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Mechanism of decidualization of human endometrial stromal cells *in vitro*

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MECHANISM OF DECIDUALIZATION OF HUMAN ENDOMETRIAL STROMAL CELLS *IN VITRO*

by

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Abstract

MECHANISM OF DECIDUALIZATION OF HUMAN ENDOMETRIAL STROMAL CELLS *IN VITRO*

by

Baiqing Tang

Advisor: Professor Erlio Gurpide

Decidualization of the human endometrial stroma plays an important supporting role in embryo implantation and subsequent pregnancy. This process involves changes in cell morphology and the expression of products characteristic of decidual tissue. In order to investigate the molecular mechanism and regulation of the differentiation events, human endometrial stromal cells were exposed to different hormones and agents and decidualization of the cells was evaluated by observation of their morphologic change to decidual phenotype and determination of the expression of

decidual products, such as prolactin (PRL), insulin-like growth factor binding protein-1 (IGFBP-1), heat shock protein-27 (HSP-27), desmin, laminin, and fibronectin.

Induction of PRL was detected in the cells treated with dibutyryl cAMP (db-cAMP) by using immunocytochemistry, immunoprecipitation and Western blotting procedures. Time and concentration dependence of db-cAMP induced increases PRL protein output was determined by using RIA and ELISA. Dibutyryl-cAMP also promoted a time dependent increase in levels of PRL mRNA, normalized to cyclophilin mRNA. Prolactin production was also observed in stromal cells cultured with 8-Br-cAMP or forskolin. MPA (medroxyprogesterone acetate) synergistically enhanced the inductive effects of db-cAMP, 8-Br-cAMP or forskolin on PRL production, as measured by Western blotting or ELISA. Transcription studies indicated that MPA also increased the rate of the PRL gene transcription in stromal cells cultured with db-cAMP. Estradiol (E_2) had no significant effect on db-cAMP induction of PRL production. In addition to its effects of PRL synthesis, db-cAMP induced morphologic change and expression of IGFBP-1, HSP-27, desmin, laminin, and fibronectin in cultured stromal cells. Intracellular cAMP levels was elevated in the

stromal cells cultured with gonadotropins. Furthermore, the treatment with gonadotropins induced PRL expression and morphologic changes of the stromal cells.

These results suggest a model to dissect the molecular mechanisms of decidualization involving gonadotropins, cAMP, steroids, and PRL expression. The synergistic regulation of PRL production in human endometrial stromal cells by MPA and cAMP suggests that these compounds regulate PRL expression and decidualization through interdependent pathways. Our results indicate that a cAMP mediated signal transduction pathway is involved in decidualization of human endometrial stromal cells.

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List of Abbreviations

BSA	bovine serum albumin
cAMP	cyclic adenosine 3′:5′-monophosphate
db-cAMP	N^6-2′-0-dibutyryl-cAMP
8-br-cAMP	8-bromo-cAMP
DCC	dextran-coated charcoal
DTT	dithiothreitol
EDTA	ethylenediamine N,N,N′,N′-tetraacetic acid
E_2	estradiol
ER	estrogen receptor
FBS	fetal bovine serum
ct-FBS	dextran-coated charcoal treated FBS
FSH	follicle stimulating hormone
hCG	human chorionic gonadotropin
HBSS	Hank's balanced salt solution
HSP 27	heat shock protein 27
IGFBP-1	insulin-like growth factor binding protein 1
LH	luteinizing hormone
MPA	medroxyprogesterone acetate
PRL	prolactin
PRLR	prolactin receptor

List abbreviations - continued

P	progesterone
PR	progesterone receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMS	phenylmethylsulfonylfluoride
RLX	relaxin
RLXR	relaction receptor
SDS	sodium dodecyl sulfate

I. INTRODUCTION

The focus of biochemical research on the human endometrium appears to have shifted, in recent years, from the epithelium to the stroma. One reason for this shift has been the realization of the multiplicity of bioactive products secreted by the human decidual cells and the potential role that these products may play during implantation and development of the embryo and even in parturition. The interest in endometrial stromal elements is further enhanced by experimental evidence revealing paracrine and autocrine interactions between stromal and epithelial cells and within the stroma. Moreover, in fertile cycles and throughout the duration of pregnancy, decidual cells can interact with the trophectoderm of the invading embryo and later with the extravillous or anchoring trophoblast and chorion cells of the fetal membranes (1).

Decidualization of the stroma is one of the remarkable events occurring in the human endometrium. During this process fibroblast-like mesenchymal cells undergo morphologic changes and extensive biochemical differentiation reflected by the expression of a

multiplicity of new products (2). The decidualization of endometrial stromal cells is observed in humans during the second half of the luteal phase of the normal menstrual cycle, about 1 week after ovulation, throughout the duration of normal pregnancy and even during ectopic pregnancy in the absence of intrauterine fetal tissue (3,4).

From a biochemical viewpoint, the processes involved in the differentiation of proliferating stromal cells to decidual cells are of considerable mechanistic interest, as they suggest the involvement of steroids, growth factors, peptide hormones and cytokines. Some of these paracrine influences are expected to involve modulation of the extracellular matrix (ECM) composition and function during pregnancy (1).

From a clinical point of view, decidualization and paracrine communication between decidual cells and chorionic elements are pertinent to *in vitro* fertilization programs, in which the correct preparation of the endometrium for implantation of the transferred embryo is essential and the synchronized development of the stroma and epithelium during the postovulatory phase is desirable. Moreover, decidual cells may contribute to the maintenance of pregnancy and be responsible for

pathology resulting in pregnancy loss (1).

The objective of the doctoral dissertation project was to obtain biochemical information concerning the process of decidualization. The studies were focused on the endometrial stromal cells, aiming to investigate decidualization mechanism, *viz* the sequence of events that take place during the transforming of endometrial fibroblast-like stromal cells into the decidual cell phenotype. These studies were conducted under *in vitro* conditions using cells isolated from human endometrial tissue obtained from diagnostic dilatation and curettage procedures or after hysterectomies. Several criteria of decidualization, e.g. decidual morphologic change of stromal cells and production of decidual products such as laminin, desmin, fibronectin, insulin-like growth factor binding protein-1 (IGFBP-1), heat-shock-protein 27 kDa (HSP-27), and prolactin (PRL) were used to evaluate decidualization of human endometrial stroma.

On the basis of a rationale to be discribed, the specific aims of this research were focused on the investigation of effects of PRL, cAMP, gonadotropins and progestins on decidualization of human endometrial stroma. The role of PRL in the decidualization process was determined by exposing human endometrial stromal

cells in culture to hPRL and differentiation of the stromal cells was documented by using a variety of biochemical end points as well as morphologic criteria. The actions of cAMP on PRL expression were evaluated by incubating human endometrial stromal cells in medium containing cAMP derivatives or forskolin; PRL production was detected by using different methodologic approaches. The effects of female sex steroids on cAMP-induced PRL expression of human endometrial stromal cells were studied by Western blotting analysis, enzyme-linked immunosorbent assay, Northern blotting analysis and nuclear run-on procedure. The induction of decidualization of human endometrial stromal cells by cAMP was investigated by evaluating morphologic changes to the decidual phenotype and expression of decidualization-related products. The effectiveness of gonadotropins (FSH, LH, hCG) as physiologic generators of cAMP in human endometrial stromal cells was determined by measuring cAMP levels. The participation of gonadotropins in the differentiation of human endometrial stromal cells was evaluated by documenting morphologic changes to the decidual phenotype and expression of PRL.

Prolactin was considered to be an appropriate

marker for the decidualization of human endometrial stroma since it was biochemically detected in both decidual cells (5) and decidualized stromal cells during the menstrual cycle (6), and since PRL expression coincided with the appearance of morphologic changes to the decidual phenotype (7). A large increase in estrone sulfate sulfatase activity during decidualization was also evident from comparisons of the enzymatic activity in endometrial tissue collected during the menstrual cycle with the activity in decidual tissue obtained by scraping fetal membranes after term delivery (8-10). Furthermore, early *in vitro* experiments utilizing cultures of decidual cells isolated from term pregnancy decidual tissue, showed that addition of PRL to the culture medium further increased the estrone sulfate sulfatase activity (11). The presence of PRL receptors in human decidual cells has been recently reported by Maskant and Bryant-Greenwood (12).

Of particular relevance to the studies described here was the observation that addition of PRL to cultures of stromal cells isolated from human proliferative endometrium also resulted in increases in estrone sulfate sulfatase activity concurrent with morphologic change to the decidual phenotype and

appearance of other products characteristic of decidual cells (2,13). These experimental results suggested that PRL may be involved in the process of endometrial stroma decidualization by acting as an autocrine or paracrine regulator. The effects of PRL on the decidualization of human endometrial stromal cells was investigated at the beginning of this dissertation project and our experimental results indicated that PRL is capable of promoting the decidualization of endometrial stromal cells *in vitro* by observing the appearance of decidual morphologic change of stromal cells and detecting the productions of decidual products such as laminin, desmin, HSP-27, IGFBP-1 and fibronectin (2).

The biochemical properties of PRL, produced by decidualized stromal cells or decidual cells, and the pituitary are identical but the factors that regulate their synthesis and secretion are different in these two tissues. In cells of the human anterior pituitary, the expression of PRL gene is affected by cAMP (14). The intact CRE (cAMP response element, TGACGTCA) that specifically binds with CREB (CRE binding protein), the effector mediating cAMP-induced transcriptional activity in other systems (15,16), is not present in the promoter of the PRL gene. However, binding sites for Pit-1

(pituitary-specific factor-1), which mediates the induction of PRL expression under cAMP stimulation in human pituitary have been reported (14). One of the Pit-1 binding sites in the promoter of PRL gene contains the TGACG motif corresponding to the first 5 nucleotides of CRE. Disruption of the DNA elements in the PRL promoter similar to CRE in plasmid constructs transfected into GH3 human pituitary tumor cells results in a loss of PRL promoter activity stimulated by cAMP (14).

The TGACG motif is also found in 5'-untranslated region of the human decidual PRL gene (17,18). The involvement of cAMP in the decidual cell reaction in the mouse or rat uterus were reported (19-22). These experimental results postulated that cAMP may be also participated in the regulation of PRL gene expression and of decidualization in human endometrial stromal cells. In order to investigate the mechanisms regulating the differentiation events in the human endometrium, stromal cells isolated from human proliferative endometrium were cultured with cAMP derivatives (dibutyryl-cAMP and 8-Bromo-cAMP) or forskolin (stimulator of adenylate cyclase). Inductive effects of cAMP on expression of PRL gene in human endometrial stromal cells were demonstrated during this dissertation

project. In addition to inducing PRL production, participation of cAMP in the decidualization process was further provided by the expression of other decidual products such as HSP-27, laminin, desmin, and IGFBP-1, as well as the appearance of decidual morphologic change, in cAMP treated human endometrial stromal cells.

The belief that P was solely responsible for decidualization of human endometrial stromal cells was challenged by a series of studies on *in vitro* decidualization of stromal cells isolated from human endometrium. These studies showed only moderate effects of medroxyprogesterone acetate (MPA) on PRL production, even in the presence of estradiol (E_2), while much larger effects on PRL output were elicited in the same system by using a mixture of porcine relaxin (p-RLX) and MPA, with or without E_2 (23-25). Similarly to these observations on PRL production, the activity of steroid aromatase, which is elevated during *in vivo* decidualization of human endometrial stroma, was found to be much higher in the stromal cells cultured with MPA plus forskolin than in those exposed to MPA alone (26,27). The interaction of ovarian steroids on the cAMP induced-PRL expression during decidualization of human endometrial stromal cells was investigated by incubating

the stromal cells with dibutyryl-cAMP (db-cAMP), 8-Bromo-cAMP (8-Br-cAMP) or forskolin in the presence or absence of MPA, E_2 or MPA+E_2. MPA treatment synergistically enhanced the effects of db-cAMP, 8-Br-cAMP and forskolin on PRL production, as measured by Western blotting, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA) procedures. The presence of MPA also resulted in higher levels of PRL mRNA relative to cAMP alone, after normalization to cyclophilin mRNA levels. Results from transcription experiments suggested that incubation with MPA also increased the effect of cAMP on transcription of the PRL gene. Conversely, E_2 had no significant effect on cAMP induction of PRL gene expression, either alone or in combination with MPA.

Adenylate cyclase was found in plasma membrane of human endometrial stromal cells (28), but the physiological factors that can elevate cAMP levels in these cells are still unclear. Although Tseng *et al* have shown that porcine RLX acted synergistically with progesterone to induce PRL production *in vitro* (24), these authors also showed that, in contrast with its effect on endometrial epithelial cells, p-RLX has no effect by itself on cAMP levels in human endometrial

stromal cells (86). Gonadotropins (follicle-stimulating hormone, luteinizing hormone and human chorionic gonadotropin) that play fundamental important roles in the regulation of human reproductive processes were reported to increase intracellular cAMP levels in different reproduction-related cell types including ovarian granulosa cells (29,30), Sertoli and Leydig cells of testis (31,32), and luteal cells of corpus luteum (33,34). As part of this dissertation project, the effects of gonadotropins on cAMP levels and decidualization of cultured human endometrial stromal cells were tested. An elevation of cAMP levels was observed in human endometrial stromal cells treated with FSH (Metrodin), FSH+LH (Pergonal) or hCG. Furthermore, the gonadotropin treatments also induced PRL production as well as decidual morphologic change in the stromal cells. As far as we know, such direct effects of gonadotropins on human endometrium or endometrial cells have not been previously reported. These results suggest that gonadotropins may also play a important regulatory role in the decidualization of human endometrial stroma through a cAMP mediated signal transduction pathway.

II. BACKGROUND

A. Decidualization of the human endometrium

Human endometrium, the tissue lining the uterine cavity, is extraordinary in its capability to undergo monthly cyclic change which affect its morphologic and biochemical characteristics. This endometrial cyclicity during a woman's reproductive years is regulated by several hormones, especially estradiol and progesterone, in order to prepare, by day 4-5 after ovulation, an intrauterine environment suitable for implantation of the embryo during fertile cycles. Once pregnancy is established, the decidua, as the gestational endometrium is called, acquires new characteristics and functions.

Several hypotheses have been proposed regarding the biologic function of decidualization. Stromal differentiation is considered to be important in the preparation of the uterus for implantation, the nourishment of the developing embryo, and the maintenance of pregnancy (35-37); in the protection of maternal tissues from excessive invasion by the trophoblasts (38-40); in providing a barrier between embryonic and maternal circulations (39) and a

structural support for the embryo (41); in the protection of embryo from immunologic rejection by the mother (37,42,43); in the promotion of endometrial hemostasis during the periimplantation period(44); in the expression and secretion of decidual proteins, some of them could act as autocrine, paracrine, and endocrine messengers for the communication between mother, placenta, and fetus (35,36,45).

Formation of human decidua in human is a rapid histogenetic process. In the absence of an implanting ovum, edema of stromal cells occurs and is followed by decidualization, first appearing in the vicinity of spiral arterioles (46). If pregnancy ensues, the decidualization persists for the duration of pregnancy; otherwise it is sloughed during menstruation. Drastic morphologic changes were observed during differentiation of human endometrial stromal cells to decidual cells (41,46-49). For instance, the cells become polyploid and occasionally binucleated, with accumulation of glycogen and lipid droplets in the cytoplasm; the number of polyribosomes and rough endoplasmic reticulum increases and parallel channels of rough-surfaced endoplasmic reticulum and small vesicles of endoplasmic reticulum are found; slender mitochondria are observed and

lysosome-like bodies appear in projections from decidual surface; fibrillar material forms an extracellular coat around cells; between cells, tight junctions (maculae, zonae, fasciae occludentes) but not true desmosomes are observed (47). These morphologic changes are accompanied by biochemical changes in metabolism of carbohydrate, lipids and proteins and in the expression of decidualization-related proteins (1,50,51).

The drastic changes in the endometrial stromal cells, described as decidualization of the endometrium, are currently considered to be triggered by progesterone secreted by ovarian granulosa cells during the ovarian luteal phase of the menstrual cycle. It is possible, however, that relaxin also secreted by the corpus luteum and reaching detectable levels in plasma even in the absence of conception (52), participates in the physiologic decidualization process. Furthermore, gonadotropins that play an important role in the regulation of reproductive function may also affect the decidualization process. FSH and LH secreted from cells of the anterior pituitary reach a peak in plasma just before ovulation (53,54) and hCG was detected in 6 to 8-cell human embryos by using in situ hybridization techniques (55). "Advanced" endometrial stromal cells

(stromal decidualization appearing earlier than during normal menstrual cycle as shown histologically in biopsy samples) was noted in women receiving exogenous gonadotropins to stimulate ovulation (56). In the *in vivo* studies, the expression of decidualization-related protein, such as PRL, IGFBP-1 and HSP-27, were immunocytochemically detected and the decidual morphologic change observed in endometrial stromal cells similar to those characteristic of the endometrium after day 23 of the menstrual cycle whereas coexisting epithelial cells showed subnuclear glycogen accumulation characteristic of endometrium at day 17 of the normalized menstrual cycle. In addition to these possible regulators, other factors or hormones could be also involved in the complex differentiation processes of human endometrial stromal cells. Endometrial stromal cells are fibroblast-like during the ovarian follicular phase, when E_2 but not P is secreted, and show only few but important differentiated characteristics, e.g. expression of E_2 and P receptors, and responsiveness to these hormones. Decidualization involves a multiplicity of phenotypic alterations, including changes in cell morphology and nuclear size, the formation of a pericellular matrix, the loss of proliferative capability, and

the synthesis of a large number of new products, as can be detected by comparing stromal cells from proliferative endometrium and decidual cells isolated from the decidua of early or term pregnancies. Table 1 lists some enzymatic activities and intracellular or secretory products of decidual cells which are not detectable in the stromal cells during the follicular phase or are present at much lower levels than those found in decidual cells.

Table 1 Products induced or enhanced during decidualization of human endometrial stromal cells.

TYPE OF PRODUCT	*PRODUCT*	*REFERENCE*
Cytokines	CSF-1	57
	TNF-α	58
	IL-1ß	59
	IL-6	60,61
ECM Components	Laminin	62,63
	Fibronectin	62,63,64
	Collagen IV	63
	Heparan Sulfate Proteoglycan	63
Hypophyseal Hormones	Prolactin	5,6,62,65
	Oxytocin	66
Neuropeptides	Somatostatin	67
	Inhibin, Activin	68
	CRF	69
Ovarian Hormones	Relaxin	52,70
Enzymes	Diamine Oxidase	71
	Aromatase	26,27,72
	Steroid Sulfatases	9,13,73
	Proteases (uPA,tPA)	74,75
	Protease Inhibitors	74
	25-OH-D_3-1α-Hydroxylase	76
	Prorenin, Renin	77,78
Miscellaneous	Platelet-activating Factor (PAF)	79,80
	Desmin	81,82
	IGF Binding Protein-1 (IGFBP-1, pp12)	83,84,85
	24 K Protein (HSP-27)	86
	Tissue Factor	44

Early *in vitro* experiments, carried out by Maslar and Riddick with human endometrial tissue (87), showed that addition of progesterone to the culture medium induced the synthesis of PRL, a pituitary hormone which was found to be also secreted by human decidual cells. Human decidual PRL, whose mRNA coding sequence is identical to that of the pituitary hormone (88), appears in different immunoreactive forms, some of which are secreted in a glycosylated form (89). In addition to PRL, a variety of other products, characteristic of decidual cells, have been shown to be induced during *in vitro* decidualization of endometrial stromal cells. Those products include laminin (63), fibronectin (62), prorenin and renin (77,78), steroid aromatase (72), desmin (82), HSP-27 (86), IGFBP-1 (85) etc. Estrone sulfate sulfatase activity which was increased during *in vivo* decidualization (9) was elevated concurrently with the production of PRL when stromal cells isolated from human proliferative endometrium were treated with progestins (13).

B. Prolactin expression during decidualization

Prolactin secretion by human endometrial stromal cells was detected at day 23 of the menstrual cycle (87)

and, on that time, the earliest decidual morphologic change of endometrial stroma was observed in the periarteriolar stromal cells (90). It was previously reported that steroid sulfatase activity in decidual cells was increased by PRL treatment (11). The stimulatory effect of PRL on steroid sulfatase activity was also demonstrated during *in vitro* decidualization of stromal cells isolated from human proliferative endometrium (13). Specific binding of PRL has been detected in human endometrium (91) and expression of PRL receptor has been recently described in human decidua (12). Treatment of human endometrial cells with human PRL resulted in translocation of PKC (protein kinase C) from cytosol to plasma membrane (92), and the same effect was observed by treating the cells with human relaxin (92) which was involved in decidualization of human endometrial stromal cells (23-25,93). These results suggested that PRL might participate in the regulation of the decidualization of human endometrial stroma *in vitro*.

