells. The staining was found to be in the basal membrane in endothelial cells. It is also associated with the extracellular matrix. All these findings imply a local function of bFGF and provide information of the possible role of bFGF in the process of pregnancy.

GENERAL DISCUSSION

The present study was conducted to examine the levels of bFGF in the luteal tissues over the course of pregnancy. Results of this experiment found the concentration of bFGF was fairly constant with advancing stages in pregnancy. There was no obvious change in bFGF levels throughout the whole pregnancy. This result was observed with both Western Blot and the immunoperoxidase staining. Moreover, immunocytochemical results suggest that the positive staining was in the extracellular matrix around the luteal cells, a few staining in the luteal cells, and the basal membrane of endothelial cells. The staining pattern was not altered by different stages in pregnancy.

All these findings indicate that the immunoreactive bFGF is most probably a low molecular weight molecule and its concentration in luteal tissue during pregnancy does not change much. Thus, our result was in contrast to that of relaxin secretion, for there is no specific pattern for bFGF secretion during pregnancy.

The molecular weight of the immunoreactive band found in Western blot was of 31 kDa. Since its presence was observed to be in the cell membrane, it seems to suggest that the bFGF is a low molecular weight molecule. A body of evidence has established that 18 kDa bFGF is in cytosol and cell membrane whereas the high molecular forms, 22 kDa, 22.5 kDa and 24 kDa are found to localize in the nucleus (Quarto *et al.*, 1991). Thus, the bFGF band found in this experiment may be the dimer of the 18 kDa bFGF molecule, which hasn't been separated in the tissue preparation or through electrophoresis. However, since the various molecular weight form of bFGF have been identified, it is quite possible that the 31 kDa

form of bFGF is a high molecular weight form bFGF in the CL tissue of pregnancy.

Another possibility is that the 18 kDa bFGF reported in previous studies is generated as a result of enzymatic degradation during tissue exaction or purification of bFGF. It remains uncertain whether the 18 kDa form of bFGF occurs in ovarian tissue under physiological conditions. Higher forms of bFGF may exist in the luteal tissue and have biological activity. Therefore, the 31 kDa bFGF found in this study could be the non-truncated form of bFGF molecule *in vivo*. Although the exact *in vivo* origins of bFGF, its regulation and physiologic significance await further investigation, it is tempting to speculate that locally produced bFGF may play autocrine or paracrine regulatory roles at or adjacent to its sites of synthesis, thereby involving in the process of cell proliferation, differentiation and interaction with other hormones and growth factors.

It has been demonstrated that endothelial cells proliferate in the presence of bFGF. The positive staining of bFGF found in endothelial cells is the evidence for its local function. It further supports the finding that bFGF is a growth factor responsible for the growth and development of vascular tissue in CL. The other cell type producing bFGF might be the luteal cells. Since there is no proliferation of luteal cells to bFGF, its presence might be related to other functions in the tissues. Extracellular matrix was positive to bFGF staining. This is consistent with previous studies which found bFGF binds the extracellular matrix. As a component of the extracellular matrix, bFGF may play a role in cell support and growth.

As mentioned before, some studies found that bFGF can modulate other hormone or growth factor function which include relaxin, prolactin, and FSH/LH. Our result found no

obvious concentration change throughout pregnancy, indicating that the effect of bFGF is not regulated by its level in tissue, but something else. The observation that bFGF is the component of extracellular matrix associated with cell membrane suggest the regulation of bFGF function might not be at the level of its synthesis but at release from the extracellular matrix. It could be that the receptor for bFGF has a greater regulatory effect than bFGF itself on the local control of other hormone and growth factor function. The regulation of the gene expression of bFGF receptors may be the key point in this aspect. It is likely that the 31kDa form bFGF is not the bioactive form of bFGF which is functioning in pregnant CL, so the level of the 31kDa bFGF is not essential for the bFGF function in pregnancy. Also, it might be that certain steps involved in the signal transduction pathway play a regulatory role to control the response of bFGF, although the transduction pathway for bFGF remains to be elucidated.

The level of bFGF in the tissue may be regulated by its gene expression. Although there is only little information concerning the regulation of gene expression, some experimental evidence found that the human bFGF gene promotor contains two regions representing negative regulatory domains. They also found that multiple mRNAs for bFGF, which originate from one single gene, contain the same starting site (Florkiewicz *et al.*, 1991). It was proposed that translation of the high molecular weight forms initiates from CUG codons (Hann *et al.*, 1988; Prats *et al.*, 1989) while translation of 18 kDa molecule initiates from AUG codon (Abraham *et al.*, 1986). Furthermore, there is a predicted stem-loop structure positioned between the three CUG codons and one AUG codon (Florkiewicz *et al.*, 1991). Therefore, this structure might be used to posttranscriptionally regulate or modify bFGF

gene expression when it is recognized by specific cytosolic factors under certain conditions (Florkiewicz *et al.*, 1991).

In luteal tissue during pregnancy, there could be a certain signal given by the conceptus that regulates the expression of bFGF to a certain level, or there could be a sensor in the cell to detect the concentration of bFGF and feedback information so as to modulate the level of bFGF. Additionally, the presence of high molecular weight forms of bFGF has been established. They are all exclusively localize to the nucleus. Although the physiological function of these high molecular weight forms is unknown, it is possible that their localizations has some relationship with their function. It is likely that they are involved in the regulation of low molecular weight form level by communicating with certain regulatory proteins inside or within the nucleus membrane which participate in the process of gene expression.

In summary, it is suggested that luteal cells and endothelial cells produce bFGF during pregnancy. This observation provides evidence for the local function of bFGF in luteal tissues. However, many questions about bFGF remain unsolved. How bFGF exerts its effects, what the physiological significance of bFGF is during pregnancy, and how the bFGF level in luteal tissue is regulated, await further exploration.

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