C. Effects of progesterone and relaxin on PRL production

Experiments reported by Huang *et al* (23), succeeded in demonstrating that production of PRL could be induced

in cultures of stromal cells isolated from human proliferative endometrium by adding to the insulin-containing medium a mixture of progesterone and relaxin, both produced by the ovarian granulosa cells during the luteal phase of ovarian cycle (94). In that type of experiments, P was usually replaced by MPA, a metabolically more stable progestin, and p-RLX was used rather than h-RLX, not available at that time. It was reported that neither MPA nor p-RLX alone sustain PRL production in the stromal cells isolated from proliferative human endometrium, even though PRL levels indicative of decidualization could be obtained with MPA + p-RLX (24). Prolactin levels obtained with either MPA or p-RLX were about 5% of the levels attained with the mixture) can be elicited (24). In contrast, when the same experiments were carried out with stromal cells isolated from secretory endometrium (24), a much larger effect of p-RLX on PRL production was noted (peak at culture day 15). The response to MPA alone was also larger with secretory endometrium. These results can be interpreted to indicate that the cells from secretory endometrium have already been primed with P, RLX, or other factors expressed after ovulation to make them more responsive to either MPA or RLX (24). If the

exposure to MPA of stromal cells from proliferative or early secretory endometrium is discontinued after 10 days in culture, the production of PRL increases considerably, as if the progestin, through mechanisms unknown, had an inhibitory effect after the initial stimulation (24). The inhibitory effect was further confirmed by adding the antiprogestin RU486 to the culture medium at day 10 instead of removing MPA (95).

In contrast with results showing that estradiol decreased P-induced PRL production in cultures of human proliferative endometrium (6), Irwin *et al* (62) have shown that a mixture of P and E_2 can induce PRL, laminin and fibronectin production in the cultures of human endometrial stromal cells *in vitro*. These effects were obtained in serum-free medium to which insulin, EGF (epidermal growth factor), transferrin, BSA (bovine serum albumin), linoleic acid, ascorbic acid, $FeSO_4$, $ZnSO_4$, $CuSO_4$, Na_2SeO_3 and trace elements were added.

Relaxin, produced together with P by the corpus luteum, is also synthesized by decidual cells (94,96) and by placenta trophoblast (97). This observation suggests a possible endocrine, paracrine and autocrine involvement of RLX in decidualization and PRL production of human endometrial stromal cells (98). Progesterone

and RLX synergistically inhibit human myometrial contractility, considered to be mediated by a cAMP pathway (99,100). It has been proposed that these hormones act physiologically in concert in the maintainance of early human pregnancy (101). Besides the synergistic effects of P and RLX on PRL expression in human endometrial stromal cells (24), similar effects on steroid aromatase (27), IGFBP-1 (83,85), prorenin and renin (77,78) have also been observed. Progesterone alone is not capable of substaining a significant production of PRL and may not be the single physiologic agent responsible for decidualization of the endometrial stroma. In contrast, the combination of a progestin and RLX is very effective in inducing a large and sustained output of PRL.

Since RLX is a member of the insulin/IGF-1 family (101), it is not suprising that insulin, and even more strongly IGF-1, had also been reported to increase PRL production by decidual cells (102,103). Similar effects were also observed in progestin-primed stromal cells (93). The presence of IGF-1 receptors in endometrial stromal cells has been documented (104). Although it is commonly believed that the *in vitro* changes induced by progestins plus RLX correspond to those occurring during

in vivo decidualization, synthesis of some decidual products during gestation may be also influenced by endocrine, paracrine or autocrine factors produced by endometrial epithelial, myometrial, bone marrow-derived cells residing in the endometrium and trophoblast which are not included in the *in vitro* systems.

D. Relaxin and cyclic AMP

It has been reported that p-RLX could increase cAMP levels in rat uterus (105), rhesus monkey uterine cells (106), and human endometrial epithelial cells (107). Recombinant human RLX treatment also elevated cAMP levels in human endometrial stromal cells in the presence of forskolin and 3-isobutyl-1-methyl-xanthine (108). The characteristic increases in cAMP levels stimulated by rh-RLX in cultured human endometrial cells had been applied to establish a RLX bioassay method (109).

It has been shown that PRL secretion can be increased in human decidual cells treated with 8-bromo-cAMP (110). Dibutyryl cAMP, or agents that can increase intracellular levels of cAMP, such as cholera toxin, forskolin or inhibitors of cyclic adenosine monophosphate phosphodiesterase, when combined with P or

MPA, can increase steroid aromatase activity in human endometrium (26), to levels as high as those obtained with MPA plus RLX (27). When stroma or glands isolated from proliferative endometrium were cultured in medium containing P or P+E_2, the increment of aromatase activity was observed in stroma but not in glands (72). Poisner *et al* reported that cAMP could cause prorenin release from human decidual cells, a finding suggesting its role as a 2nd messenger (108). Cyclic AMP stimulated IGFBP-1 secretion in the cultures of human decidual cells (111). These findings suggest that RLX may enhance the effect of P during the *in vivo* decidualization process by increasing intracellular cAMP levels in the stromal cells.

E. Progesterone and cyclic AMP

The interaction of progesterone and cAMP has been observed in many systems by different investigators (112-114). The effect of progesterone on cAMP generation in response to activation of ß-adrenergic receptor in rabbit lung tissue was reported by Moaawad and coworkers (115). Steroid aromatase activity was much higher in human endometrium treated with progesterone and forskolin than with either P or forskolin (26).

Adenylate cyclase activity was detected in plasma membrane of human endometrial stromal cells and forskolin or prostaglandin E (PGE_2) treatment increased cAMP levels in the stromal cells (116). Furthermore, the forskolin or PGE promoted cAMP generation was enhanced by progesterone (116). Since progesterone treatment inhibited cyclic adenosine monophosphate phosphodiesterase activity in nonpregnant and pregnant human myometrium (117), progesterone might enhance the stimulatory effect of forskolin on cAMP production in human endometrial stromal cells by inhibiting the phosphodiesterase activity or by increasing the levels of adenylate cyclase or its regulatory components (117,118).

Progesterone receptor (PR) levels in uterine cells are regulated by cAMP. Aronica and Katzenellenbogen reported that elevation of intracellular cAMP concentrations by treatment of fibroblast-like stromal cells of rat uterus with cholera toxin plus 3-isobutyl-1-methyl-xanthine (IBMX) or by addition of 8-Br-cAMP to the culture medium, results in an increase in PR levels (119). The stimulatory effects of cAMP on the PR concentration in guinea pig uterine cells was also demonstrated (120). These results suggest that cAMP

might affect the responsiveness of uterine cells to progestins, at least in part, *via* regulation of the intracellular PR content.

Local formation of progesterone during incubations of human decidual tissue or decidual cells with pregnenolone has been demonstrated (121), although the physiological plasma level of this precursor may be too low to contribute significantly to the concentration of P in these cells. Knight and his colleagues found that the synthesis of progesterone from pregnenolone by endometrial tissue of pregnant sows was enhanced by supplementation with cAMP (122).

F. Gonadotropins

Gonadotropins, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and human chorionic gonadotropin (hCG), have many chemical and structural similarities and are composed of two glycosylated peptide subunits (α and ß) that are tightly bound through noncovalent association. The α subunit of FSH, LH and hCG possess essentially the same amino acid sequence. In contrast, their ß subunits contain distinctive amino acid sequences. The different biologic function of each hormone is dictated by the specific ß

subunit associated with a common α subunit (123).

Follicle-stimulating hormone, LH and hCG play an important role in the regulation of gonadal endocrine and reproductive function. FSH stimulates granulosa cell proliferation and steroidal aromatase activity, promoting follicular growth and estradiol biosynthesis; LH induces follicular differentiation and increases production of progesterone in the mature granulosa and granulosa-lutein cells; hCG rescues the corpus luteum function resulting in enhanced production of progesterone and relaxin, which are essential for sustaining early pregnancy. In addition to sustaining corpus luteum function, hCG may have a paracrine and endocrine function in maternal-embryo interaction and communication at the time of implantation since it had been detected in the preimplantation embryo (124,125) and hLH/hCG receptor was detected in human endometrial stromal cells (126,127). Furthermore, incubation of preimplantation embryos with anti-hCG antibody prevents embryonic attachment to the endometrium (128). Human chorionic gonadotropin LH/receptor levels in stromal cells of human endometrium increase from the proliferative to the secretory phase (127), a finding suggesting that hCG and hLH, which bind to the same

receptor, may regulate human endometrial functions.

These actions of gonadotropins on granulosa cells mentioned above, are believed to be predominantly, if not solely, mediated through generation of intracellular cAMP (33,129,130). Although gonadotropins exert many biological functions related to the regulation and control of human reproductive process, a direct effect on decidualization of the human endometrium stroma and intracellular cAMP levels of human endometrial stromal cells has not been previously reported.

III. MATERIALS AND METHODS

A. Tissue collection

Endometrial tissue specimens from cycling women (30-50 years old) undergoing diagnostic dilatation and curettage or hysterectomy for a variety of medical indications, such as fibroid uterus, uterine prolapse or adenomyosis, but not endometrial cancer, were brought on ice to the laboratory in sterile Ham's-F10 medium (GIBCO, Grand Island, NY), immediately after excision. A portion of each specimen was fixed in formalin for histological dating according to the method of Noyes *et al* (131). After thoroughly cleaning and trimming the tissue fragments to remove blood clots and mucus, they were minced to less than 1 mm^3 under a laminar flow hood.

B. Separation and culture of stromal cells

As previously described (2), the cleaned, trimmed and minced endometrial tissue was digested with 0.25% (w/v) collagenase (Worthington Biochem. Co, Freehold, NJ) in Ham's-F10 medium containing 10% charcoal-treated fetal bovine serum (ct-FBS) at 37°C for about 60

minutes. The majority of cells passing through the approximate 25 μm openings of a stainless steel sieve (Newark Wire Cloth Co., Newark, NJ) were red blood cells and fibroblast-like stromal cells with small population of lymphoid cells, polymorphonuclear leukocytes, mast cells, and granulocytes. The cells passing through the sieve were pelleted by centrifugation at 500 x g for 5 min at 4°C, washed twice, and then resuspended in RPMI 1640 medium with 10% ct-FBS containing 0.2 U/ml insulin (Squibb & Sons Inc., Princeton, NJ) and 1% of an antibiotic-antimycotic mixture (GIBCO) to give a final concentration of 100 U/ml penicillin-G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B. The cells were plated in Petri dishes and incubated in a 95% air-5% CO_2 atmosphere at 37°C for about 30 minutes to allow the attachment of stromal cells to the plastic dish. After washing away unattached cells, morphologically near homogeneous stromal cell cultures were obtained, as frequently verified in this and other laboratories following this procedure. The procedure of separating stromal cells is illustrated in Fig. 1 and the morphology of the stromal cells in confluent culture is shown in Fig. 2. When the cells reached confluency in RPMI 1640 medium containing 10% ct-FBS and 0.2 U/ml

insulin, they were further incubated without or with added hormones or other agents. The collected media were kept at -80°C until thawed to perform assays.

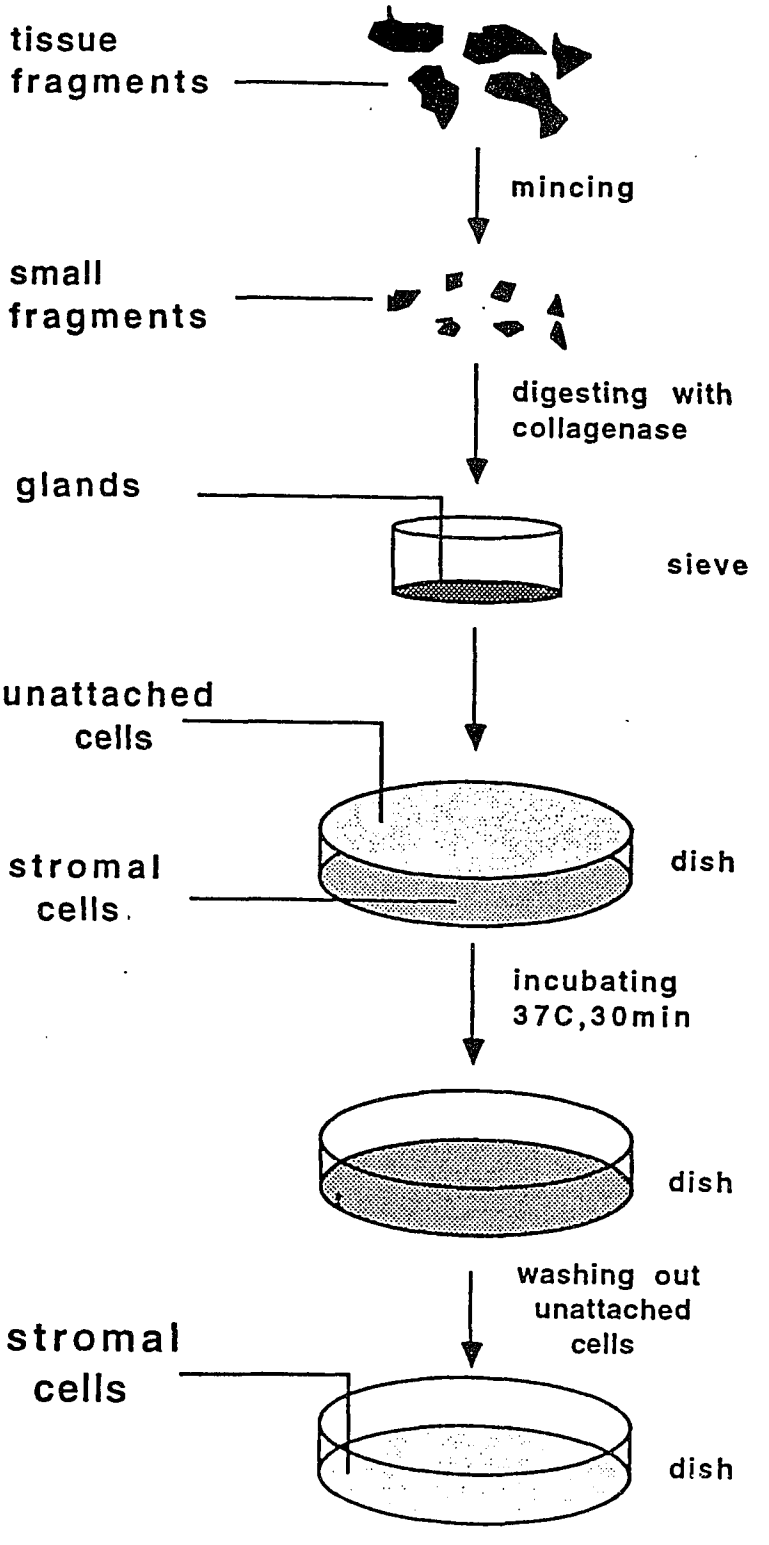


Fig. 1 Procedure for separating human endometrial stromal cells.

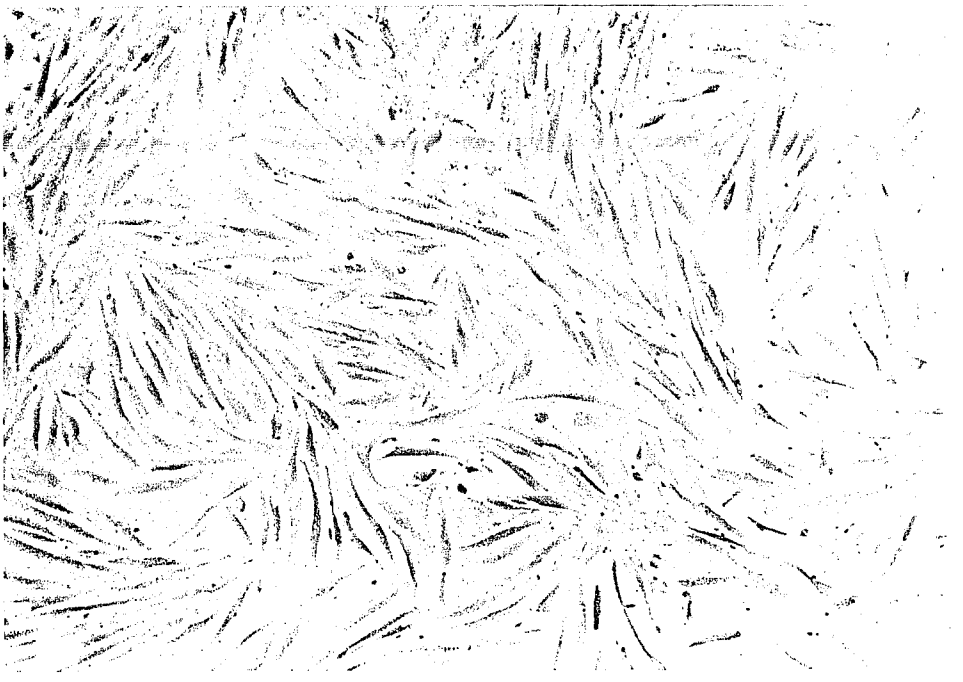


Fig. 2 Appearance of human endometrial stromal cells observed under phase contrast microscope.

C. Morphologic changes

Morphologic changes of stromal cells cultured in Petri dishes were observed under phase contrast microscopy and recorded by photography. Cells were also cultured on Lab-Tek tissue culture chambers under similar conditions, for 10 to 15 days. After aspiration of the media, cells were washed twice with Hanks' balanced salt solution (HBSS), fixed with pre-cooled acetone at -20°C for 5 minutes and then stained with hematoxylin and eosin.

D. Immunocytochemistry

Stromal cells, cultured on Lab-Tek tissue culture chambers or coverslips (Nunc, Inc., Naperville, IL) under different experimental conditions, were fixed in acetone at -20°C for 5 minutes, rehydrated and then immunocytochemically stained with rabbit anti-hPRL (polyclonal, from Dr S. Raiti, National Hormone and Pituitary Program, NIADDK), anti-IGFBP-1 (monoclonal, purchased from Medix Biochemica, Kauniainen, Finland), anti-HSP-27 (monoclonal, purchased from BioGenex Laboratories, San Ramon, CA), anti-desmin (monoclonal, purchased from Chemicon International Inc., El Segundo, CA) or anti-laminin (polyclonal, purchased from Sigma

Chem. Co., St. Louis, Missouri) antibodies by using the avidin-biotin immunoperoxidase method with the Vectastain ABC kit (Vector Laboratories Inc, Burlingame, CA). Briefly, the cells were pretreated by 0.3% H_2O_2 in methanol for 30 minutes to remove endogenous peroxidase activity and incubated with normal goat serum (horse serum when monoclonal antibodies were used) in PBS (phosphate buffered saline) for 45 minutes to block non-specific binding sites for IgG. After incubation with primary antibodies, the cells were sequentially exposed to the following solutions: biotinylated goat anti-rabbit (or horse anti-mouse when monoclonal antibodies were used) IgG for 60 minutes, and avidin-biotinylated horseradish peroxidase in PBS for 1 h. Between each step, cells were washed in PBS 4 times allowing 5 minutes per wash with gentle agitation. Peroxidase activity was visualized by exposing the cells for 3-7 minutes to 0.5 mg/ml of diaminobenzidine tetrachloride in PBS containing 0.01% H_2O_2. After rinsing in water, the Lab-Tek slides or coverslips were mounted with Gel/Mount (Biomeda Corp., Foster City, CA).

E. Prolactin assay

In order to evaluate the production of PRL by

stromal cells cultured in plastic dishes in the presence or absence of hormones and other agents, media were collected at different culture periods and centrifuged at 3000 x g to separate floating cells and debris. The supernatants were collected and kept at -80°C for further assay. After thawing, levels of PRL in the medium were measured by double antibody-RIA procedures using human PRL standards in order to determine rates of production of the hormones. In some samples, PRL concentrations in the medium were also measured by solid phase sandwich-type enzyme immunoassay with a well-characterized murine monoclonal against hPRL antibody. A goat polyclonal anti-hPRL antibody conjugated with horseradish peroxidase that converts colorless 3,3′,5,5′-tetramethylbenzidine to a colored product was used to detect the antibody-antigen complex. Optical density (OD) of the colored product was measured at 450 nm and PRL concentrations were calculated by using a standard curve. RPMI/FBS medium did not show measurable PRL levels.

F. Western blotting

Confluent stromal cells were cultured on plastic dishes in RPMI/FBS with or without addition of test

hormones and other agents in order to evaluate by Western blotting their effects on PRL output into the medium. A Bio-Rad mini-protein II electrophoresis apparatus was used to perform SDS-PAGE under reducing conditions. After electrophoresis at constant voltage (120 V) for about 1 h, proteins in the gel were transferred to a nitrocellulose membrane (Bio-Rad, Richmond, CA) at 100 V for 90 minutes under ice-cooling in a 20% methanol (v/v), 150 mM glycine and 20 mM Tris transfer buffer (pH 8.3), using a Bio-Rad mini transblot electrophoretic transfer apparatus. Bio-Rad prestained SDS-PAGE molecular standards were used for the estimation of transfer efficiency and of molecular size. Human PRL standards were run in parallel in order to compare effects of the various agents tested on PRL production. The nitrocellulose membrane was blocked by treatment for 30 minutes at room temperature with 10 mM Tris, 0.15 M of NaCl solution (pH 7.4), containing 5% nonfat dry milk (Carnation, Los Angeles, CA) and 0.2% Nonidet P-40. The membrane was then incubated with rabbit polyclonal anti-hPRL antibody at 4°C for 16 h with constant agitation. After washing out unbound primary antibody, the membrane was incubated with [^{125}I]-Protein A (250,000 cpm/ml) for 30 minutes at 25°C

with constant shaking. The bound antibody was then visualized by autoradiography. Between steps, the membrane was subjected to 2 washes with TTBS (100 mM Tris, 0.9% NaCl w/v and 0.1% v/v of Tween 20, pH 7.5) followed by 2 washes with TBS (100 mM Tris and 0.9% NaCl w/v, pH 7.5), 15 minutes per wash, at room temperature, with gentle agitation.

G. [^{35}S]-methionine incorporation and immunoprecipitation

In order to evaluate production of decidual proteins such as PRL, laminin, HSP-27, IGFBP-1, and desmin in cells and in culture media, endometrial stromal cells which have been cultured to confluency in RPMI/FBS medium with or without test hormones or other agents for a period of time were washed twice with PBS and then cultured in methionine-free and serum-free media for 15 minutes. The medium was then replaced by RPMI/FBS medium with or without test hormones or other agents containing 25 μCi/ml of [^{35}S]-methionine and the cells were cultured for 3 or 6 hours. At various intervals, medium from several dishes was collected and centrifuged at 3000 x g for 10 minutes to remove debris. At end of [^{35}S]-methionine incorporation, cells were washed twice with cold HBSS and harvested by scraping

with a rubber policemen. The cell suspension was centrifuged and the pellet was lysed with sample buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 3 mM 2-mercaptoethanol).

Aliquots with equal amounts of TCA-precipitable radioactivity (cpm) were used for immunoprecipitation. Ten volumes of buffer A (190 mM NaCl, 50 mM Tris-HCl, pH 7.4, 6 mM EDTA, 2.5% Triton X-100, 5 mM methionine, 100 μM phenyl methylsulfonyl fluoride and 0.1% BSA) was added to the cell lysates. One and one-half milliliter of media was mixed with 0.3 ml of buffer B (1.14 M NaCl, 0.3 M Tris-HCl, 36 mM EDTA, 15% triton X-100, 30 mM methionine, 0.6 mM phenylmethyl sulfonyl fluoride and 0.6% BSA, pH 7.4). Antibody to the product under testing was added to the samples of cell lysates or medium solutions in buffer A and the mixture was incubated for 12-18 hours at 4°C with constant mixing. Protein A-sepharose (PAS) was then added when rabbit polyclonal antibody was used (or goat anti-mouse IgG conjugated agarose when mouse monoclonal antibody was used) and incubated at 4°C for 3 hours to precipitate Ab-Ag complex. Immune complexes (Ag-Ab-Protein A-sepharose or Ag-Ab-goat IgG-agarose) were separated by centrifuging for 5 seconds in a Brinkmann microcentrifuge, washed 3

times with buffer C (150 mM NaCl, 10 mM Tris-HCl, pH 8.3, 5 mM EDTA, 100 uM PMSF, 0.1% Triton X-100 and 0.1% BSA), and twice with PBS. The PAS or goat IgG-agarose pellets were mixed with 30 µl of electrophoresis sample buffer (20 ug/ml bromophenol blue, 5% glycerol, 0.4% sodium dodecyl sulfate, 3 mM 2-mercaptoethanol and 12 mM Tris-HCl, pH 6.8), boiled for 5 minutes and then incubated with iodoacetamide (5 µl of a 1 M solution per tube) at room temperature in the dark for 20 minutes. After centrifugation, the supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gel under reducing conditions, running molecular weight standards in parallel. Immunoprecipitated [^{35}S]-methionine-labeled decidual proteins were visualized by autoradiography.

H. Fibronectin assay

Fibronectin in the collecting medium was measured by using a fibronectin ELISA kit according to instructions provided by the manufactures (Adeza Biomedical Corporation, Sunnyvale, CA). Briefly, one hundred of each calibrator standard or sample were pipeted in duplicate into wells of a 96 well microtiter plate that was pre-coated with affinity-purified goat

polyclonal against human fibronectin antibody. The plate was covered and incubated for 1 hour at room temperature. After discarding the solutions in the wells and washing the plate 3 times with buffer, 100 µl of alkaline phosphatase-conjugated goat anti-hFib antibody was added into each well and the plate was incubated for 30 minutes at room temperature. Following the interaction of the coated antibody with the enzyme labeled antibody-antigen conjugate, the microtiter plate was rinsed with wash buffer 4 times in order to remove unbound enzyme-labeled antibody and then incubated with phenolphthalein monophosphate (substrate of alkaline phosphatase) for 30 minutes at room temperature. The amount of substrate hydrolysed was determined spectrophotometrically in a microtiter plate reader at 550nm and the fibronectin concentration was calculated by using a calibration curve.

I. Extraction of total RNA

Human endometrial stromal cells, treated or untreated with hormones or other agents, were washed with cold HBSS before extraction of total RNA. Total RNA was isolated by the acid guanidinum thiocyanate-phenol-chloroform extraction method using

RNAzol™ B (Biotecx Laboratories, Inc., Houston, TX). Briefly, stromal cells were lysed by the addition of RNAzol™ B (2 ml per 10 cm Petri dish) and the lysates were passed through a pipette for several times in order to solubilize RNA. The lysates were transferred into 2 Eppendorf vials (1ml/vial) and 0.1 ml of chloroform (1/10 volume of lysate) was added to each vial, shaken vigorously for about half minute, incubated on ice for 5 minutes, and then centifuged at 12,000xg for 15 minutes at 4°C. The upper aqueous phase (containing RNA) was carefully transferred to new vials, mixed with an equal volume of isopropanol, shaken vigorously, kept in the vials at -20°C for 60-90 minutes. The vials were then centrifuged at 12,000 x g for 15 minutes at 4°C to precipitate RNA. The RNA pellet was washed with cold 75% ethanol by vortexing and subsequently centrifuged for 10 minutes at 7,500xg at 4°C. Finally, the precipitated total RNA was redissolved in 1 mM EDTA solution (pH 7.0) and quantified by UV spectrophotometry.

J. Analysis of PRL mRNA

Prolactin mRNA was analyzed by the conventional Northern blotting method as described by Sambrook *et al* (132). Isolated total RNAs were denatured in sample

buffer containing 6 mM sodium acetate, 1.2 mM EDTA, 24 mM HEPES (N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]), 2.2 M of formaldehyde, and 50% deionized formamide by incubation at 65°C for 15 minutes and then chilled on ice. Samples were loaded to a 1% agarose-formaldehyde gel, electrophoresed at constant 80 V, tranferred to a nylon membrane by capillary blotting, and then fixed under UV light. Plasmids containing complementary DNA clones of pituitary-hPRL (133) obtained from ATCC (American Type Culture Collection, Rockville, MD), mouse cyclophilin (134) and human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) were isolated from bacteria using the CIRCLEPREP procedure (Bio 101, Inc., La Jolla, CA). After specific cut of the purified plasmids with corresponding restriction enzymes, cDNAs were isolated by the following procedure: electrophoreses, cut single band containing insert and then recovery the insert by using ELUTIP-D-COLUMNS (Schleicher & Schuell, Keene, NH) as illustrated in Fig. 3. [^{32}P]-labeled cDNAs to PRL, cyclophilin and hGAPDH were generated by the random prime procedure (132), according to instructions provided by the manufacturer (Boehringer-Mannheim, Indianapolis, IN). Hybridizations were carried out in the conditions described by Dr.

Guller *et al* (135). In brief, membranes were hybridized with [^{32}P]-labeled cDNAs for 16-18 h at 65°C in a solution containing 7% SDS, 1 mM EDTA, 0.5 M NaH_2PO_4 buffer, pH 7.2 (NaPi). Blots were then washed (65°C, 30 minutes per wash) twice with a solution containing 5% SDS/40 mM NaPi and twice with a solution of 1% SDS in NaPi. The hybridization signal on the membrane was detected by using X-ray film, exposed for 2-4 days at -80°C. In order to evaluate RNA integrity and to normalize for the amount of loaded RNA, the same membrane was rehybridized with cyclophilin or hGAPDH cDNA probe after stripping out the PRL probe.

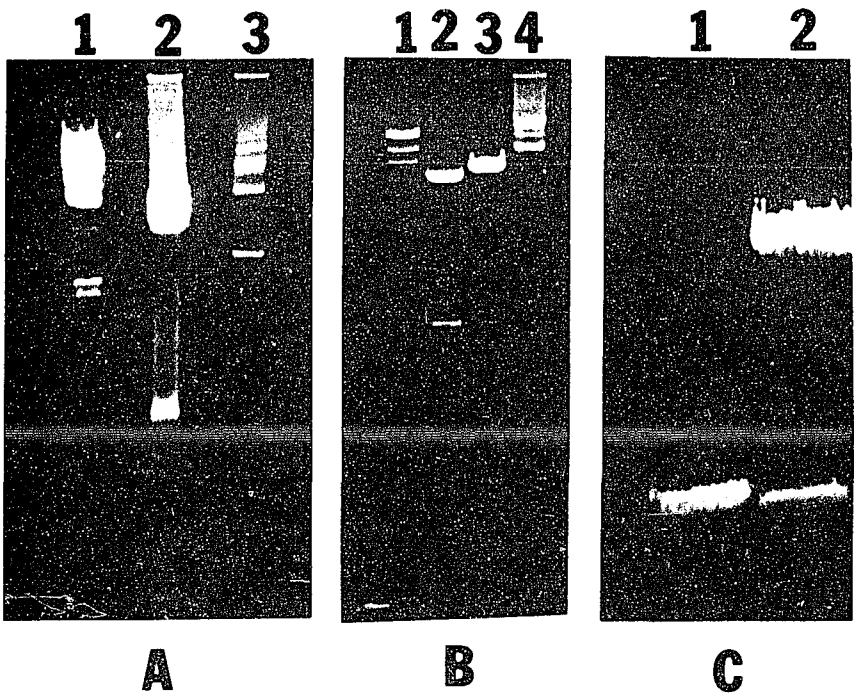


Fig. 3 Electrophoreses of hPRL and hGAPDH cDNA inserts on 1% agarose gels with ethidium bromide. Lane 1 (panels A and B): DNA M_r standards. Panel A shows DNA fragments of plasmid containing hPRL cDNA insert after incubated with (lane 2) or without (lane 3) Pst I. Panel B represents DNA fragments of plasmid containing hGAPDH cDNA insert after incubation with Pst I and Xba I (lane 2), Pst I (lane 3), and no enzyme (lane 4). Panel C shows the separation of hGAPDH cDNA: purified hGAPDH cDNA (lane 1) and DNA fragments of plasmid containing hGAPDH cDNA insert after digestion with Pst I and Xba I (lane 2).

K. Nuclear run-on assay

1. Isolation of nuclei

Confluent stromal cells were cultured under different conditions in 10 cm plastic Petri dishes for 7 days as mentioned before under III. B. At the end of the culture period, cells were washed with ice-cold HBSS and immediately harvested in 2 ml cold RNase-free buffer A per dish (buffer A: 24 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 10 mM Tris-HCl, pH 8.0) by gently scraping with a rubber policeman. The cell suspension was centrifuged at 1500 rpm for 5 minutes at 4°C and the cell pellet was gently tapped before resuspension to prevent clumping. Cells resuspended in 0.5 ml of buffer A and diluted with an equal volume of buffer B (buffer A containing 1% Nonidet P-40) were left on ice for 5-10 minutes in order to disrupt the cell membrane. Cell disruption was monitored by phase contrast microscopy to confirm the integrity of nuclei. Intact nuclei appear flat, dark and scruffy, while cells appear bright and refractile. Nuclei were separated from cytoplasm and cell debris by carefully layering the suspension over 0.4 ml of buffer C (buffer A containing 0.4 M sucrose) and then centrifuging at 1000xg for 10 minutes at 4°C. After

removing supernatant, 80 µl of buffer D (5 mM $MgCl_2$, 100 µM EDTA, 50% glycerol and 50 mM Tris-HCl, pH 8.3) was added and the nuclei were stored in liquid nitrogen.

2. Transcription in vitro

Isolated and frozen nuclei (about 100 µl) were thawed on ice and then immediately mixed with 51 µl of transcription reaction buffer and 49 µl of 24 µM [α-^{32}P]UTP (specific activity: 800 Ci/mmol, 10 mCi/ml). The transcription reaction buffer was freshly preparated from stock solutions as listed in Table 2. The transcription reaction mixture was incubated for 40 minutes at room temperature with constant shaking.

Table 2 Composition of transcription reaction buffer.

Stock Solution	µl/vial	Concentration*
1 M $MgCl_2$	0.5	2.5 mM
1 M $MnCl_2$	0.2	1 mM
1 M ammonium sulfate	8	40 mM
1 M DTT	0.25	1.25 mM
1 M Tris-HCl, pH 8.3	4	20 mM
Glycerol (enzyme-free)	32	16%
100 mM ATP	1.2	0.6 mM
100 mM CTP	0.5	0.25 mM
100 mM GTP	0.5	0.25 mM
200 mM phosphocreatine	1	1 mM
3.5 mg/ml creatine phosphokinase**	1	17.5 µg/ml
0.156 U/µl nucleoside 5′-diphosphate kinase	6.5	3.5 U/ml
66 mM EDTA	0.15	50 µM
40 U/µl RNasin	0.6	120 U/ml

*: Final concentrations in transcription reaction mixture (200 µl), including nuclei.

**: Prepared immediately before use.

3. Isolation of RNA

After incubation, the reaction mixture was treated with 40 µl of 1,000 U/ml RNase-free DNase I for 5 minutes at 30°C and then with 2 µl of 20 mg/ml proteinase K for 15 minutes at 37°C. Ten microliter of 10 mg/ml yeast tRNA were added, RNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and then centrifuged at 10,000xg to separate the aqueous phase from the heavier organic phase. The aqueous layer was carefully removed and transferred to an Eppendorf vial. RNA was precipitated from the aqueous phase by adding 3 volumes of absolute ethanol and 1/10 the final volume of 3 M sodium acetate (final concentration 0.3 M), letting it stand at -80°C for at least 30 minutes. The RNA pellet was dissolved in 300 µl HSB buffer (0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.4), 20 µl of 1,000 U/ml RNase-free DNase I were added and then the mixture was incubated for 5 minutes at 30°C. After addition of 200 µl SDS/Tris buffer (5% SDS, 0.125 M EDTA, 0.5 M Tris-HCl, pH 7.4) and 10 µl of 20 mg/ml proteinase K, the solution was incubated for 30 minutes at 42°C. RNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated from the aqueous phase with ethanol as mentioned before.

After washing with ice-cold 75% ethanol 3 times, the RNAs were ready for hybridization.

4. DNA slot blotting

DNA inserts or vectors serving as negative controls were denatured by heating in 0.4 M NaOH containing 10 mM EDTA solution at 100°C for 10 minutes and immediately chilled on ice. The denatured DNAs were immobilized onto a Zeta-Probe membrane by using a slot-blot apparatus (Bio-Rad, Richmond, CA), according to the manufacturer's instruction. Sheets of 3 MM blotting filter paper and 1 sheet of Zeta-Probe membrane were wetted with distilled water and then placed in the slot-blot apparatus. After tightened the screws of the apparatus under vacuum to prevent cross contamination, the slots were rinsed with distilled water (0.5 ml per slot). Water was sucked under gentle vacuum but the slots were not dried. Three hundred microliter of the denatured DNA were loaded into each slot and allowed to adsorb to the Zeta-Probe membrane by gravity or by using very low vacuum. All slots were washed with 0.4 M NaOH (500 µl/slot) and vacuum was then applied until all slots were thoroughly dry. After disassembling the apparatus, the membrane was rinsed briefly in twice with SSC (0.15 M sodium

chloride, 15 mM trisodium citrate) and dried under air. DNA was fixed on the membrane by cross-link under UV light and the membrane was stored between two sheets of filter paper wrapped with aluminum foil and placed in a desiccator at room temperature for later hybridization.

5. RNA-DNA hybridization

The Zeta-Probe membrane blotted with DNAs was incubated in prehybridization solution (50% formamide, 1.5X SSPE (0.18 M NaCl, 10 mM Na_2HPO_4, 1 mM EDTA), 1% SDS, 0.5% BLOTTO, 200 μg/ml yeast tRNA and 500 μg/ml denatured-herring sperm DNA) at 50°C for 4 hours. The isolated [^{32}P]-labeled RNAs were resuspended in hybridization solution (prehybridization solution without yeast tRNA and herring sperm DNA) and two 1 μl aliquots were taken for determination of TCA precipitable radioactivity by spotting them on filter paper, washing with ice-cold 10% TCA, 5% TCA and 100% ethanol in sequence and then placing the filters in a scintillation counter. [^{32}P]-labeled RNAs containing equal TCA precipitable radioactivity were transferred to new microfuge vials, yeast tRNA and herring sperm DNA were added to a final concentration 0.2 mg/ml and 0.5

mg/ml, respectively, denatured by incubation at 70°C for 5 minutes, followed by rapid cooling on ice. Hybridization was performed by replacing prehybridization solution with hybridization solution containing the denatured RNA probes, complementing with hybridization solution to a final volume of 400 µl, mixing the contents thoroughly and then incubating at 50°C for 48-72 hours with continuous shaking. After completion of the hybridization, the membranes were transferred to new vials, rinsed briefly with 2X SSC and then washed successively by constant agitation for 15 minutes at room tempereture in 0.1% SDS solutions containing 2X SSC, 0.5X SSC and 0.1X SSC. After drying the hybridized membranes under air, they were exposed to X-ray film for 5-7 days at -80°C. Rates of transcription were estimated by densitometric scanning of autoradiographs and radioactivity counting of the products eluted from the membranes by incubation with 40 mM NaOH for 4 hours.

L. Cyclic AMP determination

Media from cultures were collected in vials containing 3-isobutyl-1-methyl-xanthine (final concentration 1 mM), mixed and centrifuged at 3000xg for

10 minutes at 4°C. Supernatants were transferred to new vials and stored at -80°C for later measurement of extracellular cAMP levels. At the end of the culture period, cells in 6 cm Petri dishes were washed twice with cold HBSS, added with 1.5 ml of 0.1 N HCl and then incubated for 30 minutes at room temperature to extract intracellular cAMP. After centrifugation of the extract at 3000xg for 10 minutes at 4°C, the supernatants were collected in new vials and neutralized with 0.1 N NaOH. Cyclic AMP levels were measured by using a cAMP ELISA kit according to instructions provided by the manufacturer (Advanced Magnetics, Inc., Cambridge, MA). The method was based on the competitive binding between a variable amount of cAMP (either in samples or standards) and a fixed amount of cAMP conjugated with alkaline phosphatase with a limited number of binding sites on the rabbit anti-cAMP antibody. Briefly, one hundred microliter of standard or sample solutions were pipetted in duplicate into wells of a 96-well polystyrene microtiter plate precoated with goat anti-rabit antibody and then 100 μl of polyclonal rabbit against cAMP antibody was added to each well. After gently agitating the plates on a shaker (Dynatech Laboratories, Inc., Chantilly, VA) for at least 20

seconds, the wells were covered with a sheet of plate sealer and incubate at 3-7°C for 2 hours. One hundred microliter of cAMP alkaline phosphatase conjugate were pipetted into each well and the plate was sealed as mentioned above before incubation for another hour at 3-7°C. At the end of the incubation period, the sealer was removed and the solution was aspirated. The plate was washed 6 times with wash buffer and then inverted and blotted on paper towel after final wash. Three hundred microliter of para-nitrophenyl phosphate solution (substrate of alkaline phosphatase) were pipetted into each well, the plate was covered and then incubated at 37°C for 3 hours. After incubation, fifty microliter of 0.2 M NaOH were added to each well and mixed immediately by gently tapping the plate to stop the reaction. Absorbance at 405 nm was measured with a 96 well microtiter plate reader and the cAMP concentration was calculated by using standard curves.

M. Densitometry

Autoradiographs were scanned using a laser densitometer (UltraScan XL, LKB, Bromma, Sweden) and the intensities of bands in Western and Northern blots were expressed as absorbance units per mm (AU x mm).

N. Protein and DNA assays

Protein concentration in samples was determined with a Bio-Rad protein assay kit based on the colorimetric method of Bradford (136), using bovine serum albumin as standard.

The total DNA content was quantified by the method of Hinegardner (137), using calf thymus DNA (type 1) as standard. In brief, the method is based on the reaction of 3,5-diaminobenzonic acid (DABA) with deoxyriboses exposed after removing the purine bases of DNA by hot acid hydrolysis. Cells were lysed by addition of 0.2 ml of a 0.1% SDS solution. Fifty microliter aliquots of lysates were mixed with 100 µl of a DABA solution (80 mg/ml) and incubated at 60°C for 30 minutes. After termination of the reaction by adding 1.5 ml of 1 M HCl, DNA concentrations were determined by measuring fluorescence at 502 nm after excitation at 410 nm in a spectrofluorometer (Perkins Elmers, Model LS-5).

O. Statistical analysis

Statistical significance of differences between means was estimated by Student's paired t-test and $P < 0.05$ was considered significant.

IV. RESULTS

In order to investigate the mechanism by which endometrial fibroblast-like stromal cells differentiate into the decidual phenotype, we separated this type of stromal cells from human proliferative endometrium and cultured them with or without test hormones and other compounds of interest. Decidual morphologic changes, similar to those occurring in human endometrial stromal cells *in vivo*, were observed under a phase contrast microscope during the culture. In addition, several end-points of decidualization, such as production of HSP-27, IGFBP-1, desmin, laminin, fibronectin and PRL were detected by using a variety of techniques, such as immunocytochemistry, immunoprecipitation, Western blotting analysis, radioimmunoassay, enzyme-linked immunosorbent assay, Northern blotting analysis and nuclear run-on assay.

A. Effects of prolactin on decidualization of human endometrial stromal cells

Decidual morphologic change of human endometrial stromal cells

Morphologic change of confluent stromal cells cultured on Petri dishes were observed by phase contrast microscopy when PRL was added to the culture medium. In the cultures without added PRL, the cells remained elongated or spindle-shaped, exhibiting morphologic features considered typical for fibroblast-like endometrial stromal cells. Treatment of the stromal cells with PRL resulted in decidual morphologic changes by which the cells appeared rounder and occasional binucleated. Figure 4 shows the decidual morphologic changes induced by PRL in human endometrial stromal cells.

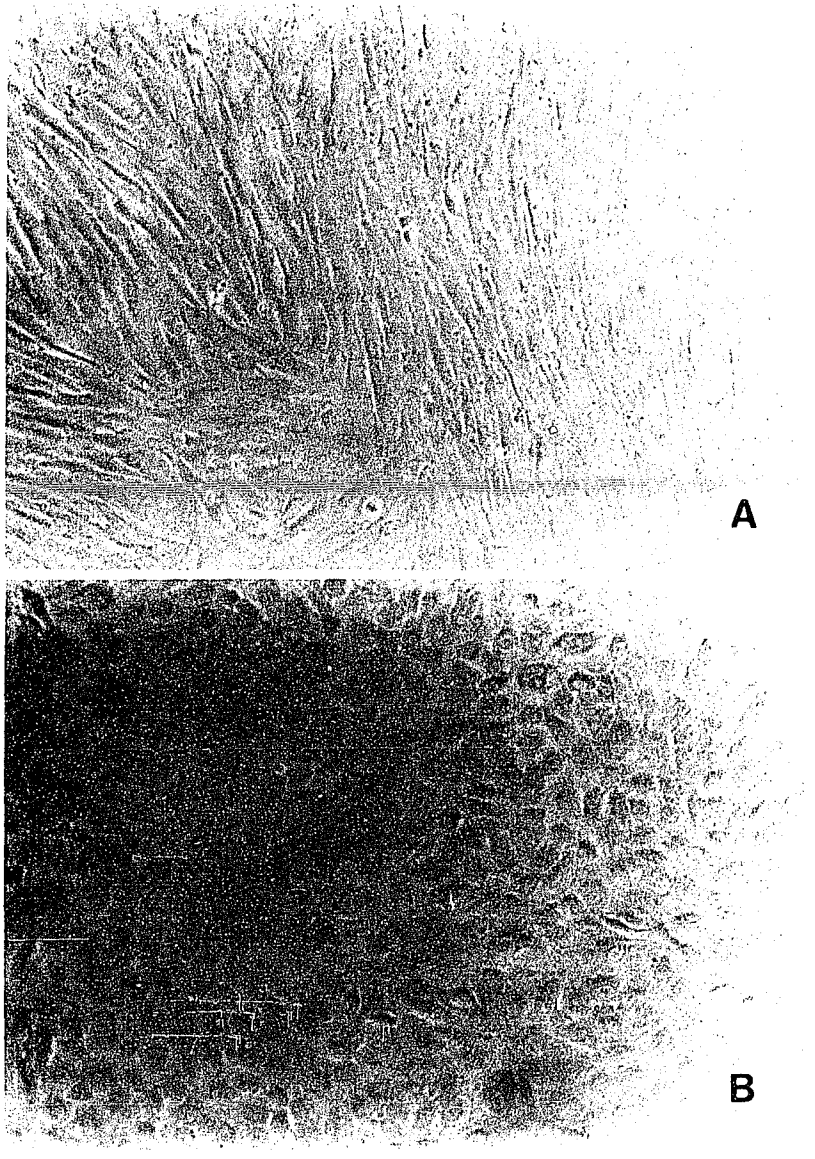


Fig. 4 Morphologic change of human endometrial stromal cells cultured with prolactin observed under phase contrast microscopy (10x10).

Panel A, human endometrial stromal cells were cultured without the addition of PRL and panel B, the stromal cells were cultured with the addition of prolactin.

Effects of prolactin on the production of decidual products in human endometrial stromal cells

Products characteristic of decidual cells, such as desmin, laminin, IGFBP-1 and HSP-27, were immunocytochemically detected in confluent stromal cells cultured with PRL on Lab-Tek tissue culture chambers. After incubation with the respective primary antibody, positive immunostaining for these proteins were observed in the stromal cells treated with PRL, but not in the cells cultured without addition of PRL. When primary antibody was replaced with non-immune serum, no staining was found in the cells. These immunocytochemical results are shown in Fig. 5 and summarized in Table 3.

Fig. 5 Immunocytochemical staining of laminin, HSP-27, IGFBP-1, and desmin in human endometrial stromal cells treated or untreated with prolactin *in vitro*.

A & B, positive immunostaining for laminin was noted in the stromal cells cultured with PRL (B), but not in the cells without PRL (A).
C & D, positive immunostaining for HSP-27 was noted in the stromal cells cultured with PRL (D), but not in the cells without PRL (C).
E & F, positive immunostaining for IGFBP-1 was noted in the stromal cells cultured with PRL (F), but not in the cells without PRL (E).
G & H, positive immunostaining for desmin was noted in the stromal cells cultured with PRL (H), but not in the cells without PRL (G).
No staining was observed under the same conditions in preparations of stromal cells incubated with non-immune serum.

Fig. 5

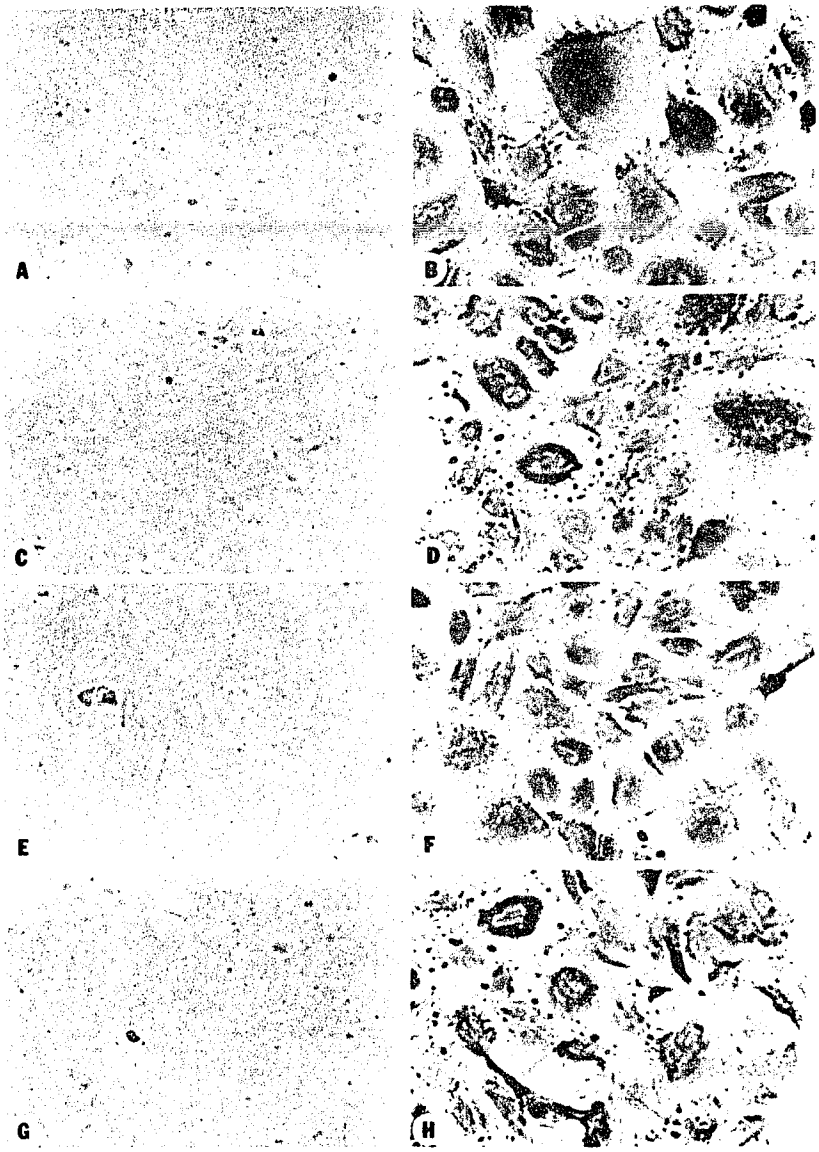


Table 3 Immunocytochemical detection of desmin, HSP-27, IGFBP-1, and laminin production in human endometrial stromal cells cultured with or without prolactin.

Products tested

Exp.#	Desmin		IGFBP-1		HSP-27		Laminin	
	A	B	A	B	A	B	A	B
1	-	+	-	+	-	+	NT	NT
2	-	+	-	+	-	+	NT	NT
3	-	+	-	+	-	+	-	+
4	-	+	-	+	-	+	-	-
5	-	+	-	-	+	+	-	+

NT: Not tested.

A: Cells cultured in control medium.

B: Cells cultured in control medium containing 500 ng/ml of prolactin.

Production of fibronectin by human endometrial stromal cells cultured with prolactin

Fibronectin, one of decidual products, was quantitatively determined by using solid-phase, enzyme-linked immunosorbent assay kits obtained from Adeza Biomedical Corporation (Sunnyvale, CA) with a detection limit of 5 ng/ml. A standard curve is shown in Fig. 6. The production of fibronectin by stromal cells was significantly higher ($P<0.05$) in the presence of PRL (500 ng/ml) than in its absence. The inductive effect of PRL on the production of fibronectin by human endometrial stromal cells is illustrated in Fig. 7.

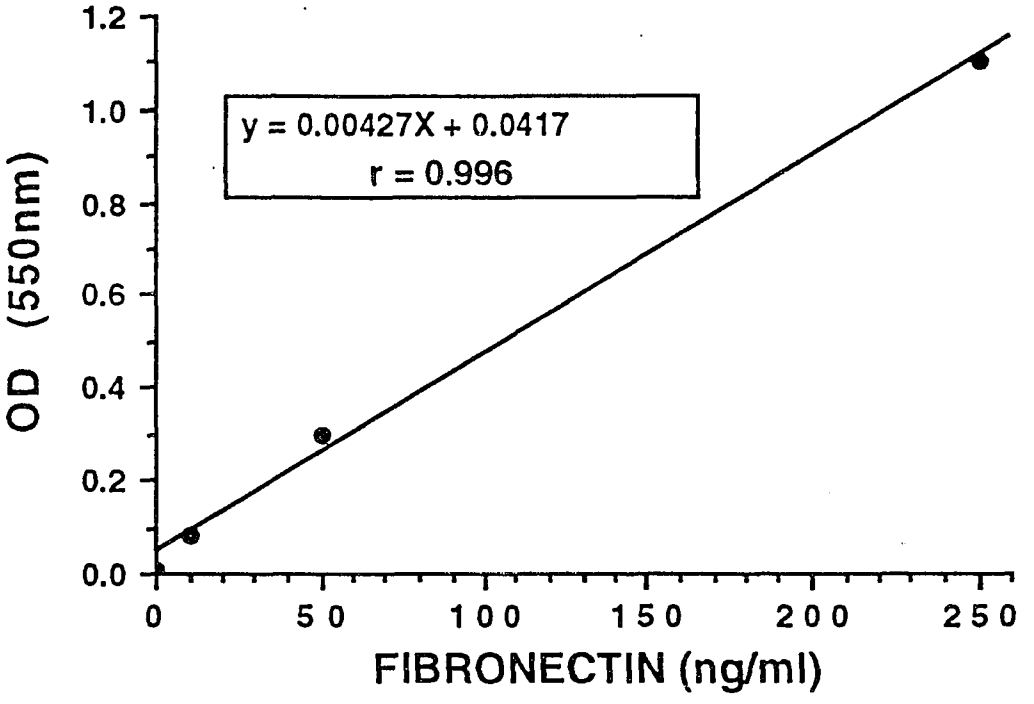


Fig. 6 Standard curve for fibronectin determination.

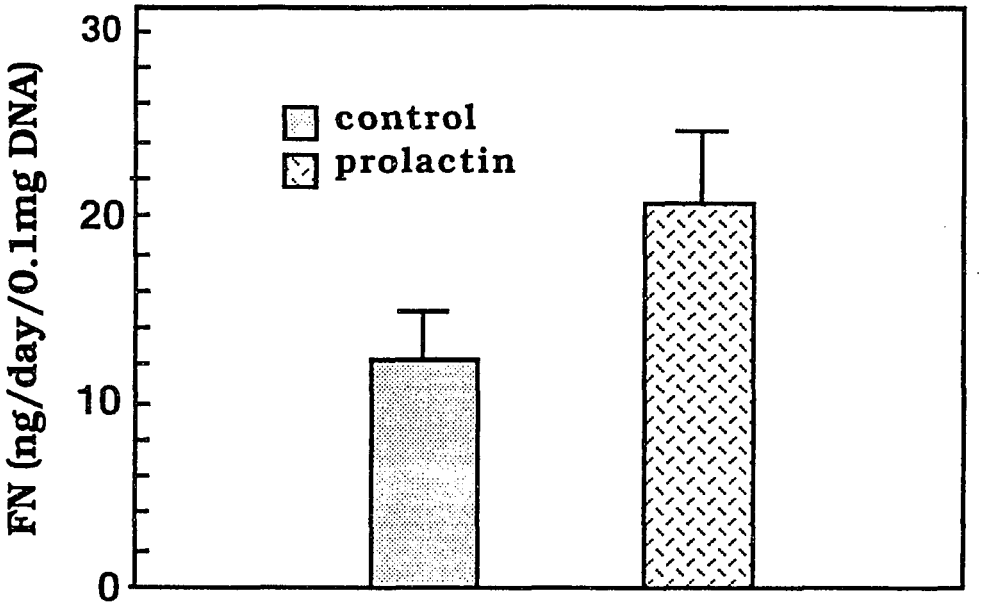


Fig. 7 Effect of prolactin on fibronectin production by human endometrial stromal cells.

B. Effects of cAMP on prolactin production by human endometrial stromal cells in vitro

Effects of db-cAMP, 8-Br-cAMP and forskolin on the rate of PRL output into the medium and on the synthesis of PRL mRNA were demonstrated by following several experimental approaches. Production of PRL by cultures of stromal cells isolated from human proliferative endometrium in response to added cAMP derivatives or foskolin were evident from immunocytochemical staining, Western blotting analysis, immunoprecipitation of [^{35}S]-methionine-labeled PRL followed by gel electrophoresis, and from radioimmunoassay of PRL secreted into the medium; increased PRL mRNA expression was documented by Northern blotting and transcription run-on assays.

Immunocytochemical determination of prolactin production by human endometrial stromal cells in the presence or absence of db-cAMP

Positive immunostaining for PRL was observed by using polyclonal rabbit anti-hPRL antibody in the stromal cells cultured with db-cAMP, but not in the cells cultured in RPMI/FBS, as shown in Fig. 8.

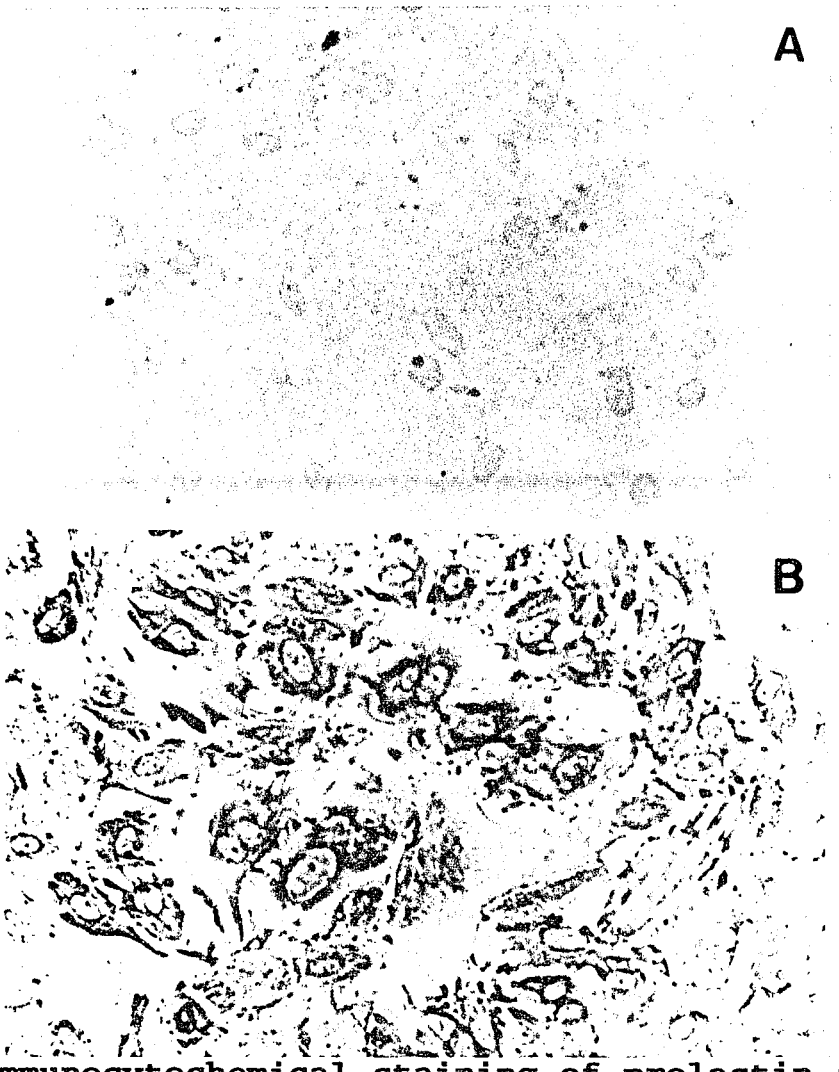


Fig. 8 Immunocytochemical staining of prolactin in human endometrial stromal cells cultured with or without db-cAMP.

Positive staining was observed in the stromal cells cultured with db-cAMP (B), but not in cells cultured without db-cAMP (A). No staining was observed under the same conditions in preparations of stromal cells incubated with non-immune serum.

Prolactin immunoblot analysis

Production of PRL induced by cAMP in human endometrial stromal cells was further investigated by using Western blotting analysis.

Stromal cells isolated from human proliferative endometrium were cultured in RPMI/FBS medium with or without db-cAMP (0.5 mM), collecting media every 48 h and saving these fractions at -80°C for further analysis. Equal volumes of media collected during different incubation periods were used for SDS-PAGE. Western blotting, performed with rabbit polyclonal anti-hPRL antibody, demonstrated that secreted immunoreactive PRL was of 2 sizes, the most abundant with M_r approx. 23 kDa and the other of approx. 25 kDa. The migration of antibody-detected PRL in the samples corresponded to standard human PRL run in parallel (23 kDa). Similar sizes of immunoreactive PRL were detected by Zhu *et al* in human endometrial stromal cells cultured with P + E_2 + p-RLX (24).

The intensity of the radioactive band visualized following exposure of the blots to anti-hPRL antibody and [^{125}I]-Protein A increased as the length of exposure of the cells to db-cAMP was extended from 3 to 7 days, but did nor increase further between days 7 and 11. No

PRL was detectable in the absence of the cyclic nucleotide. These immunoblot analysis results are presented in Fig. 9.

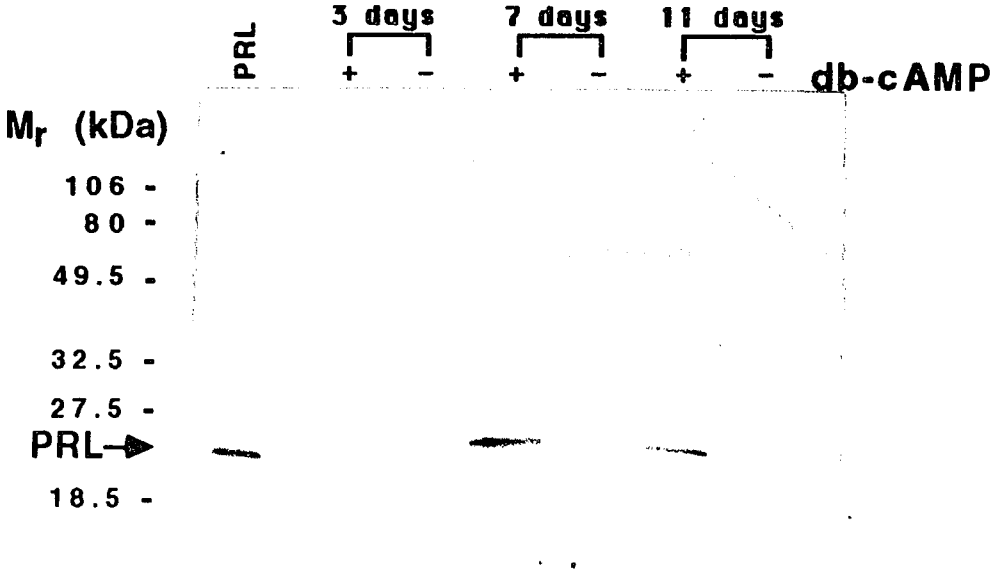


Fig. 9 Western blotting analysis of PRL production by human endometrial stromal cells cultured with db-cAMP.

Stromal cells were cultured in the absence or presence of db-cAMP (0.5 mM) for 3, 7 and 11 days, respectively. Media were collected and subjected to SDS-PAGE. PRL was specifically detected by anti-hPRL antibody and visualized by using [^{125}I]-labeled Protein A following radioautography.

Time dependence of db-cAMP effects on PRL output

Figure 10 shows the effect of db-cAMP on the concentrations of PRL in the culture medium, as determined by RIA. Under the assumption that storage or metabolism of PRL was not altered by the addition of db-cAMP, the increase in rates of PRL secretion was evident after 2 days of exposure to db-cAMP (0.5 mM), reaching a maximum at day 5-6. No PRL output was detected in the absence of the cyclic nucleotide.

Concentration dependence of db-cAMP effects on PRL production

Figure 11 indicates that db-cAMP induces PRL production in a concentration dependent manner, with maximal effects observed at approximately 0.5 mM and a decline noted at higher levels.

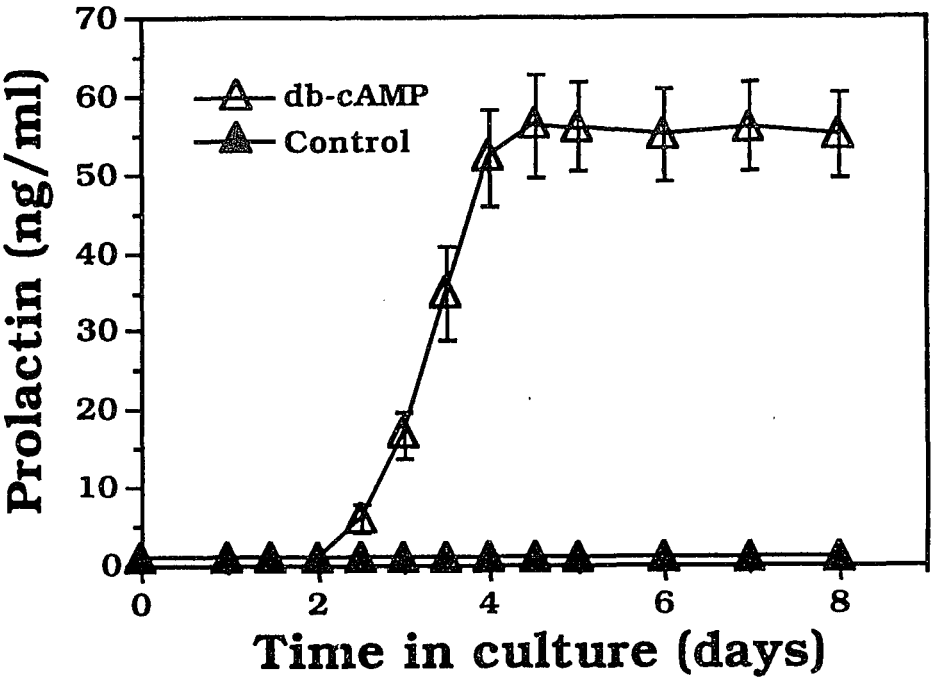


Fig. 10 Time dependence of PRL induction by db-cAMP in human endometrial stromal cells.

Confluent stromal cells were cultured in RPMI 1640 + 2% ct-FBS, with or without db-cAMP (0.5 mM) for 8 days. Medium was replaced every 24 h and PRL concentrations in the collected media were determined by RIA. The values represent the mean ± SD of duplicate determinations from three separate experiments. The concentrations of PRL in cultures of cells not treated with db-cAMP were less than 2 ng/ml. No significant temporal differences in DNA content were found.

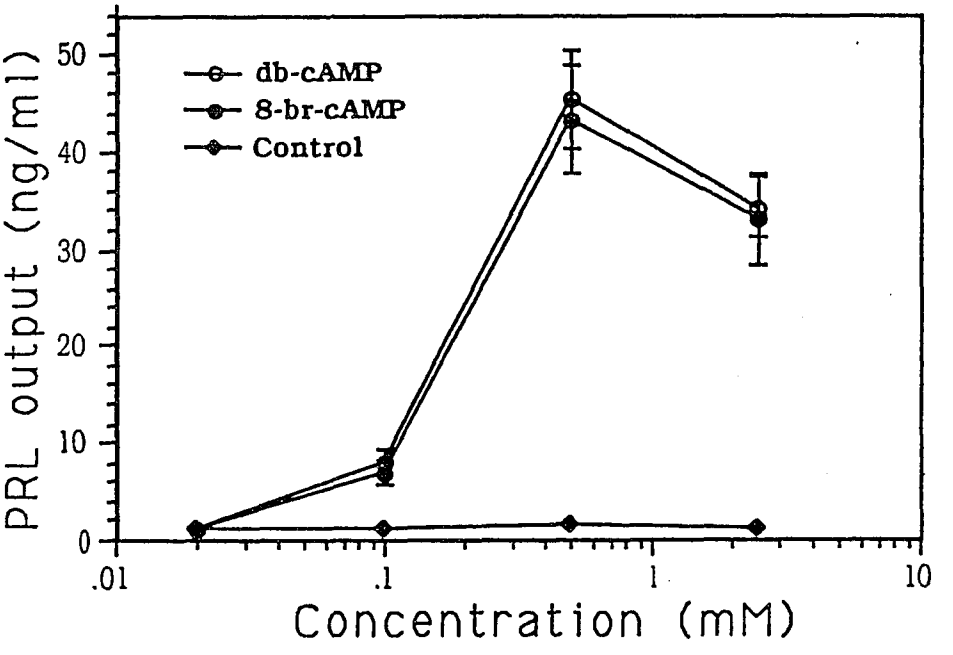


Fig. 11 Dose-response curve of cAMP-induced PRL output from stromal cells isolated from human proliferative endometrium.

Stromal cells were cultured for 10 days with db-cAMP or 8-br-cAMP at 0, 0.02, 0.1, 0.5 or 2.5 mM concentrations. Prolactin levels were determined in media collected during the final 24 h. The results show mean ± SD values of duplicate determinations from two separate cultures.

Decline of PRL output following removal of db-cAMP from the culture medium.

Figure 12 shows at t=0 daily rates of PRL output by stromal cells exposed to db-cAMP (0.5 mM) for various periods of time (48 to 96h), as reflected by PRL concentrations at the end of a preceding 24h incubation period. The graph shows the decline in PRL output during consequent 24h culture periods in the absence of db-cAMP.

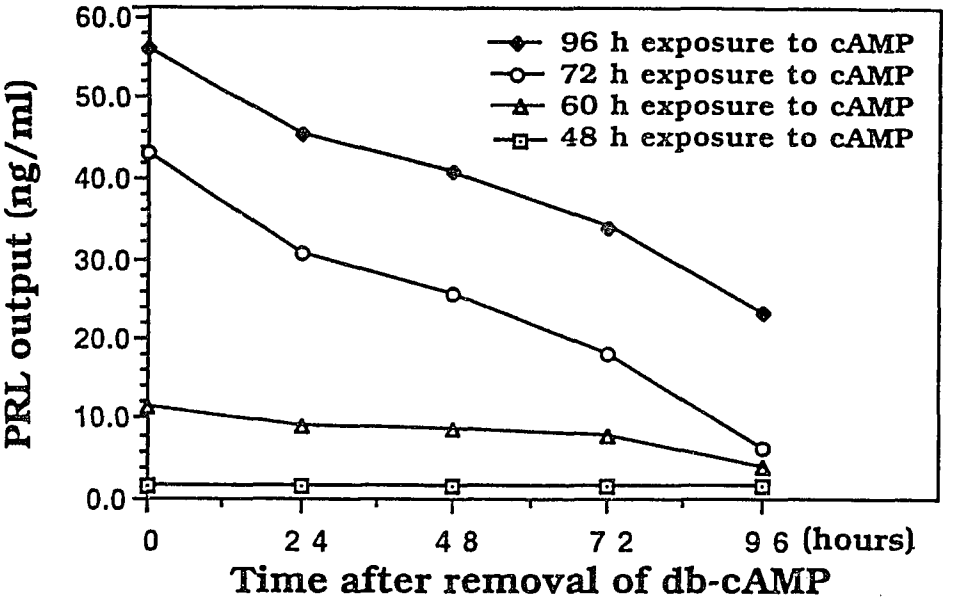


Fig. 12 Time dependence of PRL output following removal of db-cAMP from the medium.

Different levels of PRL production were obtained in stromal cells from proliferative endometrium by exposure to db-cAMP (0.5 mM) for increasing time periods, from 48 to 96 h. After removal of db-cAMP from the medium (t=0), PRL output was followed for the next 96 h. Levels of PRL in media from cultures of stromal cells in the absence of db-cAMP, or in the presence of the cyclic nucleotide for only 48 h, were undetectable. No significant temporal differences in DNA content were found.

Synthesis of immunoprecipitable [^{35}S]-methionine-labeled prolactin

The synthesis of PRL was investigated by labeling the db-cAMP treated and untreated cells with [^{35}S]-methionine. Immunoreactive PRL was precipitated by incubation with polyclonal rabbit anti-hPRL antibody. The presence of [^{35}S]-labeled PRL in the precipitate was indicated by autoradiography after SDS-polyacrylamide gel electrophoresis. Figure 13 illustrates the inductive effect of db-cAMP (0.5 mM) on the synthesis of [^{35}S]-labeled PRL during incubation of stromal cells from proliferative endometrium cultured in medium containing [^{35}S]-methionine. A radioactive band, corresponding to PRL (23 kDa) was obtained in samples of cells exposed to db-cAMP. No PRL was observed in the cells cultured with RPMI/FBS medium alone.

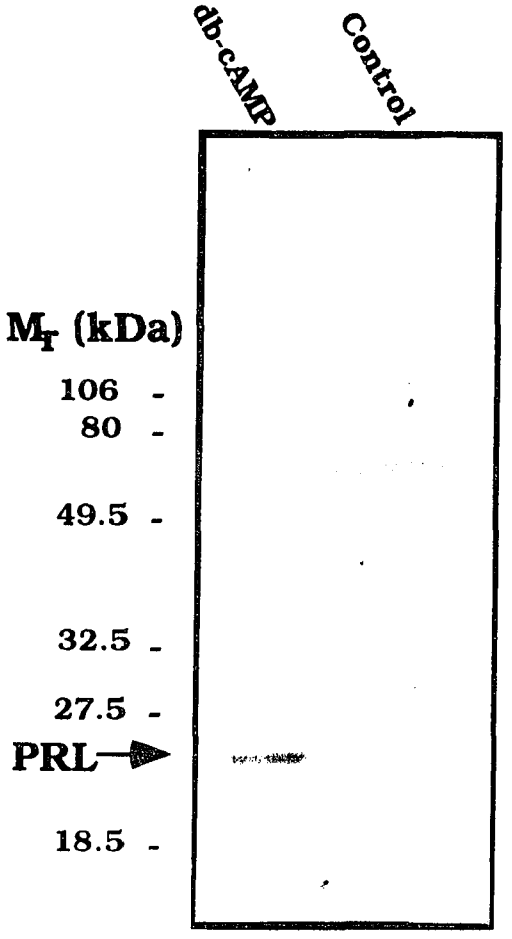


Fig. 13 **Immunoprecipitation of [^{35}S]-labeled PRL produced by human endometrial stromal cells.** Cells were cultured with or without db-cAMP (0.5 mM) for 5 days and then labeled with [^{35}S]-methionine for 6 h. Media were collected and the output of [^{35}S]-methionine labeled PRL was evaluated by SDS-PAGE and autoradiography after immunoprecipitation with rabbit polyclonal anti-hPRL antibody. Three separate experiments yielded similar results.

Induction of PRL mRNA by db-cAMP

Total RNA was extracted from the stromal cells cultured with db-cAMP for 2, 6 and 10 days in 10 cm Petri dishes, respectively. A [^{32}P]-labeled probe of pituitary hPRL cDNA was used to hybridize with hPRL mRNA in 0.5 M NaH_2PO_4, 1 mM EDTA, 7%(w/v) SDS, pH 7.2 buffer at 65°C for 16 h. Figure 14 illustrates results from Northern blot analysis of PRL mRNA induced by db-cAMP in the stromal cells. Hybridization with the [^{32}P]-labeled hPRL probe demonstrated the presence of a 1.1 kb PRL mRNA in extracts from cells exposed to db-cAMP (0.5 mM) for 6 and 10 days. The transcript has a similar size as the reported hPRL mRNA from decidua and from IM-9-P3 lymphoblast cells (138). No PRL mRNA was detected in samples from cells cultured in the absence of db-cAMP or exposure to the cyclic nucleotide for only 2 days (Panel A). The level of PRL mRNA was normalized based on the level of cyclophilin mRNA (Panel B). The time dependence of relative PRL mRNA increases in the cells cultured with db-cAMP were shown in panel C. Based on cyclophilin mRNA levels, the hPRL mRNA was 8 and 11 folds increased in the cells cultured with db-cAMP for 6 and 10 days determined by density scanning (Table 4).

Fig. 14 Northern blot analysis of db-cAMP effects on hPRL mRNA in human endometrial stromal cells. Stromal cells cultured in the presence or absence of db-cAMP (0.5 mM) for 2, 6 and 10 days, respectively. Total RNA was extracted and subjected to 1% agarose-formaldehyde gel electrophoresis. Panel A: induction of PRL mRNA by db-cAMP as measured by hydridization of a Northern blot with a [^{32}P]-labeled hPRL cDNA. Panel B: membrane rehybridized with [^{32}P]-labeled cyclophilin cDNA probe after stripping out the PRL probe to normalize levels of PRL mRNA. Panel C: ratio of density values of PRL mRNA and cyclophilin mRNA in stromal cells exposed to db-cAMP for 2, 6 and 10 days.

Fig. 14

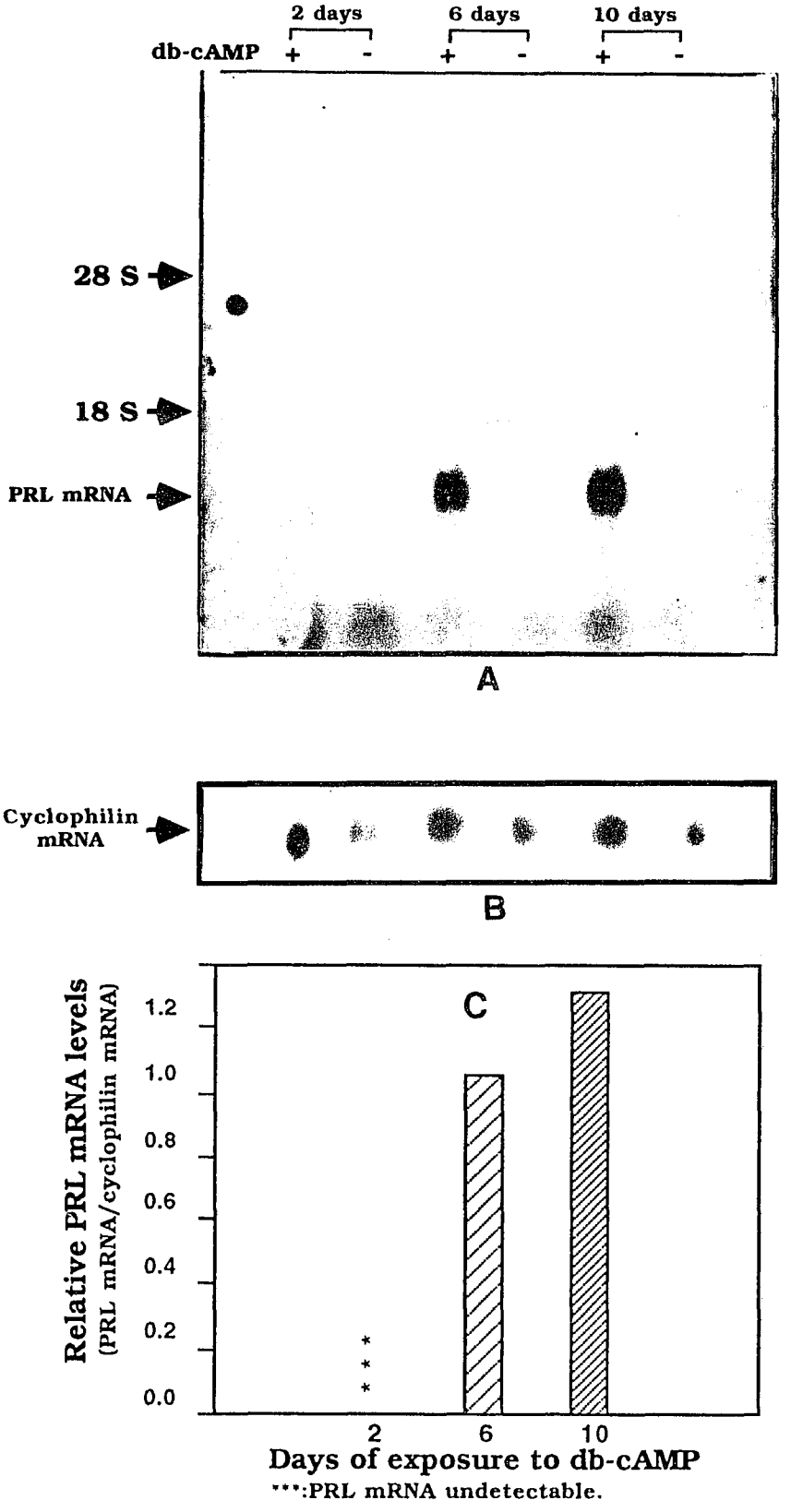

2 days
6 days
10 days
db-cAMP
+
-
+
-
+
-
28 S
18 S
PRL mRNA
A
Cyclophilin mRNA
B
C
Relative PRL mRNA levels
(PRL mRNA/cyclophilin mRNA)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
6
10
Days of exposure to db-cAMP
***:PRL mRNA undetectable.

Table 4 Effect of db-cAMP on prolactin mRNA levels in the cultures of human endometrial stromal cells

	2 days		6 days		10 days	
dbcAMP	-	+	-	+	-	+
PRLmRNA	0.381	0.666	0.369	3.568	0.354	4.516
CYCmRNA	3.503	8.111	5.324	6.359	6.039	6.905
$\frac{\text{PRLmRNA}}{\text{CYCmRNA}}$	0.109	0.082	0.069	0.561	0.059	0.654
$\frac{\text{+dbcAMP}}{\text{-dbcAMP}}$	NE		8		11	

dbcAMP: N^6-2′-O-dibutyryl-cAMP.

PRL: Prolactin.

CYC: Cyclophilin.

NE: No effect.

C. Effects of female sex steroids (estrogens and progesterone)on cAMP induced PRL production of human endometrial stromal cells

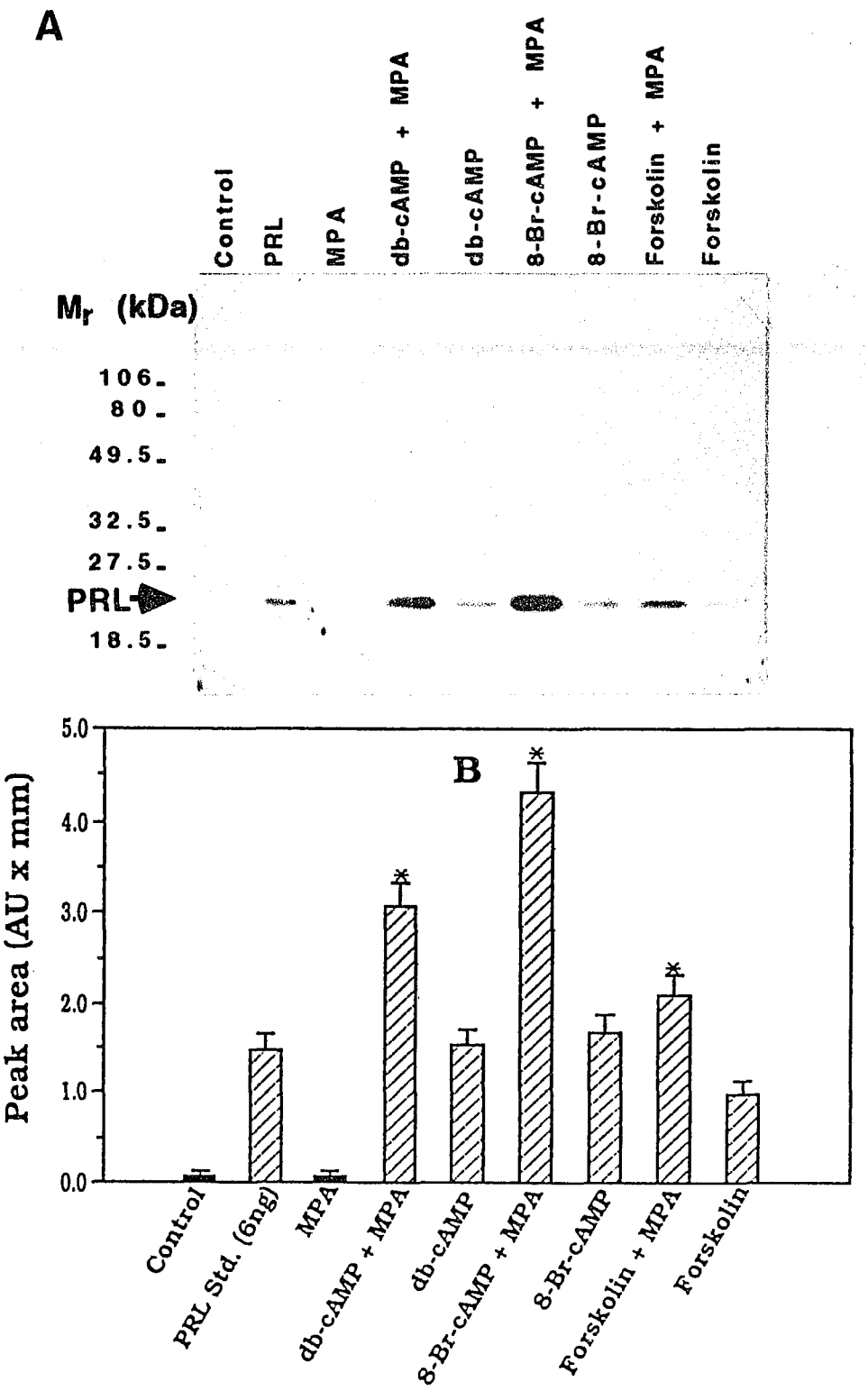
Effects of MPA on db-cAMP, 8-Br-cAMP or forskolin induced PRL production by human endometrial stromal cells

Panel A of Fig. 15 presents Western blots demonstrating the inductive effects of db-cAMP, 8-Br-cAMP and forskolin on PRL production and showing that MPA enhances the cAMP effect even though it was by itself incapable of eliciting detectable PRL production under these experimental conditions. Panel B of Fig. 15 shows that PRL output by stromal cells cultured with db-cAMP + MPA, 8-Br-cAMP + MPA or forskolin + MPA was significantly higher than the output in the absence of the synthetic progestin.

Fig. 15 Effects of db-cAMP, 8-Br-cAMP, forskolin, db-cAMP + MPA, 8-Br-cAMP + MPA and forskolin + MPA on PRL output from human endometrial stromal cells.

Media collected during the last 24 h culture were subjected to 10% SDS-PAGE, Western blotting, immunodetection and densitometric scanning. Panel A: PRL was detected by anti-hPRL antibody and [^{125}I]-protein A; human PRL standard was run in parallel to indicate its position. Panel B: PRL output was evaluated by densitometric scanning; mean ± SD for various treatments in three separate experiments were shown and statistical significance of the differences in the presence or absence of MPA were determined by Student t test (*: $P < 0.05$).

Fig. 15


A
Control
PRL
MPA
db-cAMP + MPA
db-cAMP
8-Br-cAMP + MPA
8-Br-cAMP
Forskolin + MPA
Forskolin
Mr (kDa)
106.
80.
49.5.
32.5.
27.5.
PRL
18.5.
B
Peak area (AU x mm)
5.0
4.0
3.0
2.0
1.0
0.0
*
Control
PRL Std. (6ng)
MPA
db-cAMP + MPA
db-cAMP
8-Br-cAMP + MPA
8-Br-cAMP
Forskolin + MPA
Forskolin

Effect of E_2 on db-cAMP or db-cAMP + MPA induced prolactin production of human endometrial stromal cells

The effect of E2, alone or in combination with MPA, on db-cAMP induced PRL production by human endometrial stromal cells was investigated by measuring PRL output using an ELISA procedure. A standard curve for PRL ELISA analysis is shown in Fig. 16. No significant differences in PRL output were observed between stromal cells cultured in medium with or without E2. Results from three separate experiments are shown in Fig. 17.

Effect of db-cAMP, db-cAMP + E_2, db-cAMP + MPA, and db-cAMP + MPA + E_2 on prolactin gene transcription

Confluent stromal cells were cultured with db-cAMP, db-cAMP + E2, db-cAMP + MPA, or db-cAMP + MPA + E2 in RPMI/FBS medium for 6 days. At the end of the culture period, cell nuclei were isolated and PRL gene transcription rates were evaluated by using the nuclear run-on assay technique. Medroxyprogesterone acetate treatment synergistically enhanced the effect of db-cAMP on PRL gene transcription, as shown in Fig. 18 and summarized in Table 5.

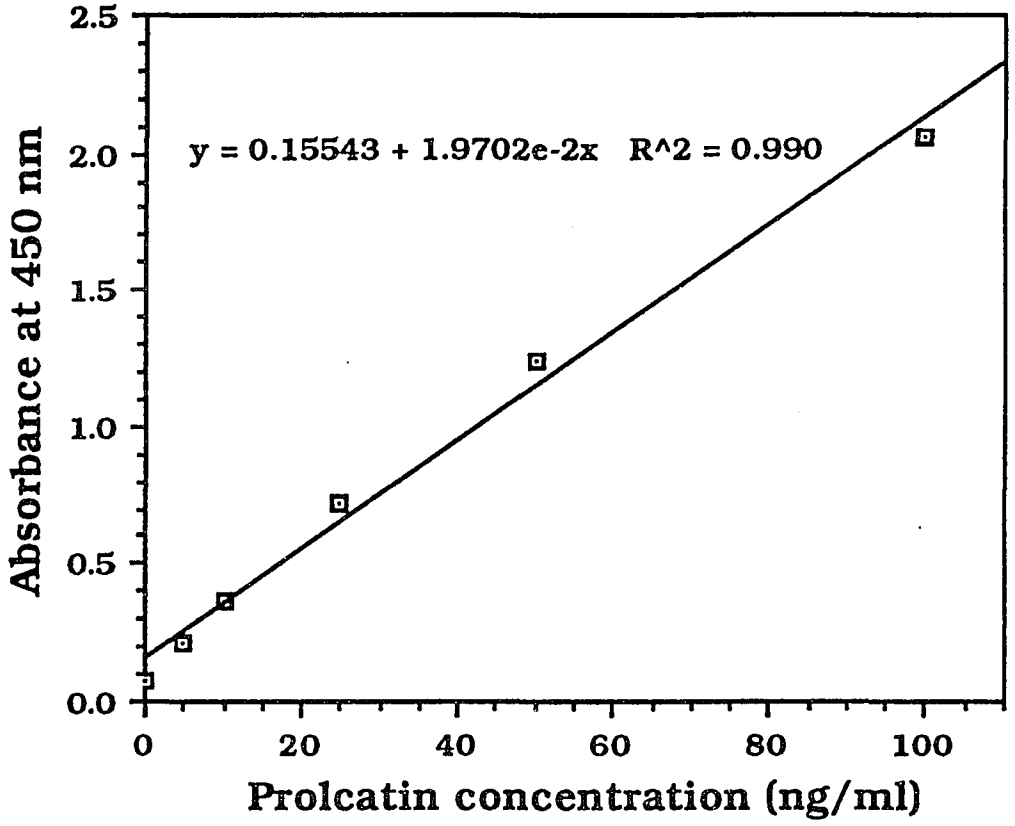


Fig. 16 Standard curve for PRL ELISA.

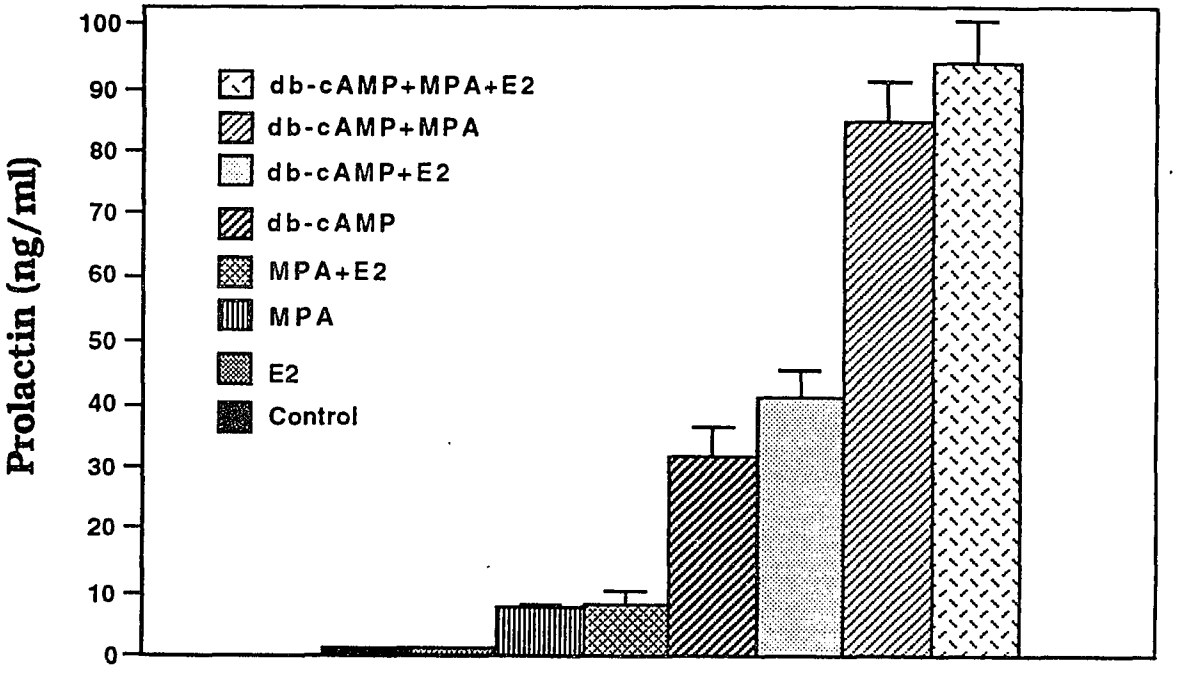
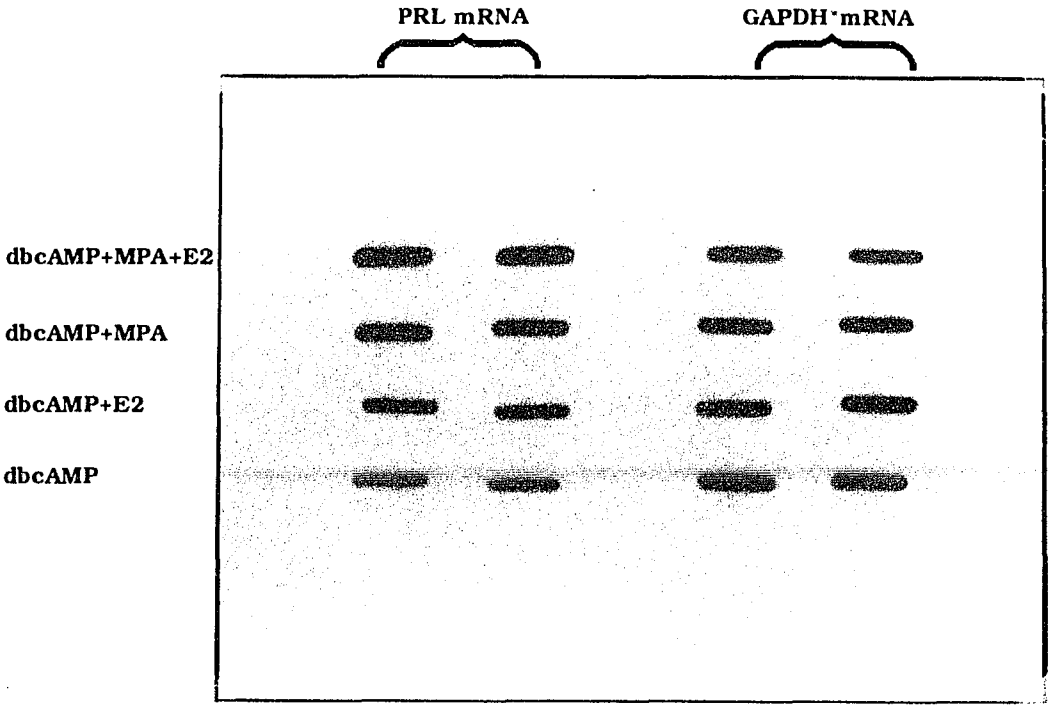
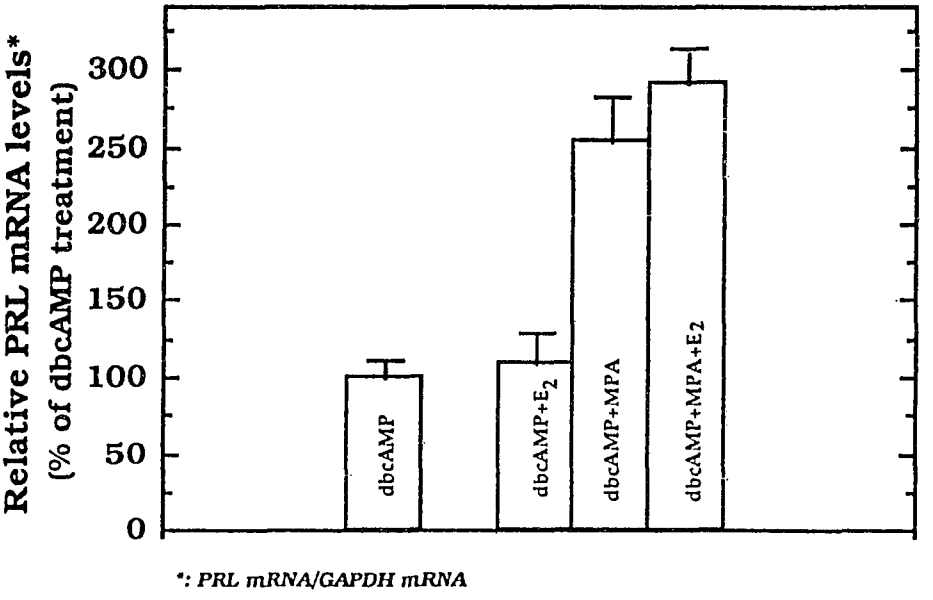


Fig. 17 ELISA analysis of E_2 effect on db-cAMP or db-cAMP + MPA induced PRL production of human endometrial stromal cells



*: Glyceraldehyde 3-phosphate dehydrogenase.



*: PRL mRNA/GAPDH mRNA

Fig. 18 Effects of db-cAMP, db-cAMP + E_2, db-cAMP + MPA, and db-cAMP + MPA + E_2 on the rate of transcription of prolactin gene in human endometrial stromal cells

Table 5 Rate of transcription of prolactin gene determined by densitometric scanning

TEST	PRLmRNA (AU X mm)	GAPDHmRNA (AU X MM)	PRLmRNA / GAPDHmRNA	Ratio to dbcAMP
dbcAMP +MPA+E_2	2.78 2.39	1.58 1.40		
Aver.	2.58	1.49	1.73	3.06
dbcAMP +MPA	2.23 1.92	1.49 1.55		
Aver.	2.08	1.52	1.37	2.40
dbcAMP +E_2	1.23 1.11	1.73 1.84		
Aver.	1.17	1.79	0.65	1.14
dbcAMP	1.16 1.04	2.09 1.80		
Aver.	1.10	1.94	0.57	1.00

PRL: Prolactin.

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

D. Effects of cAMP on decidualization of human endometrial stromal cells

In addition to the induction of PRL expression, the effects of cAMP on the decidualization of human endometrial stromal cells were evident from several experimental approaches. Decidual morphologic changes were observed in human endometrial stromal cells cultured in RPMI/FBS containing cAMP derivatives. Biochemical differentiation of the stromal cells was further demonstrated by the detection of decidual products, such as desmin, laminin, IGFBP-1, HSP-27, and fibronectin.

Decidual morphologic change of human endometrial stromal cells cultured with RPMI/FBS or RPMI/FBS + db-cAMP

The morphologic decidualization of stromal cells isolated from human proliferative endometrium and cultured in plastic dishes in the presence of db-cAMP was observed under phase contrast microscopy. Figure 19 shows the morphologic changes resulting from exposure to db-cAMP (0.5 mM) of stromal cells isolated from human proliferative endometrium and cultured in RPMI/FBS medium. In the culture with db-cAMP, the spindle-shaped,

fibroblast-like stromal cells became rounder and occasionaly binucleated, phenotypic characteristics of decidual cells. In the culture with RPMI/FBS medium without db-cAMP, the cells kept spindle-like shape characteristic of fibroblast-like human endometrial stromal cells.

Immunocytochemical detection of expression of IGFBP-1, HSP-27, desmin, and laminin in human endometrial stromal cells treated with db-cAMP

Positive immunostaining for desmin, laminin, IGFBP-1 and HSP-27 was observed in stromal cells cultured with db-cAMP as shown in Fig. 20. Neither cells cultured in RPMI/FBS and incubated with the respective first antibody nor cells cultured in RPMI/FBS + db-cAMP and incubated with non-immune serum showed immunostaining. These results are summarized in Table 6.

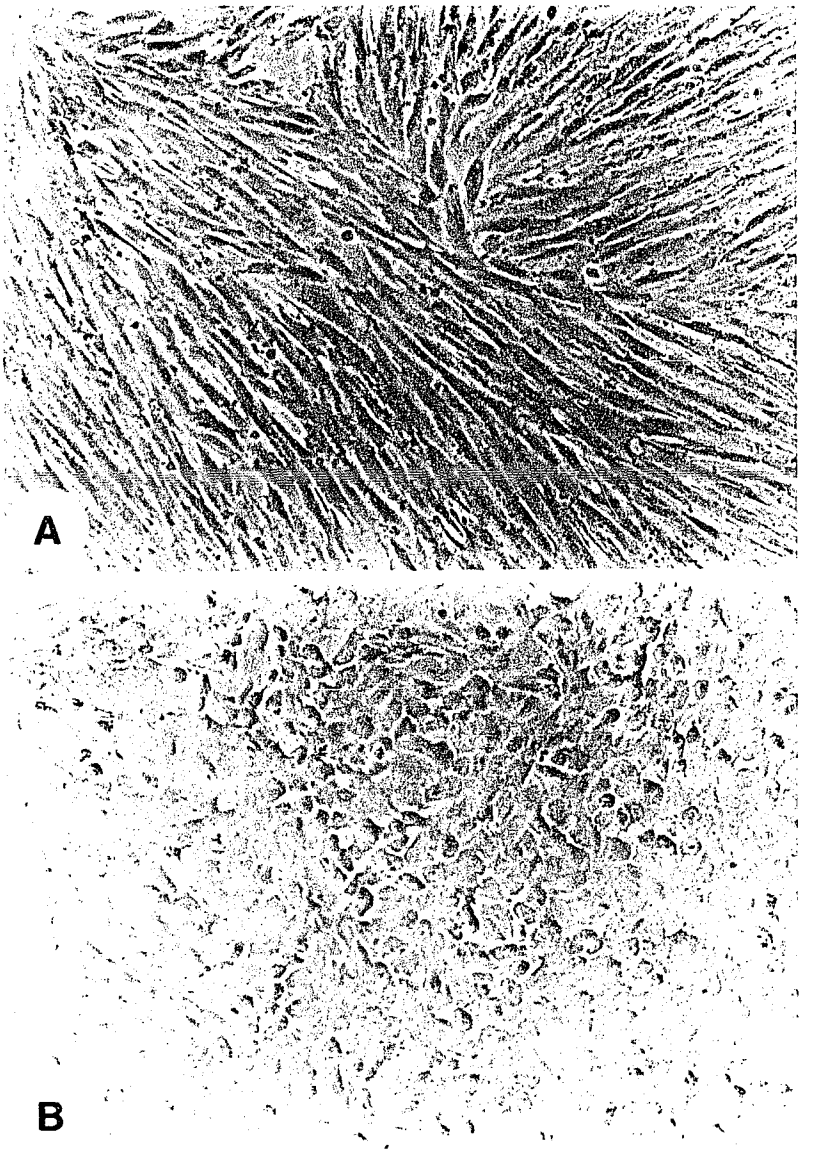


Fig. 19 Effect of db-cAMP on the morphologic appearance of human endometrial stromal cells.

Stromal cells were cultured in RPMI/FBS medium without (A) or with 0.5 mM db-cAMP (B) for 12 days and then photographed under phase contrast microscopy (10 x 10).

Fig. 20 Immunocytochemical staining of desmin, HSP-27, IGFBP-1, and laminin production in the human endometrial stromal cells cultured with or without db-cAMP.

A and B: positive staining for PRL was observed in the stromal cells cultured with db-cAMP (B), but not in the cells cultured without db-cAMP (A). C and D: positive staining for IGFBP-1 was observed in the stromal cells cultured with db-cAMP (D), but not in the cells cultured without db-cAMP (C). E and F: positive staining for laminin was observed in the stromal cells cultured with db-cAMP (F), but not in the cells cultured without db-cAMP (E). G and H: positive staining for desmin was observed in the stromal cells cultured with db-cAMP (H), but not in the cells cultured without db-cAMP (G). I and J: positive staining for HSP-27 was observed in the stromal cells cultured with db-cAMP (J), but not in the cells cultured without db-cAMP (I). No staining was observed under the same conditions in preparations of stromal cells incubated with non-immune serum.

Fig. 20

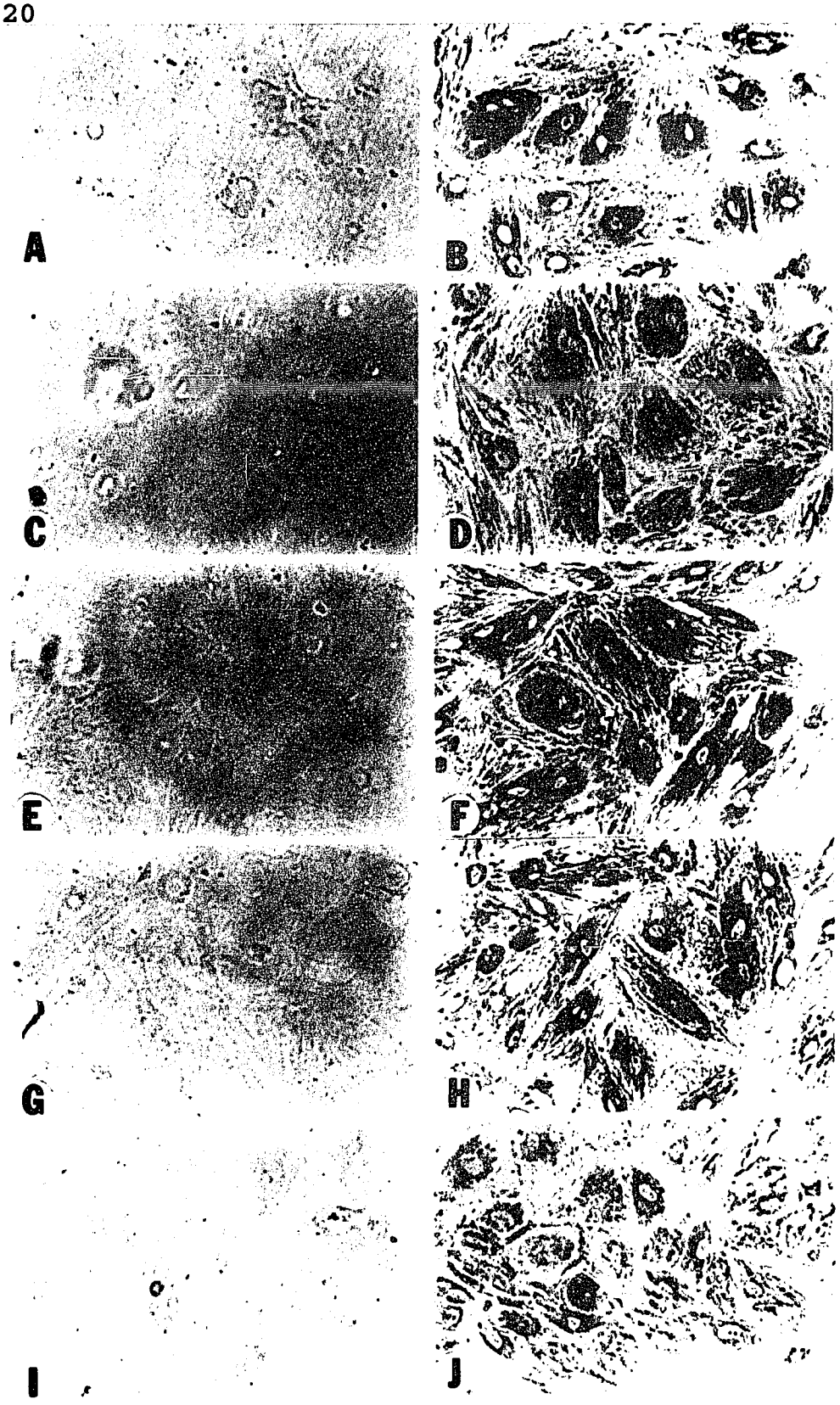


Table 6 Immunocytochemical detectability of desmin, HSP-27, IGFBP-1, and laminin production in the human endometrial stromal cells cultured with or without db-cAMP.

Products tested

	Desmin		Laminin		IGFBP-1		HSP-27	
Exp.#	A	B	A	B	A	B	A	B
1	-	+	-	+	-	+	-	+
2	-	+	-	+	-	+	-	+
3	+	+	+	+	-	+	+	+
4	-	+	-	+	-	+	-	+
5	-	+	-	+	-	+	-	-
6	-	+	-	+	-	+	-	+
7	-	+	-	+	-	+	-	+
8	-	-	-	-	-	+	-	-

A: Cells cultured in control medium.

B: Cells cultured in control medium containing 0.5 mM of db-cAMP.

Immunoprecipitation of [^{35}S]-labeled laminin, desmin, IGFBP-1, and HSP-27 from human endometrial stromal cells cultured with or without db-cAMP

The production of laminin, desmin, IGFBP-1 and HSP-27 were also detected by immunoprecipitation in the cells cultured with db-cAMP, but not in the cells cultured with RPMI/FBS medium. Figure 21 shows the effect of db-cAMP on production of laminin, desmin, IGFBP-1 and HSP-27 by human endometrial stromal cells.

Effect of db-cAMP on output of fibronectin in human endometrial stromal cells

The production of fibronectin was evaluated with solid-phase, enzyme-linked immunosorbent assay kits obtained from Adeza Biomedical Corporation (Sunnyvale, CA) with a detection limit of 5 ng/ml. The production of total fibronectin by stromal cell cultured with db-cAMP was significantly higher ($P<0.05$) than its production by cells cultured in RPMI/FBS medium only. The effect of db-cAMP on the production of fibronectin is shown in Fig. 22.

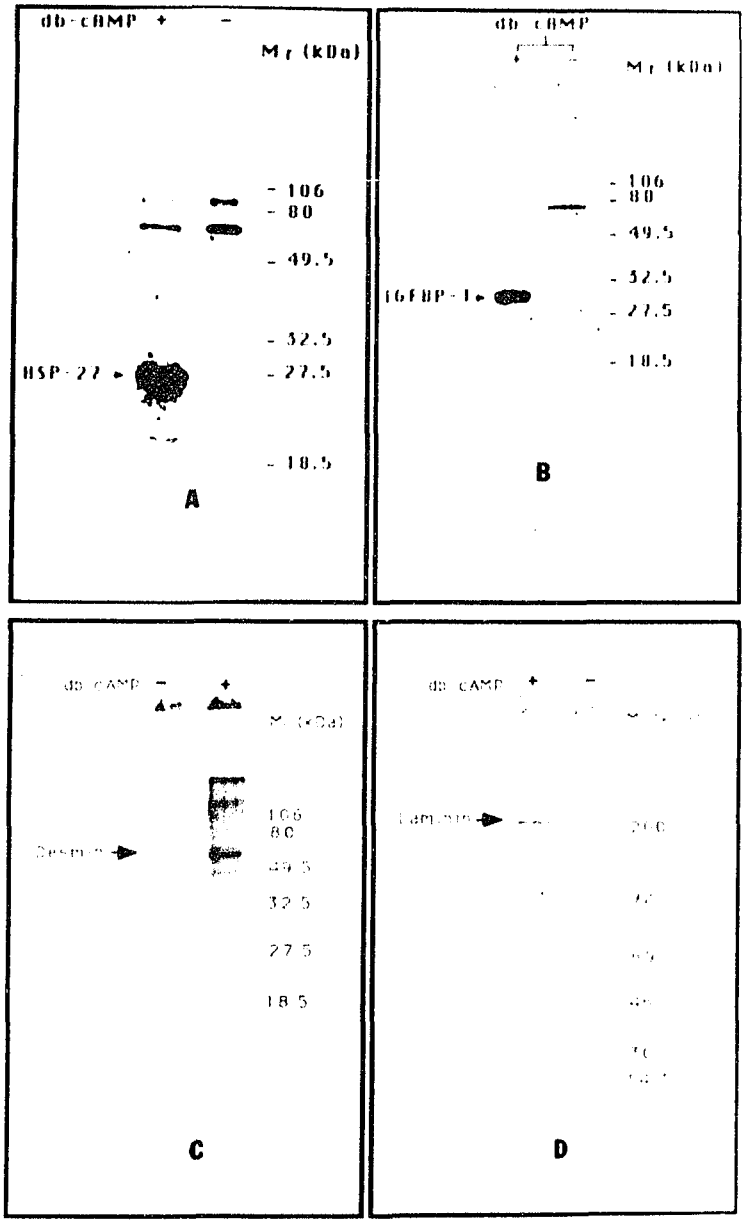


Fig. 21 Immunoprecipitation of [^{35}S]-labeled laminin, IGFBP-1, HSP-27 and desmin.

Panels A, B, C, and D: HSP-27, IGFBP-1, desmin, and laminin were detected in human endometrial stromal cells incubated with db-cAMP, but not in cells incubated without db-cAMP.

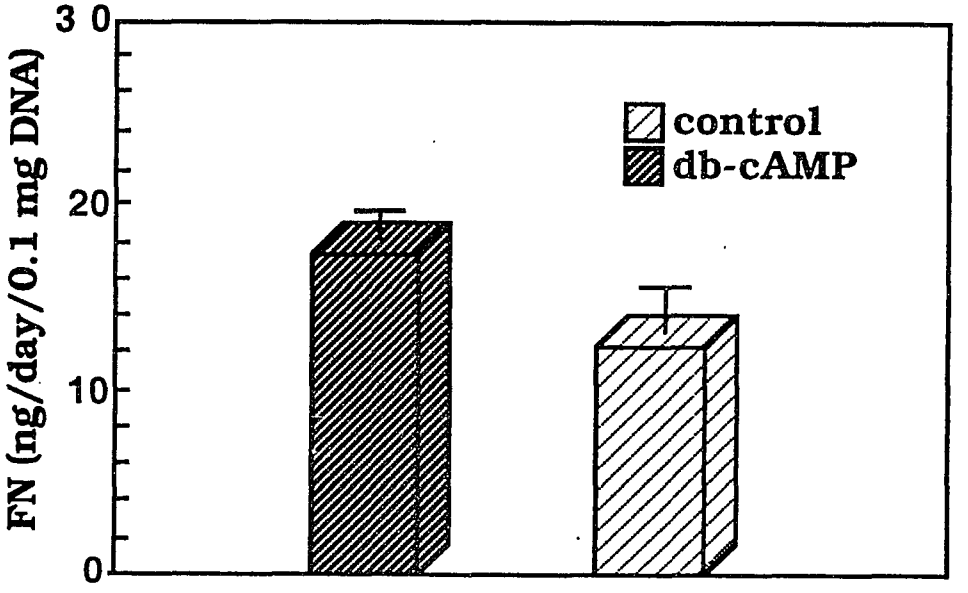


Fig. 22 Effect of db-cAMP on production of fibronectin by human endometrial stromal cells in culture.

E. Effects of gonadotropins (FSH, FSH+LH and hCG) on cAMP levels and decidualization of human endometrial stromal cells

Effects of gonadotropins on cAMP concentrations in human endometrial stromal cells was investigated by measuring cAMP levels, as determined by ELISA. Decidualization of human endometrial stromal cells induced by gonadotropin treatment was demonstrated by the morphologic changes to the decidual phenotype and by the expression of PRL in stromal cells exposed to FSH (Metrodin), FSH+LH (Pergonal), and hCG.

Effect of gonadotropins on cAMP levels of human endometrial stromal cells

Levels of cAMP in cultured stromal cells were measured by ELISA. A cAMP standard curve is shown in Fig. 23. The effects of gonadotropins on cAMP levels in human endometrial stromal cells cultured in RPMI/FBS medium are presented in Fig. 24. Cyclic AMP levels were significantly higher in the stromal cells treated with gonadotropins than in untreated cells.

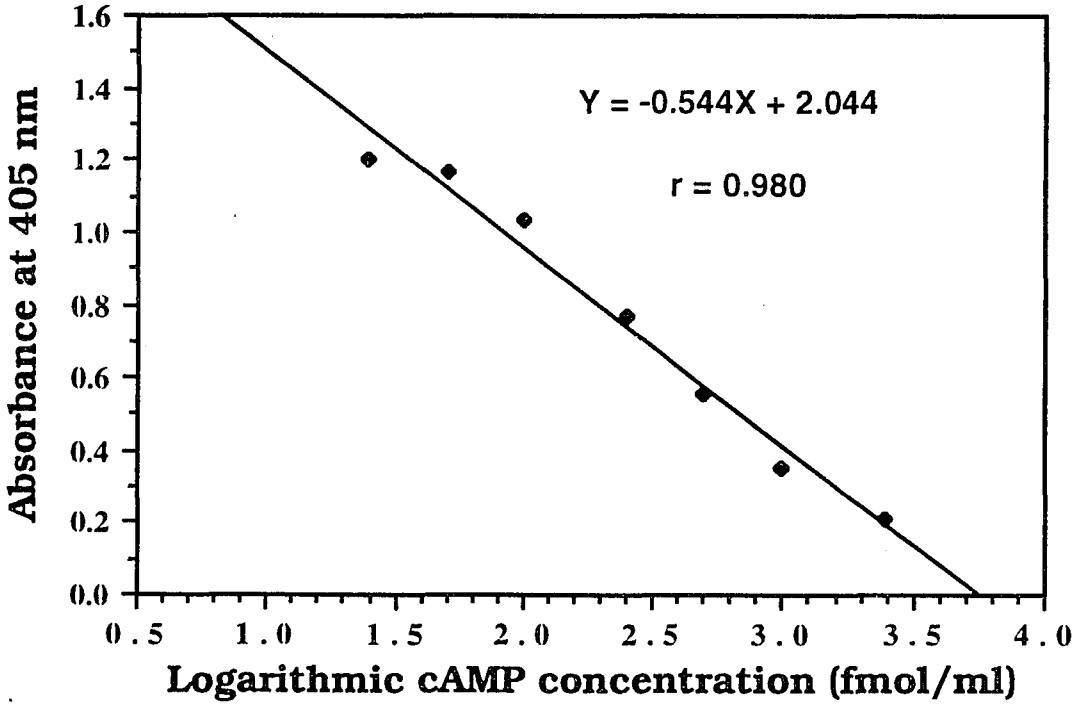


Fig. 23 Standard curve for cAMP ELISA analysis.

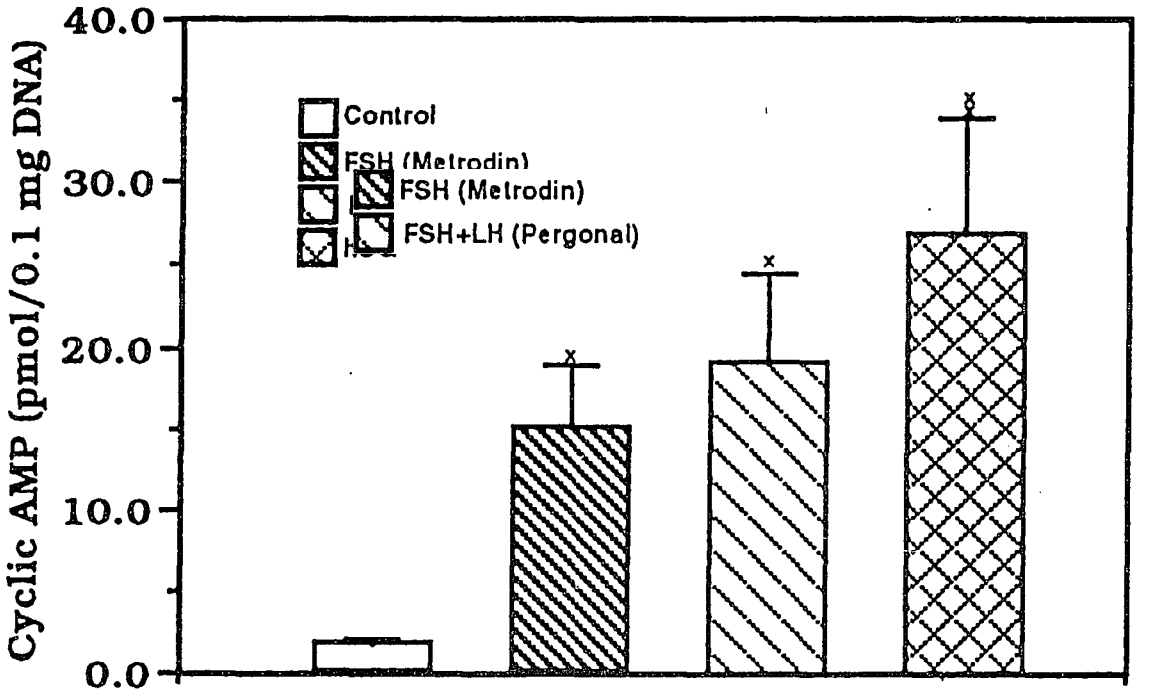


Fig. 24 Effect of gonadotropins on cAMP levels of human endometrial stromal cells.

Confluent human endometrial stromal cells were cultured in RPMI/FBS medium containing with or without 0.1 IU/ml of FSH (Metrodin), 0.1 IU/ml of FSH+LH (Pergonal), and 5 IU/ml of hCG for 6 days and cAMP levels were determined by ELISA.

Morphologic differentiation of human endometrial stromal cells induced by gonadotropins

Morphologic changes of human endometrial stromal cell cultured in the presence of gonadotropins were observed by phase contrast microscopy. Treatment of the stromal cells with gonadotropins resulted in decidual morphologic changes by which cells appeared rounder and occasional binucleated. In the stromal cells cultured without addition of gonadotropins, cells remained elongated or spindle-shaped, exhibiting morphologic features considered typical for fibroblast-like endometrial stromal cell. Figure 25 shows the morphologic change of stromal cells induced by gonadotropins.

Immunocytochemical detection of PRL expression induced by gonadotropins in human endometrial stromal cells

Positive immunostaining for PRL was observed by using polyclonal rabbit anti-hPRL antibody in the stromal cells cultured with gonadotropins, but not in the cells cultured in RPMI/FBS only, as shown in Fig. 26.

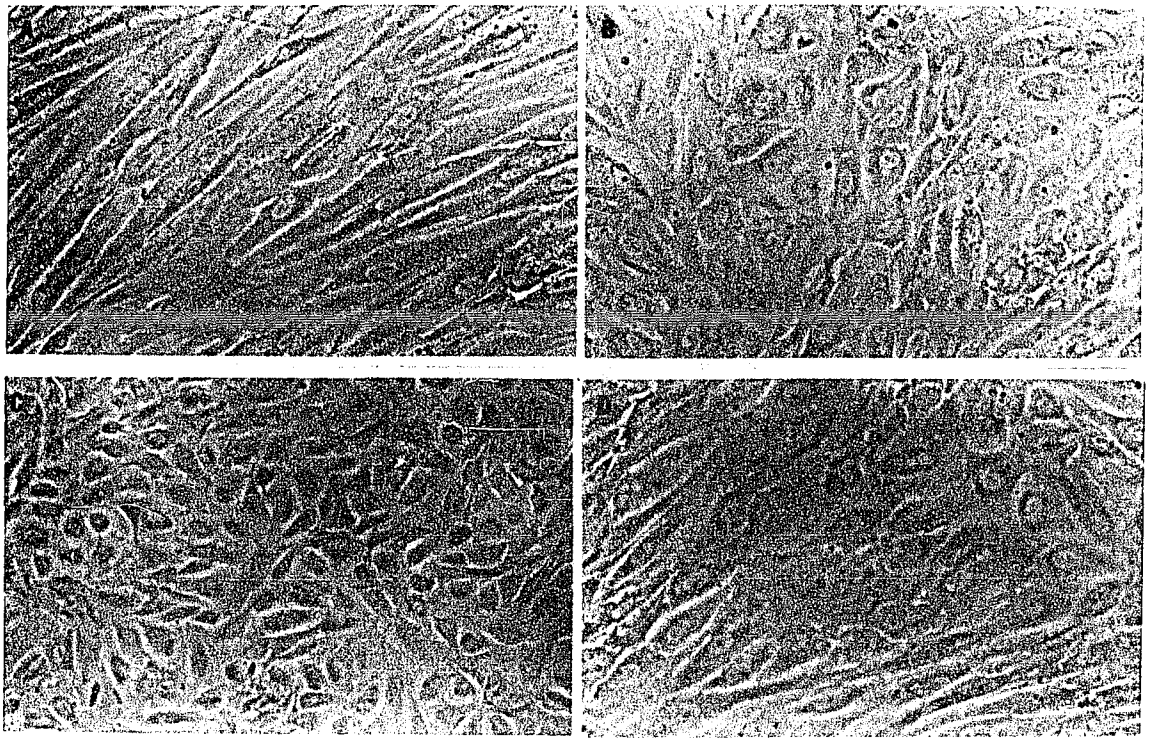


Fig. 25 Morphologic change of the endometrial stromal cells cultured with gonadotropins observed under phase contrast microscopy (10x10).

Panel A: human endometrial stromal cells were cultured in RPMI/FBS medium without the addition of gonadotropins. Panels B, C, and D: the stromal cells were cultured in the medium containing hCG (5 IU/ml), FSH [0.1 IU/ml (Metrodin)], and FSH+LH [FSH 0.1 IU/ml, LH 0.1 IU/ml (Pergonal)], respectively.

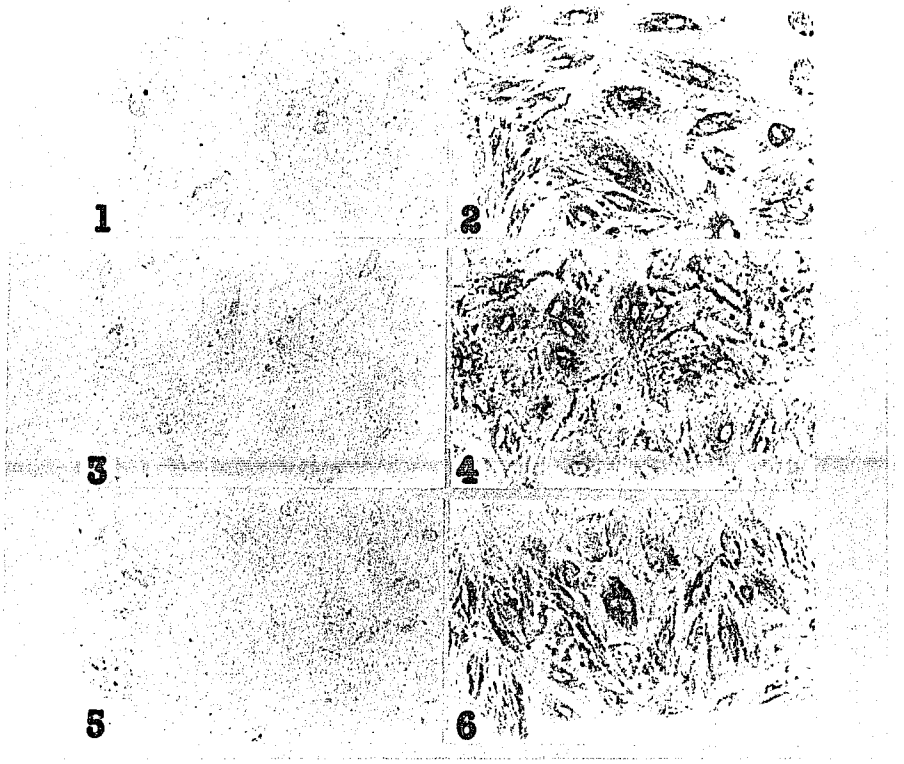


Fig. 26 Immunocytochemical staining for prolactin in human endometrial stromal cells cultured in RPMI/FBS with or without gonadotropins.

Panels 2, 4, and 6: positive staining was observed in the stromal cells cultured with LH+FSH (0.1 IU/ml for each, Pergonal), FSH (0.1 IU/ml, Metrodin), and hCG (5 IU/ml).

Panels 1, 3, and 5: No immunochemical staining was observed in stromal cells cultured without addition of gonadotropins.

No staining was observed under the same conditions in control preparations with non-immune serum.

Western blot of PRL expression induced by gonadotropins in human endometrial stromal cells

Immunoblot analyses were performed to determine whether the immunoreactive species of PRL observed by immunocytochemistry in stromal cell cultures coresponded to the electrophoretic mobility of previously characterized human PRL. Figure 27 demonstrates that the pituitary PRL obtained from NIADDK and the decidual PRL produced by human endometrial stromal cells under various culture conditions migrated at a similar 23 kDa apparent molecular mass.

ELISA Determination of PRL production by human endometrial stromal cells cultured in medium containing gonadotropins.

The inductive effect of gonadotropins on PRL production was further documented by the quantitative measurement of PRL output. ELISA results showed that exposure to gonadotropins (Pergonal, Metrodin, and hCG) led to drastic increase in PRL production. In parallel experiments, no PRL output was observed in cells cultured with RPMI/FBS without gonadotropins. Figure 28 shows results of duplicate determinations from two separate experiments.

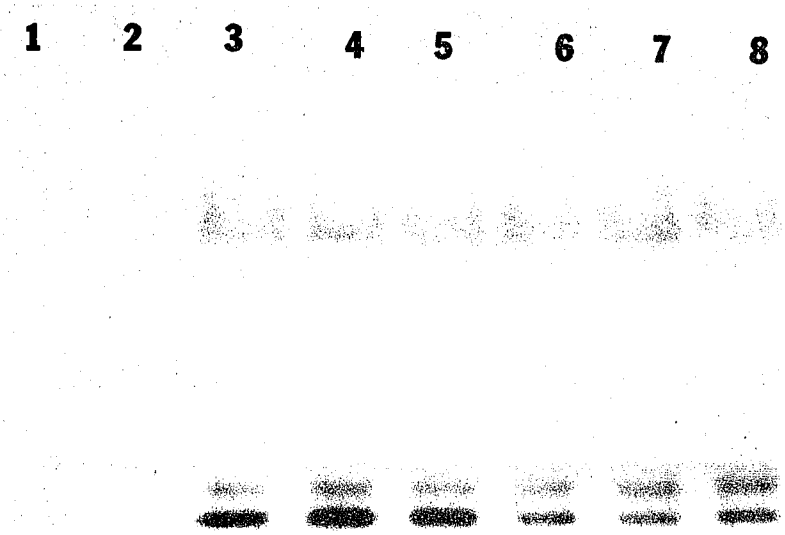


Fig. 27 Western blotting analysis of inductive effect of gonadotropins on production of prolactin by human endometrial stromal cells.

Endometrial stromal cells were cultured in RPMI/FBS medium (lane 2), 0.1 IU/ml of FSH (lanes 3 and 6), 5 IU/ml of hCG (lanes 4 and 7), and 0.1 IU/ml of LH+FSH (lanes 5 and 8) for 4 (lanes 6,7,8) and 6 (lanes 2,3,4,5) days. Media were collected and subjected to SDS-PAGE. Unglycosylated human pituitary PRL was used as standard (lane 1). PRL was specifically detected by anti-hPRL antibody and visualized by using [^{125}I]-labeled Protein A followed by autoadioautography.

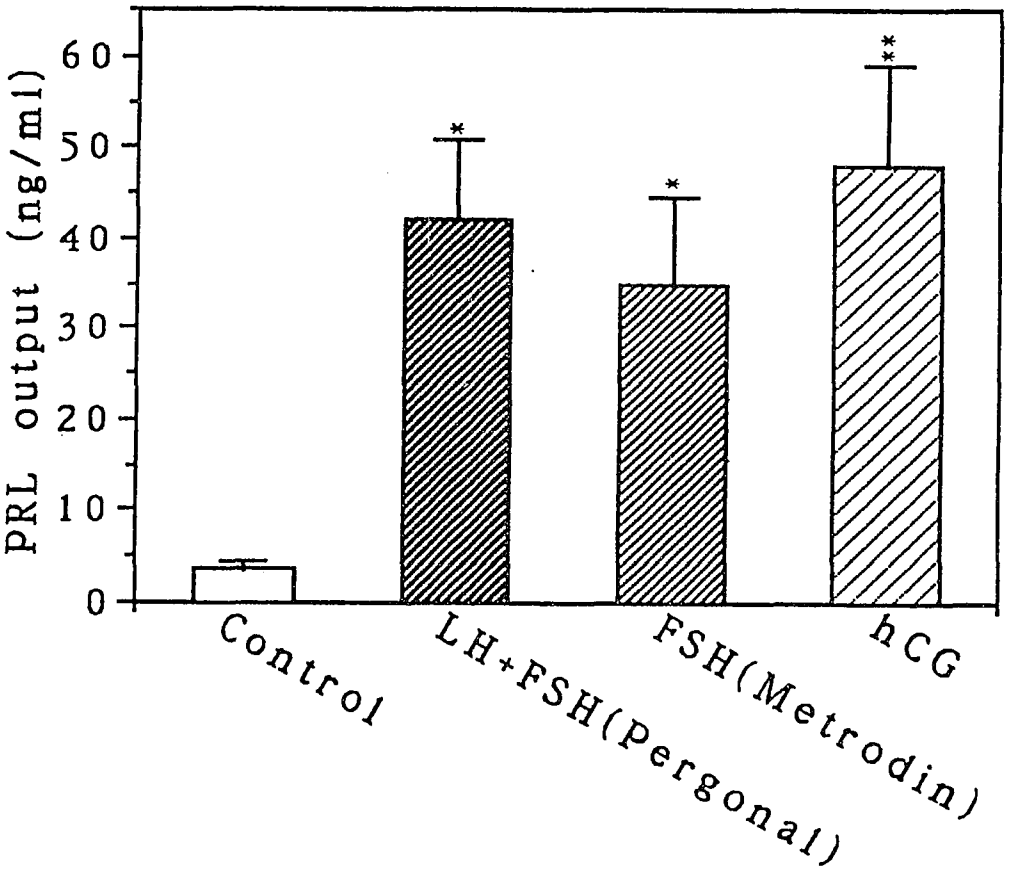


Fig. 28 Effect of gonadotropins on PRL output of human endometrial stromal cells.

Endometrial stromal cells were cultured in RPMI/FBS with or without FSH 0.1 IU/ml (Metrodin), LH+FSH 0.1 IU/ml for each (Pergonal), and hCG (5 IU/ml) for 6 days. Media were collected and subjected to ELISA procedures to measure PRL output.

V. DISCUSSION

A. Prolactin and decidualization

The change in morphology and the induction of laminin, desmin, HSP-27, fibronectin, and IGFBP-1 in human endometrial stromal cells cultured with PRL suggests the effect of this hormone on differentiation of human endometrial stromal cells, acting either as an intracellular intermediate in the decidualizing effects of cAMP or as a paracrine effector released by neighboring decidualized stromal cells.

The possibility of a physiologic involvement of PRL in decidualization of the endometrium is supported by several previous observations, such as the localization and temporal coincidence of PRL and PRL-receptor expression in endometrium and decidua (12,87,90,91), stimulatory effects of PRL on the activities of estrone sulfate sulfatase (11,13) and α_2-macroglobulin (142), and the similarity in the effects of PRL and RLX on activation of PKC in endometrial stromal cells (92). Prolactin secretion by human endometrial stromal cells has been detected at day 23 of the menstrual cycle (87), at a time when the earliest clear morphologic changes

of the endometrial stroma to the decidual phenotype is observed (90). Specific binding sites for PRL have been detected in human endometrium (91), and expression of PRL receptor in human decidua has been recently reported (12). Estrone sulfate sulfatase, an enzyme whose activity is increased during *in vitro* decidualization of human endometrial stromal cells, is stimulated by PRL in cultures of stromal cells isolated from human proliferative endometrium (1,13).

A proposed physiologic function of decidualization is to limit trophoblast invasion. However, the mechanism by which decidua controls trophoblast invasion is not known. The expression of proteolytic enzymes by trophoblastic cells has been related at least in part to their invasive behavior (139), and the antiinvasive function of decidua may be related to its ability to secrete proteinase inhibitors such as α_2-macroglobulin (α_2MG), a potent inhibitor of a wide range of proteinases, which would neutralize trophoblastic-derived proteolytic enzyme activities (140,141). Recently, Gu *et al.* reported that PRL treatment causes a marked increase of α_2MG in rat decidua (142). Although it is not clear whether PKC is involved in the decidualization process of human endometrium, treatment

of human endometrial stromal cells with human PRL or RLX resulted in activation of PKC (92). The participation of RLX in decidualization of human endometrium has been reported by different investigators (2,23-25).

B. Cyclic AMP and PRL expression in human endometrial stromal cells

Although the regulation of PRL gene expression has been primarily studied in anterior pituitary cells, the molecular mechanisms and regulation of PRL production by endometrial stromal cells is still unclear. As part of this dissertation project, cAMP effects on PRL expression by endometrial stromal cells was investigated. The time and concentration dependence of the effects of db-cAMP on PRL production (Figs. 10 and 11), as well as the similarity of the actions of db-cAMP, 8-Br-cAMP and forskolin in this system, demonstrated inductive actions of cAMP on PRL expression. In human pituitary cells, PRL gene transcription is dramatically stimulated within minutes of treatment with cAMP (15). Such fast response suggests a direct stimulatory pathway, most likely involving activation of transcription factors by phosphorylation. In contrast, the response of endometrial stromal cells

to db-cAMP, evaluated from increases in PRL synthesis and output into the medium is slow, taking longer than 2 days (Fig. 10). *In vitro* decidualization of stromal cells under the conditions described in this report provides a model in which the molecular mechanisms of regulation of endometrial PRL production by cAMP can be dissected. The novel finding from our studies, *viz* cAMP effects on the expression of the PRL gene in stromal cells of the human endometrium, sharpened the focus for the search of cis- or trans-acting regulatory factors and responsive elements involved in the regulation of endometrial PRL production. It is of interest to test with the endometrial decidualization system the possibility that Pit-1, a regulatory factor identified in Carter Bancroft's laboratory as influencing PRL expression in rat pituitary, might be operational in the human endometrial system. Further studies at the gene level are neccessary to understand the mechanism of cAMP action on endometrial PRL gene expression. Such studies, facilitated by the recently published report on cloning and partial sequencing of the human decidual PRL mRNA (18), may suggest different approaches to the regulation of decidualization of endometrial stroma.

C. Female sex steroids and cAMP induction of PRL expression

The convergence and cross-talk between steroid and peptide hormones mediated signal transduction pathways of eucaryotic cells has been reported by many research groups. It was reported that cAMP generation in response to stimulation of adrenergic receptor in various cell types *in vivo* and *in vitro* was modulated by steroid hormones such as testosterone (143,144), glucocorticoids (145,146), androgen (147,148), estrogen (115,149,150), and progesterone (115,147,149). Although it is generally believed that steroid hormone actions rely on the activation of the hormone receptor by binding with its cognate ligand, certain steroid receptor mediated reactions can be initiated by membrane receptor in the absence of steroid ligand. *In vivo* animal experiments carried out by Beyer *et al.* demonstrated that mating behavior elicited by administration of progesterone in rats could also be achieved by db-cAMP treatment (151). Progesterone-stimulated, PR-mediated transcription using *in vitro* transient transfection assay systems could be mimicked either by the activation of a cAMP-dependent protein kinase A with 8-Br-cAMP or through the inhibition of protein phosphatases 1 and 2A with okadaic

acid (112). Phosphorylation of chicken PR by cAMP-dependent protein kinase (cAMP-PK) was observed and the PR was proved to be a good substrate of cAMP-PK (152-154). Similar results for phosphorylation of PR were also obtained in mammalian PR such as calf uterine PR (155). These results suggested that some steroid effects could be also elicited by other intracellular second messengers, perhaps by phosphorylation of steroid receptors (156,157). In our *in vitro* experiments with human endometrial stromal cells, MPA enhanced the inductive effect of cAMP on PRL expression whereas E_2 had no significant effect, whether employed alone or in combination with MPA. These experimental results were consistent with the moderate effects of MPA on PRL production when it was added separately to cultures of endometrial stromal cells, even in the presence of E_2, while much larger effects on PRL output were elicited in the same culture system by using a mixture of MPA and RLX, a peptide that can drastically increase cAMP levels in human endometrial cells in the persence of forskolin and IBMX (109), with or without E_2 (23-25). Progesterone can also participate in the process of decidualization by inhibiting phosphodiesterase activity (116), by stabilizing PRL mRNA as observed in other systems

(158,159), or by other cAMP/progesterone concerted mechanisms (117).

D. Cyclic AMP and decidualization

Cyclic AMP effects on decidualization of rodent uteri have been reported (160-162). Treatment of mouse, rat, or hamster uterus with IBMX, cholera toxin, forskolin or cAMP derivatives resulted in the induction of decidual cell reactions as well as cAMP generation (163-165). Increased rat endometrial vascular permeability, a pre-requisite for decidualization (166), was observed in rat uteri treated with cholera toxin. Rat endometrial stromal cells cultured in medium containing forskolin, cholera toxin, IBMX, or db-cAMP showed an increase in alkaline phosphatase activity (160,167,168), an enzyme whose activity was elevated during *in vivo* decidualization observed in the mouse (169,170), rat (171,172), ewe (173), and rabbit (173-175). The participation of cAMP in the process of human endometrial stromal cell decidualization was evident in the present study by the appearence of decidual morphologic change and expression decidualization-related proteins such as PRL, IGFBP-1, HSP-27, desmin, laminin, and fibronectin in the stromal cells after

exposure to the cyclic nucleotide. Since we have shown that PRL promotes decidual-type morphologic change and expression of other decidual cell products (2) and cAMP induces PRL expression in human endometrial stromal cells, it can be suggested that the effects of cAMP on decidualization of human endometrial stromal cells might be mediated at least partially by PRL.

E. Gonadotropins and decidualization

The finding that cAMP induces PRL production and differentiation of endometrial stromal cells offers the opportunity to search for physiologic or pharmacologic agents which could influence decidualization by altering cAMP levels. Such agents could thereby modify the uterine environment and, consequently, be of relevance to embryo implantation and fertility. In a search for physiological inducers of cAMP, we have demonstrated that human pituitary and placental gonadotropins, known to generate cAMP in human ovarian granulosa cells (176), are also able to increase cAMP concentrations in human endometrial stromal cells. Furthermore, treatment with gonadotropins resulted in decidualization of human endometrial stromal cells, as characterized by decidual morphologic changes and induction of PRL production. The

physiologic relevance of the results on gonadotropin-induced PRL production in our stromal cell culture system is supported by the "advanced" stromal changes observed in women receiving gonadotropins for ovarian stimulation as part of *in vitro* fertilization procedure. Histologic and immunocytochemical studies on endometrial biopsies obtained during gonadotropin treatment showed the coexistence of subnuclear glycogen accumulation in epithelial cells (characteristic of the first days of the secretory phase) with positive staining for PRL in stromal cells (day 23 of the normal menstrual cycle), an indication of decidualization (56). These observations may be explained by the novel finding of gonadotropin effects on decidualization of human endometrial stromal cells *in vitro*.

F. Model of human endometrial stromal cell decidualization

From the experimental results reported in this dissertation, a novel mechanism of decidualization was proposed to involve, as shown in Figure 29, concatenated actions of gonadotropins or other physiologic cAMP generators (RLX, PGE_2), cAMP and PRL to induce the morphologic and biochemical changes characterizing the

differentiation of human endometrial stromal cells to the decidual phenotype. The role of progesterone in the decidualization process appears to involve an enhancement of the effect of cAMP on PRL expression and differentiation of the endometrial stroma. Further investigations could elucidate the molecular mechanisms of the cAMP induced-PRL production of human endometrial stromal cells by using PKA inhibitors. The synergistic effect of progesterone and cAMP on PRL gene transcription could be elucidated by searching for cis- or trans-acting regulatory factors and responsive elements involved in the control and regulation of PRL gene expression by human endometrial stromal cells. The relative importance of different physiologic cAMP generators as sources of the cyclic nucleotide in human endometrial stromal cells remains to be identified; the effects of lipocortins, phospholipase A inhibitors preventing prostaglandin synthesis (177,178), may serve to evaluate the effect of PGE_2 in this system. The role of PRL as an obligatory intermediate in the decidualization process need to be further documented by using PRL antisense oligonucleotides (anti-RNA or anti-DNA) and/or anti-PRLR antibody to block PRL effects on the process.

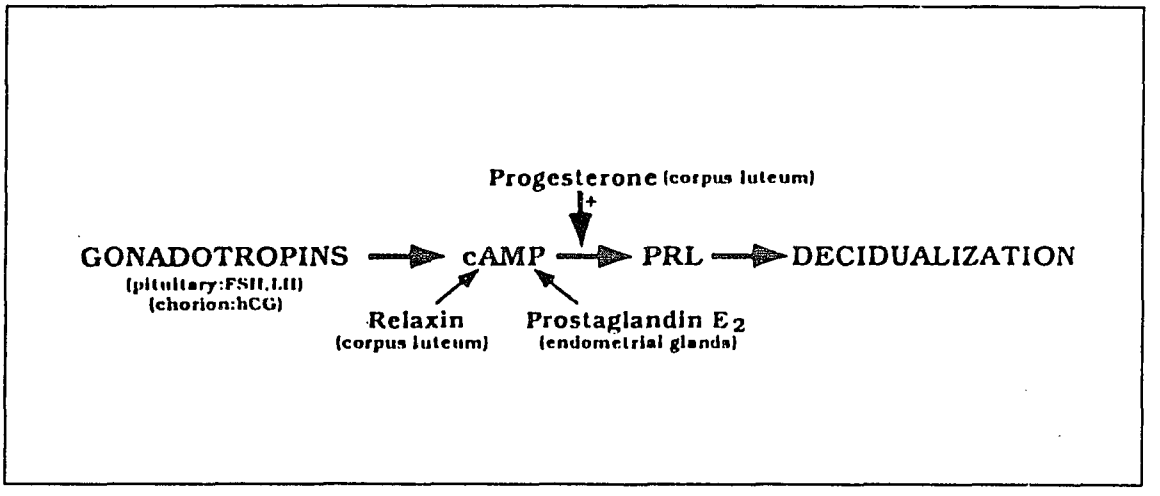


Fig. 29 Model for a mechanism of human endometrial stromal cell decidualization.

This hypothetical scheme features a decidualizing effect of cAMP and progesterone mediated by PRL. According to this scheme, cAMP is generated in human endometrial stromal cells under the influence of gonadotropins (FSH, LH+FSH, hCG) or other cAMP generators (RLX, PGE_2) to induce PRL expression and decidualization which is enhanced by progesterone.

VI. CONCLUSIONS

Our *in vitro* experiments carried out on human endometrial stromal cells in culture yielded the following results:

1. Cyclic AMP induces PRL expression and decidualization of human endometrial stromal cells.

Prolactin expression was detected by applying immunocytochemistry, immunoprecipitation and Western blotting, RIA, ELISA, and Northern blotting procedure. Decidualization was evident from morphologic changes and the expression of decidual products. The results indicate that a cAMP mediated-signal transduction pathway is involved in decidualization of the human endometrial stroma.

2. Prolactin promotes decidualization of the human endometrial stromal cells and may mediate the differentiating effects of cAMP.

The *in vitro* effect of PRL on decidualization of human endometrial stromal cells was evident from their morphologic changes to the decidual phenotype and the

expression of decidualization-related proteins, such as IGFBP-1, HSP-27, desmin, laminin, and fibronectin. The results suggested that PRL may act as a mediator in the cAMP-induced process of human endometrial stromal cell decidualization.

3. The inductive effect of cAMP on PRL expression by human endometrial stromal cells is synergisticaly enhanced by MPA.

The synergistic effect of the progestin and cAMP was determined by using western blotting, ELISA, and nuclear run-on analysis. This result may explain the physiologic role of ovarian progesterone on decidualization. It also suggests that these compounds regulate PRL production and decidualization through interdependent pathways.

4. Gonadotropins (hCG, FSH) increase cAMP levels and induce decidualization of human endometrial stromal cells.

Elevation of cAMP levels was determined by ELISA and decidualization of stroma cells was documented on the basis of morphologic changes and PRL expression. These results indicate that gonadotropins may act

directly on the endometrium to induce PRL expression and decidualization through a cAMP mediated-signal transduction pathway. A direct effect of gonadotropins on the endometrium may be of clinical and pharmacologic relevance to reproduction and endometrial physiology.

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